Current Topics in Developmental Biology

Volume 72

Edited by
Gerald P. Schatten

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Volume 72

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1

Defending the Zygote: Search for the Ancestral Animal Block to Polyspermy

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Fertilization is the union of a single sperm and an egg, an event that results in a diploid embryo. Animals use many mechanisms to achieve this ratio; the most prevalent involves physically blocking the fusion of subsequent sperm. Selective pressures to maintain monospermy have resulted in an elaboration of diverse egg and sperm structures. The processes employed for monospermy are as diverse as the animals that result from this process. Yet, the fundamental molecular requirements for successful monospermic fertilization are similar, implying that animals may have a common ancestral block to polyspermy. Here, we explore this hypothesis, reviewing biochemical, molecular, and genetic discoveries that lend support to a common ancestral mechanism. We also consider the evolution of alternative or radical techniques, including physiological polyspermy, with respect to our ability to describe a parsimonious guide to fertilization. © 2006, Elsevier Inc.

I. Introduction

Successful embryonic development is enhanced when the zygote is protected from lethal microorganisms or parasites, including additional sperm. Therefore, it is not surprising that all metazoans that reproduce through the fusion of two haploid gametes have adopted a means to establish a postfertilization barrier between the zygote and the environment.

Most physical blocks to polyspermy are derived from material stored by the egg during oogenesis (reviewed in Shapiro *et al.*, 1989).[1] In most animals, construction of a barrier against sperm and other microorganisms uses both the egg's extracellular matrix (ECM) and the egg's secretory vesicles that contain structural proteins and/or enzymes. The terms used for the egg ECM vary widely, including the *zona pellucida* (ZP) (subsequently referred to as *zona* in this chapter) in mammals; the *chorion* in fish; the *vitelline envelope* in amphibians, mollusks, and crustaceans; the *perivitelline layer* in birds; the *vitelline coat* in ascidians; the *vitelline layer* in echinoderms; and the *vitelline membrane* in dipterans. All serve the same basic functions, however. Before

[1]Most animals do not formally ovulate haploid eggs, but oocytes arrested in various stages of meiosis. For clarity, however, the term *egg* is used in this chapter to refer to the cell that is ovulated and the term *oocytes* for the cells that are still developing within the ovary.

fertilization, this matrix provides a supportive substrate for the oocyte or egg. During fertilization, this network of proteins is a primary target for the reception, activation, and binding of sperm. After fertilization, this matrix is often modified to inhibit further sperm binding, thereby avoiding the potentially lethal outcome of polyspermy.

The deleterious effects that multiple cytoplasmic sperm nuclei have on subsequent cleavages maintain a selective pressure in animals to prevent polyspermy. The mechanisms employed to curb supernumerary sperm fusion, however, vary greatly across phyla. For instance, some animal eggs (e.g., those from avians and urodeles) allow multiple sperm to fuse, only later to suppress all but one from merging with the egg pronucleus. Such physiological polyspermy is limited in the animal kingdom; most animals instead employ rapid physical modifications that alter the affinity of sperm for the fertilized egg. A core set of modifications have been described on the molecular level in all animals that use the latter category of physical blocks, suggesting that renovating the egg ECM may follow a common process. If mechanisms to block polyspermy are shared, we would expect that homologous proteins and enzymes are also involved, especially if this type of physical block diverged from a single ancestor. The current state of the field, however, suggests that establishment of a physical block might have convergently evolved through independent routes.

A significant distinction among animal blocks to polyspermy is the composition and modification of the physical barrier. In all vertebrates, members of the ZP protein family comprise most of the egg ECMs (Spargo and Hope, 2003). Thus, any modifications that result in the physical block occur on these ZP proteins. Little is known about the proteins or enzymes responsible for biochemical alterations to the matrix, but the majority of activity is believed to derive from CGs or accessory reproductive organs such as the oviduct. In contrast, very little is known about the constituents of the invertebrate egg ECM. Only a handful of proteins have been isolated and characterized—primarily from ascidians, sea urchins, bivalves, and the dipteran *Drosophila*—and these proteins bear little resemblance to one another or the vertebrate ZP proteins. Variations in the types of morphological modifications that occur are also prominent during animal fertilization, showing dramatic lifting of the egg ECM far from its original location at the egg surface in echinoderms and some anurans, to no observable changes at all in ascidians, mollusks, avians, and mammals. Yet all of these animals exhibit monospermic fertilization. Thus, we immediately see that the evolution of fertilization-related blocks to polyspermy can use a wide variety of mechanisms to achieve the same outcome.

This chapter discusses the role of the egg ECM and CGs during fertilization, focusing on data that implicate molecules and processes involved with the successful transition from an egg ECM to the zygotic block to polyspermy

(see also Bellairs, 1993; Elinson, 1986; Gould and Stephano, 2003; Shapiro *et al.*, 1989; Shur *et al.*, 2004; Yamagami *et al.*, 1992). We draw primarily on observations and molecular data from animals whose process of fertilization is well documented—mammals, amphibians, fish, ascidians, mollusks, and echinoderms—but we include observations from other animals such as birds (see Bellairs, 1993), dipterans (see Bloch Qazi *et al.*, 2003; Fitch *et al.*, 1998), decapods, and the nematode *Caenorhabditis* (reviewed in Singson, 2001), when applicable (see Tables I and II). We also present primary observations and perspectives that highlight the most basic requirements necessary for successful fertilization, consistent with the hypothesis that the present range of species–specific gamete interactions radiated from a common ancestor.

II. Egg Extracellular Matrix

The egg ECM is known by many names in animals. These include the *zona* in mammals, the *chorion* in teleosts, the *perivitelline layer* in birds, the *vitelline envelope* in amphibians, mollusks, and crustaceans, the *vitelline layer* in echinoderms, and the *vitelline membrane* in dipterans. The function of this ECM is multifaceted, providing both a biochemical interface between the oocyte and associated follicle cells and a protective structural barrier to the egg. During oogenesis, the matrix is no more than a thin layer of glycoproteins that defines the boundary between the oocyte and surrounding support cells (Anderson, 1968; Breed and Leigh, 1990; Hedrick and Nishihara, 1991; Mate, 1998; Sinowatz *et al.*, 2001). As oogenesis progresses and oocyte cytoplasm accumulates, the ECM thickens (Figs. 1 and 2), reflecting its increasingly important role in cell–cell signaling between the oocyte, its support cells, and eventually the sperm (Dean, 2004). Outside of the ovary, the primary function of the ECM changes from a supportive to a directive role, acting as a signaling platform for the complex series of events directive up to fertilization, including initiation of the sperm acrosome reaction, sperm orientation, and sperm binding (reviewed in Rankin and Dean, 2000). Following gamete fusion, the egg ECM is modified once more, transforming it into a physical barrier against environmental insults such as additional sperm or microbes. The success of this extracellular block is dependent on the components and their inherent organization within the final structure.

For purposes of this chapter, we focus on the latter events of ECM maturation that result in the protection of the egg from an environment less nurturing than the ovary. Here, we compare the ultrastructure of and highlight the major protein domains associated with each egg ECM used by different animal orders (see Table I). Although this section acts as a primer to familiarize us with the range of descriptives used to define different ECMs, it also introduces potential aspects conserved among the matrices both during the assembly process and

Table I Hierarhy of Select Genera within the Animal Kingdom[a]

Phylum (subphylum)	Class	Subclass	Order	Family	Genus	Animal
CHORDATA (Vertebrata)	Mammalia	Eutheria	Carnivora	Canidae	*Canis*	Dog
					Vulpes	Fox
				Felidae	*Felix*	Cat
				Mustelidae	*Mustela*	Weasel
			Cetartiodactyla	Suidae	*Bos*	Cow
					Sus	Pig
			Lagomorpha	Leporidae	*Oryctolagus*	Rabbit
			Perissodactyla	Equidae	*Equus*	Horse
			Primates	Callitrichidae	*Callithrix*	Marmoset
				Cercopithecidae	*Macaca*	Macaque
					Papio	Baboon
				Hominidae	*Homo*	Human
			Rodentia	Caviidae	*Cavia*	Guinea pig
				Muridae	*Lagarus*	Lemming
					Mesocricetus	Hamster
					Microtus	Vole
					Mus	Mouse, common
					Notomys	Mouse, hopping
					Pseudomys	Mouse, desert
					Rattus	Rat
		Metatheria	Dasyuromorphia	Dasyuridae	*Sminthopsis*	Dunnart
			Didelphimorphia	Didelphidae	*Monodelphis*	Opossum
			Diprotodontia	Macropodidae	*Macropus*	Kangaroo
				Phalangeridae	*Trichosurus*	Possum, brush-tail
				Phascolarctidae	*Phascolarctos*	Koala
				Pseudocheiridae	*Pseudocheirus*	Possum, ring-tail
			Peramelemorphia	Peramelidae	*Isoodon*	Bandicoot
	Aves	Neognathae	Galliformes	Phasianidae	*Coturnix*	Quail
					Gallus	Chicken
	Amphibia	Batrachia	Anura	Bufonidae	*Bufo*	Toad
				Discoglossidae	*Discoglossus*	Frog, painted
				Leptodactylidae	*Eleutherodactylus*	Frog, tree
				Pipidae	*Xenopus*	Frog, clawed
			Caudata	Salamandroidae	*Cynops*	Newt
			Urodela	Hynobiidae	*Hynobius*	Salamander

(*Continued*)

Table I *Continued*

Phylum (subphylum)	Class	Subclass	Order	Family	Genus	Animal
CHORDATA (Vertebrata)	Actinopterygii	Neopterygii (Teleostei)	Anguilliformes	Anguillidae	*Anguilla*	Eel
			Beloniformes	Adrianichthyidae	*Oryzias*	Medaka
			Clupeiformes	Clupeidae	*Clupea*	Herring
			Cypriniformes	Cyprinidae	*Barbus*	Barb, rosy
					Carassius	Goldfish
					Cyprinodon	Pupfish
					Cyprinus	Carp
					Danio	Zebrafish
					Pimephales	Minnow
					Rhodeus	Bitterling
					Tribolodon	Barbel fish
			Perciformes	Gobiidae	*Pomatoschistus*	Goby
				Malacanthidae	*Lopholatilus*	Tilefish
				Sparidae	*Sparus*	Seabream
			Pleuronectiformes	Pleuronectidae	*Pseudopleuronectes*	Flounder
				Scophthalmidae	*Scophthalmus*	Turbot
			Salmoiformes	Salmonidae	*Oncorhynchus*	Trout
					Salmo	Salmon
					Salvelinus	Char
			Scorpaenis forme	Liparidae	*Liparis*	Snailfish
			Jetraodontiformes	Tetraodontidae	*Tetraodon*	Pufferfish
		Chondrostei	Acipenseriformes	Acipenseridae	*Acipenser*	Sturgeon
				Polyodontidae	*Polyodon*	Paddlefish
	Hyperoartia		Petromyzontiformes	Petromyzontidae	*Lampetra*	Lamprey
(Urochordata)	Ascidiacea		Phlebobranchia	Cionidae	*Ciona*	Tunicate
			Stolidobranchia	Pyuridae	*Halocynthia*	Ascidian
ECHINODERMATA	Stelleroidea	Asteroidea	Forcipulatida	Asteriidae	*Asterias*	Starfish
			Paxillosida	Astropectinidae	*Astropecten*	Starfish
			Spinulosida	Asterinidae	*Asterinas*	Bat starfish
	Echinoidea	Echinacea	Arbacoida	Arbadiidae	*Arbacia*	Sea urchin
			Echinoida	Strongylocentrotidae	*Strongylocentrotus*	Sea urchin
			Temnopleuroida	Toxopneustidae	*Lytechinus*	Sea urchin

(Hexapoda)	Insecta	Pterygota	Diptera	Culicidae	*Anopheles*	Mosquito
				Drosophilidae	*Drosophila*	Fly, fruit
				Muscidae	*Musca*	Fly, house
				Tephritidae	*Dacus*	Fly, fruit
ARTHROPODA (Crustacea)	Malacostraca	Eucarida	Decapoda	Palaemonidae	*Palaemon*	Shrimp
				Rhynchocinetidae	*Rhynchocintes*	Prawn
				Sicyoniidae	*Sicyonia*	Shrimp
				Nephropidae	*Homarus*	Lobster
				Majidae	*Libinia*	Crab, spider
					Maia	Crab, spider
				Ocypodidae	*Uca*	Crab, fiddler
				Portunidae	*Carcinus*	Crab, common
(Chelicerata)	Merostomata		Xiphosura	Limulidae	*Limulus*	Crab, horseshoe

MOLLUSCA	Gastropoda		Orthogastropoda	Haliotidae	*Haliotis*	Abalone
				Trochidae	*Tegula*	Teguline
	Bivalvia	Hetaroconchie	Veneroida	Mactridae	*Spisula*	Clam
		Heteroconchia	Unionoida	Unionidae	*Unio*	Unio bivalve
		Paleoheterodonta	Mytiloida	Mytilidae	*Mytilus*	Mussel
			Ostreoida	Ostreidae	*Crassostrea*	Oyster
	Cephalopoda		Sepiida	Sepiidae	*Sepia*	Cuttlefish
	Polyplacophora		Neoloricata	Chitonidae	*Tonicia*	Chiton
				Ischnochitonidae	*Challochitin*	Chiton

ANNELIDA	Polychaeta	Palpata	Canalipalpata	Chaetopteridae	*Chaetopterus*	Annelid

NEMATODA	Chromadorea		Rhabditoida	Rhabditoidae	*Caenorhabditis*	Nematode

[a]All genera used in this chapter are listed. Approximate phylogenetic position of each order is listed from most recent (top) to most basal (bottom). Description of animals in the text uses common variants of the phylum, class, order, or family where applicable. Metatherians are referred to as "marsupials" in the text.

Table II Summary of the Molecular Components Predicted to be Involved at Particular Stages of Fertilization in Select Animals

	Decapods	Dipterans	Chiton	Bivalves	Gastropods	*Echinoderms*
Fertilization type	External/internal	Internal	External	External	External	External
% polyspermy	0%	0–11%	0%	0%	0%	0%
Egg ECM(s)	Vitelline envelope (1)	Vitelline membrane Chorion	Vitelline envelope Jelly	Vitelline envelope Jelly	Vitelline envelope Jelly	Vitelline layer Jelly
Source of ECM	Oocyte Follicular cells	Oocyte Follicular cells	Oocyte	Oocyte Follicular cells	Oocyte	Oocyte
ECM components		[dec-1], fc106, 125, 177 [cor-36], sV23/sV17 [fs(2)QJ42], s18, s36		gp180 gp273	VERL	p160 EBR1 Rendezvin^{VL}
Accessory cells ovulated	None	None	Follicle cells	None	None	None
Oviduct contribution	75-kDa trypsin target	Cerotoxin				Jelly Speract, resact ARIS, coARIS, asterosap
Cortical granule contents		n/a			n/a	CGSP1 Ovoperoxidase Transglutaminase Proteoliaisin SFE1 SFE9 Rendezvin^{CG} Hyalin
Epididymal contributions to sperm surface		*N*-acetylglucosaminidase				
Acrosome contents					Lysin	Bindin Metalloprotease
Chemoattractant				Hexapeptides	Amino acids	resact
Egg primary sperm receptor		*N*-acetylglucosamine			VERL	FSP, sialoglycan, speract ARIS, coARIS, asterosap
Sperm primary egg ligand		*N*-acetylglucosaminidase Mannosidase				ERJ1, ERJ2, ERJ3, PC2
Egg secondary sperm receptor					VERL	EBR1
Sperm secondary egg ligand					Lysin	Bindin
Sperm penetration through the ECM	Acrosomal filament	Acrosomal process	Acrosomal filament	Acrosomal filament	16-kDa Lysin	Acrosomal process
Egg contribution to fusion						Metalloprotease
Sperm contribution to fusion					18-kDa Lysin	Bindin
Transient change in membrane potential	Hyperpolarization					Depolarization
Postfertilization egg ECM modifications	Formation of vitelline envelope 2 from cortical granule secretions	Isopeptide bond formation Di-tyrosine cross-linking		Aminopeptidase disruption of sperm receptor		Isopeptide bond formation Di-tyrosine cross-linking Adsorption of cortical granule protein to the ECM

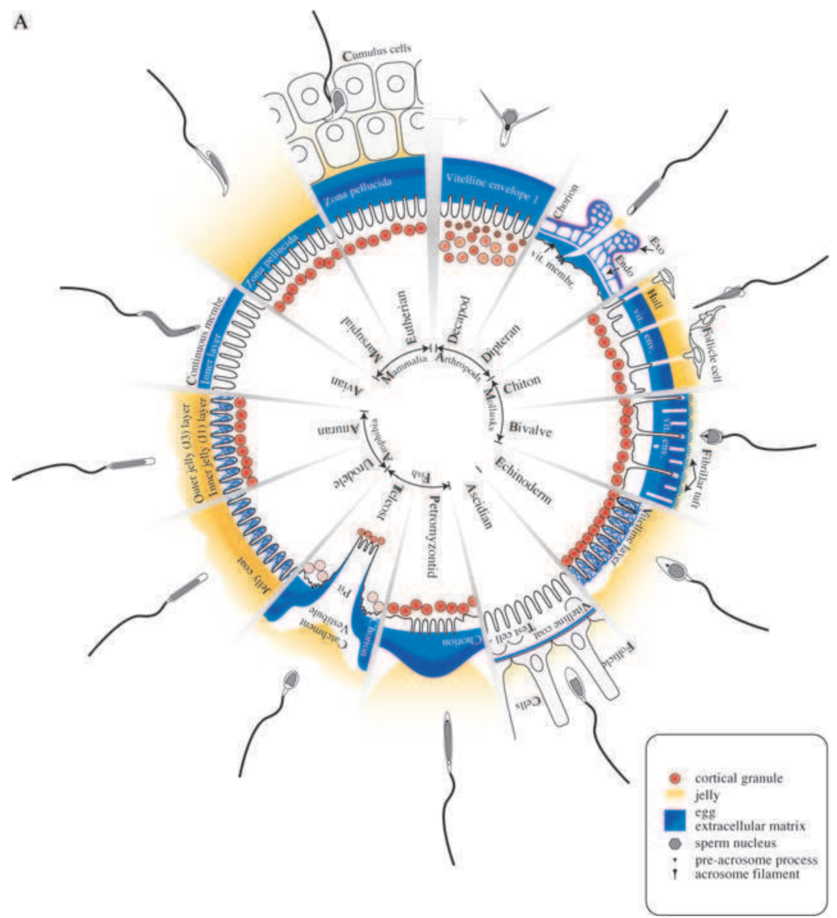
Table II *Continued*

Ascidians	Chondrostreans	Teleosts	Urodeles	Anurans	Avians	Marsupials	Eutherians
External	External	External	External	External	Internal	Internal	Internal
0%	0%	0%	100%	0%	100%	5%	0%
Vitelline coat	Chorion Jelly	Chorion Jelly	Vitelline envelope Jelly	Vitelline envelope Jelly	Perivitelline layer	Zona pellucida	Zona pellucida
		Oocyte Liver	Oocyte Granulosas cells	Oocyte Granulose cells	Oocyte Liver		
VC70	ZP orthologs (>two?)	ZP orthologs (>three)	ZP orthologs (>one?)	ZP orthologs (five)	ZP orthologs (four)	ZP orthologs (>two?)	ZP orthologs (>three?)
Test cells Follicle cells	None	None	None	None	None	Corona radiata	Corona radiata
			Jelly Cleavage of ZPC	Jelly Cleavage of ZPC	Outer egg shell	Glycoproteins	Glycoproteins
n/a	Peroxidase activity	Peroxidase activity Transglutaminase Cystatin FEO-1 FLS CLS Hyosophorin	n/a	Zinc metalloprotease Peroxidase activity XL35/CGL			Protease activity Peroxidase activity HL1 (lectin) p56/AM67 p62
							Spermadhesin
Acrosin Spermosin 20S proteosome 26S proteosome							Acrosin Spermosin TESP5 PH-20 MC41 Proteosome?
SAAF		PMIF		allurin			OGPs progesteone
VC70				ZPC oligosaccharide		PC oligosaccharide	ZPC oligosaccharide
							SVI Mannosidase Hexokinase AWN-1 RSA
	ZP homolog (not ZPA)	ZP homolog (not ZPA)					ZPA-ZPC
							GalTase I TESPs PH-20 sp56/AM67 SED1/p47
Proteolysis	Acrosomal process	n/a			Mechanical oscillation?	Mechanical force	Sperm head oscillation
Metalloprotease							CD9 CD81
				xMDC16			Izumo TRPCs M29 mMN9 Fertilin/PH30 NL1
Depolarization		Freshwater, depolarization Marine None	None	Depolarization	None	None	None
Deglycosylation and proteolysis of VC70		Proteolysis at micropyle Isopeptide bond formation	Hydration-dependent hardening	Calcium-dependent XL35 precipitation ZPA proteolysis [by Zinc metalloprotease] ZP deglycosylation	Application of oviductal coating	Application of oviductal coating	ZPA protolysis ZP deglycosylation

within the organization of the final structure. We focus on the contributions of a selection of protein domains to the egg ECM, using this information to assess whether this structure is conserved on the molecular level. We also ask which types of selective forces associated with fertilization may be influencing the evolution and adaptation of these motifs in different animals.

A. Construction of an Egg Extracellular Matrix

Assembly of a functional egg ECM ultimately depends on the oocyte, but in many animals, this construction requires input from various somatic cells. In some animals, synthesis of some egg ECMs is accomplished entirely by the oocyte (Epifano *et al.*, 1995; Haines *et al.*, 1999; Kanamori, 2000;



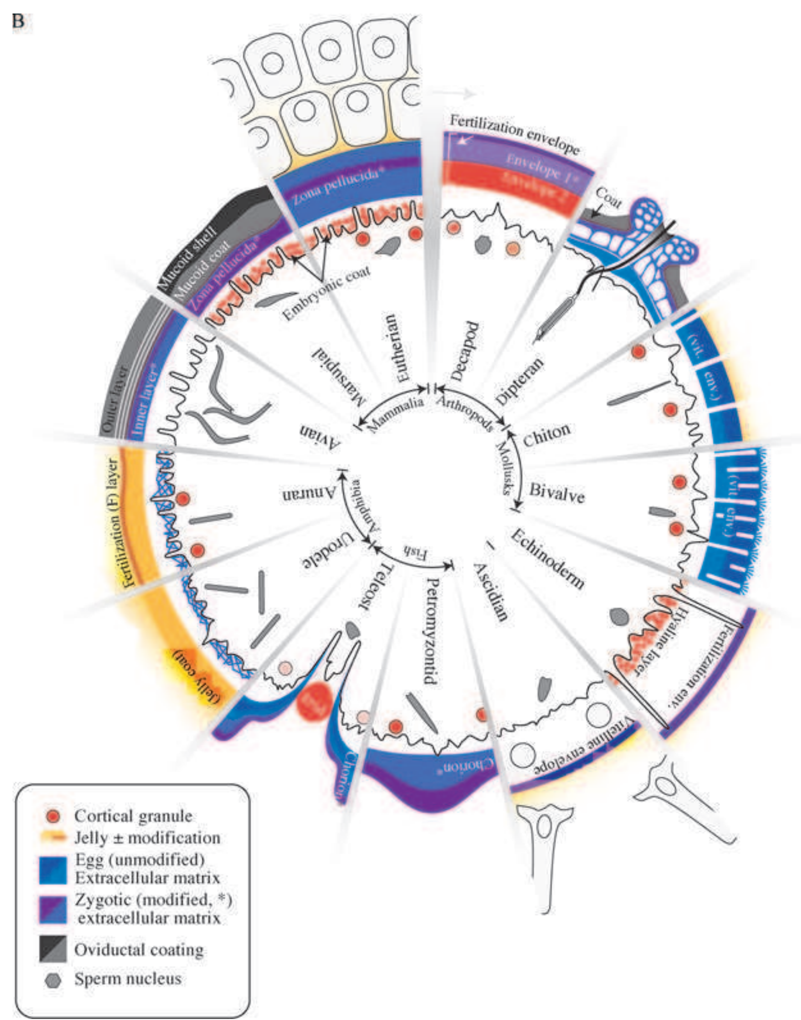


Figure 1 Direct comparison of animal egg cortices before and after fertilization. Representative animal egg cortices before (A) and after fertilization (B). Shown are the most fusion-competent domains of the respective egg (see also Fig. 4). Colors are associated with structures as in the legend: Cortical granule contents are shown in pink/red; the extracellular matrix is indicated in blue (egg) and purple (modified by cortical granule contents); jelly is shown in yellow; and postfertilization coats usually applied by external sources such as oviductal epithelium are in dark gray. Sperm nuclei are shown in light gray. Major structures and ultrastructural divisions, when discernable, are labeled. In (B), structures labeled primarily refer to structures altered at fertilization (including the use of an asterisk if the name itself does not change). endo, endochorion; exo, exochorion; vit. membr., vitelline membrane; vit. env., vitelline envelope.

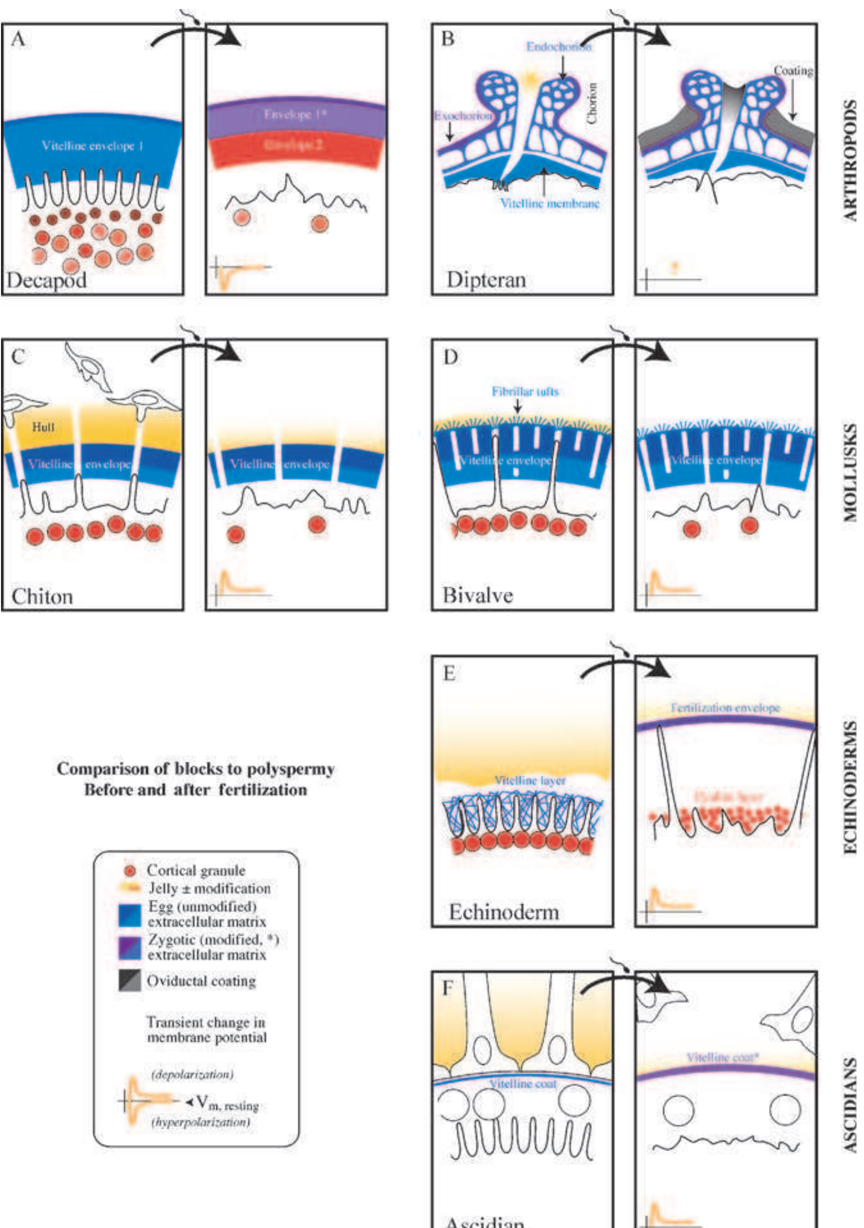


Figure 2 Juxtaposition of egg extracellular matrices before and after fertilization. Detailed side-by-side pairs of animal egg cortices before (left) and after (right) fertilization. Structures and legend generally follow those of Fig. 1. Included are the presence or absence of a fast electrical block to polyspermy (membrane voltage change, in orange). Animal orders separated

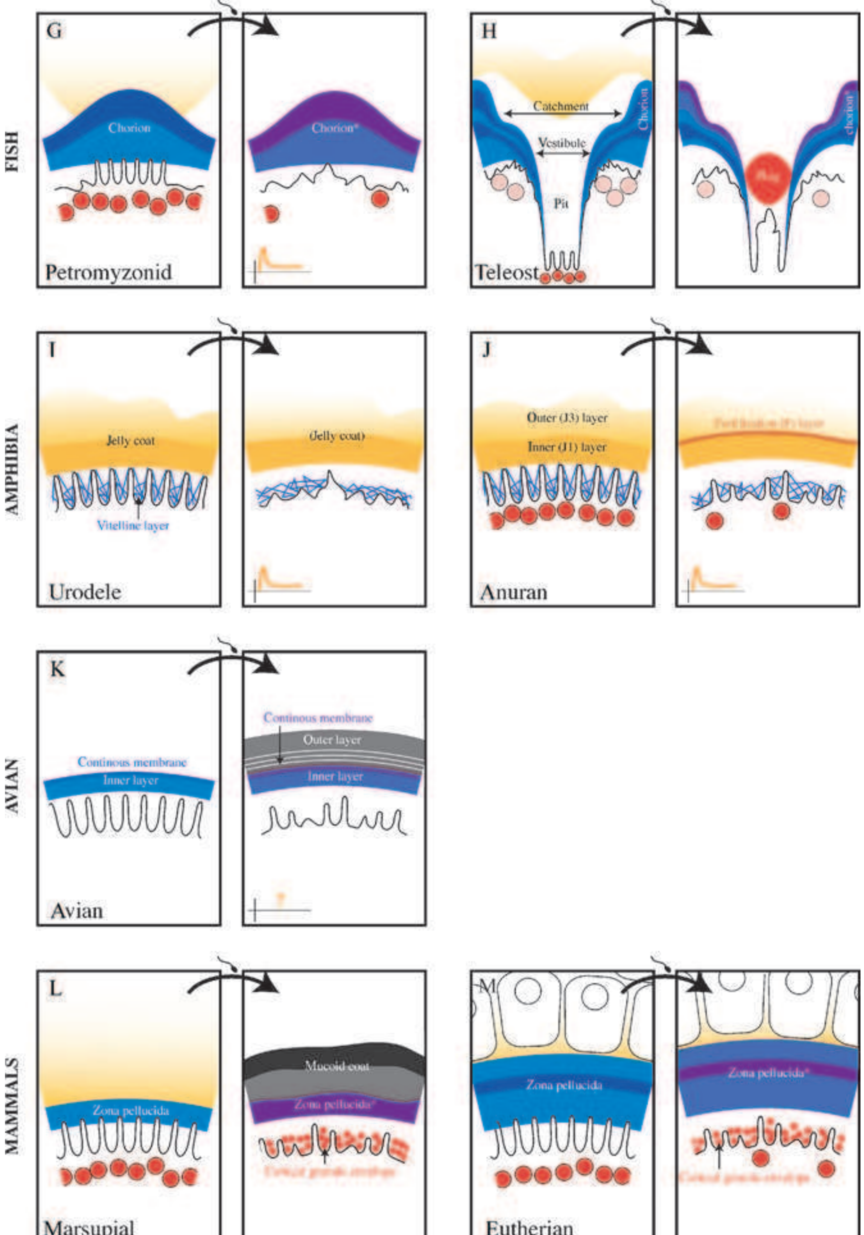


Figure 2 (*Caption continued*) by vertebrate/invertebrate classifications and arranged by their relative phylogenetic position are as follows: decapods (A), dipterans (B), chiton (C), bivalves (D), echinoderms (E), ascidians (F), petromyzontids (G), teleosts (H), urodeles (I), anurans (J), avians (K), marsupials (L), and eutherians (M).

Voyle *et al.*, 1999; Wassarman, 1988; Yamagami *et al.*, 1992), whereas in other animals, synthesis is supplemented by somatic cells (Dunbar *et al.*, 1994; Haines *et al.*, 1999; Kolle *et al.*, 1996; Martinez *et al.*, 1996; Mate, 1998; Okumura *et al.*, 2004; Takeuchi *et al.*, 1999; Vaccaro *et al.*, 2001; Wolgemuth *et al.*, 1984). Such variability of protein sources among animals reflects differences in the robustness and organizational complexity of the specific matrices. In general, simpler ECMs consist of oocyte-derived proteins, whereas the more elaborate matrices may require additional contributions from somatic tissue. The latter situation is often associated with mechanically protective roles, such as resistance to environmental factors like osmotic shock and desiccation. For example, the liver is enlisted in animals whose egg ECMs are extremely robust, such as for avians (Bausek *et al.*, 2000; Okumura *et al.*, 2004) and fish (Chang *et al.*, 1999; Hyllner *et al.*, 2001; Lyons *et al.*, 1993; Murata *et al.*, 1995, 1997; Yamagami *et al.*, 1992). These liver glycoproteins make the long journey to the ovary via the bloodstream, somehow staying in solution until they arrive between the follicle cells and the oolemma, where they self-assemble within the ECM under construction. Whether these distantly–derived proteins are modified by the follicle cells before their incorporation into the egg ECM remains to be determined.

Most ECMs are organized into concentric layers surrounding the egg, which in many cases may reflect its temporal construction. The retention of this lamellar substructure—versus a more homogenized mature ECM—does, however, suggest the possibility that these distinct layers serve a mechanical role in protecting the egg. For example, the eutherian zona can be optically dissected into three layers with orthogonal birefringence: The inner layer possesses radial filaments, the outer tangential, together sandwiching a middle layer of low retardance (Keefe *et al.*, 1997) (Figs. 1–3). This optical deconstruction of the zona agrees with the trilaminar arrangement observed by transmission electron microscopy (TEM) during mouse oogenesis (El-Mestrah *et al.*, 2002) and by immunocytochemical staining for specific glycoproteins in the rabbit zona (Wolgemuth *et al.*, 1984). This alternating, orthogonal organization of parallel fibers arranged in sheets provides greater mechanical strength than a comparably thick mat of parallel fibers (compare layers of plywood vs. fiberboard), supporting the structural role these proteins have in protecting the egg. The teleost chorion layers also appear to be subdivided into a zona radiata interna and externa (Hart and Donova, 1983; Hart *et al.*, 1984). The most inner layer (zona radiata) consists of alternating electron dense and lucent layers, the middle layer is electron lucent, and the thinnest most outer layer (zona externa) is electron dense. Amphibians also possess lamellar vitelline envelopes, a thin structure with at least four subdomains containing two layers of fibers running in parallel with the cell surface, whereas the others are thicker and more scattered (Figs. 1 and 2).

These four layers together participate in initial sperm attraction, binding, and the final block to polyspermy (Arranz and Cabada, 2000; Bonnell *et al.*, 1996; Campanella *et al.*, 1997; Infante *et al.*, 2004; Talevi and Campanella, 1988). Distal to the amphibian egg surface and outside of the vitelline envelope lies a prominent jelly layer composed of a network of fibers and globules (Fig. 3). The lamellar ultrastructure of jelly is a consequence of chronological deposition during the egg's passage through the amphibian oviduct. In anurans, this jelly layer consists of an inner J1 and outer J3 layer sandwiching a concentric stratified middle J2 layer (Bonnell and Chandler, 1996) (Figs. 1 and 3), whereas urodele jelly is composed of four structurally distinct layers with a hyaline or fibrillar ultrastructure (Jego *et al.*, 1986).

In general, the structure of an invertebrate's egg ECM is more variable than the vertebrate analog. One reason for this diversity may lie in the much greater diversity of reproductive methods used by invertebrates, which could result from the wider evolutionary distance separating these animals from the diverged clade of vertebrates (see Table I). Although a vertebrate's egg ECM is a series of glycoprotein shells that surround the egg, those of invertebrates prove more complex (Figs. 1 and 2). An invertebrate egg ECM morphologically most similar to a vertebrate's belongs to echinoderms, the most basal deuterostome order. Its thin vitelline layer consists of a dense fibrillar reticulum proximal to the oolemma that drapes over microvilli, with a second electron-lucent layer found just outside this glycoprotein shell (Bonnell *et al.*, 1994). The entire vitelline layer is synthesized by the oocyte during oogenesis (Runnstrom, 1966). As in amphibians, a more substantial jelly layer is applied by follicle cells over the vitelline layer upon oocyte maturation that serves to attract and activate sperm (Santella *et al.*, 1983; Tegner and Epel, 1976; Tosti, 1994). A similar laminar organization is present in the ascidian vitelline coat, the filamentous glycoprotein layer separated from the egg by a wide perivitelline space (De Santis *et al.*, 1980). Upon ovulation, the follicle cells remain attached to the exterior face of the vitelline coat while the interior perivitelline space is inhabited by test cells, membrane-bound extrusions from the oocyte that appear upon maturation (Rosati, 1985). Follicle cells are thought to regulate the penetrance of sperm during fertilization; the function of test cells, however, is not clear. Retention of follicle cells over the ECM is also seen in chiton. These basal mollusks are spawned with a thin layer of follicle cells surrounding an elaborate jelly hull (Buckland-Nicks and Hodgson, 2000; Buckland-Nicks *et al.*, 1988). Unlike eutherian cumulus cells or ascidian follicle cells, the chiton follicle cells may shrivel upon contact with the seawater, revealing pores in the hull that are nearly continuous with pores of the vitelline envelope. Together, these continuous tunnels facilitate a sperm's access to the egg surface. Consistent with the morphology of the egg ECM in most higher mollusks (e.g., gastropods, bivalves, and cephalopods), the chiton vitelline envelope pores are occupied by elongated microvilli that extend

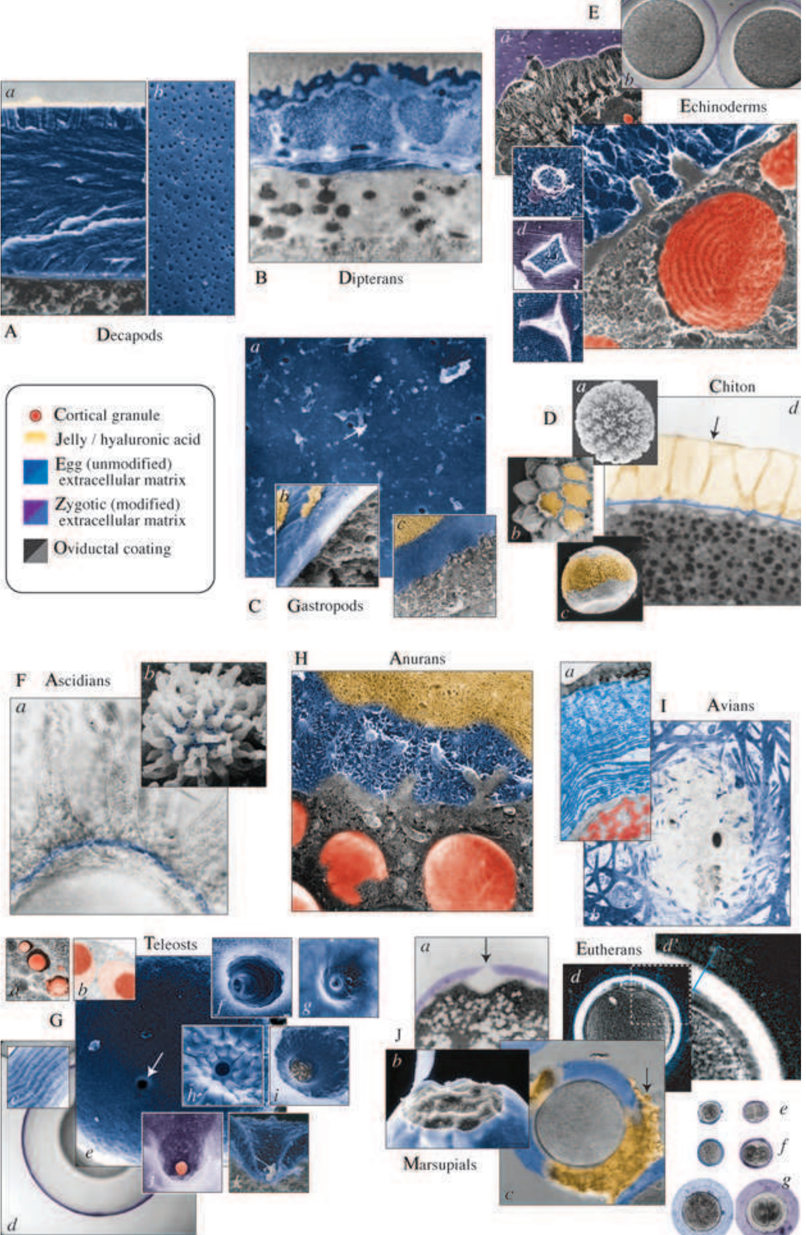


Figure 3 Representative animal egg cortices and extracellular matrices. Photomicrographs of egg cortices and extracellular matrices (ECMs) from light and electron microscopy, clustered by animal order: (A) decapods, (B) dipterans, (C) gastropods, (D) chiton, (E) echinoderms, (F) ascidians, (G) teleosts, (H) anurans, (I) avians, and (J) mammals. Colors indicate specific

to the ECM surface (Alliegro and Wright, 1983; Buckland-Nicks *et al.*, 1988; Mozingo *et al.*, 1995). Thus, passage through the tunnel affords the mollusk sperm direct access to the egg surface. Sperm access is spatially restricted in the bivalve *Unio*, whose vitelline envelope is attached to the egg only at the vegetal pole (Focarelli *et al.*, 1988) (Fig. 4). This attachment point is the sole site of sperm binding and fusion, the functional equivalent to a micropyle. Such a regional specialization of the egg ECM is ubiquitous in dipteran insects, whose eggshell is specifically molded for sperm entry only at the anterior pole (Degrugillier and Leopold, 1976; Mouzaki *et al.*, 1991; Turner and Mahowald, 1976). The dipteran chorion is composed of two substructures: the vitelline membrane, an intimate ECM surrounding the insect egg, and the chorion, an outer cavernous structure composed of an egg-proximal endochorion and a distal exochorion (Degrugillier and Leopold, 1976; Mouzaki *et al.*, 1991;

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organelles, as in Figs. 1 and 2. (A) SEM of a fractured *Limulus* egg cortex (Aa) and a surface view showing the abundance of channels found in the vitelline envelope (Ab; Brown and Humphreys, 1971). (B) Transmission electron microscopy (TEM) cross-section through the anterior region of the *Drosophila* chorion (Pascucci *et al.*, 1996). (C) *Haliotis* egg vitelline envelope, showing surface views of pores (a; arrow) and SEMs of the egg cortex (Cb–c) including the ability to distinguish the ECM from the jelly coat (Mozingo *et al.*, 1995). (D) SEM of chiton hull morphology for *Mopalia* (Da), *Lepidochitona* (Db; Buckland-Nicks, 1993), and *Challochiton* (Dc; Buckland-Nicks and Hodgson, 2000). Also, corresponding *Challochiton* bright-field image (Dd) shows the depth and extensive number of pores within the hull (arrow) (Buckland-Nicks and Hodgson, 2000). (E) Images of the sea urchin egg (f) and zygote (a–e). (Ea) SEM of a fractured *Strongylocentrotus* zygote, including the fertilization envelope (Chandler and Heuser, 1980). (Eb) DIC image of *Lytechinus* zygotes. (Ec–e) SEM series of the ovoperoxidase-dependent transition in the microvillar casts of the *Strongylocentrotus* vitelline layer over time (Larabell and Chandler, 1991). (Ef) Cortical SEM view of a fractured *Strongylocentrotus* egg (courtesy D. E. Chandler). (F) Bright-field (Fa) and SEM (Fb) of *Ciona* eggs, including follicle cells overlying the vitelline coat (De Santis *et al.*, 1980). (G) Collection of images from teleosts, including a DIC (Gd, *Danio*) and a whole chorion SEM (Ge; *Oryzias*, Hart *et al.*, 1984). Arrow indicates location of micropyle. Freeze-fracture SEM of the cortex (Ga; *Danio*, Hart and Collins, 1991). Corresponding cross-sectional TEM image of the cortex (Gb; *Danio*, Hart and Donova, 1983) and chorion (Gc; *Oryzias*, Hart *et al.*, 1984). (Gf–i) SEMs of micropyles from various species (Gf, *Oryzias*; Gg, *Lopholatilus*, both courtesy of N. H. Hart; Gh, *Danio*, Hart and Donova, 1983); Gi, *Rhodeus*, Ohta and Iwamatsu, 1983). Also included is a micropyle populated by sperm, separated from the egg (Gk; *Danio*, Wolenski and Hart, 1987) and an exterior view into the micropyle after fertilization (Gj; *Rhodeus*, Ohta and Iwamatsu, 1983). (H) SEM of fractured *Xenopus* egg cortex (courtesy D. E. Chandler). (I) Images of *Gallus* eggshell, especially a TEM cross-section of the inner layer of the vitelline membrane (Ia; Bellairs *et al.*, 1963) and a surface view of the eggshell overlying the germinal disc (Ib; Okamura and Nishiyama, 1978a). (J) Assorted images of mammalian eggs. (Ja) TEM cross-section of a *Trichosurus* zygotic zona displaying a hole where the sperm penetrated (arrow) (Jungnickel *et al.*, 1999). (Jb) SEM of zona torn from a freshly ovulated *Sminthopsis* egg (Breed *et al.*, 2002). (Jc) DIC image of *Homo* egg, with cumulus cells partially attached (arrow). (Jd) Polarized microscope image of *Homo* egg, and (Jd′) detail showing laminar difference in retardance (courtesy J. Trimarchi). (Je–g) Paired images of egg (left) and two-cell embryo (right) from *Mus* (e), *Mesocricetus* (f), and *Oryctolagus* (g) (Eakin and Behringer, 2004).

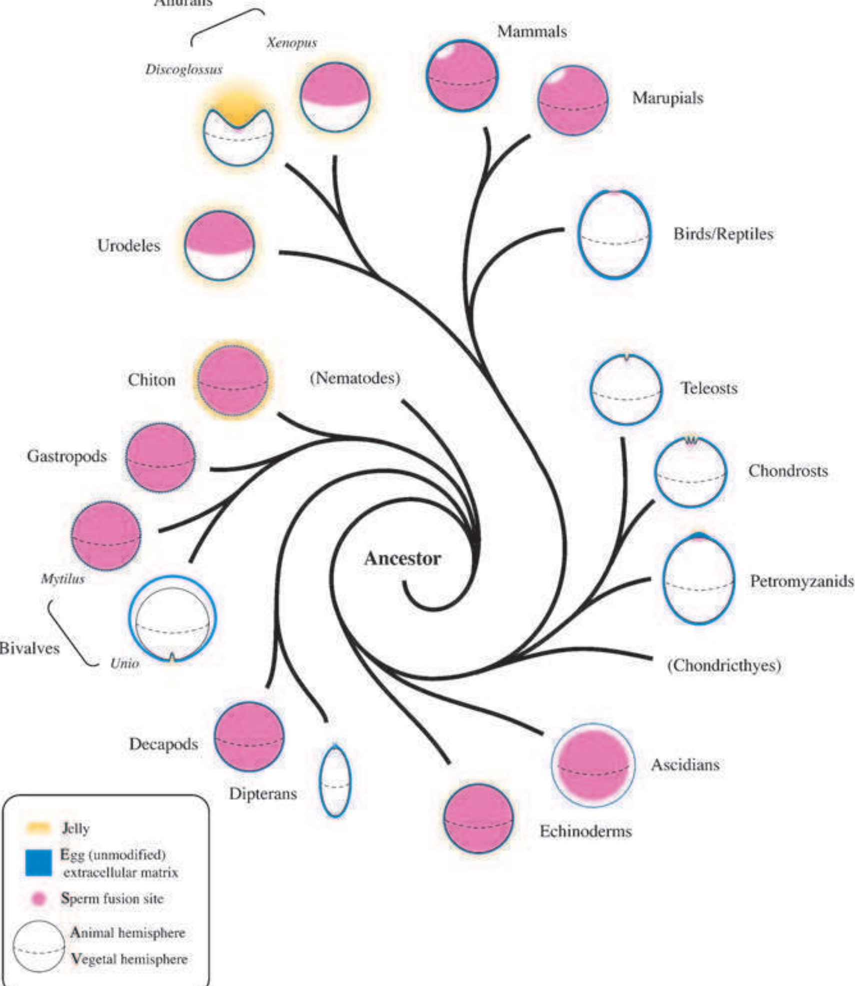


Figure 4 Polarization and fusogenic sites of eggs across animal phylogeny. Representations of eggs of different orders, indicating polarization of sperm fusion sites, superimposed onto a predicted phylogenetic tree of animal evolution. Eggs are not to scale. Where appropriate, genera are shown to represent diversity among animal orders.

Pascucci *et al.*, 1996; Turner and Mahowald, 1976) (Figs. 1 and 2). The chorion is synthesized by surrounding follicle cells and serves to protect the egg from desiccation and mechanical stress after it is laid. Thus, the diversity of egg ECMs includes their ultrastructural appearance, their molecular composition, their functions, the strategy of sperm interaction with them, and their fate

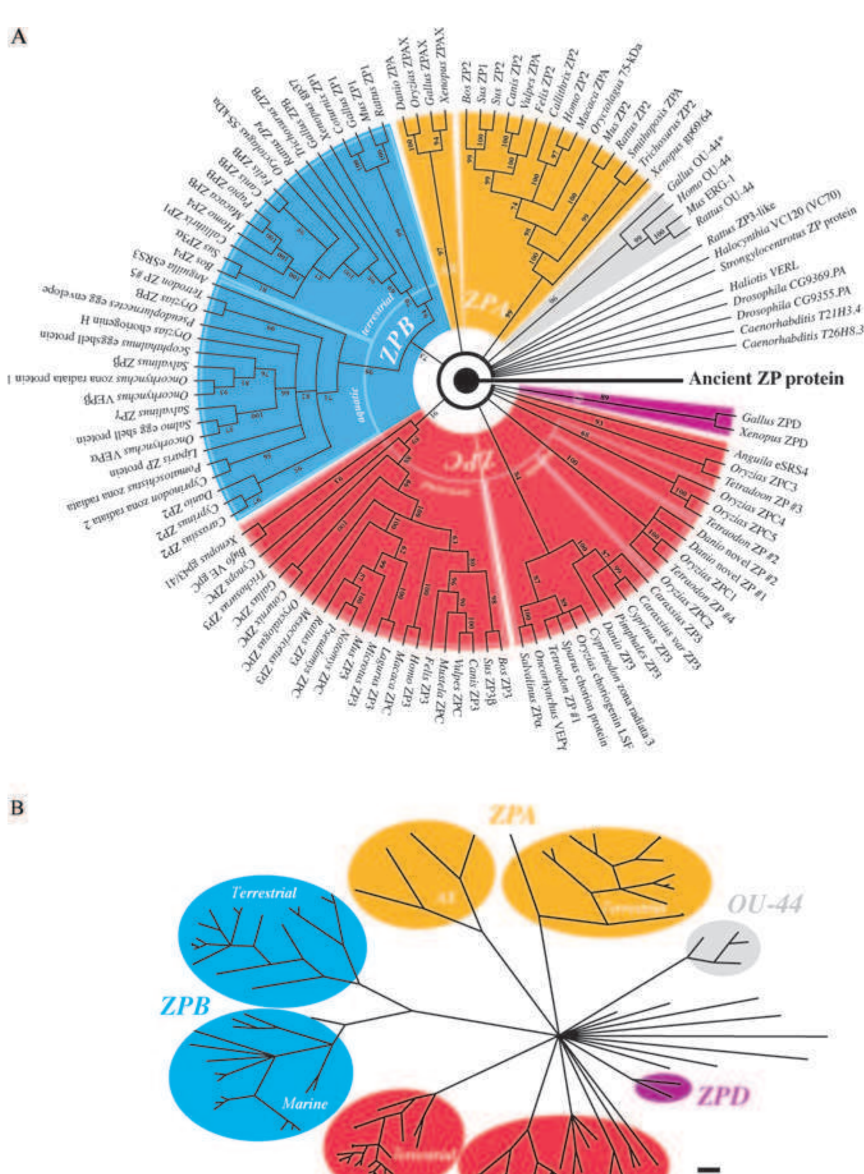
in the zygote. Perhaps, then, it is not surprising that these structures are so varied despite their involvement in a simple event: sperm–egg interaction.

B. Zona Pellucida Homologs

Vertebrate egg ECMs are predominantly composed of proteins with a single ZP domain (Bork and Sander, 1992; Breed *et al.*, 2002; Spargo and Hope, 2003). A ZP domain is about 260 residues in length and contains eight positionally-conserved cysteine residues that disulfide-bond intramolecularly to generate the typical ZP fold (Bork and Sander, 1992). The ZP fold is essential for maintaining the structural integrity of the matrix it is found in, whether the fold is part of the mammalian kidney, pancreas, avian tectorial membrane, or egg ECM (Huynh *et al.*, 2001; Jovine *et al.*, 2002; Leong *et al.*, 2004; Rankin and Dean, 2000). This domain is usually located at the carboxy-terminal end of a highly glycosylated ECM protein. Proper expression and incorporation of ZP proteins in the murine zona requires a conserved hydrophobic patch of residues just upstream of the carboxyl transmembrane domain, presumably to aid in its intracellular trafficking and to promote polymerization (Zhao *et al.*, 2003). Once at the surface of the oocyte, all ZP homologs are cleaved from the cell surface. This enables the polymerization of the ZP proteins in the absence of the steric and electrical hindrances found near the egg surface (Jovine *et al.*, 2002). All ovarian-expressed ZP homologs (Fig. 5; Table III) are cleaved at a site close to the recognition sequence for protein convertase but use a protease distinct from this family of enzymes (Boja *et al.*, 2003). The diffusible amino-terminal ectodomain then rapidly polymerizes with other ZP family members present in the perivitelline space between the oocyte and the follicular cells (Jovine *et al.*, 2002).

Historically, the best-characterized members of the ZP family are those from the protein family's namesake, the *mammalian zona*. This thick ECM (Eakin and Behringer, 2004) (Figs. 1 and 2) gradually accumulates during oogenesis through the synthesis of three major proteins belonging in the ZPA, ZPB, and ZPC subclasses (Bleil and Wassarman, 1980; El-Mestrah *et al.*, 2002; Mate *et al.*, 2003; McCartney and Mate, 1999; Sinowatz *et al.*, 2001; Spargo and Hope, 2003). In most ECMs using ZP family proteins, ZPA heterodimerizes with ZPC and these pairs polymerize into chains, whereas ZPB dimers bridge these protofilaments together via a trefoil motif (Dean, 2004; Moller *et al.*, 1990; Rankin *et al.*, 1999; Wassarman, 1988; Wolgemuth *et al.*, 1984). The major zona constituents, ZPA and ZPC, are freely soluble upon exocytosis (Martic *et al.*, 2004) and are differentially expressed during oogenesis (Epifano *et al.*, 1995), allowing for distinct configurations of the ZPA–ZPC polymers. Release of *Homo* ZPB from the cell surface of a

recombinant expression system, on the other hand, requires coexpression with both ZPA and ZPC (Martic *et al.*, 2004). Surprisingly, ZPB may be dispensable during fertilization: In the absence of murine ZPB, the zona is fully functional at fertilization, albeit morphologically distorted, suggesting that although each ZP member within the egg ECM is structurally and



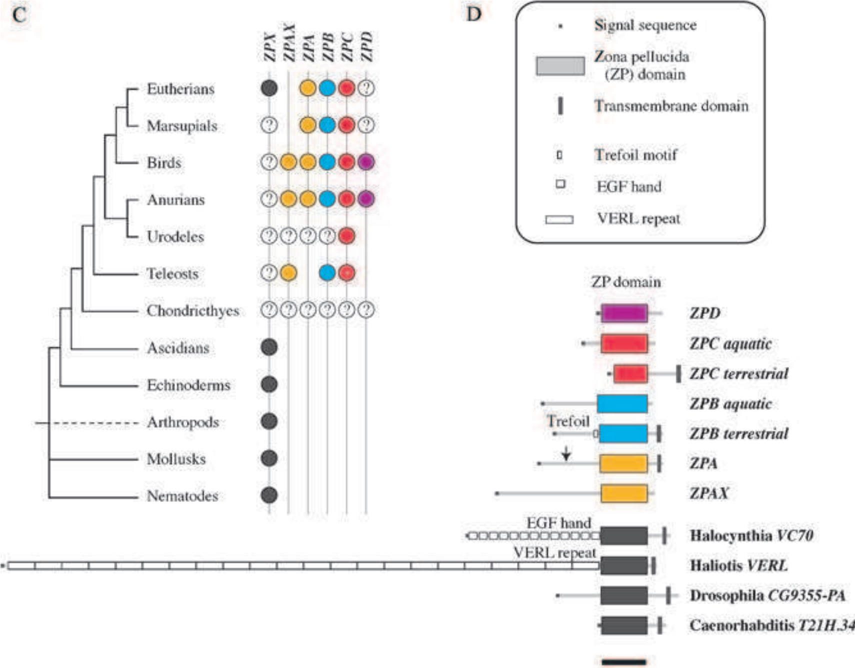


Figure 5 Phylogeny of zona pellucida family members. (A) Unrooted dendrogram of zona pellucida (ZP)–containing proteins in various animal genera. Primarily ZP family members expressed in the ovary were included in the analysis. Most protein sequences used are derived from complementary DNA (cDNA) sequences (see Table III), with the exception of *Drosophila* and *Caenorhabditis* genomic sequences predicted to encode open reading frames. Note that the *Homo* ZP4/B sequence has been annotated as a closer relative to mouse ZP1 (Hughes and Barratt, 1999); no true *Homo* ZP1 sequence has been reported. The four independent subgroups of the aquatic ZPC clade were compiled based on their relationships in other runs using lower bootstrap cutoffs than shown. Numbers represent bootstrap values following 1000 replicates, discarding relationships with a bootstrap value of 60 (60% similar). (B) Unrooted cladogram representing the same phylogenetic data from (A). Bar equals 50 changes. (C) Distribution of different ZP family classes across representative animal orders discussed in the text. (D) Comparison of ZP protein primary structures from the major clades. Colors of the ZP domains correspond to the different clades as in (A). Bar equals 300 residues.

mechanically significant, not all are essential to the fundamental processes of fertilization (Rankin *et al.*, 1999). Thus, the role of ZPB may be to organize the supramolecular structure of the zona in a species-specific fashion (Rankin *et al.*, 2003). Such a function is consistent with the low sequence similarity among the ZPB homologs (Breed *et al.*, 2002; Howarth, 1992; Iwamatsu *et al.*, 1997; Lindsay *et al.*, 2003; Spargo and Hope, 2003).

Molecular evidence from various orders of animals has identified ZP domains within the invertebrate egg ECM as well, although none are

Table III Table of zona Pellucida Homologs Used to Build Phylograms in Fig. 5

Animal	Protein	Accession number
Anguilla japonica	eSRS3	BAA36592.1
	eSRS4	BAA36593.1
	ZP2	BAB21482.1
Bos taurus	ZP3	AAA74385.1
	ZP4	BAB21481.2
Bufo arenarum	VE gpC	AAO25742.1
Caenorhabditis elegans	T21H3.4	AAB65365.2
	T26H8.3	CAB04859.1
Callithrix jacchus	ZP1	CAA71786.1
	ZP2	CAA71740.1
	ZP2	BAA08097.1
Canis familiaris	ZP3	BAA08098.1
	ZPB	AAS78984.1
Carassius auratus	ZP2	CAA96576.1
	ZP3	CAA88838.1
Cyprinus carpio	ZP2	CAA96575.1
	ZP3	CAA88736.1
Coturnix japonica	ZP1	BAB47585.1
	ZPC	BAB86301.1
Cyprinodon variegatus	ZR2	AAT51698.1
	ZR3	AAT51699.1
Cynops pyrrhogaster	ZPC	n/a
	novel ZP #1	CAD60857.1
	novel ZP #2	CAD60856.1
Danio rerio	ZP2	AF095456.1
	ZP3	AF095457.1
	ZPA	AAM34737.1
Drosophila melanogaster	CG9355.PA	NP_511130.2
	CG9369.PA	NP_572747.1
	ZP2	BAA08095.1
Felis catus	ZP3	BAA08096.1
	ZPB	AAA74389.1
	UO-44 (predicted)	XP_421804.1
	ZP1	CAC16087.1
Gallus gallus	ZPAX	CAG27604.1
	ZPB	BAA76739.1
	ZPC	BAA13760.2
	ZPD	BAD13713.2
Haliotis rufescens	VERL	AAL50827.1
Halocynthia roretzi	VC120 (VC70)	BAB72021.1
	UO-44	AAP15461.1
Homo sapiens	ZP2	AAB67599.1
	ZP3	CAA01398.1
	ZP4	AAA74391.1
Lagurus lagurus	ZP3	AAM54024.1
Liparis atlanticus	ZP protein	AAS55643.1
	ZP1	CAA71409.1
Macaca radiata	ZP2	CAA71693.1
	ZP3	CAA57961.1
Mesocricetus auratus	ZP3	AAA37079.1
Microtus brandti	ZP3	AAG18455.1
	ZP1	AAB60507.1
Mus musculus	ZP2	AAA40586.1

Table III *Continued*

	ZP3	AAB18629.1
Mustela erminea	ZPC	AAT67173.1
Notomys alexis	ZPC	AAL79568.1
Oncorhynchus mykiss	zona radiata protein 1	AAD56572.1
	VEPα	AAF71258.1
	VEPβ	AAK97529.1
	VEPγ	AAF71260.1
Oryctolagus cuniculus	55-kDa	AAA31501.1
	75-kDa	AAA31502.1
	ZPC	AAA74392.1
Oryzias latipes	choriogenin H	AAM91821.1
	choriogenin L	AAM91819.1
	ZPAX	AAN31186.1
	ZPC1	AAN31188.1
	ZPC2	AAN31189.1
	ZPC3	AAN31190.1
	ZPC4	AAN31191.1
	ZPC5	AAN31192.1
	ZPC2	AAN31189.1
Papio cynocephalus	ZPB	AAP13260.1
Pimephales promelas	ZP3	AAG28398.1
Pomatoschistus minutus	ZR protein	CAC94865.1
Pseudomys australis	ZPC	AAL79569.1
Pseudopleuronectes americanus	egg envelope	AAC59642.1
	UO-44	AAB71895
Rattus norvegicus	ZP1	BAA24486.1
	ZP2	BAA24487.1
	ZP3	BAA24456.1
	ZP4	AF456325.1
Salmo salar	egg shell protein	CAC04221.1
Salvelinus alpinus	ZPα	AAR87393.1
	ZPβ	AAR87394.1
	ZPγ	AAR87395.1
Sminthopsis crassicaudata	ZPA	AAF73044.1
Sparus aurata	chorion protein	CAA63709.1
Strongylocentrotus purpuratus	ZP protein	n/a
Scophthalmus maximus	eggshell protein	AAP15042.1
Sus scrofa	ZP1	AAB33431.2
	ZP2	BAA08092.1
	ZP3α	AAA50164.1
	ZP3β	BAA08093.1
Tetraodon nigroviridis	ZP #1	CAG11366.1
	ZP #2	CAF93676.1
	ZP #3	CAF93677.1
	ZP #4	CAF97713.1
	ZP #5	CAF92944.1
Trichosurus vulpecula	ZP2	AAC28737.1
	ZP3	AAC28736.1
	ZPA	AAF73043.1
	ZPB	AAF73042.1
Vulpes vulpes	ZPA	AAT37676.1
	ZPC	AAT37677.1
Xenopus laevis	gp37	BAA90655.1
	gp43/41	BAA13117.1
	gp69/64	AAD12172.1
	ZPAX	AF225906.1
	ZPC	AAB39079.1
	ZPD	AAA91467.1

orthologous to the vertebrate ZPs (Fig. 5; Table III). This paradoxical retention of a specific protein fold in the context of completely different proteins implies that specific characteristics of the ZP domain may be critical in all animal egg ECMs. One aspect may be its ability to maintain matrix integrity for proper sperm–egg contact. For example, the ascidian vitelline coat is composed of a dense fibrous network of proteins spatially separated from the egg by a perivitelline space. Of about 20 fucose-rich glycoproteins within the vitelline coat (Rosati, 1985), a single 70-kDa protein purified from the ascidian egg ECM is able to associate with sperm (Lambert, 1989) and to inhibit gamete associations *in vitro* (Matsuura *et al.*, 1995). By mass, this inhibitor is identical to vitelline coat protein VC70, a member of the vertebrate ZP family that contains 12 epidermal growth factor (EGF)-like repeats, a ZP domain, and a carboxy-terminal transmembrane domain (Sawada *et al.*, 2002a). The ZP domain and transmembrane domains are, like vertebrate ZP proteins, separated by a furin-like cleavage site that presumably allows for the separation of ecto-VC70 from the egg surface before its incorporation into the vitelline coat. Analogous to VC70, the abalone vitelline envelope receptor for lysin (VERL) is the major protein present in the vitelline envelope, representing about 30% of the entire mass, and is responsible for sperm associations (Swanson *et al.*, 2001a; Swanson and Vacquier, 1997). This glycoprotein is an oligomer of subunits that each contains 22 nearly homogenized repeats with a carboxy-terminus structurally very similar to the ZP family of proteins, including a predicted ZP domain, a furin-like cleavage sequence, and a transmembrane region positioned in tandem (Galindo *et al.*, 2002; Swanson *et al.*, 2001a). The functional homolog of the abalone lysin receptor is hypothesized to be vertebrate ZPA based on the observation that, upon binding of its sperm-derived ligand lysin, the conformation of VERL and associated proteins is altered to allow the sperm to reach the egg membrane.[2] Finally, a partial cDNA clone of a ZP domain was obtained from a sea urchin ovary expression library (M. L. Leguia and G. M. Wessel, unpublished observations), and genome databases indicate that ZP family homologs are present in echinoderms, dipterans and nematodes (Fig. 5; Table III; data not shown), although the expression profiles are not known. Thus, ZP family members are present in both deuterostomes and protostomes. In most animals, these ZP homologs appear to be used in the construction of the egg ECM, implying that this domain may be critical for gamete recognition.

Assembling the ZP-containing proteins involved in reproduction into a phylogram reveals distinct segregation of all the current, known homologs into five major subclasses (Fig. 5; Table III): ZPA[X], ZPB, ZPC, ZPD, and

[2]The use of the word "receptor" in this chapter refers exclusively to the egg-derived proteins, whereas "ligand" refers to the sperm complement in a receptor-ligand pair.

a more ancient group ZPX. The two largest ZPC subgroups, here listed as independent clades, cluster together 53% of the time (data not shown), whereas the smaller ZPC clades show less similarity to either larger group (Fig. 5B). Inherent in each ZP clade is a separate grouping of orthologs split between terrestrial versus aquatic vertebrates. The primary distinction between these sister clades is the presence or absence of an encoded transmembrane domain: The aquatic ZP genes do not encode transmembrane domains, whereas most terrestrial ZP orthologs do. This characteristic may correspond to the protein's origin in specific animals. For instance, these completely soluble ZPs could be synthesized by somatic cells such as ovarian follicle cells or hepatocytes, and then deposited in the perivitelline space, where they polymerize with nascent oocyte ECM proteins. This is consistent with ZP expression in teleosts, which occurs in the liver and must travel to the ovary via the circulatory system (Chang *et al.*, 1999; Hyllner *et al.*, 2001; Lyons *et al.*, 1993; Murata *et al.*, 1995, 1997; Yamagami *et al.*, 1992). Since the timing of ZP expression likely coincides with vitellogenesis, the soluble ZP proteins can be co-transported to the ovarian follicles with vitellogenic proteins (see Callard *et al.*, 1990a,b; Polzonetti-Magni *et al.*, 2004; Schneider, 1996), thereby minimizing ZP protein precipitation in circulation and maximizing the movement of essential proteins for oogenesis.

What types of selective forces may have resulted in such clear distinctions between the different ZP clades (Fig. 5)? One likely method is functional conservation, specifically the maintenance of orthologs across phlya primarily for their ability to fill a specific role within the ECM. Historically, when mammalian ZP proteins were the best-characterized proteins, this hypothesis held true because *in vitro* studies had clearly defined roles for ZPA, ZPB, and ZPC homologs during fertilization (see Dunbar *et al.*, 1994; Wassarman, 1999). However, these functional distinctions between mammalian ZPA and ZPC subclasses have been questioned. The observation that chimeric *Mus–Homo* zona still exclusively bind sperm homotypically and yield viable embryos suggests that the diversification of these protein sequences is not the sole explanation of their function during fertilization (Dean, 2004; Doren *et al.*, 1999; Rankin *et al.*, 1998, 2003). Similarly, the ZPX subclass contains members from egg ECMs of distantly related animals, including *Drosophila*, abalone (Galindo *et al.*, 2002), sea urchins, ascidians (Sawada, 2002), anurans (Lindsay *et al.*, 2002, 2003), teleosts, and mammals. Members of this group share primary structural organization at the carboxyl-terminus, specifically the ZP domain, but little identity at amino-termini (Fig. 5). Might the ZPX subclass represent the most primitive domains necessary to be an egg ECM protein, relying on other non-ZP proteins to build the ECM? Could the other ZP homologs represent specializations in the vertebrate lineage that correspond to a replacement of non-ZP homologs from the egg ECM? Compare,

for example, the variety of non-ZP proteins and one putative ZPX homolog needed to form the echinoderm vitelline layer (Gache *et al.*, 1983; Haley and Wessel, 2004a; M. L. Leguia, L. M. Varghese, and G. M. Wessel, unpublished observations; Niman *et al.*, 1984), the abalone vitelline envelope (Galindo *et al.*, 2002; Swanson and Vacquier, 1997), or the *Drosophila* eggshell (Nogueron *et al.*, 2000; Pascucci *et al.*, 1996) versus the handful of ZPA, ZPB, and ZPC homologs accounting for nearly the entire vertebrate egg ECM (Breed *et al.*, 2002; Spargo and Hope, 2003). Clearly a single invertebrate ZP homolog is sufficient to achieve monospermic fertilization, so why further limit the diversity of proteins to only ZP homologs? Is the assortment of non-ZP members in invertebrate ECMs extraneous in vertebrates? Might restriction to ZP homologs represent a more efficient process of expression, with diversification of the ZP subfamilies through gene duplication providing the diversity necessary for proper assembly? Or perhaps the ZP domain does not function at all during sperm–egg interactions; rather, it participates in a different essential process during oogenesis or development?

C. Using Homologs to Enhance Structural Diversity

ZP domains interact with each other directly, thereby enhancing the polymerization of ZP-containing proteins (Jovine *et al.*, 2002). Protofilaments formed by such ZP proteins appear to be organized in a conserved fashion, generating a right-handed double helix with frequent branch nodes to create a reticular network. Different ZP sequences can thus interact heterospecifically, allowing for a diverse assembly of proteins within a reticular network of these protofilaments. For example, both a urinary and a cochlear ZP protein are able to incorporate within the mouse zona so long as the entire ZP domain and adjacent carboxy-terminus are unperturbed (Jovine *et al.*, 2002). The auto-aggregation and polymerization of ZP family members provides a distinct advantage for the construction of ECMs because any additional motifs associated with the ZP domain in a particular protein can be incorporated without structurally interfering in matrix assembly. Thus, the quantity of ZP family paralogs expressed by an oocyte may correlate with the variety of different egg ECM ultrastructures and thicknesses represented throughout the animal kingdom. For example, the different ZPB genes might be used to organize the ZPA–ZPC fibers into the discrete layers created in each vertebrate ECM. Compare the thinner nonrefractive internal organization of the mollusk vitelline envelope that is subtly trilaminar in cross-section (Hylander and Summers, 1977; Mozingo *et al.*, 1995) but contains only a single ZPX homolog (Galindo *et al.*, 2002) (Fig. 5) to the clearly birefringent trilaminar organization of the mammalian zona under circularly polarized light (El-Mestrah *et al.*, 2002; Keefe *et al.*, 1997). The different permutations

of homodimers and heterodimers allowed by the mammalian ZPA, ZPB, and ZPC homologs could account for the three essential combinations necessary for the differential zona ultrastructure (Boja *et al.*, 2003; Shabanowitz and O'Rand, 1988). Pulse-chase autoradiography in mice has shown that the zona is constructed radially from the oocyte, laying the inside layers before the outer ones (Wassarman, 1988). Assembly of the zona appears to occur with a stepwise increase in expression of individual ZP homologs, in the chronological order ZPC, ZPB, and ZPA, as observed in the primate *Macaca* (Martinez *et al.*, 1996). Together, these observations predict that ZPC is present throughout the zona, ZPB is necessary for organization of the middle layer, and ZPA is enriched in the most distal layer—all consistent with immunogold labeling of the mouse zona (El-Mestrah *et al.*, 2002). Likewise, the pentalaminar ultrastructure of the teleost chorion (Kudo, 1988) could be built from the increased variety of ZP homologs expressed by these oocytes. More complex structures would require further diversification of the ZPB family or incorporation of other ZP subclasses. This is observed in the complex layering of the *Oryzias* chorion, which incorporates two ZPB paralogs, whereas other teleosts use only one. Alternatively, teleosts may use various ZPCs to distinguish layers of the chorion. Both zebrafish and medaka have at least four ZPC paralogs, allowing each of the chorionic layers to have a different ZPC composition. Combine this with a range of possible ZPX paralogs (Mold *et al.*, 2001), which probably polymerize with the ZPCs in a manner analogous to the ZPA–ZPC fibers formed in mammals, and it is theoretically possible to assemble the various subdomains of a multilaminar chorion without duplicating ZP pairings. As in mammals, this could be achieved by staggering expression of the individual members during the ordered assembly of the chorion from the outside in (Hart *et al.*, 1984; Yamagami *et al.*, 1992).

Is there an evolutionary advantage to the diversification of the teleosts' ZPC subfamily in the absence of the "classic" ZPA (Fig. 5), whereas an equivalent deletion in mammals is lethal (Dean, 2004; Rankin *et al.*, 2001)? Might the selective enrichment and duplication in teleosts for ZPC paralogs, with a subsequent loss in ZPAs, be a consequence of their divergent fertilization technique compared to mammals? The eggs of many fish are released from the ovary immediately into the environment, where they would be fertilized. Unlike internal mammalian fertilization, the fluid that a teleost egg experiences is not isoosmotic to female oviductal fluid. Thus, a sturdier ECM may be necessary to buffer the teleost egg against the immediate osmotic shock. Additional, specialized chorionic layers might provide more protection or at least delay the effects of the new environment until the eggs are fertilized (Gilkey *et al.*, 1978). This delay is critical because the change in osmolarity and cation concentrations is sufficient to spontaneously activate some fish eggs within 1 min after the change in salinity, thereby preventing

fertilization altogether (Lee *et al.*, 1999; Ohta and Iwamatsu, 1983; Wolenski and Hart, 1987; Yamamoto, 1954). Of course, the enhanced structural integrity provided by the multiple chorion layers would also provide protection from mechanical forces found in the more turbulent water column into which they are spawned. Thus, the selective advantage of more ZPC proteins in favor of ZPA may be representative of a mechanical advantage: Because the ZP domain alone can dictate how polymerization occurs, heterodimers of ZPC–ZPC paralogs may pack more efficiently than ZPA–ZPC heterodimers. Favoring the incorporation of the smaller ZPC subfamily would thus allow for better compaction of layers than its larger ZPA counterpart, yielding a more resilient ECM.

D. Diversity of Non-ZP Structural Proteins

The exclusive use of ZP homologs in the vertebrate ECM may not be sufficient to restrict monospermic fertilization without the presence of additional physical barriers such as a micropyle or follicle cells. Incorporation of non-ZP family members in the egg ECM for the purpose of restricting the sperm's access to the egg is common in most animals. The process is simplest in vertebrates who, despite an entire egg ECM proper composed of ZP homologs, use oviduct derivatives to enhance monospermy. This process is distinct from the application of jelly, as observed in amphibians and other invertebrates, because the egg ECM itself is modified rather than coated with an additional layer of glycoprotein.

The most common ECM alteration is adsorption of proteins to the matrix, a process that appears to enhance binding efficiency of homospecific sperm to freshly ovulated mammalian zona. For example, oviductal contributions to the ovulated egg are thought to promote fertilization by enlarging the target ECM for sperm, an outcome that is efficacious for both external and internal fertilization. Many externally fertilized eggs are released with additional coats of jelly that serve complementary roles as sperm chemoattractants for the large volume and retardants at close range due to the number of potential sperm that the egg could encounter. Similarly, oviductal contributions are used on internally fertilized eggs, particularly in animals whose egg ECMs are thin such as the marsupial zona (Breed and Leigh, 1990). These additional chemoattractive coats do not directly impact the performance of the ECM, per se, but increase the likelihood of a sperm–egg interaction. Estrogen-inducible oviductal glycoproteins (OGPs) from the mammalian oviduct epithelium, on the other hand, directly intercalate within the zona and can influence how sperm behave toward the ECM. In the absence of OGPs, homotypic sperm binding and capacitation are reduced, and the rate of polyspermy is enhanced (Buhi, 2002; Buhi *et al.*,

2000; O'Day-Bowman *et al.*, 2002; Rodeheffer and Shur, 2004; Schmidt *et al.*, 1997). The participation of the OGP oligosaccharides is critical for maintaining sperm viability, and for regulating sperm–zona binding duration (Buhi, 2002; Dubuc and Sirard, 1995; Rodeheffer and Shur, 2004), suggesting that these sugar moieties may be affecting how sperm respond to zona binding and subsequently determining fertilization success. This is particularly useful in eutherians because estrogen-dependent OGP expression (Buhi, 2002) favors fertilization when the uterus is in the appropriate luteal phase. Thus, the simple presence or absence of a protein adsorbed within an egg ECM can influence fertilization success.

Rather than applying proteins to the ECM *ex post facto*, invertebrates instead intercalate potential sperm-regulating proteins into their ECMs during oogenesis. In contrast to ascidians and abalone, which use ZP family members for homotypic sperm binding, echinoderms employ the egg bindin receptor (EBR1) (Kamei and Glabe, 2003). This 300-kDa glycoprotein contains a metalloendoprotease domain and EBR repeats composed of paired thrombospondin type 1 (TSP-1) repeats and CUB domains (Adams, 1997; Bork and Beckmann, 1993; Kamei and Glabe, 2003). These EBR repeats contain motifs implicated in protein–protein binding and cell aggregation, consistent with the function of EBR1 in gamete interactions: The TSP-1 repeat is a calcium-dependent fold that homo-multimerizes (Adams, 1997); CUB domains may homodimerize or heterodimerize to form carbohydrate-binding pockets or protein-interactive surfaces through anti-parallel β-strands that are stabilized by up to four positionally conserved disulfide bonds (Bork and Beckmann, 1993; Romero *et al.*, 1997; Varela *et al.*, 1997). Together, these binding motifs functionally mimic the polymerizing properties of the ZP domain (Jovine *et al.*, 2002). Like ascidian VC70 (Sawada *et al.*, 2004) and abalone VERL (Galindo *et al.*, 2002; Galindo *et al.*, 2003), sea urchin EBR1 contains a large number of tandem, homogenous repeats that appear to be species specific (Kamei and Glabe, 2003). How such extensive tandem repeats contribute to the function of the invertebrate sperm receptor remains unresolved.

Unlike constituents of the vertebrate ECM, the structural proteins of invertebrate egg ECMs do not appear to be related across animal taxa. The absence of significant molecular data on these proteins, however, limits a rigorous analysis of their phylogeny. Little has been reported on the non–sperm receptor constituents of the ascidian vitelline coat or the abalone vitelline envelope. In the bivalve *Unio*, the single structural glycoprotein gp180 is found throughout the entire vitelline envelope; the sperm receptive gp273 is localized to the crater (Focarelli and Rosati, 1995). Similar roles have been assigned to the products of three genetic loci in the dipteran *Drosophila* that are required for assembly of the vitelline layer and chorion: the *chorion-36* (*cor-36*), *fs(2)QJ42*, and *defective chorion 1* (*dec-1*) loci

(Pascucci *et al.*, 1996; Perotti *et al.*, 2001). The X chromosomal *cor-36* encodes proteins necessary for early chorion formation, whereas *fs(2)-QJ42* encodes the vitelline membrane protein sV23 that is later cleaved into sV17 (Pascucci *et al.*, 1996). The *dec-1* locus encodes an alternatively spliced gene whose products include proteins that share an amino-terminal sequence but vary in their carboxy-terminal motifs (Badciong *et al.*, 2001; Nogueron *et al.*, 2000). The three major *dec-1* proteins—fc106, fc125, and fc177—are expressed differentially during oogenesis and are posttranslationally proteolyzed in a manner that promotes their incorporation into either the vitelline membrane or the endochorion (Nogueron *et al.*, 2000). Finally, two structural protein products of the endochorion, s18 and s36, are synthesized late in oogenesis by the follicle cells to assemble into the chorionic pillars (Pascucci *et al.*, 1996). Most of these *Drosophila* proteins are evenly distributed throughout the eggshell rather than localized at the micropyle, suggesting that these proteins primarily maintain structural integrity rather than participating in gamete interactions, a role supported by the loss-of-function phenotypes associated with the genetic elimination of these loci (Pascucci *et al.*, 1996).

Of the estimated 25 major glycoproteins in the sea urchin vitelline layer (Gache *et al.*, 1983; Longo, 1981; Niman *et al.*, 1984), two have been shown to play a structural role: p160, a 160-kDa, transmembrane vitelline post protein clustered at the tips of microvilli (Haley and Wessel, 2004a) and rendezvin^{VL}, a splice-variant of the oocyte-specific rendezvin gene (Wong and Wessel, 2006). Like the sperm-receptive EBR1, both of these proteins contain CUB domains that may aid assembly and maintenance of the vitelline layer (Bork and Beckmann, 1993; Romero *et al.*, 1997). This enrichment of CUB domains in the sea urchin egg ECM is consistent with the functional sensitivity of the vitelline layer to disulfide bond reducing agents (Aketa and Tsuzuki, 1968). Expression of p160, however, is not oocyte specific. p160 links the vitelline layer to the egg plasma membrane. Cleavage of this linker protein must occur in order for the vitelline layer to separate from the egg surface during the establishment of one sea urchin block to polyspermy. It reappears before the second cell division and persists on the apical ectodermal cell surfaces, suggesting that p160 is not solely involved with the permanent block to polyspermy (Haley and Wessel, 2004a). Instead, this matrix protein may have a more general role in retaining the intimacy between a cell and its ECM. Rendezvin^{VL} (Wong and Wessel, 2006), on the other hand, is retained in the modified egg ECM after it lifts off the egg surface, serving as a core scaffold protein from the vitelline layer that organizes fertilization envelope assembly (Carroll *et al.*, 1986; Kay and Shapiro, 1985; Ruiz-Bravo *et al.*, 1986). Thus, unlike vertebrate eggs, no clear selective pressure is known to exclude a particular family of structural

proteins from the invertebrate egg ECM. Instead, the proteins retained over time appear to be ones that maintain structural integrity of the respective ECM.

E. Divergence or Convergence in Egg ECM Phylogeny?

When compared to the extreme diversity of egg ECMs, the assortment of traits shared among all animal eggs suggests that the selective pressures of speciation that favor divergence of ECM morphology have molded how the ECM evolves. For example, architectural diversity in chiton egg ECM has been proposed as a gauge of evolutionary relatedness among species within the phylogeny of this animal (see Buckland-Nicks and Hodgson, 2000). The organization and articulation of the chiton hull, like anatomical traits, have been scrutinized in order to taxonometrically categorize different species into specific clades. Could such a critical comparison of different egg ECM attributes be used to evaluate the phylogeny of animals? If so, what might this indicate about the relationship between the egg and speciation?

Looking at both the ultrastructural and the molecular components of the egg ECM among taxa, we see a gradual simplification in ECM structure during the progression from protostomes (e.g., decapods, dipterans, and mollusks such as chiton and bivalves) to deuterostomes (e.g., echinoderms, ascidians, and vertebrates) (Fig. 1). The elaboration of microvillar pores in mollusks gives way to a more uniform structure that is devoid of extraneous articulations, as found in fish chorions and the mammalian zona. The palette of proteins utilized is also significantly narrowed, from an array of up to 25 proteins in the echinoderm vitelline layer (Gache *et al.*, 1983; Niman *et al.*, 1984), including a ZP family member (M. L. Leguia, L. M. Varghese, and G. M. Wessel, unpublished observations), to the exclusive use of ZP homologs by most vertebrates. Are these trends merely coincidence, and hence counter to the molecular diversity seen elsewhere in these animals, or do they represent the influence of various selective forces implicit to reproduction? For example, might the diversity observed in egg ECM morphology reflect mechanisms the egg uses to minimize sperm–egg ratios? If so, could these also be related to differences between external and internal fertilization, independent of the position an animal holds on the phylogenetic tree? Broadcast spawners, including most mollusks and echinoderms, often dilute their gamete populations in the ocean. By elongating the microvilli through a sperm-receptive ECM, the eggs of these animals significantly increase their chances of being fertilized. In contrast, the concentrated deposition of sperm into the uterus or oviduct of internally fertilized animals such as mammals

greatly increase the probability that the few eggs ovulated per cycle will be fertilized. To counter the potential onslaught of sperm in these animals, a simple yet substantial ECM is employed to mechanically dilute the number of simultaneous egg encounters.

Numerous exceptions to this simplified comparison of external versus internal fertilization exist, however. Decapod eggs are ovulated with a thick unadorned vitelline envelope surrounding decapod eggs (Talbot and Goudeau, 1988). This ECM most closely resembles the mammalian zona in its simplicity yet requires a significantly greater effort from the sperm to penetrate (Brown and Humphreys, 1971; Goudeau and Becker, 1982; Tsai and Talbot, 1993). The utility of creating such a modification is linked to the method of fertilization employed by decapods, involving the simultaneous release of eggs from the gonophore, whereas sperm are released from storage in the thelycum into the seawater (Lindsay *et al.*, 1992a; Talbot and Goudeau, 1988). This process results in a single decapod egg encountering a large number of sperm at once, hence the selection of a more substantial ECM to "filter" sperm quantities. Another exception to the internal-versus-external hypothesis involves the independent evolution of micropyles in animals such as the mollusk *Unio* (Focarelli *et al.*, 1988), dipterans (Degrugillier and Leopold, 1976; Mouzaki *et al.*, 1991; Turner and Mahowald, 1976), and teleosts (Hart, 1990). The micropyle physically restricts the number of sperm capable of binding to an egg, implying that it is particularly useful for limiting sperm–egg ratios. This is likely its role in *Unio*, whose fertilization occurs through the release of gametes into the seawater, followed by concentration of sperm/egg ratio by "sucking" them into the suprabrachial chambers of the gills (Focarelli *et al.*, 1988). Similarly, fish sperm are released externally in concert with a clutch of eggs, so the ratio of sperm to eggs is significantly higher than compared to other broadcast spawners such as echinoderms or ascidians. In contrast, sperm numbers are reduced in internally fertilized dipterans through the spermathecae, a sperm storage organ adjacent to the oviduct that limits the release of sperm to a few at a time (Neubaum and Wolfner, 1999). In these animals, a micropyle is not necessary for limiting sperm–egg encounters, but has still been retained. This likely reflects a separate role for the micropyle, perhaps as a way to ensure that fertilization and embryonic gas exchange occur in the presence of a chorion selected for minimizing desiccation of the embryo following terrestrial oviposition (Li *et al.*, 1996). Thus, while the morphology of an egg ECM generally appears to be governed by the range of sperm concentrations that an egg may encounter, it is not a hard-and-fast rule. Rather, the ecological challenges of reproduction certainly influence the evolutionary selection on the egg ECM.

III. Cortical Granules

Cortical granules (CGs) are secretory vesicles synthesized during oogenesis and released following gamete fusion (reviewed in Cran and Esper, 1990; Wessel *et al.*, 2001). These oocyte- and egg-specific organelles are abundant, ranging from 8000/egg in mice (Ducibella *et al.*, 1994) to 15,000/egg in sea urchins (Laidlaw and Wessel, 1994), and are always enriched within the outermost region of the egg's cortex, subjacent to the plasma membrane. The shear number of granules per egg and their secretion en masse following fertilization implies that CG contents significantly alter the local extracellular environment upon exocytosis, easily transforming a sperm-competent egg ECM into a physical barrier against additional sperm. The mammalian zona, for example, does not undergo any significant histological changes, although biochemical modifications occur following CG exocytosis that reduces its affinity to sperm (Hoodbhoy and Talbot, 1994) (Figs. 1 and 2). In echinoderms, on the other hand, CG exocytosis causes a physical separation of the vitelline layer from the egg plasma membrane, resulting in the formation of a fertilization envelope of approximately fourfold greater surface area than the original vitelline layer (Figs. 1 and 2; reviewed in Kay and Shapiro, 1985). The force necessary for this lifting is thought to be a result of the hydration of mucopolysaccharides derived from the echinoderm CGs (Schuel *et al.*, 1974) but also requires proteolysis of egg–ECM linkages to ensure full release of the vitelline layer (Haley and Wessel, 1999).

The range of ECM modifications that occur as a consequence of CG exocytosis suggests that the contribution of these organelles to the block to polyspermy is both enzymatic and structural in nature. In this section, we review the major constituents of animal CGs (Table II) and briefly describe the functions of some of these proteins after fertilization. A more contextual description of these CG components, however, can be found in Section IX, later in this chapter.

A. Cortical Granule Enzymes

Historically, proteolysis is the one enzymatic activity considered to be of general importance in the block to polyspermy (Boldt *et al.*, 1988; Hatanaka *et al.*, 1992; Hoodbhoy and Talbot, 1994; Moller and Wassarman, 1989). The types of proteases involved may be quite variable among animals, but cleavage or removal of the sperm receptor from the ECM is thought to be a common outcome. Serine protease activity common to the trypsin family has been reported in sea urchins (Haley and Wessel, 1999, 2004b) and mammals (Cherr *et al.*, 1988; Hoodbhoy and Talbot, 1994). The CG serine protease

(CGSP1) is the *only* protease activity detected in the CGs of the sea urchin (Carroll and Epel, 1975b; Haley and Wessel, 1999). This is significant because many functions have been ascribed to this protease, including removal of the sperm receptor, modification of the vitelline layer, and even egg activation (Carroll and Epel, 1975a; Carroll and Jaffe, 1995; Runnstrom, 1966; Vacquier *et al.*, 1973), yet it appears that only CGSP1 is responsible for this extensive range of activities. This CG protease appears to be selective, suggesting that it has specific roles and/or regulators that are not compatible at any other time during development (Haley and Wessel, 1999). For example, it is known that CGSP1 cleaves the vitelline post protein p160, releasing a diffusible 85-kDa ectodomain from its transmembrane domain (Haley and Wessel, 2004a), thus permitting the physical detachment of the vitelline layer from the egg surface during the formation of the fertilization envelope (Kay and Shapiro, 1985). As in sea urchins, one function of mammalian trypsin-like proteases is to facilitate the loss of sperm-binding capabilities on the ECM (Hoodbhoy and Talbot, 1994). The source of one such serine protease activity has been localized to CGs (Cherr *et al.*, 1988; Hoodbhoy and Talbot, 1994), but no further information is known about this mammalian subclass of enzymes.

Other classes of protease activity have also been traced to CGs or to organelles with similar behaviors following fertilization. For example, aminopeptidase activity is detectable around the *Mytilus* egg only after fertilization has occurred, implying that its release is due to CG exocytosis, even though the release of these granules is not documented in detail for this animal (Togo and Morisawa, 1997; Togo *et al.*, 1995). Cathepsin-like substance (CLS) is derived from *Cyprinus* CGs and is presumed to target proteins in the teleost chorion (Chang *et al.*, 1999). Its co-migration with other chorion proteins suggests that any proteolytic activity related to CLS may enhance overall protein aggregation (Chang *et al.*, 1998). On the other hand, *Xenopus* zinc-dependent protease cleaves ZPA, causing a steric shift in the protein fold that results in the hardening of the vitelline envelope (Lindsay and Hedrick, 2004). This conformational change in ZPA is the major proteolytic alteration observed in anurans and eutherians following fertilization, suggesting that zinc-dependent protease orthologs may be functionally conserved in terrestrial vertebrate zygotes (Bauskin *et al.*, 1999; Doren *et al.*, 1999; Moller and Wassarman, 1989; Moos *et al.*, 1994; Shabanowitz and O'Rand, 1988; Tian *et al.*, 1999). This is consistent with the reported insensitivity of mammalian ZPA cleavage to serine protease inhibitors (Hoodbhoy and Talbot, 1994).

Glycosidases also originate from CGs of many taxa. The first CG protein identified from sea urchins was β-1,3-glucanase, an enzyme whose primary substrate is found in algae, not the animal egg itself (Epel *et al.*, 1969). This enzyme has an unusual heritage: Its sequence similarity to bacterium suggests

that sea urchin β-1,3-glucanase was acquired by horizontal gene transfer (Bachman and McClay, 1996). The function of this glycosidase during fertilization is not known, although its accumulation in the perivitelline space following CG exocytosis suggests its target substrates may include constituents of the egg ECM and the perivitelline space (Wessel *et al.*, 1987, 2001). *N*-acetylglucosaminidase activity, on the other hand, has been detected from *Xenopus* (Prody *et al.*, 1985) and mouse CGs (Miller *et al.*, 1993a). Given the participation of oligosaccharides enriched in *N*-acetylglucosamine during vertebrate sperm–egg interactions (Miller *et al.*, 1993b; Vo *et al.*, 2003; see also Section V, later in this chapter), the hypothesized role of this CG derivative is in the abolition of sperm-binding sites within the egg ECM. Whether this occurs remains untested because there has been no definitive report indicating the release of specific sugar residues following fertilization in vertebrates.

One outcome conserved throughout most animal orders is the mechanical transformation of the egg ECM from a flexible network of glycoproteins into a hardened shell. This physical modification is often associated with enzymatic activity detected following CG exocytosis, although the enzyme(s) responsible varies. As previously mentioned, proteolysis of anuran ZPA by a zinc-dependent protease derived from CGs is sufficient to induce hardening of the vitelline envelope (Lindsay and Hedrick, 2004). A similar hardening role is suspected for teleost CLS because detergent-resistant complexes containing *Cyprinus* ZP proteins and other CG derivatives results from this enzyme's activity (Chang *et al.*, 1998, 1999). Thus, the use of protease-dependent hardening of the egg ECM may be common to all vertebrate zygotes. Yet, the exact process that such protease-dependent hardening follows has not been deciphered; only correlations have been made between the changes in physiochemical properties of the ECM in the presence or absence of protease activity (Lindsay and Hedrick, 2004).

A distinct mechanism of matrix hardening involves the formation of covalent bonds between ECM constituents. Such changes typically result from transglutaminase and peroxidase activity, both of which have been found associated with eggs at fertilization. Transglutaminase involvement in the conversion of the egg ECM following fertilization has been reported for decades, as evidenced by the isopeptide amide bonds between glutamine and lysine left in its wake, a process that fuses adjacent proteins to one another (Battaglia and Shapiro, 1988; Cariello *et al.*, 1994; Chang and Huang, 2002; Chang *et al.*, 2002; Lee *et al.*, 1994; Mozingo and Chandler, 1991; Oppen-Berntsen *et al.*, 1990; Yamagami *et al.*, 1992). This extended family of calcium-dependent enzymes generates intermolecular bonds through a cysteine-protease–like catalytic mechanism (reviewed in Lorand and Graham, 2003; Nemes *et al.*, 2005). Only a few candidate egg-derived proteins have been cloned from ovary RNA: one from the teleost *Cyprinus* (Chang *et al.*, 2002) and two from the sea urchin *Strongylocentrotus* (J. L.

Wong and G. M. Wessel, unpublished observations). Yet the subcellular source of this enzyme does not appear to be CGs, even though its activity is intimately associated with CG exocytosis. For example, a zymogenic form of a sea urchin transglutaminase activity has been reported at the egg surface (Battaglia and Shapiro, 1988). This sea urchin transglutaminase is activated within 2 min following CG exocytosis, establishing isopeptide bonds necessary for stabilizing the initial fertilization envelope assembly (Battaglia and Shapiro, 1988; Cariello *et al.*, 1994; Kay and Shapiro, 1985). CG exocytosis is a prerequisite for sea urchin egg transglutaminase activity, suggesting that the zymogenic form is activated by CGSP1 proteolysis, consistent with the observation that its morphological changes are blocked by inhibitors of either transglutaminase or serine proteases (Mozingo and Chandler, 1991). Such a cascade of events is consistent with the requisite proteolytic activation of homologs such as transglutaminase type 2 and plasma coagulation factor XIIIa (reviewed in Lorand and Graham, 2003; Nemes *et al.*, 2005). It is also reminiscent of the activity profile reported for chorionic transglutaminase of *Cyprinus*, which is active only when collected in CG exudate (Chang *et al.*, 2002), and *Tribolodon*, which is activated by a serine-protease–like sialoglycoprotein from CGs (Kudo and Teshima, 1998). In all reported cases, transglutaminase activity is enriched at the periphery of the extracellular chorion (Chang *et al.*, 2002; Kudo and Teshima, 1998; Oppen-Berntsen *et al.*, 1990). Yet, the *Cyprinus* egg-derived family member (Chang *et al.*, 2002), an ovary-expressed echinoderm transglutaminase originally identified in *Paracentrotus* blastula (Zanetti *et al.*, 2004; J. L. Wong and G. M. Wessel, unpublished observations), and one *Strongylocentrotus* homolog with 35% primary sequence identity to the *Homo* vertebrate type I/keratinocyte isoform (J. L. Wong and G. M. Wessel, unpublished observations) do not possess a signal peptide downstream of their putative initiating methionine—a characteristic of other secreted transglutaminases (Lorand and Graham, 2003). Thus, how these enzymes end up in the extracellular space where their target substrates reside remains a mystery.

Peroxidases catalyze the formation of dityrosine bonds between adjacent proteins through a free radical intermediate (Gross, 1959). Although peroxidase activity is present in mouse CGs and in the perivitelline space following fertilization (Gulyas and Schmell, 1980a,b), the observation that a zinc-dependent protease is sufficient for hardening the amphibian ECM (Lindsay and Hedrick, 2004) suggests that this peroxidase activity may be supplemental and/or specific to murids. Reports of dityrosine residues in the dipteran chorion imply that peroxidases are also responsible for ECM maturation in these invertebrates (Li *et al.*, 1996; Mouzaki *et al.*, 1991). Likewise, peroxidase activity is present in *Tribolon* and *Cyprinus* chorion, in layers that participate in fertilization envelope formation, but whether the activity derives from CGs has yet to be determined (Kudo, 1988). On the other hand,

CG-derived ovoperoxidase is definitely required for the hardening of the sea urchin fertilization envelope, specifically at the intercast regions found between the microvillar caps identifiable within the vitelline layer scaffold (Deits *et al.*, 1984; Foerder and Shapiro, 1977; Hall, 1978; LaFleur *et al.*, 1998; Mozingo and Chandler, 1991; Nomura and Suzuki, 1995; Showman and Foerder, 1979). This myeloperoxidase-like family of enzymes is specifically transcribed in oocytes and packaged into CGs. Upon secretion, it is separated from the egg surface by a tethering protein that keeps it associated with the elevating vitelline layer, thereby restricting its cross-linking activity to the ECM undergoing modification (Mozingo *et al.*, 1994; Somers *et al.*, 1989). In addition to their proposed roles immediately following fertilization, peroxidase activity may act as a temporary antimicrobicide (Klebanoff *et al.*, 1979; Kudo, 1988) whereas transglutaminase activity may alter fertilization envelope adhesivity (Chang *et al.*, 2002; Cheng *et al.*, 1991).

Why is proteolysis sufficient for the hardening of the egg ECM in mammals and anurans, whereas both peroxidase and transglutaminase are required in fish and sea urchins? Consider the differences in environmental complexity that each embryo experiences. Eutherian cumulus cells help buffer the embryo from mechanical forces within the oviduct, at least until implantation. Similarly, the formation of a second calcium-induced precipitate adjacent to the anuran jelly may provide a barrier between the embryo and the environment. But teleosts and sea urchin embryos do not acquire such a supplemental shell. Might the use of both peroxidase and transglutaminases ensure that a hardened barrier will be in place within the first 10 min of gamete fusion? If so, then what other environmental factors could be significantly influencing the selection of enzymatic activities employed during the construction of a physical block to polyspermy?

B. Nonenzymatic Proteins of Cortical Granules

The major protein mass released from an animal's CGs is nonenzymatic, yet it significantly contributes to the ECM remodeling required to establish a permanent block to polyspermy. The most is known about this process in echinoderms and anurans, whose permanent blocks to polyspermy are observable by low-power light microscopy. For example, of the 12 proteins derived from sea urchin CGs (Wessel *et al.*, 2001), the major proteins visible by Coomassie staining are directly incorporated into the fertilization envelope (Wong and Wessel, 2004). The total mass of these proteins account for the fourfold increase in surface area observed as the vitelline layer is dramatically "lifted" from the egg surface during CGs exocytosis (Runnstrom, 1966; Shapiro *et al.*, 1989). Similarly, part of the permanent block in the anuran *Xenopus* is clearly visible as the accumulation of a

refractive CG-derived precipitate between the vitelline envelope and inner jelly (J1) layer (Grey *et al.*, 1974; Shapiro *et al.*, 1989). Using electron microscopy, the contents of decapod CGs can clearly be seen accumulating within the vitelline envelope over the hour-long exocytotic process (Talbot and Goudeau, 1988). Thus, structural proteins that transform the egg ECM are clearly released from CGs. But how conserved are these nonenzymatic content proteins across animal taxa?

The dramatic formation of the sea urchin fertilization envelope is a rich source of raw material for biochemical analysis of CG content proteins. Consequentially, the most is known about the structural proteins responsible for modifying the vitelline layer (Wong and Wessel, 2004). Five genes encode the majority of CG proteins that comprise the fertilization envelope, including *proteoliaisin* (Somers and Shapiro, 1991; Somers *et al.*, 1989), *SFE-1* (Laidlaw and Wessel, 1994; Wessel *et al.*, 2000), *SFE-9* (Laidlaw and Wessel, 1994; Wessel, 1995), and *rendezvin* (Wong and Wessel, 2006). The proteins can rapidly self-assemble within the vitelline layer scaffold to form the fertilization envelope within minutes of their release. This biochemical property is likely due to the tandem arrangement of common protein-binding motifs in all these proteins. Rendezvin contains an abundance of CUB domains that likely participate in protein–protein interactions, perhaps with other CUB domain proteins found in the vitelline layer (Wong and Wessel, 2005b). The high percentage of tyrosine residues in rendezvin^{CG} also suggests that it is a target of ovoperoxidase activity (Wong and Wessel, 2006). Proteoliaisin, SFE-1, and SFE-9, on the other hand, are abundant in low-density-lipoprotein receptor type A (LDLrA) repeats, containing up to 28 tandem LDLrA repeats, in some orthologs (Wessel, 1995; Wessel *et al.*, 2000; Wong and Wessel, 2004). In addition to LDLrA repeats, SFE-1 and SFE-9 contain low-complexity repeats whose sequence and length differ between orthologs (Wong and Wessel, 2004), suggesting that their binding partners may be rapidly changing. Isolation of an SFE-9 ortholog from the starfish *Asterina* (J. L. Wong and G. M. Wessel, unpublished observations), a distant echinoderm ancestor that split from the sea urchin more than 500 million years ago (Hinman *et al.*, 2003), exhibits 45% primary sequence identity to the sea urchin orthologs over the 240 residues initially cloned. Further identification and characterization of other echinoid orthologs such as SFE-9 will provide a great deal of information pertaining to selective pressures influencing the evolution of nonenzymatic CG proteins.

Formation of the anuran fertilization (F) layer also requires the deposition of a significant quantity of CG protein. As in echinoderm fertilization envelope formation (Bryan, 1970b), assembly of this layer requires high concentrations of extracellular calcium (Nishihara *et al.*, 1986). The major protein contributed to the F layer is the *Xenopus* CG-derived lectin XL35/CGL (Chamow and Hedrick, 1986; Chang *et al.*, 2004; Lee *et al.*, 1997; Nishihara

et al., 1986; Quill and Hedrick, 1996). This oligosaccharide-binding protein favors sulfated sugars found along the inner surface of the J1 jelly layer (Bonnell *et al.*, 1996; Tseng *et al.*, 2001), sterically blocking the penetration of sperm into the perivitelline space (Hedrick and Nishihara, 1991; Larabell and Chandler, 1991). A human ortholog of XL35/CGL, HL-1, has been found, but it is not expressed in the ovary (Lee *et al.*, 2001). This observation suggests that, like members of the ZP family of proteins (see Section II.B and II.C, earlier in this chapter), XL35/CGL may be a specialized member of a more ubiquitous lectin family that happens to function at fertilization.

Other than anuran XL35/CGL, the nonenzymatic contents of vertebrate CGs that contribute to the permanent block to polyspermy are not known. The primary reason is a relatively low abundance of protein per CG compared to animals with more significant morphological changes, such as echinoderms and anurans. Even though the teleost chorion is a substantial structure assembled prior to fertilization, it has been difficult to isolate proteins derived from the CGs because most of the content proteins remain in the perivitelline space, leaving only a small fraction that interact with the ZP proteins of the egg ECM (Hart, 1990). Like echinoderms (Runnstrom, 1966; Santella *et al.*, 1983; Wessel *et al.*, 2001), teleost CGs have separate regions of electron density that appear to contain different acidic glycoproteins based on the regional differences in lectin affinity (Hart and Donova, 1983; Hart, 1990). Three *Cyprinus* CG proteins contribute to the postfertilization chorion in a calcium-dependent fashion. These include fertilization envelope outer layer protein-1 (FEO-1) (Chang *et al.*, 1999), fibroin-like substance (FLS) (Chang and Huang, 2002), and cystatin (Chang *et al.*, 1998). FEO-1 is an alternatively expressed protein homologous to a chicken vitelline membrane protein that settles at the outer layer of the fertilization envelope (Chang *et al.*, 1999, 2002). FLS is enriched in glycine, alanine, and serine residues, suggesting that it is extremely elastic and may be responsible for the inherent flexibility of the outer fertilization envelope layer (Chang and Huang, 2002). During its discharge, FLS is believed to associate with cystatin, an inhibitor of cysteine proteases (Chang *et al.*, 1998), and CLS, together forming a complex that facilitates the trapping of FEO-1 within the outer fertilization envelope (Chang *et al.*, 2002). This four-protein complex is directly cross-linked by transglutaminase to the ZP orthologs within the chorion, ensuring the complete transformation of the teleost ECM (Chang *et al.*, 2002).

The precipitation of CG contributions within the egg ECM following fertilization is calcium dependent in three animals (Bryan, 1970b; Chang and Huang, 2002; Chang *et al.*, 2002; Nishihara *et al.*, 1986). Why calcium rather than another cation? Does this divalent cation somehow control the behavior of these proteins? Based on optimal *in vitro* monospermic fertilization of porcine eggs in culture media, an estimated calcium concentration is 1.5–2.0 mM in oviductal fluid (Herrick *et al.*, 2003). Eggs of amphibians and

fish laid in freshwater are exposed to roughly equal concentrations of magnesium and calcium (about 3–5 mM of each) under optimal survival conditions (Godfrey and Sanders, 2004). Thus, the use of one cation versus another in freshwater spawners does not depend on bioavailability. Seawater, on the other hand, contains 53 mM of magnesium versus 10 mM of calcium, making the bioavailability of calcium fivefold less than magnesium. Yet the conservation of calcium-binding LDLrA repeats and EF hands in sea urchin CG proteins implies that the larger ion has been selected for its structural contributions to protein folding (Wong and Wessel, 2004). A similar situation could be predicted for saltwater fish, which experience the same concentrations of magnesium and calcium.

In most animals, CG exocytosis also results in the deposition of proteins essential for embryogenesis. For example, hyalin is released from the echinoderm CGs upon exocytosis (Matsunaga *et al.*, 2002; Vater and Jackson, 1990). This large calcium- and magnesium-sensitive glycoprotein constitutes the bulk of the hyaline layer (Bryan, 1970a; Chandler, 1991; Rimsay and Robinson, 2003; Wessel *et al.*, 1998) and serves as the main substrate for blastomere attachment and cell signaling (Matsunaga *et al.*, 2002; Wessel *et al.*, 1998). Epitope similarities to echinoderm hyalin are also found in vertebrate CGs, including mice and hamsters (Hoodbhoy *et al.*, 2000, 2001). This eutherian homolog was first described by its cross-reactivity with the polyclonal antiserum ABL2, which recognizes CG contents of many species (Hoodbhoy *et al.*, 2001). ABL2 antibodies specifically detect the *Mesocricetus* CG proteins p62 and p56 (Hoodbhoy *et al.*, 2001) and a 75-kDa *Mus* CG protein (Pierce *et al.*, 1990), all found in the CG envelope, an embryonic matrix found in the perivitelline space following fertilization (Figs. 1 and 2). The teleost analog to this family of embryonic signaling molecules is hyosophorin, a heterogeneous protein that possesses high calcium affinity and is deposited along the membrane surface following CG secretion (Hart, 1990; Tsao *et al.*, 1999). Like echinoderm hyalin, hyosophorin is enriched with oligosaccharides and has a tendency to attract water, facilitating hydration of the perivitelline space (Tsao *et al.*, 1999). Its highly repetitive domains are also thought to participate in the gelation process of its embryonic matrix (Tsao *et al.*, 1999). The presence of hyalin-like proteins in the CGs of most deuterostomes reinforces the dual properties of these organelles, participating in both the rapid renovation of the egg ECM and deposition of a new extraembryonic one.

IV. Courtship, Gamete Attraction, and Sperm/Egg Ratios

Selection of a particular type of block to polyspermy depends on a number of factors, including the type of mating used by an animal and the environment within which fertilization occurs. Motile animals have significantly more

mating options than their sessile counterparts, as reflected in the diversity of mating strategies used by mobile organisms. Generally, two types of insemination are employed that can be distinguished by the site of gamete release, "spawning" and "copulation." Subsequent fertilization can thus occur externally or internally, relative to a female's anatomy. *Spawning* is the simplest method of mating, whereby an individual's gametes are released into the environment (usually aquatic) in hopes that a complementary gamete will be nearby. Sessile animals frequently employ this technique, although fish, amphibians, echinoderms, and some mollusks also spawn. Mating choice is often not a consideration in spawning by sessile animals because gamete interaction happens by chance. Their meeting is enhanced by pheromones, which encourage a local female and male spawn at overlapping times, but the union of the individual gametes is dependent on luck. Some vertebrates do, however, actively seek a mate before spawning their gametes into the environment, thereby increasing the probability of fertilization because of the higher sperm/egg ratios (O'Rand, 1988). Fertilization of spawners often occurs externally, although some species such as the bivalve *Unio* draw a mixed population of gametes into a respiratory chamber to undergo fertilization and later release the zygotes into the ocean (Focarelli *et al.*, 1988). *Copulation*, on the other hand, requires deposition of sperm by the male into the female reproductive tract. Individuals of the mating pair involved often seek one another by choice and must come in close contact to complete the transfer of sperm. The sperm is often stored in the female reproductive tract until needed, and the female can use the sperm for either internal or external fertilization (Neubaum and Wolfner, 1999). Internally, sperm-release into the oviduct is controlled by female anatomy and is timed to optimize the chances that the free sperm will encounter a freshly ovulated egg. Externally, sperm are released into the water at the same time as the eggs. Mammals, birds, dipterans, and nematodes usually copulate, with subsequent internal fertilization. Decapods commonly copulate to transfer sperm, but the female simultaneously releases both gametes into the environment when she is ready. Therefore, mating styles and fertilization techniques are interchangeable. In this section, we explore the impact these behaviors have on successful monospermic fertilization.

A. Behavior, Anatomy, and Monospermy

Polyspermy can be achieved in normally monospermic eggs by simply increasing the number of sperm encountering an egg (Alliegro and Wright, 1983; Grey *et al.*, 1982; Lambert *et al.*, 1997; Snook and Markow, 2002; Yu and Wolf, 1981). Several mechanisms are employed by animals to reduce sperm/egg ratios, including the type of insemination and timing of gamete

release. Broadcast spawners naturally minimize sperm/egg ratios by diluting gametes in the environment, a method that does not guarantee that each gamete will encounter its complement. Hence, the more gametes produced and spawned at a given time, the better an individual's success in reproduction. Yet making such large quantities of gametes can prove to be a substantial burden to an animal's energy resources. Strategies that enhance gamete interaction among broadcast spawners with less energy expenditure include locally concentrating the gametes before releasing the zygotes into the open water, as observed in the bivalve *Unio* (Focarelli *et al.*, 1988). Reproductive success of the spawning male also requires a prime location and proper timing to ensure that the largest, fittest eggs are inseminated with his sperm rather than his competitor's (Marshall *et al.*, 2004). An alternative strategy is to actively seek a mate, thereby ensuring that a higher percentage of gametes will be fertilized by one male due to an enhanced sperm/egg insemination ratio (O'Rand, 1988). Of course, one caveat to this behavioral modification is that the ratio is consistently too high, thus favoring polyspermy. Fish and amphibians have minimized this deleterious outcome by incorporating mating choice with external egg insemination. This partially dilutes the sperm to optimize the sperm/egg ratio, but this does not preclude the selection of additional mechanisms on a per-egg basis that further modify the sperm/egg ratio (see Section IV.B and IV.C, later in this chapter).

Deposition of sperm within the female reproductive tract through copulation is the most efficient way a male can enhance reproductive success, but the outcome of such a high sperm/egg ratio could be devastating for the progeny. Consequently, female anatomy has evolved methods to cope with this plethora of sperm, namely by controlling the release of sperm per ovulation. Like other aquatic vertebrates, decapods use the additional dilution factor provided by spawning into the environment to reduce the number of sperm available per egg (Talbot and Goudeau, 1988). Terrestrial animals, on the other hand, control the quantity of sperm released from storage through reproductive organs (Neubaum and Wolfner, 1999). For example, mammalian oviductal crypts store sperm along the oviduct epithelium until ovulation occurs, when only fertilization-competent sperm are released into the lumen (Eisenbach, 1999; Rodger and Bedford, 1982a). Spermathecae, a sperm storage organ that can release only from one to a few sperm per ovulation, are used by nematodes (Singson, 2001), dipterans (Fitch *et al.*, 1998; Snook and Markow, 2002; Turner and Mahowald, 1976), and urodeles (Elinson, 1986) to limit the sperm/egg ratio within the oviduct. Again, further specializations in the morphology or biochemistry of the egg ECM can significantly pare down the sperm/egg ratio almost to unity to maximize successful fertilization (see Singson, 2001).

B. Egg Attraction

Successful fertilization requires the union of two compatible gametes, a conspecific pairing of sperm and egg. Behavior and mating are crude methods to ensure that individuals of the same species will copulate; broadcast spawning has little guarantee of success unless the timing and distance of spawning is optimal. To overcome some of these negative influences, eggs use soluble chemical factors to guide conspecific sperm toward them. Thus, sperm have been selected for their phenotypic response toward a chemoattractant gradient (Brown, 1976; Eisenbach, 1999; Elinson, 1986; Garbers *et al.*, 1986; Hansbrough and Garbers, 1981; Hirohashi and Vacquier, 2002a,b; Hoshi *et al.*, 2000; Koyota *et al.*, 1997; Oda *et al.*, 1995, 1998; Olson *et al.*, 2001; Ramarao *et al.*, 1990; Riffell *et al.*, 2002, 2004; Suzuki *et al.*, 1988; Zatylny *et al.*, 2002).

Most gametes are released into aqueous environments, making microfluid dynamics a significant factor in the behavior of chemoattractants released by an egg. The efficacy of a particular sperm chemoattractant is dictated by the chemical's diffusion constant (mass vs. solvation properties) and local fluid turbulence (Xiang *et al.*, 2005). These two variables determine how steep and how long a gradient can be maintained in a form that is conducive to attracting homotypic sperm toward an egg. Additional factors that may influence the gradient itself include the source and abundance of the chemoattractant. For example, egg jelly is used as a chemoattractant in many animals. This glycoprotein coat is often applied over the egg ECM by ovarian follicle cells (Buckland-Nicks and Hodgson, 2000; Santella *et al.*, 1983) or oviduct epithelium as the egg travels toward the uterus (Elinson, 1986; Hedrick and Nishihara, 1991; Jego *et al.*, 1986; Lindsay *et al.*, 2003; Olson *et al.*, 2001; Schmidt *et al.*, 1997). Upon ovulation, jelly immediately, albeit passively, dissolves into the environment because of local convections and its relatively low chemoattractant retention constant (Arranz and Cabada, 2000; Olson *et al.*, 2001; Ward *et al.*, 1985; Xiang *et al.*, 2005). The single layer of jelly means that only a finite amount of chemoattractant is available, limiting the duration that the egg will be attractive to sperm. This is in contrast to actively released chemoattractants, such as the amino acids used by gastropods (Riffell *et al.*, 2002). In these mollusks, attraction gradients can be maintained for extended periods and may be modified in response to the environment to enhance the likelihood of attracting the correct sperm.

Eggs and oocytes of both protostomes and deuterostomes use a range of substances to attract conspecific sperm. Molecularly, the simplest chemoattractant is L-tryptophan, used by the gastropod *Haliotis rufescens* (Riffell *et al.*, 2002, 2004). The uniform presence of L-tryptophan actively released by the egg

activates *H. rufescens* sperm motility and orients it toward the source (Riffell *et al.*, 2002). Yet sperm from the closely related abalone *H. fulgens* are indifferent to L-tryptophan, the first indication that L-tryptophan is a bona fide chemoattractant that facilitates conspecific gamete interaction in animals whose spawning geographies may overlap (Riffell *et al.*, 2004). To further the complexity, and hence the specificity, some animals use peptides and small proteins as chemoattractants. For example, the cuttlefish *Sepia officinalis* egg uses the modified pentapeptide PIPGVamide to attract sperm toward the egg (Zatylny *et al.*, 2002). Surprisingly, sperm behavior toward this peptide can work both ways: The concentration gradient of *S. officinalis* PIPGVamide initially attracts sperm released from the female copulatory pouch toward the freshly spawned egg, but following fertilization or egg activation, this same peptide is trapped in the surrounding capsule, causing the accumulation of the peptide to concentrations that are repulsive to sperm (Zatylny *et al.*, 2002). This biphasic sperm-response elegantly toggles between promoting sperm interactions and inhibiting them to avert polyspermy.

Sperm chemoattraction also plays a critical role in deuterostome fertilization. Starfish asterosap, a glutamine-rich tetracontapeptide found in the jelly coat, can activate and reorient sperm through receptors along the sperm tail (Hoshi *et al.*, 2000; Neill and Vacquier, 2004). Its functional homologs in sea urchin eggs include members of the speract (Hansbrough and Garbers, 1981; Ramarao *et al.*, 1990; Suzuki *et al.*, 1988) and resact families of peptides (Garbers *et al.*, 1986; Ward *et al.*, 1985). Both of these sea urchin peptides originate from the jelly coat and diffuse away from the egg. When bound by sperm receptors found on the tail and midpiece of sperm, both peptides conspecifically increase sperm respiration, but only resact can reorient the sperm toward the source of the chemoattractant (Kaupp *et al.*, 2003; Neill and Vacquier, 2004; Ward *et al.*, 1985). In contrast to echinoderms, sperm chemoattractant has only recently been identified in higher deuterostomes. For example, chemotaxis of *Xenopus* sperm is achieved using the 21-kDa allurin, a glycoprotein member of the cysteine-rich secretory protein (CRISP) family of sperm-binding proteins (Olson *et al.*, 2001). It is responsible for 88% of sperm the chemoattractive behavior elicited by unfractionated egg jelly (Arranz and Cabada, 2000; Bonnell *et al.*, 1996; Olson *et al.*, 2001). This anuran chemoattractant diffuses from the outermost layer of *Xenopus* jelly (J3) (Figs. 1 and 2) into the surrounding fluid at a rate similar to smaller chemoattractants (Xiang *et al.*, 2005). Together, these observations imply that *Xenopus* allurin is a bona fide sperm attractant. A similar activation profile is observed for the *Ciona* sperm activating and attracting factor (SAAF), a sulfated steroid derivative that serves as a chemoattractant, albeit as a heterospecific one because the identical molecules are used by *C. intestinalis* and *C. savignyi* (Yoshida *et al.*, 2002). Such absence of conspecificity, however, may be balanced by the follicle cells

attached to the vitelline coat surface since these cellular gatekeepers regulate which sperm may interact with the ascidian vitelline coat (Lambert, 2000) (Figs. 1 and 2). Acid hydrolysis of the sulfur groups on SAAF abolishes activity, suggesting that the additional charges provide hydrophilicity to the steroid, thereby allowing it to participate in signaling on the sperm surface (Yoshida *et al.*, 2002). Use of the steroid progesterone is also implicated in chemoattraction toward mammalian eggs, but the data do not indicate that this ubiquitous steroid triggers chemotaxis per se; it may only activate sperm (Eisenbach and Tur-Kaspa, 1999). Could the addition of sulfate groups to mammalian progesterone convert this steroid into a functional chemoattractant? Or might progesterone represent a speract homolog, with a more potent chemotactic factor also required to reorient the sperm?

The involvement of various classes of molecules in chemoattraction points to the enormous diversity acquired during the initial phases of gamete interaction. Yet, a pattern exists throughout phylogeny with regards to the classes of chemoattractants used: Lower protostomes, who are often broadcast spawners, use single molecules (amino acids or modified peptides) to achieve a conspecific chemotactic response from sperm, whereas more recently evolved deuterostomes use more complex molecules (groups of peptides, proteins, steroids). Might chemoattractant complexity be linked to organism diversity, under the presumption that species specificity will increase upon addition of more variable in the mix of chemoattractants? How does the type of mating used by an animal factor into the selection of chemoattractant properties? Broadcast spawning might favor simpler molecules, such as L-tryptophan or peptides, because of the reduced cost of synthesizing them in bulk. Meanwhile, the pressure to co-evolve species-specific chemoattractive molecules may have relaxed as a consequence of reproductive isolation, either through geographical separation of populations (e.g., ascidians) or by acquisition of conspecific mating behavior (e.g., vertebrates). Additional data from other taxa that implicate specific molecules in conspecific versus generic sperm chemotaxis will be critical to assess how gamete chemoattraction ranks within the hierarchy of gamete interactions and prefertilization events.

C. Cell-Mediated Reduction in Sperm Quantity

Chemoattractants guide sperm toward a receptive egg, but an appropriate outcome falls within a defined range of sperm/egg ratios to achieve monospermy. Thus, a balance exists between effective chemoattraction and limited sperm number. In many animal eggs, the same structure that distributes the chemoattractant is responsible for retarding the progression of sperm toward the egg. The thick jelly coat of amphibian, echinoderm, xiphosurid,

and mollusk eggs helps to orient sperm perpendicular to the egg membrane. In the process, the proteoglycan constituents invariably retard sperm progress, reducing the number of sperm that successfully reach the egg surface at one time, delaying fertilization (Brown and Humphreys, 1971; Elinson, 1986) and/or selecting against prematurely activated sperm (see Section V, later in this chapter) (Hylander and Summers, 1977; Mah *et al.*, 2005). Chiton use their elaborate jelly coat hull, a structure synthesized and shaped by follicle cells in the ovary, as a chemoattractant and an obstacle that masks available sites of sperm binding (Buckland-Nicks and Hodgson, 2000; Buckland-Nicks *et al.*, 1988) (Figs. 1 and 2).

In a select few animals, follicle cells ovulated with the egg also participate in reducing the sperm/egg ratio. For example, the same follicle cells that construct the chiton's hull are ovulated with the egg. In some species, these follicle cells retract upon contact with the hyperosmotic seawater, revealing evenly ordered channels that guide sperm toward the receptive egg surfaces; in other species they remain steadfast, directing sperm to those productive tunnel openings found between cells (Buckland-Nicks, 1993; Buckland-Nicks and Hodgson, 2000; Buckland-Nicks *et al.*, 1988). In both situations, chiton follicle cells passively obstruct sperm access to the egg ECM (Figs. 1–3). Ascidian follicle cells, on the other hand, actively participate in promoting sperm–egg interactions. The ascidian egg is spawned with a vitelline coat separated from the egg by a significant perivitelline space and a tight epithelial layer of follicle cells coating the outer surface of the vitelline coat (Figs. 1 and 2). Upon insemination, sperm must pass through the layer of follicle cells to access the ECM. To do so, two mechanisms have been proposed where sperm are either phagocytosed by the cells and transported across to contact the vitelline coat (De Santis *et al.*, 1980) or they penetrate through lateral junctions shared by adjacent cells (Lambert, 1989); neither has been observed *in vivo*. Regardless of which method is used to transit this cellular barrier, an active decision is made by the follicle cell on a per–sperm basis, and part of this decision is dependent on the species of the sperm (Lambert, 2000). Eutherian sperm are also required to transit a stratified layer of cumulus cells before contacting the zona (Figs. 1–3). Unlike ascidian or chiton follicle cells, the stratified organization of these cumulus cells helps them surround themselves with a viscous ECM enriched in hyaluronic acid and chondroitin sulfate A (Tatemoto *et al.*, 2005). The presence of these two matrix molecules impedes sperm progression through the cumulus layer and requires functional hyaluronidase, PH-20, on the sperm head (Dean, 2004; Hunnicutt *et al.*, 1996b; Myles and Primakoff, 1997; Primakoff and Myles, 2002; Tatemoto *et al.*, 2005). It is quite probable that other molecules on the sperm surface are co-opted by the egg to both retard and survey incoming sperm, thereby optimizing the sperm/egg ratio for successful fertilization.

Many animals utilize other methods to minimize sperm quantities that do not involve cellular gatekeepers, but utilize spatial restrictions instead. In birds, an egg ovulates from the ovarian capsule with the germinal disc, the preferred site of sperm binding and fusion, facing the infundibulum (Bramwell and Howarth, 1992; Okamura and Nishiyama, 1978b). Because fertilization occurs at the time of rupturing, the remainder of the large egg is anatomically blocked from sperm access, thereby avoiding polyspermy from occurring at a site other than the germinal disc (Bramwell and Howarth, 1992; Harper, 1904). Limiting the site of sperm fusion along the egg surface is common in many animals (see Section VII.A, later in this chapter) (Fig. 4). Often this spatial restriction is associated with complementary morphological modifications to the egg ECM. Some eggs have evolved radical polarizations in their ECM architecture compared to their sister taxa. For example, the anuran *Discoglossus* designates a patch of membrane at the animal pole as the major site of gamete fusion (Campanella *et al.*, 1992; Caputo *et al.*, 2001). Sperm are directed to this patch of microvillar-rich membrane by an extensive, chemoattractive jelly plug impinging on the animal hemisphere (Campanella *et al.*, 1997; Talevi and Campanella, 1988) (Fig. 4). The chemoattractive role of this jelly is most obvious when comparing fertilization to dejellied *Discoglossus* eggs, in which sperm are found to bind anywhere along the vitelline envelope but can fuse only at the dimple (Caputo *et al.*, 2001). A similarly radical polarization can be found in the bivalve mollusk *Unio*, whose egg is attached to the vitelline envelope only at the vegetal-most tip (Focarelli *et al.*, 1988) (Fig. 4). Here lies an elaboration of the ECM, where a crater composed exclusively of the sperm receptive gp273 marks the only fusogenic region of the egg; the remainder of the vitelline envelope consists of the inert structural glycoprotein gp180 (Focarelli and Rosati, 1995; Focarelli *et al.*, 1988).

The most elaborate reduction in sperm-accessible surface is found in eggs with polarized, impenetrable ECMs with a narrow channel, or micropyle, that guides sperm to the only receptive site on the egg. Such a specialization has convergently evolved in at least two animal orders with surprisingly different methods of insemination, namely dipterans and teleosts. Dipteran eggs are fertilized internally as the egg traverses down the oviduct. Sperm/egg ratios are limited anatomically by the controlled release of sperm from the spermatheca (Bloch Qazi *et al.*, 2003; Neubaum and Wolfner, 1999), so it is surprising to find that dipterans such as *Drosophila*, *Dacus*, and *Musca* utilize a micropyle (Degrugillier and Leopold, 1976; Mouzaki *et al.*, 1991; Turner and Mahowald, 1976) (Figs. 1 and 2). This paradox likely reflects the role of the chorion in minimizing desiccation following egg deposition (Li *et al.*, 1996), leaving the micropyle not as an elaboration whose primary role is not to block polyspermy, but as a feature that enhances gamete interactions while favoring gas exchange during embryogenesis. This

micropyle is marked by a thickened chorion, formed with the help of follicle cells, and a tuft of glycoproteins that distally seal the micropylar pore from the oviduct (Mouzaki *et al.*, 1991; Perotti *et al.*, 1990; Turner and Mahowald, 1976). To achieve fertilization, sperm must pierce this tuft before traveling through the canal separating the endochorion and the vitelline membrane (Degrugillier and Leopold, 1976). The molecular composition of this tuft suggests that it is the initial site of species-specific gamete interaction (Cattaneo *et al.*, 1997, 2002).

Unlike their dipteran counterparts, female teleosts do not have the ability to anatomically regulate the number of sperm per insemination; rather, the localized receptivity and the narrowness of the chorionic micropyle must be sufficient to deter excessive sperm entry. How might this have evolved, particularly because the more ancient chondroichthyes use sperm storage and anatomically regulated release of sperm (Neubaum and Wolfner, 1999)? The lineage of present-day fish provides some clues to this evolutionary process (see Fig. 4). In the most primitive bony fish, such as the petromyzontid *Lampetra*, the animal pole is covered with a small region of thickened chorion decorated with a tuft of jelly (Figs. 1 and 2). This jelly guides and orients the sperm toward the most fusogenic patch of egg membrane (Kobayashi and Yamamoto, 1994). Chondrostean fish such as *Acipenser* or *Polyodon* use a more advanced specialization, specifically a cluster of multiple channels that transect the chorion (Ciereszko *et al.*, 2000; Hart, 1990) (Fig. 4). The micropylar catchment of each channel participates in sperm attraction and binding (Cherr and Clark, 1986). Finally, the most recently diverged teleosts, whose eggs meiotically activate when they transit from the oviduct into the environment regardless of the state of fertilization, possess a single micropylar interruption in their chorion (Hart, 1990; Lee *et al.*, 1999; Yamagami *et al.*, 1992). Only the micropylar region is attractive to sperm and remains so for only a short window of time outside of the body before environmental activation, a time scale on par with the tens of seconds–long period of sperm motility (Hart, 1990; Tosti, 1994; Wolenski and Hart, 1987). Osmotic egg activation results in complete hardening of the chorion, as well as a depression or loss in micropylar chemoattractiveness to sperm (Amanze and Iyengar, 1990; Iwamatsu *et al.*, 1997; Wolenski and Hart, 1987).

The morphology of the fish micropyle is dependent on the activity of a micropylar follicular cell found in the ovary. This cell dictates how the chorion will be locally molded to form the micropyle (reviewed in Hart, 1990). Micropyle architecture ranges across the species from a simple tunnel traversing the chorion, marked by a local elevation of the zona radiata externa (Hart *et al.*, 1984), to a more elaborate structure consisting of an outer sperm catchment area or vestibule that funnels the sperm into a canal or pit (Amanze and Iyengar, 1990; Cherr and Clark, 1986; Hart, 1990; Yamagami *et al.*, 1992). The nuances of micropylar adornments within each

region vary among species (Fig. 3). For example, the catchment of *Barbus* has 7–10 grooves and ridges that radiate from the micropylar pit, accounting for 0.01% of the total surface area of the rosy barb chorion (Amanze and Iyengar, 1990). On the other hand, the *Danio* vestibule is covered with folds arranged in a right-hand spiral toward the pit (Hart and Donova, 1983). The pit ends above a circle of egg membrane enriched in fusogenic microvilli (see Section VII.A, later in this chapter) (Hart, 1990; Hart and Donova, 1983; Ohta and Iwamatsu, 1983). The pit diameter is only wide enough to accommodate a single sperm head, thus making monospermic fusion more likely (Amanze and Iyengar, 1990; Cherr and Clark, 1986; Hart, 1990; Hart and Donova, 1983; Ohta and Iwamatsu, 1983; Wolenski and Hart, 1987).

Teleost sperm use different intracellular signaling cascades to distinguish where they are within the micropyle, shifting the sperm behavior from a "seek" to a "follow" mode as necessary (Iwamatsu *et al.*, 1997; Murata *et al.*, 1995). *Clupea* sperm use the chemokinetic molecule herring sperm activating protein (HSAP) to initiate the "seek" mode of sperm (Oda *et al.*, 1995, 1998). HSAP is an 8-kDa water-soluble protein that readily diffuses from the outer chorion layer and can alter sperm motility at short ranges (Oda *et al.*, 1995). HSAP is found throughout the chorion, except at the micropyle. Its ability to initiate chemokinetic activity in sperm is consistent with a role in directing sperm *away* from the less productive chorion proper and *toward* the micropyle (Oda *et al.*, 1998). Following their activation, the sperm are attracted to the micropylar pit by a gradient of insoluble sperm motility initiation factor (SMIF) immobilized within the micropylar catchment (Griffin *et al.*, 1996). The ability of sperm to bind a solubilized form of herring SMIF *in vitro* (Griffin *et al.*, 1996) suggests that this normally immobile glycoprotein can facilitate the sperm's switch to "follow" mode, tracking along the grooves or folds lining the catchment to enter the vestibule and micropyle, as documented in other teleosts (Amanze and Iyengar, 1990; Hart and Donova, 1983). Might these structural micropylar grooves expose other deeper layers of the chorion, providing additional molecules for the sperm to maintain contact during the "following" stage? If so, then the participation of additional ZPC homologs (see Fig. 5; Section II.B and II.C, earlier in this chapter) could be a factor in the sperm's behavioral changes.

Is one of the mechanical obstacles, including viscous jelly, micropyles, and cellular gatekeepers, more effective than the others at keeping conspecific sperm/egg ratios low? Considering the wide range of methods used by species within the same taxon (Figs. 1, 2, and 4), the best mechanism is clearly the most adapted for a particular mating style. The dual chemoattractant–retardant properties of jelly serve spawning animals well because dilution of sperm in the media sufficiently lowers the ratio of interacting gametes per volume, particularly at the rate gametes are spawned in the wild. The use of a single micropyle ensures a sperm/egg ratio of unity but does not guarantee

that every egg will be fertilized (Hart, 1990; Snook and Markow, 2002). Likewise, chemoattraction and insemination do not guarantee that conspecific sperm will fertilize the egg; these processes only favor this outcome. The involvement of follicle cells in distinguishing between nonspecific and conspecific sperm (Lambert, 2000) clearly trumps the limitations imposed by the passive micropyle. Even though cellular gatekeeping requires more energy expenditure per egg than micropyles, the selectivity afforded by the initial sperm–follicle cell recognition phase may outweigh such costs, particularly in sessile animals like ascidians, for whom heterospecific gamete interactions are more likely (Lambert *et al.*, 2002). It is important to point out that selecting for a slightly higher sperm/egg ratio may be favorable to yield high fertilization percentages because of the successive series of gamete recognition steps that must occur before fusion.

V. Initial Gamete Contact

The ECM is a critical mediator of cell–cell communication in many tissues, including gamete interactions. The initial contact with the egg ECM triggers a cascade of changes in the sperm, including increased metabolism, greater motility, and the acrosome reaction, when the contents of the sperm's only secretory vesicle are released into the local environment (reviewed in Neill and Vacquier, 2004; Okamura and Nishiyama, 1978a; Tulsiani *et al.*, 1998; Wassarman, 1999). In most animals, these events are the first indications of successful homotypic recognition between gametes. Among animals, the types of molecules required to achieve this state of sperm activity vary significantly and often involve a combination of overlapping receptor–ligand interactions. This complexity not only reinforces species specificity between gametes prior to fertilization, but also proves to be more difficult for the egg to deal with when a block to polyspermy must be established.

In this section, we survey the major receptors and ligands responsible for initial gamete recognition (see also Table II). Particular emphasis is made on the candidates in the egg ECM or at the sperm surface that are likely modified during the establishment of a block to polyspermy. We also discuss the impact of these essential proteins on speciation and radiation within the animal kingdom.

A. Variability in Locations of Initial Sperm Contact

The site of initial conspecific sperm–egg interaction is highly variable among animals (Figs. 1 and 2). For example, a sperm's chemokinetic and chemotactic response to attraction factors (see Section IV.B, earlier in this chapter) are often indistinguishable from metabolic changes resulting from initial

sperm–egg contact. The critical difference between attraction and physical contact lies in the status of the sperm acrosome: Only primary sperm–egg contact will initiate the appropriate cascade of events that result in the acrosome reaction. Thus, the acrosome reaction has become a benchmark for the full activation of sperm and the achievement of initial gamete contact. The only taxon exhibiting an exception to this generality is teleosts, whose sperm do not possess acrosomes (Hart, 1990) (Figs. 1 and 6).

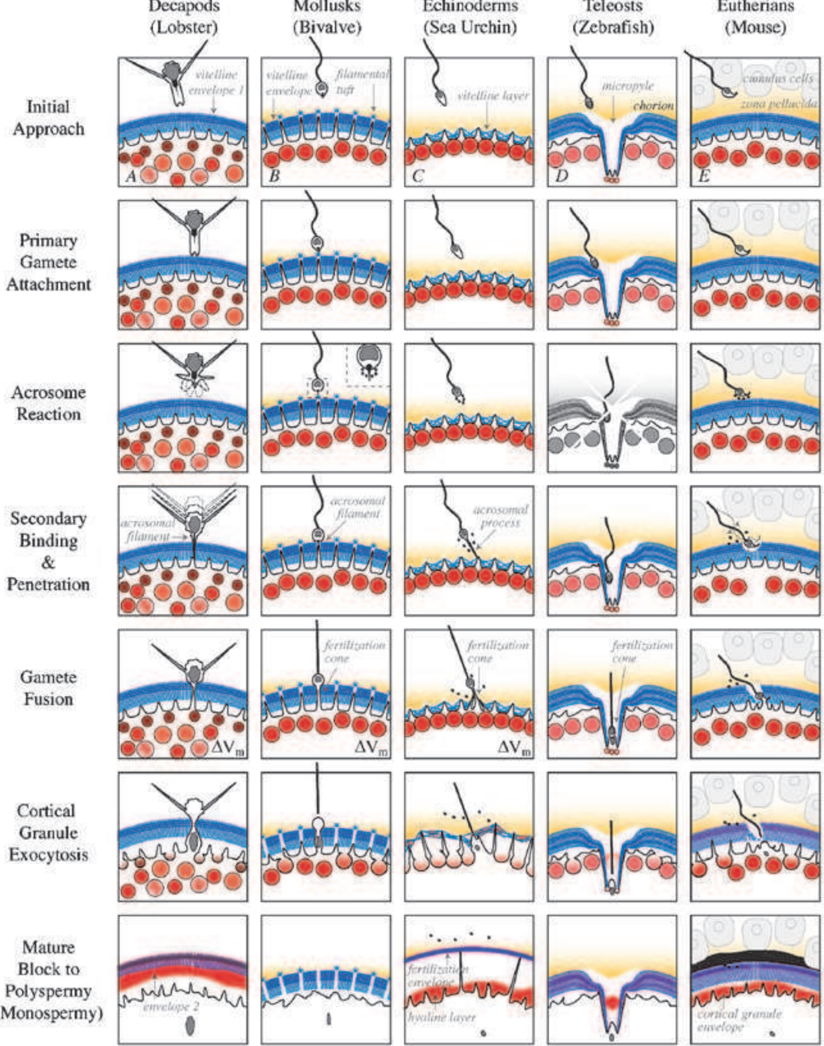


Figure 6 *Continued*

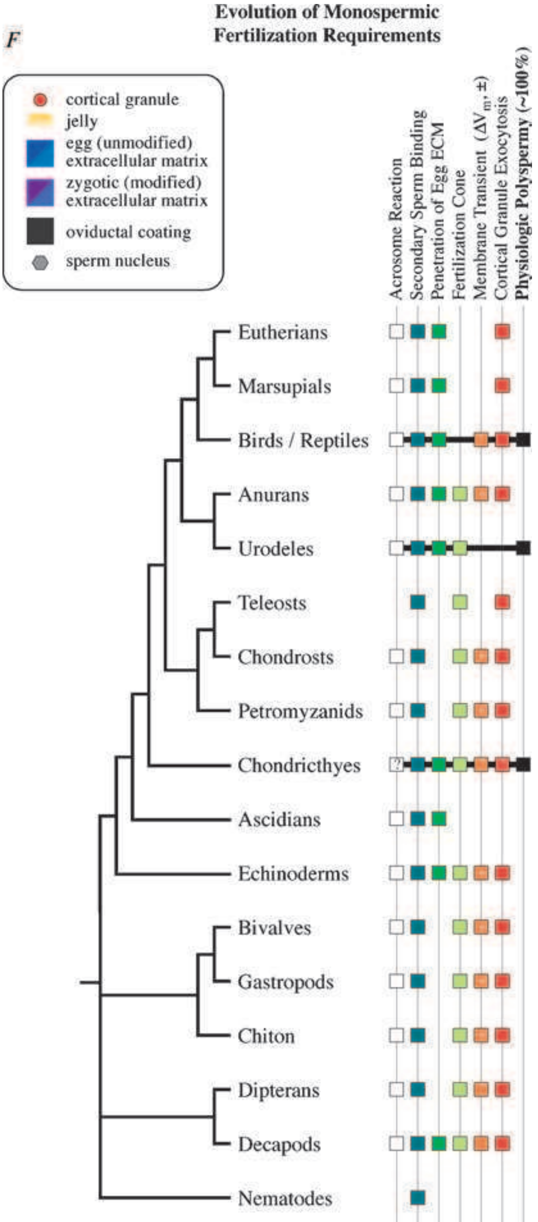


Figure 6 Diversity of mechanisms used during animal fertilization. Representations of sequential steps required for fertilization used by a selection of animals, including decapods (A), bivalves (B), echinoderms (C), teleosts (D), and eutherians (E). Steps are listed vertically in chronological order. Colors are as represented in the figure legend, following the scheme of

Egg jelly is often bifunctional, acting as both chemoattractant and sperm activator. The different effects of echinoderm jelly on sperm activity have been traced to separate molecules found in the heterogeneous mix of jelly glycoproteins (reviewed in Neill and Vacquier, 2004). The chemoattractive peptides encoded by starfish asterosap increase sperm motility in a heterospecific fashion and potentiate the acrosome reaction (see Section IV.B, earlier in this chapter) (Hoshi *et al.*, 2000; Matsumoto *et al.*, 2000). Starfish simultaneously present a greater than 2000-kDa acrosome reaction–inducing substance (ARIS) also found within the jelly coat that activates sperm conspecifically (Hoshi *et al.*, 2000; Koyota *et al.*, 1997; Miller and Crawford, 1994). The essential fragment of pronase-digested *Asterias* ARIS contains at least 10 repeats of a sulfated pentasaccharide oligomer that can compete with full-length ARIS for binding to a 0.2-μm patch of the anterior sperm head (Hoshi *et al.*, 2000; Koyota *et al.*, 1997; Longo *et al.*, 1995). Under physiological conditions, however, ARIS alone cannot induce the acrosome reaction; normal activation is achieved only when sperm are presented with a complex containing asterosap, ARIS, and coARIS, a class of saponins covalently linked to sulfated steroids (Hoshi *et al.*, 2000; Matsumoto *et al.*, 2000). A triad of sea urchin jelly components is also essential for maximum rates of sperm activation. Like starfish asterosap, the speract family of peptides initiates sea urchin sperm chemokinesis (see Section IV.B, earlier in this chapter; Garbers *et al.*, 1986; Hansbrough and Garbers, 1981; Ramarao *et al.*, 1990; Suzuki *et al.*, 1988; Ward *et al.*, 1985). The acrosome reaction, however, is conspecifically potentiated by a glycoprotein complex anchored within the jelly by the 380-kDa fucose sulfate polymer (FSP) (Bonnell *et al.*, 1994; Hirohashi and Vacquier, 2002b; Keller and Vacquier, 1994; Vacquier and Moy, 1997; Vilela-Silva *et al.*, 1999). FSP contains more than 50% carbohydrate by mass, and 97% of this is fucose (Vacquier and Moy, 1997). Like its starfish analog ARIS, FSP alone can trigger the acrosome reaction species specifically at a pH of 8.0 (Hirohashi and Vacquier, 2002a; SeGall and Lennarz, 1979). In the presence of additional globular sialoglycans that reside on the branches of FSP, the acrosome reaction can be initiated at a pH of 7.8 (Bonnell *et al.*, 1994; Hirohashi and Vacquier, 2002a,b; Keller and Vacquier, 1994). When presented with speract, the FSP complex is able to trigger the acrosome reaction and to maintain sperm activity in more than 90% of all sperm, all at a significantly lower pH of 7.0 (Hirohashi and Vacquier, 2002a). This pH sensitivity is

Figs. 1 and 2. Grayed image indicates an absence of that particular step during fertilization in the respective animal. (F) Phylogenetic comparison of steps or structures used by different animals during fertilization. Where applicable, colors correspond to panels A–E; green tones indicate additional processes. ?, no data reported; −/+, direction of the fast electrical block (?V_m), either hyperpolarizing (−) or depolarizing (+).

curious because the pH of sea urchin egg jelly is identical to the surrounding media (Holland and Cross, 1983). The neutrality of the paleontological ocean is hypothesized to be a major force that selected for the maintenance of this triad of egg jelly proteins for sustaining sperm activation (Hirohashi and Vacquier, 2002a).

Based on the functional parallels between starfish and sea urchin jelly components (asterosap-speract; ARIS-FSP; coARIS-sialoglycans), it is tempting to speculate that jelly may be partly responsible for the divergence of these echinoderms. Both asterosap and speract represent families of peptides that increase sperm activity, yet sequence and size differences among these functional homologs are indicative of divergence. Similarly, the high degree of glycosylation in ARIS and FSP is probably held over from a common ancestor, whereas the size of each subunit could be a consequence of speciation. The synergists coARIS and sialoglycan, however, share little resemblance: The active backbone of coARIS is a sulfated steroid-liked saponin (Hoshi *et al.*, 2000), whereas sialoglycans predominantly consist of oligosaccharides (Hirohashi and Vacquier, 2002b). Yet the effects of each synergist on sperm activity are similar: Co-presentation of ARIS and coARIS increases intracellular calcium levels (Hoshi *et al.*, 2000), whereas co-presentation of FSP and sialoglycans raises intracellular pH and potentiates calcium-dependent acrosome exocytosis (Hirohashi and Vacquier, 2002b). Could speciation alone be responsible for the divergence in the synergists used? Might this switch be a memento of the earliest changes that resulted in the separation of these sister taxa more than 500 million years ago (Hinman *et al.*, 2003), with the divergence of ARIS and FSP following soon after? Perhaps the expression profile of sperm has also influenced the use of saponins versus sialoglycans. The absence of definitive sperm ligands for each molecule of the triad, however, makes this hypothesis difficult to evaluate.

Diffusion, the one factor that makes jelly a good chemoattractant, limits its utility as a trigger of sperm activation (Xiang *et al.*, 2005). In echinoderms, jelly is generously applied over each egg even though only a thin layer at the egg surface is necessary for successful fertilization; the excess is probably used to establish a large chemoattractive territory to lure other spawned sperm. A similar bifunctional role in both sperm attraction and activation has been proposed for anuran jelly. Such duality is supported by the absence of fertilization in anuran eggs that have been exposed to spawning media too long, a phenotype attributed to the visibly swollen and thinned jelly (Hedrick and Nishihara, 1991). The observation that mechanically dejellied anuran eggs are fertilization incompetent also strongly suggests that jelly is a significant contributor to initial sperm binding (Caputo *et al.*, 2001; Elinson, 1986). In *Discoglossus*, the most distal jelly layer (J3) is responsible for triggering the acrosome reaction, whereas the glycoproteins

that comprise the jelly plug primarily direct the reacted sperm toward the dimple (Campanella *et al.*, 1997; Talevi and Campanella, 1988). Similarly, cross-species fertilization can be achieved by simply passing a *Xenopus* egg through the oviduct of the species akin to the desired sperm donor (Elinson, 1986). Yet all anurans do not appear to use jelly to initiate a conspecific acrosome reaction. Only the vitelline envelope proteins are capable of species-specific exocytosis of the acrosome in *Bufo* (Barisone *et al.*, 2002) and the presence of jelly only enhances fertilization by 10% in *Eleutherodactylus* (Toro and Michael, 2004). One reason for this difference in jelly-mediated activation may lie in the aquatic versus terrestrial environment that anurans inhabit; semiaquatic frogs may use the outer jelly layer to induce the acrosome reaction because the spawned sperm are less numerous upon contact with the jelly compared to their terrestrial counterparts. By using the outer jelly layer, the egg establishes its attractiveness to the more dilute sperm earlier in the process, thereby favoring its own fertilization. In contrast, the role of jelly in terrestrial anuran eggs may be to prepare the sperm for interaction with the egg ECM, perhaps enhancing the sperm's affinity for the components found within the vitelline envelope, while reducing the number of sperm that finally contact the egg by selecting against prematurely acrosome-reacted individuals.

Consistent with terrestrial anurans, sperm activation and the acrosome reaction in most animals occur at the closely associated ECM made by the oocyte. More often than not, mechanical removal of jelly or other extraneous glycoprotein coats does not drastically impair sperm activation or fertilization *in vitro*, but removal of the ECM does (Talbot and Chacon, 1982). Thus, components in the egg ECM of most animal taxa mediate conspecific sperm–egg interactions. In many vertebrates, the major glycoproteins responsible for primary sperm–egg interactions are members of the ZPC subfamily (Infante *et al.*, 2004; Iwamatsu *et al.*, 1997; Kerr *et al.*, 2002; Mengerink and Vacquier, 2001; Moller *et al.*, 1990; Murata *et al.*, 1995; Tulsiani, 2000a; Vo and Hedrick, 2000; Wassarman, 1987, 1999) (Fig. 5). Two regions of ZPC, found at the most amino- and carboxy-terminal domains of the ectodomain, are under positive selection, whereas the primary sequence of the whole protein is under negative selective pressure (Swanson *et al.*, 2001b). Such negative selection on the ZP domain suggests that its conformation is essential for ZPC to intercalate within the ECM, a model supported by experimental deletions (Jovine *et al.*, 2002) and exon swapping (Kinloch *et al.*, 1995). Thus, the orientation of ZPC within the ECM may be essential for successful sperm binding, perhaps using the ZP domain to anchor itself within the matrix while the divergent regions are left exposed. Conformational dependence of ZPC for sperm-activating behavior is supported by the requirement for oviductal modification prior to achieving sperm binding to the egg ECM in anurans (Gerton and Hedrick, 1986; Infante

et al., 2004; Kubo *et al.*, 1999), urodeles (Makabe-Kobayashi *et al.*, 2003), marsupials (Breed *et al.*, 2002), and mammals (Boja *et al.*, 2003; Kiefer and Saling, 2002).

Additional factors found within the ECM may also participate in optimizing access of incoming sperm to the appropriate sperm receptor. For example, adsorption of oviductal glycoproteins to freshly ovulated eggs can enhance conspecific sperm–egg binding efficiency (Brown and Cheng, 1986; Buhi, 2002; Buhi *et al.*, 2000; O'Day-Bowman *et al.*, 2002; O'Rand, 1988; Schmidt *et al.*, 1997), possibly by modulating the binding affinities of ZPC (Rodeheffer and Shur, 2004). Endogenous ZP proteins may also be critical for their proper exposure: Oviductal cleavage of ZPA is required for functional sperm binding to the anuran vitelline envelope (Caputo *et al.*, 2001; Infante *et al.*, 2004; Tian *et al.*, 1997, 1999; Vaccaro *et al.*, 2001), and the simultaneous presentation of ZPB–ZPC dimers is required for sperm binding to *Sus* zona (Yurewicz *et al.*, 1998). The identification of ZPD family members (Fig. 5) also supports a model of increased complexity in sperm-binding conformations through heterodimerization of ZP proteins (Lindsay *et al.*, 2002; Okumura *et al.*, 2004). The chicken perivitelline layer, for example, is primarily composed of three major ZP homologs: ZP1 (Bausek *et al.*, 2000), ZPC (Takeuchi *et al.*, 1999), and ZPD (Okumura *et al.*, 2004) (a minor fourth protein can be detected [Okumura *et al.*, 2004] and may represent the ZPAX ortholog identified in the *Gallus* genome, but this link has not been made experimentally). Of these three, ZPD is readily removed from the ECM by sonication; ZP1 and ZPC require acid and chaotropic denaturation for separation (Okumura *et al.*, 2004). Yet only dimeric ZP1 and monomeric ZPD can induce the acrosome reaction in sperm at 50% and 95% of the frequency of the intact perivitelline layer, respectively (Okumura *et al.*, 2004). As with *Xenopus* ZPD, the presence of the small molecular weight *Gallus* ZPD was initially masked by an overwhelming quantity of ZPC under non-reducing gel electrophoretic conditions (Lindsay *et al.*, 2002; Okumura *et al.*, 2004). Because initial sperm-binding and sperm-activating assays in vertebrates were accomplished with gel-purified ZPC, the ZPD contaminant was never considered functionally relevant. Although chicken ZPC follows the pattern of the archetypical mammalian ZPC ortholog, namely homology and abundance in the perivitelline layer, its inability to induce the acrosome reaction *in vitro* leaves room for other contributing ECM components such as ZPD (Lindsay *et al.*, 2002; Okumura *et al.*, 2004). The exclusive presence of ZPD orthologs in avians and anurans (Fig. 5), however, suggests that vertebrates procured ZPD late in evolution as animals became terrestrial but was subsequently lost in the mammals. One hypothesis for this loss may be the transition to a broader sperm-receptive surface on the egg (see Section VII.A, later in this chapter) (Fig. 4): Avian eggs fertilize primarily at the germ disc (Bramwell and Howarth, 1992; Okamura and Nishiyama,

1978b), anurans fertilize in a restricted region of the animal pole (Elinson, 1986), but mammalian eggs can be fertilized anywhere along the egg surface except over the metaphase spindle (Gulyas, 1980; Myles, 1993; Wassarman, 1999) (Fig. 4). In animals that retain ZPD expression (Fig. 5), this soluble protein might work in conjunction with ZPC to enhance sperm receptivity by locally lowering the threshold for sperm activation (Okumura *et al.*, 2004).

Where does the acrosome reaction occur in invertebrate eggs that also contain bona fide ZP homologs in their egg ECM? In hermaphroditic ascidians, the acrosome reaction is triggered upon contact between the sperm and vitelline coat (De Santis *et al.*, 1980; Rosati, 1985). Immobilized VC70, the major protein of the vitelline coat, is able to bind conspecific sperm *in vitro* (Sawada *et al.*, 2004), whereas soluble VC70 can block sperm–vitelline coat binding (Matsuura *et al.*, 1995). In addition to the carboxy-terminal ZP domains, VC70 has six paired EGF–like repeats separated by regions that exhibit a high frequency of allelic polymorphisms among individuals (Sawada *et al.*, 2004). The potential of each polymorphic linker region to modulate how one allele of VC70 folds is fundamental to discriminate between self and nonself gametes, a property significantly more stringent than the species specificity required for sperm–ECM interactions in other animals. Similarly, the acrosome of abalone sperm remains intact until the gamete interacts with conspecific VERL filaments (Mozingo *et al.*, 1995). VERL, a member of the ZPX subfamily (Fig. 5), contains 28 tandem repeats of 150 residues in length (Galindo *et al.*, 2002). Between species, only the first two VERL repeats exhibit positive selection compared to the neutral purifying selection occurring over the remaining 26 repeats (Galindo *et al.*, 2003; Swanson *et al.*, 2001a; Swanson and Vacquier, 1998). Thus, like vertebrate ZPCs, both ascidian and abalone sperm receptors are diversifying while maintaining their overall structure by limiting sequence modifications to only a few regions selected for sperm interaction.

What does regional diversification in primary sperm receptor sequence imply about the assembly and evolution of egg ECMs, particularly in light of sexual conflict? The sexual conflict hypothesis postulates that an endless coevolutionary chase between the sexes is driving speciation through behavioral, anatomical, and molecular interactions (see Haygood, 2004; Swanson *et al.*, 2001b). Viewing the final criterion in light of gamete interactions, the egg should be dictating the diversification of a species because eggs are so outnumbered by sperm. Yet, critically analyzing the phylogeny of primary sperm receptors questions the penetrance of this process in gamete evolution. The ZP family contains at least three members that serve as primary sperm receptors throughout phylogeny, namely mammalian/anuran ZPC, ascidian VC70, and abalone VERL. As is required for species specificity in gamete interactions, these proteins exhibit regional positive selection in their sequences. Yet the diversifying domains are extremely restricted and are

found between otherwise conserved structural folds such as ZP domains, EF hands, and VERL repeats. In keeping with the overall conservation of the ECM assembly process within animal orders, the selection to retain the sperm receptor's overall structure clearly outweighs the need to diversify. But are the scattered regions under positive selection large enough to sweep into a population such that their sequences will lead to speciation? In the abalone, the 300 amino terminal residues of VERL repeats no. 1 and 2 are clearly sufficient to promote diversification without detriment to the remaining 3400 residues (Galindo *et al.*, 2002), and evidence from its complementary ligand shows that sperm are able to keep up with the changes (see Kresge *et al.*, 2000b; Lee *et al.*, 1995; Vacquier *et al.*, 1997). But what about the two patches of ZPC that together represent nearly 20% of the whole primary sequence? Is the conservation of the ZP domain fold sufficient to preserve protofilament assembly, even if most of these sequences are altered? Experimental evidence suggests that this is possible so long as none of the 260 residues encoding the ZP domain are affected (Jovine *et al.*, 2002), and natural selection would favor the loss of eggs that were not able to sustain such conservation (Dean, 2004; Rankin and Dean, 2000). Thus, the egg successfully maintains its advantage in the arms race against sperm by elegantly incorporating potentially sweeping changes into an otherwise conserved structure.

B. Oligosaccharide Sperm Receptors

The minimal sequence diversity in conserved sperm receptors cannot fully account for the plethora of animal species. Do other molecular components participate, specifically ones that significantly alter the surface profile of the sperm receptor without compromising the protein's structural conformation? Of all the protein modifications possible, animal eggs appear to rely on oligosaccharides to readily increase diversity. Given the appropriate transferases, a single chain of 1,6-linked sugar units can be synthesized using any available monosaccharide. Further complexity can be gained along the same polysaccharide backbone by alternative branching positions, covalent linkages made at 1,3 and 1,4 positions of a terminal or internal sugar unit that expands the oligosaccharide into the second dimension. Within the Golgi apparatus, branched or linear oligosaccharides are often covalently linked to proteins at asparagine (*N*-linked), serine, or threonine (both *O*-linked) residues found at select surfaces along a folded protein. The permutations available for oligosaccharide structure alone are more than sufficient to account for the number of species in most animal orders. But if monosaccharide composition and branch structure do not provide enough diversity, a simple nonsynonymous mutation to a putative *N*- or *O*-linked residue or a change in glycosylation

position could dramatically alter the surface profile and glycosylation pattern of a protein. Furthermore, specializations including acetylation, methylation, and sulfation of sugar residues are also possible, modifying the electrical charge on the oligosaccharide in a manner that could modulate ligand affinities. Thus, it should not be surprising that animals utilize specific oligosaccharides as the cofactors that mediate initial sperm–egg interaction (reviewed in Mengerink and Vacquier, 2001; Shalgi and Raz, 1997; Tanghe *et al.*, 2004; Tulsiani, 2000a).

A single protein can be linked to any number of oligosaccharides. Identifying which ones are responsible for sperm reception requires careful dissection of the composition of the protein. Similar methods have been used in all animals to assess which fragments and oligosaccharides may be critical for binding. These include the following binding assays: (1) competition, where sperm are challenged with oligosaccharides or glycopeptide fragments while assessing binding affinities to endogenous ECM proteins; (2) sperm activation, where sperm are preincubated with oligosaccharide or glycopeptides followed by scoring their degree of specific binding or the frequency of sperm activation as indicated by acrosome exocytosis; (3) loss of binding, where the ECM glycoprotein is enzymatically stripped of oligosaccharides and asked how effective they are at competing for sperm binding to control eggs; and (4) chimeras, where transgenic animals are created to test the role of specific protein backbones in the context of another species' glycosylation machinery. Once a fragment or an oligosaccharide has been identified functionally, the structure of the sugar may be determined using (5) lectin affinity analysis, which tests various plant lectins to determine the branching pattern and approximate composition of the candidate oligosaccharide, (6) monosaccharide release following glycosidase treatment, or (7) mass spectroscopy or NMR to establish a more detailed map of the molecule, including information on composition and branch points. The extensive data from this field of gamete glycobiology is beyond the scope of this chapter, but we include a partial listing of key findings from different animals (Table IV). In the following paragraphs, we highlight some of the observations made that further extend our understanding of the conservation of initial sperm–egg interactions and the sexual conflict of gamete receptor–ligand pairs.

The oligosaccharides presented to sperm are frequently composed of a variety of specific sugar residues. This enrichment reflects a process unique to oogenesis because the recombinant ZPC expressed in conspecific non-ovarian cell lines is not sperm receptive (Martic *et al.*, 2004), whereas *in situ* expression in eggs of heterospecific ZPC does yield functional protein (Doren *et al.*, 1999; Kinloch *et al.*, 1992; Moller *et al.*, 1990; Rankin *et al.*, 1998, 2003). Although it is theoretically possible to generate these moieties *de novo*, evidence suggests that animals directly incorporate dietary monosaccharides into their glycoproteins (Berger *et al.*, 1998; Martin *et al.*, 1998).

Table IV Table of Monosaccharides in Animal Egg Extracellular Matrices: Monosaccharide Reported to be Present in the Structure of Specific Molecules of Animal Egg[a]

Genus	Source	Linkage	L-Fucose	D-Galactose	Galactosamine	*N*-acetylgalactosamine	Glucosamine	*N*-acetylglucosamine	D-Mannose	D-Xylose	*N*-acetylneuraminic acid	*N*-glycolyneuraminic acid	Sialic acid	Other	Assay	Citation
Sus	ZPB	N-	■					■	■						5	Kudo *et al.*, 1998
	ZPC	N-		■				■					■ *		5	Yurewicz *et al.*, 1991
		N- (a)		■				■	■		■	■	■		5	Noguchi and Nakano, 1992
		N- (n)		■				■	■						5	Noguchi *et al.*, 1992
		O-		■		■	■								5	Yurewicz *et al.*, 1991
Oryctolagus	zona		■											D-Glucose*	1, 5	O'Rand *et al.*, 1988
Homo	zona		■												5	Lucas *et al.*, 1994
									■					D-Fructose	1	Mori *et al.*, 1993
Mus	n/a					■		■	■						2	Loeser and Tulsiani, 1999
	zona							■	■						1	Cornwall *et al.*, 1991
	ZPA	N-	■	■				■	■				■		5	Tulsiani, 2000
	ZPA / C	O-		■					■		■	■	■		5, 7	Easton *et al.*, 2000
	ZPC	N-	■	■			■	■					■		5	Tulsiani, 2000
		O-				■									1, 3	Florman and Wassarman, 1985
		O-	■	■											1, 6	Bleil and Wassarman, 1988
		O-													4	Kinloch *et al.*, 1995
		O-													1, 4	Chen *et al.*, 1998
Rattus	zona		■						m						1	Shalgi *et al.*, 1986
Sminthopsis	zona			■		■	■	■	■		■				5	Chapman *et al.*, 2000
Monodelphis	zona			■		■		■	■		■				5	Chapman *et al.*, 2000
Macropus	zona			■		■		■	■		■				5	Chapman *et al.*, 2000
Trichosurus	zona			■		■		■	■		■				5	Chapman *et al.*, 2000
Phascolarctus	zona			■		■		■	■		■				5	Chapman *et al.*, 2000
Pseudocherius	zona			■		■		■	■		■				5	Chapman et al., 2000
Isoodon	zona			■		■		■	■		■				5	Chapman *et al.*, 2000
Discoglossus	ZPA		■	■		■		■	■		■				5	Caputo *et al.*, 2001
		N-							■						5	Vaccaro *et al.*, 2001
		O-				■									5	Vaccaro *et al.*, 2001
	ZPC			■		■		■	■		■				5	Caputo *et al.*, 2001
	gp106		■												5	Maturi *et al.*, 1998
			■	■		■		■	■		■				5	Caputo *et al.*, 2001
	gp118		■												5	Maturi *et al.*, 1998
				■		■			■		■				5	Caputo *et al.*, 2001
	gp200, 260		■												5	Maturi *et al.*, 1998

Xenopus	jelly (J2, J3)							■			■				5	Bonnell and Chandler, 1996
	ZPC	N-	■	■				■	■		■				6	Vo *et al.*, 2003
Oncorhyncus	micropyle				■	■								Ceramide	1, 3	Yu *et al.*, 2002
Ciona	vit. coat		■				■	■	■		■				5	Rosati *et al.*, 1978
							■		■						5	De Santis *et al.*, 1980
			■												1	Rosati, 1985
Halocynthia	vit. coat	N-				■		■	■						1, 5	Baginski *et al.*, 1999
		O-		■ *		■		■	■	■ *				D-Arabinose	1, 5	Baginski *et al.*, 1999
Asterias	ARIS		■ *	■	■					■				D-Glucopyranose	2, 7	Koyota *et al.*, 1997
	coARIS		■	■	■					■				D-Glucopyranose	7	Koyota *et al.*, 1997
Arbacia	FSP		■ *												2, 7	Alves *et al.*, 1997
	FSP	O-	■												2, 3, 7	SeGall and Lennarz, 1979
	sialoglycan		■										■		2, 3, 7	SeGall and Lennarz, 1979
Echinometra	FSP													L-Galactose	2, 7	Alves *et al.*, 1997
Lytechinus	FSP		■												2, 7	Alves *et al.*, 1997
		O-	■ *												2, 3,	SeGall and Lennarz, 1979
			■												2	Biermann *et al.*, 2004
	sialoglycan		■										■		2, 3, 7	SeGall and Lennarz, 1979
Strongylocentrotus	FSP		■												2, 7	Keller and Vacquier, 1994
			■ *												2, 7	Vilela-Silva *et al.*, 1999
			■												2	Biermann *et al.*, 2004
		O-	■ *												2, 3, 7	SeGall and Lennarz, 1979
	sialoglycan		■										■		2, 3, 7	SeGall and Lennarz, 1979
				■		■		■	■						2, 7	Keller and Vacquier, 1994
Drosophila	micropyle		■	■		■		■	■		■				5	Perotti *et al.*, 1990
	vit. memb.							■	■						5	Perotti *et al.*, 1990
Limulus	vit. env.		■					■							1, 5	Barnum and Brown, 1983
Haliotis	VERL		■	■			■		■					D-Glucose	7	Swanson and Vacquier, 1997
Unio	gp273	N- (n)					■	■	■		■				7	Di Patrizi *et al.*, 2001
		O-													3	Focarelli and Rosati, 1995
		O-	■	■		■									7	Di Patrizi *et al.*, 2001

[a]Grayed boxes indicate the presence of the sugar residue.

(a) = acidic oligosaccharide chains; (n) = neutral oligosaccharide chains; * = sulfated residue; m = methylated residue; Fuc = L-fucose; Gal = D-galactose; GalNAc = *N*-acetylgalactosamine; GlcNAc = *N*-acetylglucosamine; Gln = Glucan; Glt = Galactan; (L-galactose); Glc = Glucosamine; Glu = Glucose; Man = D-mannose; NeuNAc = *N*-acetylneuraminic acid; NeuNGc = *N*-glycolyneuraminic acid; Sial = Sialic acid; * = Sulfated; Cer = Ceramide; Xyl = D-xylose; Gan = Galactosamine.

(1) Competition; (2) Sperm activation w/acrosome reaction; (3) Loss of binding to stripped ECM; (4) Chimera; (5) Lectin affinity analysis; (6) Monosaccharide release; (7) Gas chromatography, mass spectroscopy, or NMR.

In light of the abundance of rare monosaccharides in the animal egg ECM, such as L-fucose and *N*-acetylglucosamine (GlcNAc) (Table IV), might direct utilization of dietary saccharides be favored by oocytes? Such a hypothesis is consistent with the correlations reported between animal fitness and reproduction—specifically, the more physically fit an animal is, the more likely it will produce viable offspring. Because one significant influence of animal fitness is the quality of the diet, the observed enrichment of dietary sugars in the egg ECM suggests one method that gametes may use to exhibit the fitness of the individual they came from. For example, the predominant monosaccharide used by sea urchins is L-fucose (Alves *et al.*, 1997; Biermann *et al.*, 2004; Keller and Vacquier, 1994; SeGall and Lennarz, 1979; Vilela-Silva *et al.*, 1999). Because the main source of food for sea urchins is algae, a prominent source of L-fucose (Vanhooren and Vandamme, 1999), the selective enrichment of this hexose in the primary sperm receptor FSP (see Section V.A, earlier in this chapter) is compatible with the dietary and fitness contributions to fecundity.

The ability of sea urchins to conspecifically regulate the interaction of gametes based on the branching chain geometry of fucan (Alves *et al.*, 1997; Biermann *et al.*, 2004) represents an elegant form of oligosaccharide-based interactivity that is likely extended to other animals by the use of more complex glycan chains. It has been reported that most animals use L-fucose in their egg ECMs (Table IV). This hexose alone may serve as a backbone for further elaboration, consistent with the ability to generate more than 750 different isomers using a single tetra-fucan root (Alves *et al.*, 1997). Variability in the expression pattern of fucosyltransferase orthologs among different animals (Staudacher *et al.*, 1999) is consistent with distinct fucosylation profiles on the egg surface. The presence of D-galactose, D-xylose, and D-glucopyranose in addition to L-fucose on the primary starfish sperm receptive proteins ARIS and coARIS (see Section V.A, earlier in this chapter) (Baginski *et al.*, 1999; Koyota *et al.*, 1997) further enhances gamete selectivity between these two echinoderm families. Incorporation of monosaccharides such as GlcNAc and D-mannose, as observed in many other animal ECMs, may reflect further diversification because use of these common dietary sugars can generate significantly more elaborate oligosaccharide moieties. Incorporation of more unusual sugars, such as L-rhabanose and L-arabinose (Baginski *et al.*, 1999) or L-galactose (Alves *et al.*, 1997), would only serve to expand the possible permutations. In the case of the hermaphroditic ascidian, the incorporation of such ecologically rare sugars facilitates the process of allorecognition already used in their receptor VC70 to distinguish self from nonself (Sawada *et al.*, 2004). Similarly, the coexpression of the nonfucosylated sea urchin sialoglycan (Keller and Vacquier, 1994) might reflect requirements for additional factors to enhance both gamete specificity and the potency of sea urchin jelly (Hirohashi and Vacquier, 2002a). Finally,

modification of the oligosaccharide by sulfation or methylation could ensure that species-specific interactions occur at the egg surface.

The additional bias in monosaccharide use across different orders (Table IV) could also reflect changes in diet that certain animals were required to make during their transition to different habitats, thus allowing speciation to follow reproductive isolation. For example, with the move to land comes a different source and abundance of essential sugars, two factors that have a direct impact on the complexity of an egg's ECM. If true, then the use of a specific collection of monosaccharides in the egg ECMs of a single family of animals could suggest evolutionary relatedness. Such a transition can be seen in anurans, who prominently display L-fucose, D-mannose, GlcNAc, and *N*-acetylneuraminic acid on the egg surface (Caputo *et al.*, 2001; Maturi *et al.*, 1998; Vaccaro *et al.*, 2001; Vo *et al.*, 2003; Vo and Hedrick, 2000). This set appears to have been maintained through the separation of terrestrial marsupials (Chapman *et al.*, 2000) but later expanded upon in eutherians with the inclusion of *N*-glucosylneuraminic acid and sialic acid moieties (Easton *et al.*, 2000a; Noguchi and Nakano, 1992; Tulsiani, 2000b; Yurewicz *et al.*, 1991). Another event that favors additional complexity in sperm receptors may be occurring in teleosts, as suggested by the incorporation of ceramide in the moiety galactose-*N*-acetyl-β4-galactose-β4-glucan-β1-ceramide that is enriched at the micropylar entrance of *Oncorhyncus* eggs (Yu *et al.*, 2002). Thus, the collection of monosaccharides present on a functional sperm receptor may provide a rough estimation of the relationship between taxa.

C. Sperm Ligand for the Egg

The vast array of oligosaccharides used by eggs for sperm binding presents a challenge to the complementary gamete. How have sperm managed to keep up? One method is by maintaining diversity in their expressed carbohydrate-binding ligands (reviewed in O'Rand, 1988; Shalgi and Raz, 1997; Sinowatz *et al.*, 1995; Topfer-Petersen *et al.*, 1998, 2000). Unlike the egg, sperm have fewer constraints requiring them to maintain one class of ligands over another because sperm contribute few cytoplasmic or surface proteins to the zygote. Nevertheless, sperm appear to prefer catalytically inactive glycolytic enzymes and lectin-like molecules to function as egg ligands.

One primary advantage to using glycosidases as the ligand of the egg's receptor(s) is the complementary nature of the interaction. Although the specific sequence of events that lead to the final glycosylation pattern of the egg receptor for sperm is specific to oocytes, it is likely that the enzymes responsible for this process are conserved in other organs or even identical in the testis. Thus, little effort would be required for sperm to adopt posttranslational,

synthesis-related glycosidases for the purposes of egg binding. The major caveat to the exploitation of these enzymes, however, is the possibility for their activation. Most candidate glycosidases have counterparts in the Golgi, an acidic organelle whose major function is the conjugation of oligosaccharides to proteins destined for the extracellular environment. Hence, the optimal pH of most of these glycosidases tends to be acidic. Considering that the normal extracellular pH is higher than 6.5, the activity of such enzymes would likely be depressed, making them respectable ligands for mediating sperm–egg interactions.

Several major glycosidases with putative monosaccharide targets on the egg ECM can be found on the sperm surface. Using enzyme activity assays, the presence of surface glycosidases has been documented in animals such as dipterans (Cattaneo *et al.*, 1997), ascidians (Lambert, 1989), and eutherians (Loeser and Tulsiani, 1999; Rebeiz and Miller, 1999; Tulsiani *et al.*, 1990). The best-studied candidate is mammalian β-(1,4)-galactosyltransferase (GalTase). Under the appropriate conditions, this surface-expressed sperm isoform is capable of binding terminal GlcNAc residues on the respective egg sperm receptor (Table IV) and covalently attaching its endogenous substrate UDP-galactose (Loeser and Tulsiani, 1999; Rebeiz and Miller, 1999). Clustering of the GalTase also effectively activates sperm, inducing acrosome exocytosis (Macek *et al.*, 1991), a phenotype that is exacerbated when GalTase is overexpressed on the sperm surface (Youakim *et al.*, 1994). Yet in the absence of this enzyme, sperm are still fertile, albeit at a lower percentage than their comparable wild-type counterparts (Lu and Shur, 1997). Thus, the role of GalTase during murine fertilization is likely complemented by other surface proteins, a model consistent with the reported low- or no GalTase-associated activity in other eutherian sperm (Larson and Miller, 1997; Tulsiani *et al.*, 1990). Alternative glycosidases found on sperm include α-D-mannosidase in rats and humans (Cornwall *et al.*, 1991; Mori *et al.*, 1993; Tulsiani *et al.*, 1989, 1990), a mouse 95/116-kDa hexokinase (Kalab *et al.*, 1994; Leyton and Saling, 1989), and *N*-acetylglucosaminidase in ascidian (Lambert, 1989) and dipterans (Cattaneo *et al.*, 1997, 2002; Perotti *et al.*, 2001). Each of these candidates participates in species-specific binding to the egg ECM, especially to the sperm receptor derivatives, but like GalTase, they do not appear to be essential for sperm binding. In fact, *Drosophila* β-*N*-acetylglucosaminidase may be active *in vivo*, suggesting that its catalytic behavior participates in sperm competition at the micropyle by removing sperm-receptive proteins before subsequent sperm may enter and activate (Cattaneo *et al.*, 1997, 2002; Pasini *et al.*, 1999; Perotti *et al.*, 2001). Thus, the participation of sperm surface glycosidases appears to be supplementary during gamete interactions, facilitating the process of recognition rather than initiating the process of sperm activation.

Another mechanism for binding oligosaccharides uses more versatile sugar-binding ligands. Like their plant-derived lectin homologs, these low molecular weight molecules exhibit a high affinity for specific sugar moieties or oligomers. One such protein first described in *Mus* is sperm EGF-discoidin protein 1 (SED1) (Ensslin and Shur, 2003). This protein is synthesized by and localized on the surface of sperm heads, specifically to regions overlying the intact acrosome. SED1 is thought to interact with both ZPA and ZPC via one of its carboxy-terminal discoidin domains (Ensslin and Shur, 2003). The absence of a taxon-specific zona binding by SED1 reminds us that the purpose of some ligands may simply be to retain sperm attachment to the egg ECM rather than for recognition or conspecificity. Another generic oligosaccharide-binding family prefers the sulfated fucose-rich proteins found within the zona of mammalian eggs (O'Rand, 1988; O'Rand *et al.*, 1985, 1988; O'Rand and Fisher, 1987) (Table IV). These ligands were first identified in a screen of autoimmune serum raised against a cluster of 13–15 kDa rabbit sperm autoantigens (RSAs) (O'Rand *et al.*, 1988). Insemination in the presence of anti-RSA Fabs causes 60% inhibition of *in vitro* sperm–zona attachment and *in vivo* fertility (O'Rand, 1981). This is consistent with the ability of purified RSA to compete for sperm–egg associations at the zona surface (O'Rand *et al.*, 1988). Upon sperm–zona binding, RSA is believed to cluster along the sperm surface, thereby initiating a signaling cascade that facilitates acrosome exocytosis (O'Rand and Fisher, 1987). Thus, as predicted for GalTase and other glycosidases, RSA may act as a liaison between the zona surface and signaling ligands.

Spermadhesins represent a second major family of sperm-specific lectin-like proteins that participate in initial gamete attachment. Zona binding by the 12–16 kDa spermadhesins occurs via their single CUB domain (Topfer-Petersen *et al.*, 1998). The conformation of these β-strand CUB domains is maintained by positionally conserved disulfide bonds, creating a structure that can either homodimerize or heterodimerize to form a carbohydrate-binding pocket or multimerize in a side-by-side configuration to create a binding plane (Romero *et al.*, 1997; Varela *et al.*, 1997). They prefer branching oligosaccharides containing D-galactose, D-glucosamine, and D-mannose (Calvete *et al.*, 1996; Dostalova *et al.*, 1995a,b; Solis *et al.*, 1998). Most spermadhesins adsorb to the surface of the sperm during ejaculation but are lost during their transit through the oviduct. Thus, most spermadhesins are thought to prevent premature activation of sperm (Dostalova *et al.*, 1994). A minor fraction of glycosylated AWN-1, AQN-3, and HSP-7 is retained at the sperm plasma membrane over the apical third of the acrosomal cap, however, suggesting that they may participate in sperm–zona interactions (Calvete *et al.*, 1994, 1996; Dostalova *et al.*, 1995a,b; Reinert *et al.*, 1996; Robinson *et al.*, 1987). Unlike other family members secreted by accessory glands, this minor population of spermadhesins are also synthesized during

spermatogenesis and are presumed to remain with the sperm through direct interactions with specific phospholipids found in the sperm plasma membrane (Calvete *et al.*, 1994; Dostalova *et al.*, 1994; Sinowatz *et al.*, 1995). This subset of sperm-derived proteins also has a high affinity for heparin-like glycoproteins found in the zona (Calvete *et al.*, 1994, 1996; Reinert *et al.*, 1996), suggesting that AWN-1 and AQN-3 may participate in carbohydrate-dependent gamete interactions involving glucosamine moieties such as GlcNAc (Topfer-Petersen *et al.*, 1998). This is consistent with the ability of anti-AWN serum to inhibit the *in vitro* association of gametes (Sanz *et al.*, 1992) but has not been observed *in vivo* (Topfer-Petersen *et al.*, 1998). Interestingly, expression of spermadhesins is limited to ungulates such as *Sus* and *Equus*, suggesting that this family of proteins evolved after the split of ungulates from other eutherians (Dostalova *et al.*, 1994; Reinert *et al.*, 1996; Topfer-Petersen *et al.*, 1998). Such restricted expression in animals is likely a consequence of changes in both female anatomy and egg ECM glycobiology.

The range of sperm ligand classes among animal taxa reinforces the dynamic relationship between evolving gametes. Thus far, diversification or specialization in sperm expressed ligands appears to be limited to a few clusters of animal classes, in whom the types and sequences of these proteins are often highly conserved (e.g., conservation of RSA epitopes in many eutherians [O'Rand *et al.*, 1985] or greater than 98% sequence similarity between spermadhesins among ungulates [Reinert *et al.*, 1996]). Yet distinctions must also occur at the species level, possibly using ligands that are just as diverse as the target sperm receptor. Teleost sperm appear to have responded to the inherent complexity ingrained in the glycobiology of the sperm receptor (see Section VI.C, later in this chapter) by using oligosaccharides as the complementary ligand. Conspecific gamete binding at the *Oncorhyncus* micropyle is achieved with sperm ligand 2-keto-3-deoxy-D-glycero-D-galactononoic acid $\alpha2\rightarrow3$-galactose-$\beta4$-galactose-$\beta4$-glucan-$\beta1$-ceramide (Yu *et al.*, 2002). Thus, involvement of sperm carbohydrates at fertilization may also reflect how male fitness is evaluated during fertilization (see Section V.B, earlier in this chapter). This may be a phenomenon specific to teleosts, however, because their sperm lack an acrosome, the one organelle that could influence the effectiveness of a second round of species specificity analysis (Hart, 1990).

Perhaps sperm do not combat the diversity in the ECM with a single ligand but instead assemble a functional complex in response to egg receptor binding. Echinoderm sperm appear to use such a clustering mechanism to recognize the egg jelly triad responsible for initiating sperm chemotaxis (see Section V.A, earlier in this chapter). Sea urchins, for example, use a family of carbohydrate-binding proteins that preferentially associate with egg jelly. This family of receptors for egg jelly/polycystins (REJ/PCs) is represented by many members, including three original REJ proteins found

on the sperm as well as more distantly related polycystin proteins (Galindo *et al.*, 2004; Hughes *et al.*, 1999; Mengerink *et al.*, 2002; Moy *et al.*, 1996; Neill *et al.*, 2004; Neill and Vacquier, 2004). The core motifs of this family include a lectin domain, a calcium-dependent carbohydrate-recognition domain (CRD), a 1000-residue REJ module containing a carboxy-terminal G-protein–coupled receptor proteolytic cleavage site (GPS) and at least one carboxy-terminal transmembrane domain. The various members encode additional motifs that likely have distinct functions during sperm activation, including one additional CRD in REJ1 (Moy *et al.*, 1996), a single region of polycystic kidney disease (PKD) repeats and two transmembrane domains in REJ2 (Galindo *et al.*, 2004), or a series of tandem PKD repeats and 11 transmembrane domains that form a cluster homologous to ion channels in REJ3 (Mengerink *et al.*, 2002). Both REJ1 and REJ3 are cleaved at the GPS site, but the extracellular portion remains associated with the plasma membrane overlying the sperm acrosome (Mengerink *et al.*, 2002; Moy *et al.*, 1996; Trimmer *et al.*, 1985). It is hypothesized that these ectodomains remain associated with the plasma membrane via heterogenic protein–protein interactions, likely anchored to the membrane via unmodified transmembrane REJ/PC members (Mengerink *et al.*, 2002; Neill *et al.*, 2004).

The role of REJ/PC proteins during sperm activation is twofold. Original observations show that REJ1 directly binds the sulfated sea urchin jelly fucan (see Section V.B, earlier in this chapter) (Alves *et al.*, 1997; Biermann *et al.*, 2004; Bonnell *et al.*, 1994; SeGall and Lennarz, 1979; Vacquier and Moy, 1997). The specificity required by this interaction (Alves *et al.*, 1997; Biermann *et al.*, 2005; SeGall and Lennarz, 1979) is probably dictated by the CRDs because clusters of positively selected residues are found on the extended loops of CRD thought to participate in receptor recognition (Mah *et al.*, 2004). A high degree of nonsynonymous polymorphisms cluster at very specific sites along the protein is observed among individuals (Mah *et al.*, 2005)—clear evidence that an individual's sperm sustain a high level of adaptation to remain compatible with eggs (see Section V.A, earlier in this chapter). Further, species selectivity may be achieved by including REJ3 into the equation because of its preference for L-galactose of sialoglycan (Hirohashi and Vacquier, 2002b; Mengerink *et al.*, 2002). Upon binding of its specific FSP-sialoglycan receptive complex, REJ1–REJ3 is thought to participate in a single transduction event that leads to sperm activation. The stable association of this REJ1–REJ3 pair might induce extracellular calcium influx via the ectodomain's association with sea urchin PC2 (Neill *et al.*, 2004) or a putative transmembrane calcium channel (Mengerink *et al.*, 2002). The similarity of the transmembrane domains encoded by REJ3 and PC2 to transient receptor potential channels (TRPCs), a family of proteins intimately involved with the maintenance of cytoplasmic calcium levels via

extracellular sources (Zhu *et al.*, 1996), suggests an elegant mechanism that accounts for the immediate activation of sperm upon exposure to egg jelly (Vacquier, 1979). This is also consistent with the reported participation of *Mus* TRPC2 in calcium-dependent sperm activation at the zona (Jungnickel *et al.*, 2001).

D. Common Thread to Initial Sperm–Egg Interactions?

The initial interaction between sperm and egg involves a complex set of highly divergent molecules. The ability to modify the glycosylation status of the egg receptor on a whim provides a challenging situation for sperm, whose respective males have clearly adapted to such conditions by introducing a high frequency of individual polymorphisms within critical ligand domains (Mah *et al.*, 2005). Indeed, this may be one reason for the advantage in large populations of cheaply produced sperm; although individual sperm are extremely diverse, the cohort as a whole exhibits enhanced recognition and subsequent activation efficiency to favor fertilization of any conspecific egg encountered. The cofactors employed are not restricted to carbohydrate-binding molecules, either. For example, a 15-kDa sperm seminal vesicle proteinase inhibitor (SVI) receptor found embedded in the murine sperm plasma membrane can, upon association with ZPC, activate a pertussis toxin–sensitive G-protein–coupled signaling cascade that results in acrosome exocytosis (Aarons *et al.*, 1991; Boettger-Tong *et al.*, 1992; Robinson *et al.*, 1987). Thus, sperm have confronted the high level of uncertainty associated with each generation of egg receptor oligosaccharide moieties by (1) increasing the polymorphism of ligands at those specific interaction surfaces that recognize divergent regions of the receptors and (2) assembling ligand complexes that, together, have a greater probability of receptor recognition over each ligand alone. An egg's response to the quantity of sperm that now have the potential to fertilize it may be the incorporation of oviduct-derived glycoproteins into the ECM, partly to foster sperm attraction, but also to act as a distraction that serves to prolong the duration of sperm–egg interaction at the egg surface (Buhi, 2002; Mate *et al.*, 2000; Rodeheffer and Shur, 2004; Schmidt *et al.*, 1997).

VI. After the Acrosome Reaction

Sperm activation causes two major changes in sperm behavior. The first is a switch to chemotactic motility toward the ECM (see Eisenbach, 1999; Kaupp *et al.*, 2003; Neill and Vacquier, 2004). The second involves release

of the sperm acrosome, whereby a collection of new membrane-associated proteins from the luminal face of the acrosome is exposed, promoting additional sperm–egg ECM binding partners, whereas soluble content proteins are released into the local environment. Some of the soluble components are known to facilitate the progression of sperm through the ECM. Here, we review the events involved with acrosome exocytosis and the consequences of its release on sperm–egg interactions (see Table II). In particular, we focus on the content of proteins released and the role of each in advancing the sperm towards the egg plasma membrane.

A. Secondary Sperm Contact

Secondary sperm binding is an important opportunity available after the acrosome reaction because it raises the stringency of species specificity. Acrosome exocytosis requires that the delimiting acrosome membrane fuse with the plasma membrane, resulting in the transformation of surface ligands on the sperm. Many of these ligands are contributed by the inner acrosome matrix, the content proteins most closely juxtaposed to the luminal face of the acrosome membrane, but some may also migrate from other unaffected sites on the sperm head. This alteration in the surface profile of the sperm provides both gametes with a second checkpoint for conspecificity.

Abalone sperm prominently display their acrosome contents on the surface, allowing these proteins to participate in subsequent interactions. Although chemoattraction and sperm activation are conspecifically regulated in these animals (Riffell *et al.*, 2002), it is still possible for heterospecific gamete interactions to occur because of the distribution of various species within the same habitat (Riffell *et al.*, 2004). Abalone sperm have selected for the acrosome-derived lysin, a 16-kDa protein with a high affinity for the filamentous VERL of the vitelline envelope. The lysin-VERL dissolution process is often conspecific in a mixed population of animals (Hellberg and Vacquier, 1999; Vacquier and Lee, 1993; Vacquier *et al.*, 1990). Specificity of this interaction is enhanced through positive selection at most of the exposed surfaces of the lysin fold (Lee *et al.*, 1995; Vacquier and Lee, 1993; Yang *et al.*, 2000) and in the first and second VERL repeats on the receptor (Galindo *et al.*, 2003). Under situations of heterospecific insemination, significantly more lysin is required to bind and dissociate VERL (Vacquier *et al.*, 1990), a quantity too high to be released by the paltry mollusk acrosome (Buckland-Nicks and Hodgson, 2000; Hylander and Summers, 1977; Togo and Morisawa, 1999; Usui, 1987); thus, heterospecific gamete fusion is averted.

The major acrosome-derived echinoderm protein responsible for secondary gamete contact is bindin, a 30-kDa protein homologous in at least 70%

of the echinoid orders (Vacquier and Moy, 1977; Vacquier *et al.*, 1995; Zigler and Lessios, 2003). Bindin contains a highly conserved core sequence enriched in nonpolar residues, flanked by two hypervariable domains, including a carboxy-terminal domain of repeats whose quantity differs in sister species (Biermann, 1998; Metz and Palumbi, 1996; Minor *et al.*, 1991; Palumbi, 1999; Zigler and Lessios, 2003; Zigler *et al.*, 2003). The carboxy-terminal tandem repeats of bindin homodimerize to form a lectin-like binding pocket with high affinity for sulfated fucan polymers (DeAngelis and Glabe, 1987; Glabe *et al.*, 1982; Ruiz-Bravo *et al.*, 1986; Vacquier and Moy, 1977; Vacquier *et al.*, 1995). The predicted characteristics of this protein match its egg receptor, EBR1, the sea urchin egg bindin receptor. EBR1 is a 350-kDa sulfated glycoconjugate found within the vitelline layer whose oligosaccharides are enriched with L-fucose, D-mannose, D-galactose, and galactosamine (Aketa *et al.*, 1968; DeAngelis and Glabe, 1987; Dhume and Lennarz, 1995; Foltz and Lennarz, 1990; Foltz *et al.*, 1993; Kamei and Glabe, 2003; Ohlendieck *et al.*, 1993; Rossignol *et al.*, 1984; Ruiz-Bravo *et al.*, 1986). The functional motifs of EBR1 include thrombospondin type 1 (TSP-1) repeats (Adams, 1997), CUB domains (Bork and Beckmann, 1993), and a highly conserved cytoplasmic tail that may participate in intracellular signaling (Foltz and Lennarz, 1993; Foltz *et al.*, 1993). Receptor aggregation is thought to occur via the TSP-1 and CUB domains (Romero *et al.*, 1997; Varela *et al.*, 1997), whereas its selectivity for bindin is linked to the type of protein interaction domains encoded by the carboxy-terminal segment of the receptor's ectodomain (Foltz and Lennarz, 1990; Foltz *et al.*, 1993; Kamei and Glabe, 2000, 2003; Ruiz-Bravo *et al.*, 1986). For example, *Strongylocentrotus franciscanus* EBR1 contains homogenous EBR1-specific repeats consisting of TSP-1/CUB tandems, whereas the orthologous region in *S. purpuratus* encodes hyalin-like repeats (Wessel *et al.*, 1998). Such complete divergence between these repeat motifs may also be responsible for the variable *O*-linked oligosaccharide composition and branching patterns observed among species (Foltz and Lennarz, 1990; Hirohashi and Lennarz, 2001) and is a likely contributor to the species specificity of the EBR1-bindin interaction (Glabe and Vacquier, 1977b; Lopez *et al.*, 1993; Minor *et al.*, 1991; Ohlendieck *et al.*, 1993; Ruiz-Bravo *et al.*, 1986). In some species, however, carbohydrate moieties do not significantly affect the affinity of the bindin–EBR1 pairing (Kamei and Glabe, 2000); in such cases, specificity may be more effectively determined at a more distal site to the egg, such as in the jelly (see Section V.A, earlier in this chapter) (Biermann *et al.*, 2004).

Vertebrate ZPA is an egg-derived glycoprotein thought to be responsible for binding acrosome-reacted sperm. This preference, however, varies across taxa. Teleosts, whose sperm lack acrosomes, may have selected variants (e.g., ZPAX) or duplications of ZPX and ZPC to replace the activity of ZPA proposed for other vertebrates (Fig. 5). In most other animals,

acrosome-containing sperm are reported to preferentially bind ZPA glycoproteins at specific sites along the sperm head, including the acrosome-derived membrane of anuran sperm (Barisone *et al.*, 2002; Maturi *et al.*, 1998; Vaccaro *et al.*, 2001; Vo and Hedrick, 2000; Vo *et al.*, 2003) and the postacrosomal domain and midpiece of acrosome-reacted murine sperm (Kerr *et al.*, 2002; Tsubamoto *et al.*, 1999). Positive selection has been observed in a region totaling about 4% of the ZPA gene (Swanson *et al.*, 2001b), implying that like ZPC and sea urchin EBR1, this member participates in species-specific discrimination. The comparative affinity of acrosome-reacted murine sperm for ZPA, however, is significantly lower than for ZPC (K_d of about 200 nM, compared to 63 nM for ZPC) (Kerr *et al.*, 2002), suggesting that the reported sperm–ZP protein preferences are not absolute. Is it possible for a relatively large sperm head to distinguish between individual ZP members within a long oligomer of ZPA–ZPC pairs (see Section II.B, earlier in this chapter)? Probably not since the acrosome-membrane–associated sperm protein sp56/AM67, named for its orthologs from mouse (sp56) and guinea pig (AM67), preferentially binds ZPC (Bleil and Wassarman, 1990; Bookbinder *et al.*, 1995; Easton *et al.*, 2000b; Foster *et al.*, 1997). Yet, this protein is exposed to the zona only after the acrosome reaction (Foster *et al.*, 1997), where it is believed to multimerize via its β-strand Sushi domains to achieve a lectin-like binding pocket that specifically recognizes ZPC oligosaccharides (Cheng *et al.*, 1994). Consistent with the participation of both ZPA and ZPC in sperm binding is the higher sperm–zona interactions of ZPB-null compared to wild-type eggs, indicative of more accessible ZPA–ZPC filaments for sperm attachment (Rankin *et al.*, 1999). Similarly, the acrosome-intact sperm surface protein SED1 does not discriminate among ZPA or ZPC proteins in the zona (Ensslin and Shur, 2003), whereas acrosome-intact *Sus* sperm show a synergistic enhancement in zona binding in the presence of the ZPB–ZPC dimer (Yurewicz *et al.*, 1998). Also consider the sperm hyaluronidase PH-20. Originally found on the posterior surface of acrosome-intact guinea pig sperm and used to penetrate the cumulus cell complex, PH-20 is thought to also be invloved with secondary sperm-binding reactions (Hunnicutt *et al.*, 1996b; Myles and Primakoff, 1997). After the acrosome reaction, a fraction of the remaining membrane-bound PH-20 migrates to the inner acrosome matrix and is cleaved (perhaps by one of the acrosome-derived proteases) (see Section VI.B, later in this chapter), exposing a nonenzymatic carboxy-terminal domain. Antibody studies suggest that the fragment of PH-20 still associated with the plasma membrane participates in secondary sperm binding (Hunnicutt *et al.*, 1996a). It is also possible that the nonenzymatic domain of PH-20 is involved with sperm attachment to the same zona receptor pre-acrosome and postacrosome reaction, thereby obviating a need for the sperm to reattach to the ECM surface after acrosome exocytosis.

It is clear that sperm do not exhibit single-receptor preferences at any stage of sperm–egg ECM interaction. This is advantageous for both gametes because the wider range of receptor-ligand pairs used will bias for conspecific fertilization. The observation that most egg receptors evolve with regional positive selection (Swanson *et al.*, 2001b) further enhances the argument that the most effective method to achieve species-specific gamete interactions enlists a collection of selective molecular mechanisms.

B. Acrosomal Proteases

The egg ECM is a fibrillar matrix that does not, at first, appear to be readily penetrable (Fig. 3), yet sperm do regularly penetrate this matrix. One hypothesis to explain this phenomenon is that sperm gain access to the egg membrane through hydrolytic digestion of the ECM fibrils, gradually creating a tunnel that eventually allows for complete penetration. Thus, it was assumed that acrosomal contents included proteases that would participate in the lysis of the egg ECM. One caveat associated with the digestion of a tunnel through the mammalian zona to accommodate the sperm head is the potential loss of secondary sperm-binding epitopes. Nevertheless, the model for proteolytic digestion of the ECM is supported by the observed depression in fertilization rates associated with the including of protease inhibitors during insemination (Ciereszko *et al.*, 1994, 1996, 2000; Dabrowski *et al.*, 2004; Jones *et al.*, 1996; Rios and Barros, 1997; Sawada *et al.*, 1984b; Takano *et al.*, 1993; Takizawa *et al.*, 1993; Yamagata *et al.*, 1998a). Hence, the search for acrosome-derived proteases was initiated.

The first candidate protease described was acrosin, a 55-kDa serine protease containing a single disulfide linkage between the amino- and carboxy-terminus of its catalytic domain (see Sawada, 2002; Topfer-Petersen *et al.*, 1990). Acrosin is stored in the acrosome in its inactive zymogenic form. Upon release into a more alkaline environment, the enzyme is activated by autocatalysis just downstream of the amino-terminal cysteine, freeing the catalytic domain from its constricted conformation without the loss of the light chain from the proteolytic heavy chain. Among ascidians and eutherians, the animals in whom acrosin is most conserved, the only major variation in primary sequence is the addition of two CUB domains at the carboxy-terminus of the ascidian ortholog (Kodama *et al.*, 2001; Sawada, 2002). Ascidian acrosin is thought to bind the vitelline coat via its CUB domains, yet the predicted separation of these CUB domains from the active chains of acrosin leaves the true function of these binding motifs in question (Kodama *et al.*, 2001). Instead, both ascidian and eutherian acrosin attach to the ECM via positionally conserved basic residues in its amino-terminus, possibly binding through sulfated fucans within their respective egg ECMs

(Baginski *et al.*, 1999; Howes and Jones, 2002; Howes *et al.*, 2001; Jansen *et al.*, 1998; Jones, 1991; Jones *et al.*, 1996; Moreno and Barros, 2000; Richardson and O'Rand, 1996; Topfer-Petersen *et al.*, 1990; Urch and Patel, 1991).

The participation of acrosin proteolysis during sperm penetration, however, remains unclear. First, no evidence has been reported that clearly shows acrosin-dependent digestion of the ascidian vitelline coat (Sawada *et al.*, 1984a). Similarly, *in vitro* insemination with a wide range of serine protease inhibitors never completely inhibits eutherian fertilization (Takano *et al.*, 1993), and acrosin-null sperm are still able to fertilize eggs, albeit with a 30-min delay in sperm penetration compared to heterozygous or wild type genotypes (Adham *et al.*, 1997; Baba *et al.*, 1994; Honda *et al.*, 2002; Nayernia *et al.*, 2002). The retention of acrosin on the surface of acrosome-reacted sperm, particularly at domains most available for ECM binding, suggests an alternative role for acrosin as a tether that maintains sperm–egg interactions (Castellani-Ceresa *et al.*, 1983; Howes *et al.*, 2001; Huang and Yanagimachi, 1984; Jones, 1991; Noguchi and Nakano, 1992; Sawada, 2002; Urch and Patel, 1991). Acrosin could also be involved with the dispersal and/or activation of acrosome proteins (Honda *et al.*, 2002; Takano *et al.*, 1993; Yamagata *et al.*, 1998b) because the presence of endogenous levels of activity during fertilization is consistently associated with a selective advantage over sperm with low levels of activity, as observed in mice (Adham *et al.*, 1997; Yamagata *et al.*, 1998a) and humans (Cui *et al.*, 2000; Shimizu *et al.*, 1997).

In light of the fertilization rates observed in acrosin-compromised sperm, a search for other acrosome-derived proteases has also identified candidates that could be involved with the protease inhibitor–dependent phenotype. One protease present in both ascidians and eutherians is spermosin, another serine protease released from the acrosome (Kodama *et al.*, 2002; Sawada, 2002). Spermosin contains a proline-rich domain at its amino-terminus and a carboxyl ECD (Glu-Cys-Asp) motif thought to enhance the enzyme's association with the ECM. This is best documented by the *in vitro* association between ascidian spermosin and both a 28-kDa vitelline coat protein and VC70 (Kodama *et al.*, 2002; Sawada, 2002; Sawada *et al.*, 1996). Like acrosin, however, the absence of detectable vitelline coat debris following spermosin exposure leaves its role in sperm penetration in question (Sawada *et al.*, 1984a). Another family of proteins postulated to participate in sperm penetration is the testicular serine proteases (TESPs), specifically TESP1, TESP2, TESP4, and TESP5 (Honda *et al.*, 2002). Of particular interest is murine TESP5, a protease that co-migrates with a suspected 42-kDa trypsin-like activity responsible for digesting the zona of both wild-type and acrosin-null mice that is present in sperm lipid rafts, structures thought to be involved with cell–cell signaling (Honda *et al.*, 2002). TESP5 is limited to

Mus, however, suggesting that this animal has co-opted the use of two different trypsin-like proteases, perhaps selecting for allotypes with lower activity compared to other eutherians (Honda *et al.*, 2002).

The first evidence that sperm-dependent ECM degradation occurs was documented during ascidian fertilization (Lambert, 1989). One mechanism for this process depends on a 35-kDa chymotrypsin-like protease that specifically depletes the outer electron-dense layer of the *Ciona* vitelline coat (Marino *et al.*, 1992; Sawada *et al.*, 1998). Extracellular proteasomes have also been implicated in the digestion of the vitelline coat (Saitoh *et al.*, 1993; Sakai *et al.*, 2003; Sawada *et al.*, 1998, 2002a,b). This family of multienzyme complexes is normally found in the cytoplasm of most cells, where it participates in general housekeeping and cell homeostasis via its ubiquitin-mediated method of degradation. Yet even the extracellular members, such as the 20S (620-kDa) and cognate 26S (930-kDa) ascidian proteasomes, are able to efficiently degrade polyubiquitinated targets like the ascidian sperm receptor VC70 (Saitoh *et al.*, 1993; Sakai *et al.*, 2003; Sawada *et al.*, 2002a,b). The conservation of extracellular proteasome in deuterostomes is implied by additional reports from sea urchin (Matsumura and Aketa, 1991) and human sperm (Morales *et al.*, 2004; Rossato *et al.*, 1999; Wojcik *et al.*, 2000). In deuterostomes, proteasome activity is associated with specific regions of the sperm head (Morales *et al.*, 2004; Sawada *et al.*, 1996; Wojcik *et al.*, 2000). Also, micrograms per milliliter of free ubiquitin, an essential cofactor of proteasomes, have been found in human seminal fluid (Lippert *et al.*, 1993; Wojcik *et al.*, 2000); application of free ATP, as suggested by the *in vivo* rise in oviductal ATP upon ovulation, to sperm during *in vitro* fertilization increases success with infertile males (Rossato *et al.*, 1999); and anti-proteasome antibodies are found in the seminal fluid of clinically infertile males (Bohring *et al.*, 2001). Yet in contrast to a direct role for proteasome-dependent degradation in ascidians (see later discussion), no ultrastructural evidence for requisite ECM proteolysis exists in mammals.

The exact mechanism of protease-dependent dissolution of the ascidian vitelline coat may proceed as follows (Lambert, 1989): Upon exocytosis of the small apical acrosome (Fig. 6), the sperm plasma membrane becomes intimately attached to the vitelline coat (De Santis *et al.*, 1980; Lambert, 1989). Following digestion of a physical hole in the vitelline coat by the proteasome, the sperm cytoplasm and nucleus are extruded, leaving the mitochondrion and the tail extending along the extracellular surface of the vitelline coat. Acrosin and spermosin may act to sterically mask potential sperm-binding sites along the vitelline coat that would otherwise impede the progression of the acrosome-reacted sperm plasma membrane as it slides through the hole, perhaps via the detached CUB domains of acrosin (Kodama *et al.*, 2001) and/or by associations along the plasma membrane that also mask or modify potential ECM-associating ligands.

A similar mechanism of sperm penetration requiring the creation of a hole in the ECM is documented in modern decapods, suggesting that another animal order depends on direct proteolysis of the egg ECM (Fig. 6). Decapod sperm lack flagellum, or any other motile organelle; their mobility is restricted to the reaction initiated by acrosome exocytosis (Hinsch, 1971; Medina and Rodriguez, 1992; Tsai and Talbot, 1993). Following primary contact with the egg ECM, the decapod sperm acrosome dehisces, causing violent hydration of its contents outside the sperm (Tsai and Talbot, 1993). The next phase of the acrosome reaction involves a circular contraction along the tip of the former anterior sperm cap, forcing the remaining plasma membrane to evert (Medina and Rodriguez, 1992; Tsai and Talbot, 1993). This process causes a preformed filament stored in the posterior end of the sperm to extend forward, carrying the nucleus with it. Thus, the sperm tip travels some 10 μm closer to the egg surface within seconds of initial contact with the ECM (Tsai and Talbot, 1993). Penetration of these immotile sperm through a dense fibrous ECM requires lysis of the matrix to create a tunnel that facilitates the sperm's progress (Hinsch, 1971; Rios and Barros, 1997). The source of this lysis is thought to be a protease released from the acrosome. In fact, a trypsin-like activity that specifically degrades a 72-kDa protein from the shrimp *Rhynochocinetes* vitelline envelope has been reported, but the absence of a definitive acrosome in its sperm leaves the source of this enzyme in question (Rios and Barros, 1997), a situation historically reminiscent of the status of the ascidian sperm until the discovery of the minute ascidian acrosome (De Santis *et al.*, 1980). Still, even at low concentrations of enzyme, the proteolytic of sperm-penetration model remains plausible in shrimp, whose acrosomal filament is not ejected until 10 min after initial sperm–ECM contact (Lindsay *et al.*, 1992a). In contrast, the rate of sperm penetration in *Homarus* and *Uca* gametes *in vitro* (on the order of seconds from primary sperm binding to complete eversion [Tsai and Talbot, 1993]) is too fast to accommodate the activity of enzymatic degradation, suggesting that a complement of processes may be required for rapid vitelline envelope penetration in these decapods. For example, these sperm may utilize the combination of a chaotropic ECM softener or hyperactive protease and the mechanical force of eversion and/or acrosomal filament extension to successfully penetrate the vitelline envelope.

C. Penetration of Egg ECM in the Absence of Proteolysis

With the exception of ascidians and *Rhynochocinetes*, the absence of identified sperm-derived protease substrates from the egg ECM brings into question the actual mechanism used by sperm to penetrate the egg ECM (see Section VI.B, earlier in this chapter). The simplest method of penetration in

the absence of protein degradation is observed in animals whose eggs possess micropyles. In teleosts such as *Danio* (Mengerink and Vacquier, 2001; Wolenski and Hart, 1987) and *Rhodeus* (Ohta and Iwamatsu, 1983), no sperm acrosome exists, so the involvement of acrosome-derived proteases is moot (Figs. 1 and 6). Instead, the process of penetration is likely a consequence of modifications in intracellular signaling cascades in response to the relative position of the sperm within the micropyle (Yu *et al.*, 2002). The same may even hold true for more primitive fish such as chondrosteans, whose sperm exocytose their acrosome at the micropylar entrance, followed by extension of an acrosomal process through the pit to rapidly contact the fusogenic egg membrane at the other end (Cherr and Clark, 1986; Kobayashi *et al.*, 1994; Kobayashi and Yamamoto, 1994). A similar reaction occurs in dipterans, whose acrosome is also released upon contact with the glycoprotein tuft at the micropylar entrance (Figs. 1 and 2), exposing an acrosomal rod that participates in sperm penetration of the tuft (Degrugillier and Leopold, 1976). The observed sperm-dependent loss in surface glycosidases following the acrosome reaction likely facilitates passage of the sperm by removing high-affinity receptors along the micropylar entrance (Cattaneo *et al.*, 1997, 2002; Perotti *et al.*, 2001).

Animals that use preestablished tunnels similar to a micropyle also do not require mechanisms to dramatically alter the integrity of the ECM. For example, the journey of flagellate mollusk sperm is made easier by channels in the vitelline envelope that terminate in fusogenic microvilli (Figs. 1–4). These sperm also use a preformed microtubule filament that extends away from the anterior face of the nucleus and transects the anterior acrosome (Buckland-Nicks and Hodgson, 2000; Hylander and Summers, 1977; Togo and Morisawa, 1999; Usui, 1987). Following the acrosome reaction, the membrane-delimited filament is exposed and coated with the acrosome content proteins (Lewis *et al.*, 1982; Usui, 1987). In general, the acrosome filament is slightly shorter than the distance from the ECM surface to the microvillar occupant, so the acrosome-reacted sperm need only push the filament a short distance into the vitelline envelope to contact the egg membrane (Buckland-Nicks and Hodgson, 2000; Hylander and Summers, 1977). Indenting the vitelline envelope surface requires more than just mechanical force, however. In the case of gastropods such as teguline or abalone, the evolutionarily divergent lysin chaotropically dissociates the vitelline envelope to facilitate the sperm head's progress through the ECM (Hellberg and Vacquier, 1999; Lewis *et al.*, 1982). The 16-kDa acrosome-derived lysin irreversibly binds its oligomeric glycoprotein receptor via hydrophilic residues found on its α-helices, thereby converting a high-affinity hydrogen-bonded interactive surface along the VERL repeat into a hydrophilic interface that is easily separated (Galindo *et al.*, 2002; Kresge *et al.*, 2000a,b, 2001). This process essentially unzips VERL macromolecules,

separating them into individual subunits, a process that can domino through the vitelline layer to provide sperm full access to the microvilli at the terminus if enough lysin is present (Kresge *et al.*, 2001; Lewis *et al.*, 1982; Vacquier *et al.*, 1990). Given the concerted evolution of the VERL repeats (Galindo *et al.*, 2002, 2003), however, this domino effect could prove detrimental because fortuitous separation of VERL along its first two repeats by a heterospecific sperm lysin could unzip the remainder of the molecule (see Section VI.A, earlier in this chapter) (Swanson and Vacquier, 1997; Vacquier and Lee, 1993; Vacquier *et al.*, 1990). Thus, the heterospecific sperm would quickly gain access to the egg and possibly fertilize it, leaving at least one embryo with incompatible haploid genomes (Kresge *et al.*, 2001; Swanson and Vacquier, 1997).

Most animals do not synthesize eggs with preestablished sites for sperm access in their ECM or use single molecules that offer lenient safeguards against heterospecific secondary sperm binding (see Section VI.A, earlier in this chapter). Instead, sperm must mechanically penetrate the matrix to access the egg plasma membrane. A dramatic example of this process occurs during fertilization of the primitive crustacean *Limulus*, whose flagellate sperm uses an acrosome filament with stored mechanical energy (Tilney, 1975). Upon binding fucan residues exposed on the vitelline envelope (Barnum and Brown, 1983; Brown, 1976), *Limulus* sperm acrosome react, forming an anterior collar that remains attached to the surface of the ECM while the actin-based acrosome filament elongates from the subacrosomal domain with a right-handed helical turn (Tilney, 1975; Tilney *et al.*, 1979). The curvature of the filament allows the sperm to screw itself into the matrix ahead, carrying the sperm nucleus closer to the egg surface while the flagellum and collar remain perpendicular and peripheral to the ECM (Brown, 1976; Tilney, 1975). Like a corkscrew inserting into cork, the actin filament would mechanically displace and fray the ECM fibers. No ultrastructural evidence for such displacement has been reported, however, suggesting either that limited fragmentation occurs or that acrosome contents participate in the dispersal of the ECM glycoproteins.

Echinoderm sperm use both the force of their acrosomal processes and the flagellar movement to penetrate the jelly and to contact the delicate vitelline layer draped over the egg's microvilli (Anderson, 1968; Chandler and Heuser, 1980; Chandler and Kazilek, 1986; Glabe and Vacquier, 1977a; Larabell and Chandler, 1991; Runnstrom, 1966) (Fig. 2). Within seconds after the acrosome reaction, cytoplasmic calcium concentrations and pH rise in echinoid sperm (Neill and Vacquier, 2004). This causes, respectively, acrosome exocytosis and polymerization of actin monomers originating from the actomere, an actin-organizing center found anterior to the nucleus and posterior to the acrosome proper (Dan *et al.*, 1964; Schatten and Mazia, 1976; Tilney, 1978). Polymerization results in the extension of a membrane-delimited acrosomal

process toward the egg surface (Dan *et al.*, 1964; Schroeder and Christen, 1982; Tilney *et al.*, 1978) (Fig. 6). The acrosomal contents coating the acrosomal process thus gain immediate access to its cognate receptors found in the vitelline layer (Moy and Vacquier, 1979). The primary protein responsible for coating this acrosomal process is bindin (Vacquier and Moy, 1977; Vacquier *et al.*, 1995). Bindin preferentially associates with EBR1 within the vitelline layer (see Section VI.A, earlier in this chapter) (DeAngelis and Glabe, 1987; Kamei and Glabe, 2003; Ohlendieck *et al.*, 1993; Rossignol *et al.*, 1984). Conspecific association of bindin and EBR1 is thought to facilitate agglutination of the gametes, whereby the sperm and egg are drawn together through the adhesive and mutually attractive forces of this receptor–ligand pair (Glabe, 1985a; Glabe and Vacquier, 1977b; Glabe *et al.*, 1982; Lopez *et al.*, 1993; Vacquier and Moy, 1977). The egg jelly protein FSP responsible for triggering the acrosome reaction is also rich in fucose (Keller and Vacquier, 1994) (Table IV). Thus, premature acrosome reaction in the jelly could retard the progression of sperm toward the egg. Given the brief 20-sec half-life a sperm has from the time of acrosome reaction to successfully fuse (Vacquier, 1979), any retardation of progress in the jelly could prove detrimental to the sperm's success yet is advantageous for monospermic fertilization since fewer competent sperm will interact with the egg.

Mechanical penetration of vertebrate egg ECMs is also possible, despite their relatively thick fibrillar matrices. The acrosome reaction of many anuran sperm occurs within the jelly (see Section V.A, earlier in this chapter) (Campanella *et al.*, 1997; Elinson, 1986; Maturi *et al.*, 1998; Vaccaro *et al.*, 2001; Vo and Hedrick, 2000; Vo *et al.*, 2003). In these animals, penetration through the remaining jelly coat likely relies on the morphology of the acrosome-reacted sperm head, a long tapered cone or pointed rod that can be used to stab through the fibrillar ECM (Arranz and Cabada, 2000; Campanella *et al.*, 1997). Such a model is also parsimonious for all anurans, even those taxa whose sperm remain acrosome intact until reaching the vitelline envelope (Arranz and Cabada, 2000; Barisone *et al.*, 2002; Toro and Michael, 2004). Further evidence for the application of mechanical force during sperm penetration of the vertebrate egg ECM has been reported in avians and mammals. Holes can be found in the avian perivitelline layer, yet the morphology of these holes is indicative of the dispersion of avian perivitelline layer filaments rather than proteolysis (Howarth, 1990; Okamura and Nishiyama, 1978a) (Fig. 3). Similarly, frayed tunnels were reported in some marsupial zonae following insemination (Breed and Leigh, 1990; Jungnickel *et al.*, 1999; Rodger and Bedford, 1982b) (Fig. 3). Combined with the observations from anurans, the absence of detectable proteolytic fragments from these vertebrates has resulted in an alternative model for vertebrate sperm penetration of the egg ECM (Breed and Leigh, 1990):

Following the acrosome reaction, content proteins such as acrosin (see Section VI.B, earlier in this chapter) or β-*N*-acetylglucosaminidase (Miller *et al.*, 1993b) adsorb to their appropriate receptors, sterically masking these sites within the ECM from the sperm to limit otherwise retardant interactions between the gametes. The morphology of the acrosome-reacted sperm head would dictate how the sperm mechanically penetrates the ECM, namely by piercing through with a long narrow head or by pushing filaments aside using an oscillating motion, as predicted for the scythe-shaped head of murine sperm (Bedford, 1998) (Figs. 1 and 6). In this way, the sperm gains access to the egg membrane without severely altering the integrity or the mass of the ECM, a result that may be essential for the assembly of a permanent block to polyspermy using components of this very same egg ECM.

VII. Climax of Fertilization

Once the sperm head has successfully penetrated the egg ECM, its remaining obstacle before completing fertilization is the egg plasma membrane. The theoretical energy necessary for fusion of two inflexible planar membranes is upwards of 93 kcal/mol for mammals at physiological temperatures (Siegel, 1993). The composition of the membrane significantly impacts both the absolute energy required to achieve such intermediates (Basanez, 2002; Kozlovsky and Kozlov, 2002; Kuzmin *et al.*, 2001; Markin and Albanesi, 2002). This is best observed in zona-free hamster eggs, who normally can fuse with all species of mammalian sperm (Talbot and Chacon, 1982). Pretreatment of these eggs with phospholipase C (PLC), however, abolishes this promiscuity, presumably as a consequence of the resultant alteration to membrane composition (Boldt *et al.*, 1988).

Catalysts have a significant impact on the rate of membrane fusion. The most effective protein catalysts include the SNARE (soluble *N*-ethylmaleimide sensitive factor attachment protein receptor) protein complexes (Bentz and Mittal, 2000; Lang *et al.*, 2001; Lentz *et al.*, 2000), viral membrane glycoproteins (Basanez, 2002; Lentz *et al.*, 2000; Shemer and Podbilewicz, 2003), and homologs of the nematode fusogenic protein *eff-1* (Shemer and Podbilewicz, 2003). The participation of such protein catalysts is consistent with the loss-of-fusion phenotypes following pronase pretreatment of acrosome-reacted human sperm (Arts *et al.*, 1997).

Two additional components that appear to be necessary for fusion include active proteases (Boldt *et al.*, 1988; De Santis *et al.*, 1992; Kato *et al.*, 1998; Roe *et al.*, 1988; Takano *et al.*, 1993) and extracellular calcium, as suggested by the reduced efficiency of fusion in the absence of this divalent cation (Glabe, 1985b; Tilney *et al.*, 1978; Yanagimachi, 1978). This cation-dependence is consistent with the participation of calcium in other

membrane-fusion events (Abbott and Ducibella, 2001; Ales *et al.*, 1999; Crabb and Jackson, 1985; Horsley and Pavlath, 2004; Lang *et al.*, 2001; Lentz *et al.*, 2000). Here, we survey the mechanisms of membrane fusion in the context of fertilization, focusing on catalysts likely responsible for the union of a sperm and an egg (see Table II).

A. Sites of Fusion and Membrane Properties

Even though a single sperm has gained access to the egg membrane, it must be near a fusion-competent site to complete fertilization. Most animal eggs use microvilli, specifically microvillar tips, as the preferred site of fusion (Buckland-Nicks *et al.*, 1988; Evans, 1999; Hart, 1990; Hylander and Summers, 1977; Longo *et al.*, 1986; Myles, 1993; Talbot and Chacon, 1982; Vigil, 1989; Wolenski and Hart, 1987). These cell-surface extensions provide the egg with two features that dramatically improve fusion efficiency: (1) an increase in overall surface area that is proportional to the square of the radial gain provided by average microvillus length and (2) discrete sites to localize and concentrate fusion machinery. One added benefit is the intimate association of microvilli with the actin cytoskeleton, which allows for rapid remodeling of the membrane in ways that can significantly increase the rate of sperm incorporation and the surface area of plasma membrane participating in a single fusion event. For example, many animals exhibit the formation of a fertilization cone at the site of sperm fusion, an extension of many local microvilli that together engulf the sperm head to rapidly complete fusion (Buckland-Nicks and Hodgson, 2000; Goudeau and Becker, 1982; Hart, 1990; Schatten and Mazia, 1976; Wolenski and Hart, 1987) (Fig. 6). In some teleosts, the extensiveness of this cone is sufficient to plug the micropylar canal, thereby ensuring that the egg is monospermic (Ohta and Iwamatsu, 1983) (Figs. 3 and 6).

Animal eggs use a wide range of fusion-competent domains along their membranes (Figs. 3 and 6). The method selected for may be related to what the sperm/egg ratios of insemination are *in vivo* (see Section IV, earlier in this chapter). For example, eggs of broadcast spawners such as mollusks and echinoderms show no distinct preferences for where the sperm may fuse, possibly because their effective dilution of gametes in the open ocean significantly reduces the number of sperm that may encounter an egg. One exception, is the bivalve *Unio* egg, which has a very small fusogenic domain that likely evolved in response to the high sperm/egg ratios encountered following gamete concentration in the suprabrachial chambers of the gills after spawning (Focarelli *et al.*, 1988). Like echinoderms, mammalian eggs are receptive over the majority of the egg surface, with the exception of the region overlying the meiotic spindle (Evans, 1999; Myles, 1993). Such a large

promiscuous fusogenic surface implies that the mammalian oviduct and egg are extremely efficient at regulating the sperm/egg ratio. Amphibians, whose method of insemination significantly increases the sperm/egg ratio, produce eggs that preferentially fuse at the animal hemisphere (Elinson, 1986). Again, the large surface area primed for fusion may be a consequence of the efficacy of the surrounding jelly in limiting the number of sperm that reach the egg surface. One exception to this generalization lies with *Discoglossus*, whose egg restricts sperm fusion to a single depression at the animal pole (Talevi and Campanella, 1988). Thus, the most restrictive sites are associated with animals that either spawn in close quarters (avian, petromyzontids) or utilize micropyles (dipterans, chondrosteans, teleosts) (Cherr and Clark, 1986; Harper, 1904; Hart, 1990; Mouzaki *et al.*, 1991; Neubaum and Wolfner, 1999; Okamura and Nishiyama, 1978b).

Sperm also appear to possess discrete fusion-competent domains. Teleost sperm, for example, generally fuse along the equatorial band (Hart, 1990; Wolenski and Hart, 1987). Similarly, echinoderms and mollusks preferentially initiate fusion at the apical tips of their acrosomal projections, although fusion can proceed anywhere along the side (Buckland-Nicks *et al.*, 1988; Hylander and Summers, 1977; Schatten and Mazia, 1976). The echinoderm sperm head, for example, is drawn towards and rotated parallel to the egg surface following initial membrane contact, presumably to increase the total surface area available to rapidly complete fusion (Schatten and Mazia, 1976). Acrosome-reacted eutherian sperm, however, prefer the equatorial segment and anterior third of the postacrosomal region (Arts *et al.*, 1993; Clark and Koehler, 1990; Myles, 1993; Talbot and Chacon, 1982; Vigil, 1989). This β-hydroxysterol–rich equatorial segment (Clark and Koehler, 1990) significantly lowers the energy threshold for fusion by increasing membrane flexibility (Basanez, 2002; Markin and Albanesi, 2002).

B. Egg Contributions to Fusion

Protein-dependent catalysis of plasma membrane fusion requires contributions from both membranes. Because the major effect these proteins have on fusion is a reduction in the energy requirements for membrane deformation, it is hypothesized that these catalysts are associated with the plasma membrane or complex with other membrane-affiliated proteins (Basanez, 2002; Bentz, 2000; Bentz and Mittal, 2000; Lentz *et al.*, 2000). Only a few candidate molecules fit these criteria in eggs. The first is the sea urchin sperm receptor EBR1 (egg bindin receptor). This freely soluble receptor is a member of the "a disintegrin and metalloprotease" (ADAM; also known as "metalloprotease/disintegrin/cysteine-rich," or MDC) family of proteins (Evans, 1999; Kamei and Glabe, 2003). This family of transmembrane

proteins classically encode a metalloendoprotease domain followed by a disintegrin loop, a cysteine-rich motif, and an EGF–like repeat within the ectodomain (Evans, 1999). EBR1 encodes a metalloprotease domain but lacks the disintegrin repeat. Association of EBR1 with the vitelline layer likely occurs through its CUB and/or thrombospondin type 1 (TSP-1) repeats (Kamei and Glabe, 2003). Its candidacy as a member of the gamete fusion complex comes first from its sequence, specifically the metalloprotease domain whose putative enzymatic activity is critical for sperm–egg fusion in sea urchins (Kato *et al.*, 1998) and ascidians (De Santis *et al.*, 1992). Second, EBR1 associates with sperm bindin, making it a possible coordinator of the bindin-dependent agglutination and fusion observed *in vitro* (see Section VII.C, later in this chapter) (Glabe, 1985a,b; Glabe and Vacquier, 1977b).

Another female-contributed protein important for membrane fusion is egg CD9, a mammalian member of the tetraspanin protein family (reviewed in Hemler, 2003; Kaji and Kudo, 2004). Tetraspanins are integral membrane proteins containing four transmembrane domains and a large central extracellular loop thought to participate in direct protein–protein interactions with other membrane-associated receptors and signaling enzymes. A subclass of tetraspanins is implicated in the process of cell–cell fusion, including *late bloomer*, involved in establishment of neuromuscular junctions in *Drosophila* (Kopczynski *et al.*, 1996); the interacting pair CD82/CD81 (C33/M38) that facilitates human T-cell leukemia virus type 1–dependent T-lymphocyte fusion (Imai and Yoshie, 1993); and CD9/CD81, which promotes myoblast fusion (Tachibana and Hemler, 1999). Within the egg, CD9 localizes specifically at the microvillar tips on the egg (Chen *et al.*, 1999). The role of CD9 in fertilization was first observed in knockout mice, whose most significant phenotype is sterility because of a failure of sperm–egg fusion (Kaji *et al.*, 2000; Le Naour *et al.*, 2000; Miyado *et al.*, 2000; Zhu *et al.*, 2002). Overexpression of CD81, a putative binding partner also implicated in plasma membrane fusion events, rescues the CD9-null phenotype (Kaji *et al.*, 2002). As with other tetraspanins, the extracellular loop—especially residues Ser^{173}-Phe^{174}-Gln^{175}—is essential for retaining CD9-dependent fusion (Zhu *et al.*, 2002). It is thought that CD9 is required for organizing egg membrane proteins like integrins and other tetraspanins, thereby enhancing cell aggregation and potentiating fusion (Maecker *et al.*, 1997; Zhu *et al.*, 2002). Such coordinated activity of CD9 would ultimately facilitate the initial stage of sperm–egg fusion by localizing all the necessary cell–cell contact and fusion machinery to a cluster of microvilli near the site of sperm–egg membrane attachment (Chen *et al.*, 1999; Kaji *et al.*, 2000, 2002).

Based on their potential interactions with the actin cytoskeleton and membrane-associated tetraspanins, the superfamily of integrins is also suspected to participate in gamete fusion (reviewed in Berditchevski, 2001;

Evans, 2001; Hemler, 2003; Maecker *et al.*, 1997). Each member of the integrin family is a heterologous receptor composed of an α-subunit and a β-subunit. Several members have been detected on the egg surface, including eutherian α_2, α_3, α_5, α_6, α_9, α_M, α_V, β_1, β_2, β_3, β_4, β_5, and β_6 (He *et al.*, 2003; Sengoku *et al.*, 2004) and sea urchin α_B- and β_C-subunits (Murray *et al.*, 2000). Based on observations made in other fusogenic mammalian cells, only the paired $\alpha_6\beta_1$ and $\alpha_9\beta_1$ heterodimeric pairs are believed to participate in CD9-dependent gamete fusion. Yet functional and genetic knockdown experiments of every integrin pairing possible in eutherian eggs, via antibody or genetic manipulations, have shown that none of the known egg surface integrins is essential for fertilization (Eto *et al.*, 2002; He *et al.*, 2003; Kaji and Kudo, 2004; Miller *et al.*, 2000; Sengoku *et al.*, 2004; Zhu and Evans, 2002). No additional reports have been presented for the sea urchin integrin, but based on the results from eutherian experiments, their participation in fusion is unlikely.

C. Sperm Contributions to Fusion

As observed in all other gamete receptor–ligand pairs, the list of candidate sperm catalysts that may participate in gamete fusion is extremely diverse compared to the shorter list of shared egg contributors. One candidate is part of the SNAREs, a family of proteins that often assemble into specific multimeric complex at the future site of vesicle-plasma membrane fusion (Bentz, 2000; Chamberlain *et al.*, 2001; Conner *et al.*, 1997; Ikebuchi *et al.*, 1998; Lang *et al.*, 2001; Pelham, 2001; Tahara *et al.*, 1998). The sperm-specific Vam6p (vesicle-associated membrane protein type-6) is present in both acrosome-intact and acrosome-reacted sperm, although its localization changes dramatically upon sperm activation (Brahmaraju *et al.*, 2004). The abundance of Vam6p over the acrosome implies that, with the assistance of SNAP, this protein participates in acrosome fusion during sperm activation. Following the acrosome reaction, the only detectable Vam6p is found at the fusogenic equatorial band (Brahmaraju *et al.*, 2004). Based on the current models of SNARE-mediated membrane fusion, Vam6p would have to lie between the two membrane faces (Bentz, 2000; Lentz *et al.*, 2000). Antibody inhibition of Vam6p blocks sperm–egg binding, consistent with an ectopic orientation (Brahmaraju *et al.*, 2004). But this phenotype does not directly implicate it in the process of fusion because it is not clear how the normally cytoplasmic vesicle-associated protein flips across the membrane to become an extracellular protein capable of binding the egg.

A broader list of sperm-derived candidates has been identified through fusion-inhibiting screens using monoclonal antibody raised against antigens from the highly-fusogenic regions of *Mus* and *Cavia* sperm (see Myles, 1993).

Two of these antigens, (IgM) M29 (Saling *et al.*, 1983) and mMN9 (Toshimori *et al.*, 1998), localize exclusively to the equatorial region of acrosome-reacted sperm. Their respective immunoglobulins inhibit gamete fusion, but not membrane attachment between the gametes (Saling *et al.*, 1983; Toshimori *et al.*, 1998). The target antigen of mMN9 is the acrosome-derived equitorin, and is functional as a contraceptive that blocks sperm fusion mechanisms (Toshimori *et al.*, 1998; Yoshinaga *et al.*, 2001). A third candidate from such a screen is PH-30, the α-subunit of eutherian fertilin (Primakoff *et al.*, 1987). Fertilin is a mammalian heterodimeric member of the ADAMs family of proteins; thus, both sperm and eggs could contribute the necessary metalloendoprotease activity required for fusion (see Section VII.B, earlier in this chapter) (De Santis *et al.*, 1992; Evans, 1999; Roe *et al.*, 1988). Fertilin consists of an α-(ADAM1) and a β-(ADAM2) subunit that both retain their ability to bind the egg surface via their disintegrin domains, together forming one potential candidate egg receptor part of the CD9-dependent signaling network (Blobel *et al.*, 1990; Eto *et al.*, 2002; Lum and Blobel, 1997; Takahashi *et al.*, 2001; Zhu and Evans, 2002). Within these domains are two functionally conserved motifs thought to participate in gamete fusion: the β-subunit's functional ECD (Glu-Cys-Asp) protein-binding motif and the α-subunit's "fusion peptide," a stretch of hydrophobic residues homologous to a viral fusion protein (Blobel *et al.*, 1990; Evans *et al.*, 1997a,b; Konkar *et al.*, 2004; Lum and Blobel, 1997; Myles and Primakoff, 1997; Nishimura *et al.*, 2002; Primakoff *et al.*, 1987; Zhu *et al.*, 2000). The acidic residues of the conserved ECD motif are necessary and sufficient for sperm–egg association (Konkar *et al.*, 2004; Zhu *et al.*, 2000), whereas the fusion peptide of fertilin α is thought to provide the impetus for membrane deformation and pore formation during fusion (Basanez, 2002; Myles and Primakoff, 1997). In the absence of functional *Mus* sperm fertilin β, both sperm binding to zona-free eggs and membrane fusion are impaired compared to wild type, although the 50% reduction in fusion is probably a compounded effect of the 13% reduction in gamete binding (Cho *et al.*, 1998; Myles and Primakoff, 1997; Nishimura *et al.*, 2001). Though not as penetrant as a CD9 knockout (see Section VII.B, earlier in this chapter), this reduced-fertility phenotype further supports the hypothesis that fertilin is involved with the final stage of fertilization (Bronson *et al.*, 1999; Nishimura *et al.*, 2001; Zhu and Evans, 2002; Zhu *et al.*, 2000).

The persistence of low levels of fusion competency following loss of sperm fertilin raises the question of how redundant the ADAMs protein function is along the surface of an acrosome-reacted sperm. Initially, the low sequence similarity among fertilin orthologs within the fusion peptide domain and the nonfunctional human fertilin α pseudogene suggests that fertilin proper is likely not involved in fusion of all eutherian gametes (see Jury *et al.*, 1997, 1998; Myles and Primakoff, 1997). The discovery of complete

fertilin α paralogs in mice (Nishimura *et al.*, 2002) and primates (Hooft van Huijsduijnen, 1998; Jury *et al.*, 1998), however, indicates that alternate forms of the fertilin heterodimer can exist. These neighboring intron-less genes encode fertilin α paralogs of different mass with one paralog displaying a carboxy-terminal truncation while another in humans lacks an appropriate active catalytic histidine (Hooft van Huijsduijnen, 1998; Jury *et al.*, 1998; Nishimura *et al.*, 2002). These isoforms are predicted to be distinctly and alternatively expressed during spermatogenesis (Nishimura *et al.*, 2002). Yet, even though deletion of any of the fertilin α paralogs is detrimental to the surface expression of the fertilin heterodimer, the observed retention of fusion competency of these sperm suggests that non-fertilin alternatives are compensatory (Nishimura *et al.*, 2004; Primakoff and Myles, 2002).

One of the proteins thought to supplement and/or compensate for fertilin during gamete fusion is the ADAM cyritestin (ADAM3) (Evans, 1999, 2001). As with fertilin, the extracellular binding loop of cyritestin is hypothesized to be critical for fusion, as shown by peptide competition assays using its extracellular loop sequence (Kaji and Kudo, 2004; Takahashi *et al.*, 2001). Although elimination of cyritestin from the sperm surface completely abolishes *in situ* fertilization following mating, these knockout sperm are still able to fuse with zona-free eggs *in vitro* (Nishimura *et al.*, 2001; Shamsadin *et al.*, 1999). These knockout results in mice are consistent with data in humans that show no impaired fertility in the absence of functional *cyritestin* transcript (Frayne and Hall, 1998; Grzmil *et al.*, 2001). Furthermore, the absence of both *Mus* fertilin and cyritestin does not further impair the fertilin β–null fusion phenotype during insemination of zona-free eggs (Cho *et al.*, 1998; Nishimura *et al.*, 2001), suggesting that cyritestin functions in the oviduct prior to fusion (Nishimura *et al.*, 2001, 2004; Shamsadin *et al.*, 1999).

Production of functional sperm ADAMs is also linked, as documented by the impaired surface expression of fertilin or cyritestin when the reciprocal protein is knocked out (Nishimura *et al.*, 2001, 2004). This sensitivity to surface protein levels suggests the importance of ADAMs in fusion (Bronson *et al.*, 1999; Evans, 2001; Takahashi *et al.*, 2001). This is further supported by competition assays that report impaired *in vitro* fertilization when the highly conserved ADAM disintegrin loop sequence RX_8DLPEF is present (Eto *et al.*, 2002). Although no known egg integrin is directly responsible for mediating cell fusion, the possibility remains that an egg-specific integrin or a mimetic found at the microvillar tips is involved (Myles and Primakoff, 1997). Localization of fertilin and cyritestin to lipid rafts (Nishimura *et al.*, 2001) implies that eutherian fusion is dependent on intracellular signaling to coordinate the complex sequence of steps. Thus, an initial sketch of the molecular mechanisms involved in gamete fusion could be described as thus:

Binding of the disintegrin loop to its egg receptor initiates a series of cytoskeletal modifications that remodel the location of tetraspanins such as CD9 or CD81. This would reorient neighboring microvilli toward the site of the bound sperm. In total, these rearrangements move the necessary proteins to sites of successive fusion events. Given the correct ADAM subunit, a functional fusion peptide could be used to initiate the membrane deformation required for fusion. Such a model is consistent with the close proximity of tetraspanins and ADAMs binding domains, as observed by the steric inhibition fertilin- or cyritestin-dependent binding using antibodies against CD9 or CD81 (Chen *et al.*, 1999; Maecker *et al.*, 1997; Takahashi *et al.*, 2001). This signaling-dependent mechanism of action is consistent with the reported ability to activate *Xenopus* eggs upon exposure to the disintegrin loop encoded by the *Xenopus* ADAM ortholog xMDC16 (Shilling *et al.*, 1998).

Although the reported involvement of metalloendoproteases during fusion of deuterostome gametes is also consistent with a role for ADAM members at fertilization (De Santis *et al.*, 1992; Kato *et al.*, 1998; Roe *et al.*, 1988), the current ADAM candidates do not appear to be likely candidates for this proteolysis. Processing during spermatogenesis is thought to cleave the metalloendoprotease domains from both fertilin subunits before encountering the egg (Blobel *et al.*, 1990; Lum and Blobel, 1997). It is possible that the fertilin β metalloendoprotease domain remains tethered to the surface by association with its transmembrane domain or other surface binding partners, as shown to occur with other sperm proteins (Neill *et al.*, 2004). But the absence of tethering evidence requires that the proteolytically competent fertilin β be ignored for now. NL1, a soluble alternative to the ADAMs, may have been selected for in eutherians instead. NL1 is a secreted sperm-specific zinc metalloendoprotease that preferentially degrades neuropeptides enriched with aliphatic and aromatic residue (Ghaddar *et al.*, 2000). Its participation in fertilization is evident from the reduced fertility of male mice lacking functional NL1 (Carpentier *et al.*, 2004). One hypothesized role for NL1 is in sperm–egg signaling, possibly via its activity on proenkephalin derivatives found in the sperm acrosome (Carpentier *et al.*, 2004; Ghaddar *et al.*, 2000; Kew *et al.*, 1990). The involvement of NL1 in cell signaling is consistent with the model that sperm proteases may be active during the rapid reorganization of the egg cortex in preparation for fusion. Alternatively, the essential enzymatic activity could be associated with the egg, as observed in sea urchins. The final step prior to fusion in these animals requires the participation of the ADAM protein EBR1 (see Section VII.B, earlier in this chapter) (Kamei and Glabe, 2003). EBR1, however, only contains a canonical metalloendoprotease domain; no disintegrin domain is present. Thus, in echinoderms, reunification of the egg metalloendoprotease with a sperm-contributed disintegrin domain might trigger the

release of a fusion peptide into the appropriate plasma membranes, thereby initiating membrane deformation and fusion.

The retention of fertilization in the absence of metalloendoproteases still implies that other proteins participate in the actual fusion event. One such candidate is Izumo, the antigen of the potent fusion-blocking monoclonal antibody OBF13 (Inoue *et al.*, 2005). Izumo is a sperm transmembrane protein containing a single extracellular immunoglobulin-like domain. It is only egg-accessible after the acrosome reaction, when it is found over the most fusogenic region of the sperm head (Inoue *et al.*, 2005). Unlike sperm ADAMs, deletion of the functional *Izumo* gene does not impair expression of other candidate sperm fusion proteins (Inoue *et al.*, 2005). Yet sperm from these knock-out males are fusion-incompetent, suggesting that Izumo is necessary and sufficient to mediate mammalian sperm–egg fusion (Inoue *et al.*, 2005). The corresponding receptor for Izumo is hypothesized to be egg CD9 (see Section VII.B, earlier in this chapter), based on this tetraspanin's promiscuous associations with other immunoglobulin family members (Hemler, 2003). Association of Izumo with other, perhaps compensatory, egg sperm receptors is likely because deletion of CD9 still retains a low percentage of fertility (Kaji *et al.*, 2000; Miyado *et al.*, 2000), whereas sperm lacking Izumo are incapable of fusing with eggs *in vitro* or *in vivo* (Inoue *et al.*, 2005).

Invertebrate gamete fusion also appears to be dependent on the activity of nonenzymatic proteins from the acrosome (see Section VI, earlier in this chapter). For example, the participation of sea urchin EBR1 in fusion depends entirely on the properties of its sperm ligand bindin; in abalone, it depends on VERL and its sperm ligand sp18, a divergent 18-kDa paralog to sperm lysin (Swanson and Vacquier, 1995a,b). Both proteins localize to the extended tips of their respective acrosomal process or filament, the structures most likely to make first contact with the egg microvilli (Buckland-Nicks *et al.*, 1988; Moy and Vacquier, 1979; Mozingo *et al.*, 1995; Swanson and Vacquier, 1995b). Bindin alone promotes cell–cell aggregation (Glabe and Vacquier, 1977b; Lopez *et al.*, 1993); both proteins can induce mixed-phase liposome aggregation followed by direct fusion (Glabe, 1985b; Hong and Vacquier, 1986; Swanson and Vacquier, 1995b). In sea urchin bindin, this latter activity is directed by the hydrophobic 18-residue core "fusion peptide" conserved in 70% of all echinoderms (Vacquier *et al.*, 1995; Zigler and Lessios, 2003), possibly by a mechanism common to viral- or SNARE protein–dependent membrane fusions (Bentz, 2000; Bentz and Mittal, 2000; Knecht and Grubmuller, 2003; Lentz *et al.*, 2000). The fusogenic properties of abalone sp18, on the other hand, are attributed to its amphipathic fold rather than a specific fusion domain (Swanson and Vacquier, 1995b). The hydrophobicity of each respective domain suggests that these fusion catalysts act to overcome the electrostatic repulsion of membranes, holding them in an intermediate

state of deformation that thermodynamically favors mixed-phase lipid bilayer fusion (Glabe, 1985b; Hong and Vacquier, 1986). Both sperm-derived proteins prefer the negatively charged fluid-phase lipid phosphatidylserine but are reciprocally affected by the presence of divalent cations; bindin-mediated fusion rates are enhanced by Zn^{2+} whereas sp18 rates are retarded by most divalent cations (Glabe, 1985b; Hong and Vacquier, 1986; Swanson and Vacquier, 1995b), properties that may be consequences of their different modes of operation. Whether the transmembrane egg receptors for bindin or lysin, EBR1 or VERL, respectively, participate in the aggregation events has not been addressed experimentally. The proposed role of sea urchin EBR1 as a metalloendoprotease (see Section VII.B, earlier in this chapter), however, suggests that proteolytic activity could initiate a conformational change that releases bindin's fusion peptide towards the egg and plasma membranes. Thus, the hydrophobic properties of bindin alone could be sufficient to achieve fusion.

D. Extracellular Calcium

The role of extracellular calcium during fusion remains questionable. Although many fusion catalyzing complexes have been shown to be activated by calcium (Conner *et al.*, 1997; Crabb and Jackson, 1985; Lentz *et al.*, 2000; Tahara *et al.*, 1998), the ubiquitous presence of calcium in most extracellular media (see Sections III.B, earlier in this chapter, and Section IX.A, later in this chapter) suggests that the functional target for calcium is intracellular. One family of proteins that could participate in the influx of such calcium may be the TRPCs. These canonical ion channels are responsible for the restoration and maintenance of intracellular calcium stores in a voltage-independent fashion, a mechanism referred to as "capacitative calcium entry" (Putney and Ribeiro, 2000; Zhu *et al.*, 1996). TRPC members have been found along the surface of the sperm head, specifically overlying the midpiece and sperm acrosome (Castellano *et al.*, 2003; Mengerink *et al.*, 2002; Neill *et al.*, 2004). Thus, a primary role of TRPC homologs may be in the activation of sperm motility (Castellano *et al.*, 2003) and during the acrosome reaction when they are required to maintain high intracellular calcium levels long enough to complete exocytosis (see Section V.C, earlier in this chapter) (Jungnickel *et al.*, 2001; Mengerink *et al.*, 2002; Neill *et al.*, 2004). A secondary role is postulated for a TRPC member during fusion based on the failure of *Caenorhabditis* sperm to fuse with the egg in the absence of TRPC-3 (Xu and Sternberg, 2003). Nematode sperm lack acrosomes (Singson, 2001), thereby obviating the need for a bona fide calcium channel that facilitates vesicle exocytosis. Of all the known TRPC members, only the TRPC-3 homolog has been implicated in gamete fusion

(Castellano *et al.*, 2003; Jungnickel *et al.*, 2001; Mengerink *et al.*, 2002; Neill *et al.*, 2004; Trevino *et al.*, 2001). Thus, one hypothesis is that the influx of ions through TRPC-3 may be responsible for local influx of calcium into the sperm head just before fusion. Because of the presence of TRPCs in lipid rafts of the sperm head (Trevino *et al.*, 2001), TRPC-3 could be a target of specific signaling pathways that respond to egg receptors or to other extracellular interactions, as proposed for sea urchin TRPC homologs (Mengerink *et al.*, 2002; Neill *et al.*, 2004). Opening of the TRPCs would promote the local elevation of calcium, leading to conformational changes in membrane-associated proteins, such as SNARE proteins, which might be involved with membrane deformation. Such deformations in the sperm plasma membrane would be translated directly to extracellular machinery, thereby lowering the threshold of energy required to complete fusion.

VIII. A Denouement

Gamete fusion is correlated with the activation of many signaling cascades that contribute to the block to polyspermy and to egg activation. Sperm alone are capable of inducing such processes in the egg, suggesting that contributions from their membrane and/or cytoplasm are sufficient to release the egg from quiescence. For example, initiation of gamete fusion in sea urchins has been correlated with a local loss of CGs as the fertilization cone expands to accommodate the sperm pronucleus (Buckland-Nicks and Hodgson, 2000; Goudeau and Becker, 1982; Longo *et al.*, 1986; Schatten and Mazia, 1976). In some animals, the rapid flux of intracellular calcium necessary for these cortical changes may also be responsible for electrically altering the voltage potential across the membrane, resulting in the temporary activation of a series of voltage-dependent ion channels that are responsible for establishing an initial block to supernumerary sperm fusion. Elevation of intracellular calcium concentrations in the zygote is also required to completely activate the beginning of development. Here, we briefly review the conservation of these processes immediately following animal fertilization, focusing on those events responsible for enhancing monospermy. More detailed coverage of the signaling events at fertilization may be found elsewhere (see Bement, 1992; Jaffe *et al.*, 2001; Santella *et al.*, 2004).

A. Fast Electrical Block to Polyspermy

The fast electrical block to polyspermy present in many animals is dependent on changes to the voltage potential across the zygotic plasma membrane. The flux of specific monovalent ions through transmembrane channels is

the major contributor to the fast electrical block, and dictates whether the membrane potential (V_m) rises (depolarization) or falls (hyperpolarization) from the egg's resting state. Upon fertilization, most animals exhibit a depolarization of the membrane (see Fig. 6) including most marine animals (Dufresne-Dube *et al.*, 1983; Goudeau *et al.*, 1994; Gould and Stephano, 2003; Hagiwara and Jaffe, 1979; Jaffe, 1976; Moccia *et al.*, 2004; Togo and Morisawa, 1999; Togo *et al.*, 1995), primitive fish (Kobayashi *et al.*, 1994), primitive urodeles (Iwao, 1989), and anurans (Charbonneau *et al.*, 1983; Cross and Elinson, 1980; Glahn and Nuccitelli, 2003; Jaffe and Schlichter, 1985; Jaffe *et al.*, 1983a; Nuccitelli *et al.*, 1988). Decapod embryos, however, exhibit a transient hyperpolarization (Goudeau and Goudeau, 1986, 1989, 1996; Gould and Stephano, 2003). The absolute change in V_m is often greater than 30 mV and may persist for 60 s to 10 min, depending on the animal and the combinations of ion channels employed (reviewed in Gould and Stephano, 2003; Hagiwara and Jaffe, 1979), but must subside before egg activation and development can progress (Iwao and Jaffe, 1989).

The ion flux that changes V_m involves specific chloride, sodium, and/or potassium channels found in the egg plasma membrane (Cross and Elinson, 1980; Dufresne-Dube *et al.*, 1983; Goudeau and Goudeau, 1986, 1989; Grey *et al.*, 1982; Iwao, 1989; Iwao and Jaffe, 1989; Jaffe and Schlichter, 1985; Kobayashi *et al.*, 1994; Nuccitelli *et al.*, 1988; Obata and Kuroda, 1987; Togo and Morisawa, 1999; Togo *et al.*, 1995). The participation of sperm-derived factors likely initiates the fast electrical block because changes in V_m are usually triggered at the site of gamete fusion and propagate rapidly and uniformly along the entire egg membrane (Fall *et al.*, 2004; Iwao and Jaffe, 1989; McCulloh and Chambers, 1992). In *Discoglossus* eggs, however, the arrangement of ion channels within the fusogenic dimple mechanically restricts the change in V_m to a region slightly wider than the dimple (Nuccitelli *et al.*, 1988; Talevi and Campanella, 1988). Thus, the potency of a fast electrical block is geographically optimized at the membrane surfaces responsible for fertilization while minimally affecting global ion homeostasis. Voltage-clamp studies of other anuran eggs, which do not exhibit such specializations for fusion, have shown that the efficacy of depolarization is instead dependent on its maximum amplitude. For example, the peak V_m achieved by one species is optimized to block supernumerary fusion of only conspecific sperm, and is not sufficient to repel less sensitive heterospecific sperm who require a higher voltage potential to be deterred or who are simply insensitive to membrane voltage potentials (Iwao and Jaffe, 1989; Jaffe *et al.*, 1983a).

To establish a timely fast electrical block requires a rapid signaling cascade that likely originates from the sperm itself (Iwao and Jaffe, 1989). In determining which factors may be responsible for initiating the fast electrical block, it is important to distinguish between electrophysiological continuity

of the membranes versus cytoplasmic continuity. Although both result in capacitance changes, the former only requires outer leaflet continuity, whereas the latter requires fusion of both leaflets (Basanez, 2002; Markin and Albanesi, 2002). Thus, the time between cytoplasmic continuity and the onset of the fast electrical block may be much shorter than reported. Nevertheless, the time delay between fusion and the onset of a change in V_m is sufficient for its initiation by a soluble acrosome-derived sperm factor that can regulate an egg's ion channels (Gould and Stephano, 1987) or by a sperm ion channel contributed to the site of membrane fusion (Gould and Stephano, 2003; McCulloh and Chambers, 1992), such as the TRPC family of calcium channels (see Section VII.D, earlier in this chapter) (Xu and Sternberg, 2003). One simple trigger of the fast electrical block could be the flux of ions from the sperm into the egg following the establishment of cytoplasmic continuity. In this case, the influx of sperm-derived calcium or protons—initially accumulated from the extracellular fluid during events leading up to the acrosome reaction (see Section V.C, earlier in this chapter) (Hirohashi and Vacquier, 2002a; Neill and Vacquier, 2004; Runft *et al.*, 2002; Shapiro *et al.*, 1990; Tosti, 1994)—could be responsible for the initial change in V_m that releases a voltage-dependent fast electrical block over the egg.

A fast electrical block has not been observed in mammals (Gianaroli *et al.*, 1994; Jaffe *et al.*, 1983b; Kline and Stewart-Savage, 1994), teleosts (Nuccitelli, 1980), or common urodeles (Charbonneau *et al.*, 1983). Gradual changes in membrane potential have been reported in mammals, however, including hyperpolarizations in hamster (Igusa *et al.*, 1983; Kline and Stewart-Savage, 1994; Miyazaki and Igusa, 1981), mouse (Igusa *et al.*, 1983), and human eggs (Gianaroli *et al.*, 1994) or a prolonged minor depolarization in rabbits (McCulloh *et al.*, 1983). But the periodicity and duration of these oscillations are too late to reasonably block the fusion of supernumerary sperm. Instead, such gradual changes may be a consequence of intracellular calcium waves that result from egg activation (see Section VIII.B, later in this chapter) (Gianaroli *et al.*, 1994; Goudeau and Goudeau, 1996; Igusa *et al.*, 1983; Kline and Stewart-Savage, 1994; McCulloh *et al.*, 1983). Both hamster and mouse eggs also lose membrane resistance rapidly following fertilization (Jaffe *et al.*, 1983b; Miyazaki and Igusa, 1982). Might this membrane change be sufficient to inhibit additional sperm from binding the egg membrane, specifically by altering global membrane flexibility (Horvath *et al.*, 1993; Lee *et al.*, 1988; Tatone *et al.*, 1994; Wolf and Hamada, 1979)?

One likely source of the different penetrance of a fast electrical block across animal phyla may lie with the environment of fertilization per taxon. Because the concentration gradient of ions across the plasma membrane influences the direction of ion flow, a major factor in the survivorship of the

zygote utilizing a fast electrical block is significantly influenced by the ion concentration of the insemination media, particularly extracellular concentrations of ions used to establish current across biological membranes, such as sodium, potassium, and chloride (Gianaroli *et al.*, 1994; Grey *et al.*, 1982). In general, individual ion concentrations in bodies of water are significantly higher than oviductal fluid (see Section III.B, earlier in this chapter, and Section IX.A, later in this chapter), so those eggs that are fertilized in higher ionic environments are more likely to use the ion potentials that exist across their plasma membranes than eggs fertilized internally. Such a model agrees with the observed changes in V_m described for most eggs, specifically the presence of a fast electrical block in animals that spawn (decapods, echinoderms, ascidians, primitive fish, and anurans; see previous discussion) versus an absence in those that undergo internal fertilization (urodeles, mammals). The major exception is with teleosts, but the physical limitations established by the micropyle are likely sufficient to achieve monospermy, so a fast electrical block was not retained (see Section IV.C, earlier in this chapter).

B. Zygotic Intracellular Calcium-Dependent Signaling

Upon gamete fusion, the egg undergoes a series of changes that release it from quiescence to incorporate the sperm nucleus and to initiate embryogenesis (Bement, 1992; Ben-Yosef and Shalgi, 1998; Dumollard *et al.*, 2004; Mellor and Parker, 1998; Miyazaki *et al.*, 1993; Patel, 2004; Talmor-Cohen *et al.*, 2002). The universal trigger of this activation process is a cytoplasmic increase in calcium levels (see Carroll, 2001; Hart, 1990; Hogben *et al.*, 1998; Kaji *et al.*, 2000; Machaty *et al.*, 2000; Miyazaki *et al.*, 1993; Pecorella *et al.*, 1993; Runft *et al.*, 2002; Santella *et al.*, 2004; Stricker, 1999; Witton *et al.*, 1999). This calcium originates from intracellular stores enriched at the egg cortex or from mitochondria (Dumollard *et al.*, 2004; Halet, 2004; Leckie *et al.*, 2003; Liu *et al.*, 2001; Putney and Ribeiro, 2000; Shen, 1995; Stricker, 1999; Thaler and Epel, 2003). Distinct patterns of calcium release have been observed in different animals, ranging from a single prolonged wave that travels across the egg along its cortex from the point of sperm entry to oscillations of high and low calcium release that persist at least until first cleavage (Runft *et al.*, 2002; Stricker, 1999). The ability to propagate and maintain such patterns of intracellular calcium requires a network of calcium storage that acts synchronously in response to the activation status of a neighboring site (Machaca, 2004; Nuccitelli *et al.*, 1988; Sardet *et al.*, 2002).

Initiation of calcium release involves a universal signaling cascade that begins with PLC (reviewed in Dumollard *et al.*, 2004; Runft *et al.*, 2002, 2004). This membrane-associated enzyme converts phosphoinositol found in the plasma membrane into the secondary messengers inositol-3-phosphate

(IP3) and diacylglycerol (DAG). IP3 directly affects the level of intracellular calcium by triggering the release of stored calcium in the cortical endoplasmic reticulum upon binding its receptor, which also acts as a calcium channel. This initial cytoplasmic flux is sufficient to propagate local calcium release along the cortex that results in calcium waves of short duration (Dupont and Dumollard, 2004; Fall *et al.*, 2004). IP3, however, is quickly inactivated, a process that promotes the re-sequestration of calcium into stores and results in a rapid lag phase at the end of each oscillation. Prolonged waves of calcium require the additional participation of calcium-induced calcium responsive (CICR) channels (reviewed in Abbott and Ducibella, 2001; Ben-Yosef and Shalgi, 1998; Dumollard *et al.*, 2004; Galione *et al.*, 1991; Hart, 1990; Lawrence *et al.*, 1997; Miller *et al.*, 1994; Putney and Ribeiro, 2000; Stricker, 1999). IP3-dependent or neighboring CICR-mediated calcium release is often sufficient to trigger an extended flux of calcium from CICR stores. A separate mechanism that controls calcium release is dependent on the outcome of DAG activity. This second messenger facilitates the phosphatidylserine-dependent activation of conventional and novel protein kinase C (PKC) signaling. In addition to its role in cytoplasmic calcium dynamics (Putney and Ribeiro, 2000; Stricker, 1999), the phosphorylation activity of specific PKC isozymes also selectively initiates downstream signaling cascades, enzymes, and processes involved with cytoskeletal reorganization and nuclear function during early development (reviewed in Bement, 1992; Halet, 2004; Mellor and Parker, 1998; Page Baluch *et al.*, 2004; Talmor-Cohen *et al.*, 2002). Unlike the transient nature of the fusion-dependent IP3 signaling, the DAG–PKC relationship has more enduring effects on early development.

Based on the required timing of PLC-dependent calcium release following gamete fusion, one general hypothesis is that the sperm activates PLC. In echinoderms, the process of activation requires the gamma isoform of PLC (PLC_γ). The translocation of normally cytoplasmic PLC_γ to the plasma membrane is thought to be triggered by a Src-like kinase via tyrosine phosphorylation (Dumollard *et al.*, 2004; Runft *et al.*, 2002, 2004). How this egg kinase is activated by the sperm, however, is still debated (Runft *et al.*, 2002; Santella *et al.*, 2004). One source of such activation could be the sperm itself, as described in mammals. Upon fusion, the soluble sperm-specific zeta isoform of PLC (PLC_ζ) is released into the egg cytoplasm, where it rapidly triggers IP3-dependent calcium release (reviewed in Kurokawa *et al.*, 2004; Swann *et al.*, 2004). Under physiological concentrations, exogenous PLC_ζ is able to initiate calcium signaling and progress through early stages of development in a wide range of deuterostome eggs (Cox *et al.*, 2002; Kouchi *et al.*, 2004; Saunders *et al.*, 2002; Yoda *et al.*, 2004). Furthermore, depletion of PLC_ζ by RNAi from murine sperm significantly affects the pattern of calcium oscillations following fusion (Knott *et al.*, 2005). The repression of its

activity by calcium concentrations higher resting levels in eggs (Kouchi *et al.*, 2004) suggests that PLC_{ζ} is optimally active upon dilution into the egg at fusion. The absence of a membrane targeting domain on PLC_{ζ} (Saunders *et al.*, 2002) implies that it is also soluble in the sperm, at least until the calcium-dependent process of sperm activation when its calcium-binding domain might target it to a sperm membrane. The persistence of high cytoplasmic sperm calcium levels following activation would keep PLC_{ζ} in a primed, but not active, state at the membrane. Only upon dilution of the local calcium levels following fusion could PLC_{ζ} then catalyze the degradation of phosphoinositol to initiate the IP3-dependent calcium release in the egg, a process that likely shuts off the sperm-derived PLC again.

A similar sperm-derived contribution effectively activates echinoderm eggs and, like mammalian PLC_{ζ}, can mark the site of sperm entry. Nicotinic acid adenine dinucleotide phosphate (NAADP), a molecule that permanently binds its target receptor, initiates a rapid rise in cortical calcium (the "cortical flash") via membrane-associated voltage-gated calcium channels located on the surface of the egg (Churchill *et al.*, 2003; Moccia *et al.*, 2004; Patel, 2004; Santella *et al.*, 2004). This sharp peak in calcium concentration likely activates the egg's PLC_{γ} signaling cascade, although CICR channels may be triggered as a consequence of the cross-talk among calcium-sensitive calcium channels found in the endoplasmic reticulum and/or the plasma membrane (Patel, 2004; Santella *et al.*, 2004). The involvement of plasma membrane–associated calcium channels could also occur via TRPCs, whose presence in the sperm plasma places them in a prominent position to play an active role during early zygotic calcium signaling (see Section VII.C, earlier in this chapter).

The first sequence of calcium transients following fusion is usually responsible for the translocation, when necessary, and secretion of CGs, as well as initial events essential for the transition to embryogenesis (Ben-Yosef and Shalgi, 1998; Cran and Esper, 1990; Ducibella *et al.*, 2002; Goudeau and Goudeau, 1996; Goudeau *et al.*, 1991). The timing and duration of CG release is species-dependent, but universally requires calcium (Abbott and Ducibella, 2001; Cran and Esper, 1990; Gilkey *et al.*, 1978; Goudeau *et al.*, 1991; Wessel *et al.*, 2001). CG exocytosis is most likely governed by calcium-responsive proteins embedded within the membrane of the organelles and plasma membrane (Crabb and Jackson, 1985), particularly cysteine string proteins (Gundersen *et al.*, 2001) and the SNARE complex, including the calcium-sensitive proteins synaptotagmin, rab3, and rabphilin-3A (Abbott and Ducibella, 2001; Conner *et al.*, 1997; Ikebuchi *et al.*, 1998; Tahara *et al.*, 1998; Wessel *et al.*, 2001). Thus, conformational changes in the protein fusion machinery induced upon calcium binding could be directly translated into forces needed to initiate membrane deformation, membrane fusion, and content exocytosis (reviewed in Bentz and Mittal, 2000).

IX. Producing the Physical Block to Polyspermy

The most effective mechanism to block supernumerary sperm from fusing with an egg is to establish a physical barrier separating the monospermic zygote from the sperm. Constructing such an obstacle at the zygote's surface is impossible without molecules, specifically enzymes or structural proteins, which alter or mask the egg's sperm-receptive ECM (see Table II). In most animals, the source of these converting factors resides at the egg cortex. The secretory granules found at the periphery of most eggs are released immediately after fertilization, usually in response to the initial prolonged elevation of intracellular calcium (see Section VIII.B, earlier in this chapter). Prior to secretion, the contents of these CGs are often organized into paracrystalline arrays whose architecture is often species specific (see Bannon and Brown, 1980; Campanella *et al.*, 1992; Cran and Esper, 1990; Hart, 1990; Talbot and Goudeau, 1988; Wong and Wessel, 2004). During exocytosis, subcomplexes of this paracrystalline architecture are expelled nearly intact, but rapidly hydrate and evenly incorporate into the egg ECM (Bryan, 1970b; Carroll *et al.*, 1986; Gulyas, 1980; Talbot and Goudeau, 1988).

The duration of CG exocytosis varies significantly across animal phyla, requiring anywhere from seconds to hours to complete (Brown and Clapper, 1980; Campanella *et al.*, 1992; Elinson, 1986; Gilkey *et al.*, 1978; Goudeau and Becker, 1982; Gould and Stephano, 2003; Hart, 1990; Kline and Stewart-Savage, 1994; Matese *et al.*, 1997; Talbot and Goudeau, 1988; Whalley *et al.*, 1995). Although the duration of CG exocytosis tends to give a good estimate of how long the permanent block to polyspermy takes to establish, the reported duration of hours required for decapods clearly suggests that construction of the physical block is not the only role of CG contents after fertilization (see Section III.B, earlier in this chapter) (Brown and Clapper, 1980; Santella and Ianora, 1992; Talbot and Goudeau, 1988).

In this section, we summarize the various biochemical modifications that occur at the egg ECM after CG exocytosis. We review and compare the different methods used by various taxa, including a discussion of those exceptional eggs whose physical blocks are constructed without the contribution of CGs.

A. Cortical Granule Contents + Egg ECM = ?

Establishment of a physical block to polyspermy requires the combination of proteins from two distinct structures originally synthesized in the egg, specifically the ECM and CGs (Table II). Although the effect of CG contents on the egg ECM is usually significant, the ultrastructural changes that occur

across phylogeny range from minor to radical. Radical modifications are inherently associated with a complete loss of sperm recognition because of the severity of the changes to the ECM surface. In those ECMs exhibiting insignificant ultrastructural changes, however, biochemical modifications prove to be critical because they are solely responsible for the reduction in sperm affinity associated with a permanent block to polyspermy. Thus, a predictable relationship exists between the probability of polyspermy and the degree of morphological change to which the CG contents contribute.

Although mammalian CG exocytosis significantly alters the receptivity of sperm at the ECM, little morphological modification is observed (Shapiro *et al.*, 1989) (Figs. 1–3). In most mammals, CG exocytosis results in a slightly greater distance between the zona and the zygotic plasma membrane, as well as the accumulation of a thin intimate CG envelope overlying the zygotic plasma membrane (Breed and Leigh, 1992; Dandekar and Talbot, 1992; Dandekar *et al.*, 1995; Hoodbhoy and Talbot, 2001; Hoodbhoy *et al.*, 2001; Jungnickel *et al.*, 1999; Talbot and Dandekar, 2003). Many lectin-reactive proteins derived from CGs are deposited at the surface of the egg, accumulate within the perivitelline space in mice (Lee *et al.*, 1988), and sometimes intercalate into the ECM, as observed in hamsters (Cherr *et al.*, 1988; Hoodbhoy and Talbot, 2001). One such glycoprotein is the antigen of the ABL2 antibody, a CG envelope protein that remains adherent to the embryo until hatching and may participate in establishing a membrane-level block to polyspermy (Hoodbhoy *et al.*, 2001; Talbot and Dandekar, 2003). Unlike eutherians eggs that remain surrounded by a cumulus cell layer, marsupial zygotes acquire two dense mucoid layers external to the zona, structures thought to be applied by oviduct epithelium as the zygote travels toward the uterus (Selwood, 1992).

The most noticeable change to the anuran egg ECM at fertilization occurs to the jelly layer (Figs. 1 and 2). As in mammals, the vitelline envelope is released from the plasma membrane but remains a smooth shell compacted against the jelly layer (Larabell and Chandler, 1991). Within minutes of CG exocytosis, a precipitate forms along the boundary between the elevated vitelline envelope and the inner J1 jelly layer (Hedrick and Nishihara, 1991; Larabell and Chandler, 1991). This fertilization (F) layer is the result of the CG-derived lectin XL35/CGL (or its orthologs) preferentially binding a sulfated galactose-rich oligosaccharide present in the J1 layer (Arranz-Plaza *et al.*, 2002; Chang *et al.*, 2004; Hedrick and Nishihara, 1991; Nishihara *et al.*, 1986; Quill and Hedrick, 1996; Tseng *et al.*, 2001). Pretreating anuran eggs with total CG exudate inhibits fertilization only when the jelly layer is intact, providing direct evidence that, in addition to its role in sperm attraction, jelly also contributes to the block to polyspermy (Barisone *et al.*, 2002; Prody *et al.*, 1985). In *Discoglossus*, on the other hand, CG exocytosis is responsible for the dissolution of the jelly plug (Campanella

et al., 1992). This process is functionally analogous to the physical obstruction of the sperm's path via the F layer produced in other anurans (Hedrick and Nishihara, 1991) because the chemoattractive jelly plug is required to guide sperm to the fusogenic dimple of the animal pole (see Section IV.C, earlier in this chapter).

Most CG-dependent morphological changes occur at the teleost micropyle, consistent with the need to alter the most fusogenic regions of an egg after fertilization (Figs. 1 and 2). For example, *Danio* eggs contain three types of CGs associated with the egg membrane, each type distinguishable by size and location in the animal or vegetal hemispheres (Hart and Donova, 1983). The patch of membrane directly below the micropylar canal is enriched in microvilli and devoid of CGs; surrounding this void, however, is a specialized cluster of smaller CGs that lie in the shadow of the micropylar tunnel (Gilkey *et al.*, 1978; Hart, 1990). These small CGs are thought to establish the modifications necessary for plugging the micropyle. Although most teleosts exhibit a propagated pattern of CG release starting at the site of fusion (Gilkey *et al.*, 1978; Hart, 1990), the micropylar population in *Danio* is the last to exocytose after environmental activation or fertilization, postponed sufficiently to allow the short-lived sperm to penetrate before the fertilization plug is formed (Hart and Donova, 1983; Ohta and Iwamatsu, 1983; Wolenski and Hart, 1987). In the end, the global outcome of teleost CG exocytosis results in both the physical separation of the chorion from the egg surface, via hydration of the CG contents secreted into the perivitelline space (Hart, 1990; Ohta and Iwamatsu, 1983; Wolenski and Hart, 1987), and the physical obstruction of the micropyle using CG lectins that form the fertilization plug (Hart, 1990) or using protease-dependent collapse of the overlying chorion (Iwamatsu *et al.*, 1997). Additional CG-dependent modifications occur over the nonmicropylar chorion, events that have been partly worked out in *Cyprinus*. Following CG exocytosis, FLS, CLS, and cystatin polymerize in a cation-dependent fashion within the chorion and/or perivitelline space (Chang and Huang, 2002; Chang *et al.*, 1998). Different FLS isoforms are spatially separated during oogenesis but polymerize during fertilization envelope formation; only this homo-oligomeric complex is able to bind CLS and cystatin. As the FLS–CLS–cystatin triad diffuses away from the egg surface, it is trapped by ZPA–ZPC protofilaments at the outer edge of the chorion, where the triad then tethers fertilization envelope outer layer protein-1 (FEO-1) to the chorion (Chang and Huang, 2002; Chang *et al.*, 1998, 1999). Such a peripheral location of cystatin is also optimal for its role as an antifungal protein that serves to protect the zygote chemically and structurally (Chang *et al.*, 1998).

As in some teleosts, the exocytosis of decapod CGs is a hierarchal process that modifies the ECM in stages. Decapod eggs synthesize at least four populations of cortical vesicles that are packaged independent of the Golgi

apparatus (Brown and Clapper, 1980; Goudeau and Becker, 1982; Goudeau *et al.*, 1991; Santella and Ianora, 1992; Talbot and Goudeau, 1988) (Figs. 1, 2, and 6). The smallest, most peripheral electron-dense granules are released within 20 min after fertilization (Bannon and Brown, 1980; Talbot and Goudeau, 1988). The contents of these first granules are thought to separate the chorion from the plasma membrane as they adsorb or intercalate within the inner layer of the chorion. Upon completing small CG exocytosis, gradual exocytosis of the remaining high-density, medium-density and ring granules—the ''secondary'' granules—follows. These secondary CGs contribute to the more intimate electron-dense embryonic envelope deposited at the surface of the zygotic membrane and hardened by mechanical processes (Bannon and Brown, 1980; Brown and Humphreys, 1971; Goudeau and Becker, 1982; Goudeau *et al.*, 1991; Hinsch, 1971; Santella and Ianora, 1992; Talbot and Goudeau, 1988). CG release can last for hours in decapods (Goudeau and Becker, 1982; Goudeau *et al.*, 1991; Santella and Ianora, 1992), suggesting that the contribution of these vesicles, particularly the secondary CGs, to an immediate physical block to polyspermy is negligible. Compared to the original vitelline envelope, however, the differences in physical characteristics of the zygotic ECM following CG exocytosis support the involvement of early CG secretion in establishing a protective barrier for development (Goudeau and Becker, 1982; Talbot and Goudeau, 1988).

The most dramatic change to egg ECMs following CG exocytosis is observed in echinoderms (reviewed in Kay and Shapiro, 1985). A nearly fourfold increase in surface area develops during the transformation of the vitelline layer into the fertilization (Figs. 1–3), a structure that is lifted from the plasma membrane by the hydration of CG-derived glucosaminoglycans (Harvey, 1909; Larabell and Chandler, 1991; Runnstrom, 1966; Tegner and Epel, 1976). Based on the sheer abundance of CG protein mass relative to the vitelline layer, it is almost guaranteed that these proteins interact with one another, sticking wherever possible along the vitelline layer scaffold (Inoue and Hardy, 1971). These protein–protein interactions probably use the abundant LDLrA repeats and CUB domains encoded by both the CG and the vitelline layer components (see Sections II.D and III.B, earlier in this chapter) (Wong and Wessel, 2004, 2006). The network of binding is not random: Initial studies reported proteoliaisin binding to the vitelline layer and ovoperoxidase (Weidman and Shapiro, 1987). Further analysis showed that the vitelline layer component of rendezvin promiscuously binds all the CG content proteins through high-affinity ionic interactions (Wong and Wessel, 2006). This implies that CUB domains of the vitelline layer are seeds that coordinate the interaction of all the CG proteins during their rapid, autonomous assembly. The role of the sea urchin LDLrA repeats, however, is not clear. Evidence from other proteins that contain similar tandem arrays of LDLrA repeats clearly indicates that these repeat motifs are essential for

the function of the ECMs they compose (Kallunki and Tryggvason, 1992; Yochem *et al.*, 1999).

A common theme in the assembly of structural components necessary for a physical block to polyspermy is the participation of calcium (see Section III.B, earlier in this chapter). Ascidians modify their plasma membrane using electron-dense, calcium-rich granules extruded from subcortical cytoplasmic vesicles (Rosati *et al.*, 1977). XL35/CGL binds a terminally sulfated, galactose-rich 250-kDa mucin of the anuran J1 jelly layer in a calcium-dependent fashion, making calcium essential to the stable precipitation of the fertilization (F) layer at the interface of the vitelline membrane and jelly coat following fertilization (Arranz-Plaza *et al.*, 2002; Chang *et al.*, 2004; Hedrick and Nishihara, 1991; Nishihara *et al.*, 1986; Quill and Hedrick, 1996; Tseng *et al.*, 2001; Wolf *et al.*, 1976). Calcium chelation motifs are also abundant in all the sea urchin fertilization envelope structural proteins (Wong and Wessel, 2004). In both teleosts (Chang and Huang, 2002) and echinoderms (Bryan, 1970b), free calcium is essential to establish a crystalline matrix within the renovated ECM that can withstand chemical and physical abuse (Harvey, 1909; Zotin, 1958). The difference lies in how this cation is primarily used: Teleosts need calcium for enzymatic activity (Yamagami *et al.*, 1992), whereas echinoderms need it to maintain the structural integrity of the matrix (Bryan, 1970b; Wong and Wessel, 2004). As observed in anurans, protein precipitation at the ECM–jelly interface may occur in sea urchins during fertilization envelope elevation, with the jelly providing a mold for CG structural proteins to efficiently assemble against (J. L. Wong and G. M. Wessel, unpublished observations). Retention of a thin layer of jelly may also prove to be important for sea urchin development, especially to reduce the agglutination of embryos and attachment of microbes (Mah *et al.*, 2005). The need to create a durable barrier within a calcium-rich environment could account for the selection of calcium-chelating motifs for structural elements by these spawning animals. For internally fertilized animals such as eutherians, on the other hand, pressure to maintain these attributes diminishes because oviductal fluid is not as abundant in calcium ($\sim$1.7 mM calcium vs. 3–5 mM in freshwater, 10 mM in seawater) (see Section III.B, earlier in this chapter). Instead, other associated modifications to the ECM or the eutherian cumulus cells likely provide equivalent protection to the developing embryo until implantation.

B. Modifying the Egg ECM by Destruction

In most animals, CGs contribute the enzymes essential for assembling a permanent block to polyspermy. The most common activity associated with the modification involves destruction of the ECM to minimize its attractiveness to tardy sperm.

Proteinases are essential for the construction of barriers that are histologically distinct from the original egg ECM. Successful development of petromyzontids to two-cell embryos requires a chymotrypsin-like activity (Dabrowski *et al.*, 2004), suggesting that this family of egg-derived enzymes participates in the permanent block to polyspermy (Kobayashi and Yamamoto, 1994). A more definitive reduction in molecular weight of major teleosts chorion components following CG exocytosis suggests that proteolysis may occur along the inner layer, facilitating the hardening process by exposing hydrophobic amino acid residues for targeted cross-linking (see Section IX.D, later in this chapter) (Yamagami *et al.*, 1992). Alternatively, proteases may facilitate the collapse of the micropylar vestibule to physically block sperm from reaching the egg (Iwamatsu *et al.*, 1997). A similarly dramatic histological change is the assembly of an echinoderm fertilization envelope, whose formation requires the separation of the egg ECM from the egg plasma membrane. Detachment of the vitelline layer is facilitated by the sea urchin serine-like protease CGSP1, which cleaves target proteins on the surface of the egg membrane (Carroll and Epel, 1975a,b; Haley and Wessel, 1999) such as the vitelline post protein p160 (Haley and Wessel, 2004a) and the 350-kDa EBR1 sperm receptor, whose fragments can be found buried in the core of the mature fertilization envelope (Carroll *et al.*, 1986; Ruiz-Bravo *et al.*, 1986).

A strong case for the involvement of proteolysis during the transformation of the egg ECM can be found in the cleavage of ZPA from anurans (Barisone *et al.*, 2002; Infante *et al.*, 2004; Lindsay and Hedrick, 2004; Wolf *et al.*, 1976) and eutherians (Bauskin *et al.*, 1999; Moller and Wassarman, 1989). One candidate originally thought to be responsible for the degradation of ZPA was anuran ovochymase, an extracellular chymotrypsin-like protease active in the perivitelline space of the *Xenopus* zygote (Lindsay *et al.*, 1992b, 1999; Lindsay and Hedrick, 1995). The ovochymase zymogen contains a single, amino-terminal CUB domain that may anchor it to the vitelline envelope until its activation (Lindsay *et al.*, 1999). A trypsin-like enzyme released upon CG exocytosis is thought to activate 0.01% of the bound ovochymase zymogen functional at fertilization (Lindsay and Hedrick, 1989; Lindsay *et al.*, 1992b, 1999). Such a weak activation potential of this CG-derived trypsin-like enzyme on ovochymase suggests that ovochymase and its sibling ovotryptases do not participate in vitelline envelope remodeling (Lindsay *et al.*, 1999). Rather, the protease responsible for cleavage of anuran ZPA is a zinc-dependent CG protease similar to bone morphogenic protein-1 (BMP-1) (Lindsay and Hedrick, 2004). This family of enzymes cleaves approximately 28 residues from the ZPA amino-terminus at a consensus sequence (X|DD/E) found in most vertebrate ZPA orthologs (Lindsay and Hedrick, 2004). Proteolysis likely disrupts the conformation of ZPA by relaxing the fold normally retained by an intramolecular disulfide

bond between the amino- and carboxyl-termini. This site-specific hydrolysis of ZPA accounts for the observed retention of the entire ZPA protein following ECM modification, albeit its electrophoretic mobility could change (Bauskin *et al.*, 1999; Lindsay and Hedrick, 2004; Moller and Wassarman, 1989; Moller *et al.*, 1990). Consequences of this structural relaxation in ZPA likely initiate an avalanche of systemic conformational changes along a ZP protofilament, ultimately terminating in the acquisition of chemical and protease resistance throughout the postfertilization ECM (Lindsay and Hedrick, 2004; Sun *et al.*, 2003).

Does the model of non-proteolytic sperm penetration fit with the hypothesized ZPA-dependent mechanism of ECM conversion in anurans? Under the non-proteolytic model of penetration, the integrity of the ECM remains the same (see Section VI.C, earlier in this chapter). Because every ZP protofilament (Jovine *et al.*, 2002) along the sperm's path through the ECM also retains its native configuration, reassembly and modification of the fibers occurs efficiently because no holes would have to be patched during remodeling. By simply rearranging the displaced protofilaments through changes in ZPA conformation, sperm tunnels are eradicated and sperm receptors are masked without the integration of new material (Barisone *et al.*, 2002; Infante *et al.*, 2004; Rankin *et al.*, 2003). This radical change in conformation would also allow for the acquisition of mechanical resilience association with the zygotic ECM (Sun *et al.*, 2003). Thus, the intramolecular conformation of ZPA affects both primary and secondary sperm receptivity by regulating ZPC and ZPA accessibility to sperm both before and after fertilization (Rankin *et al.*, 2003). Such simple protease-dependent conversion is also parsimonious with the ECM conversion in other animals. For example, cleavage of the homologous glycoproteins at the *Oryzias* micropylar catchment leaves a thin compacted outer chorion layer that is unattractive to teleost sperm (Iwamatsu *et al.*, 1997). In ascidians, too, proteasomes specifically target the putative sperm binding protein, VC70, for degradation (Sawada *et al.*, 2002a)—although the contribution of this cleavage is male derived, making its proteasome activity more like a sperm competition mechanism than a block to polyspermy (Lambert, 2000). Finally, the mollusk *Mytilus* uses an aminopeptidase to disrupt the sperm-binding affinity of the vitelline envelope proteins (Togo and Morisawa, 1997; Togo *et al.*, 1995). Unlike the proteases used in other animals, no significant morphological or biochemical modification other than loss of sperm binding has been reported in this bivalve.

Given the high degree of complexity and overlap often observed at fertilization, it is not surprising to find that other CG-derived enzymes alter the animal ECM. The universal involvement of oligosaccharides in sperm–egg interaction (see Section V.B, earlier in this chapter) provides one likely target of enzymatic modification. The source of this alteration is not known, but

the process is hypothesized to be essential for a permanent block to polyspermy (Mahowald *et al.*, 1983). This model is consistent with the reported change in lectin affinities during the transition from egg to zygotic ECM in *Drosophila*, whose vitelline membrane loses α-mannose and sperm-binding β-*N*-acetylglucosamine following fertilization (Perotti *et al.*, 1990, 2001). In some animals, the loss of primary sperm binding has been attributed to deglycosidases presumably stored in the CGs (Florman *et al.*, 1984; Prody *et al.*, 1985; Talbot and Dandekar, 2003; Vo *et al.*, 2003) but alternatively may be stored in the perivitelline space, such as in ascidian test cells or in follicle cells external to the vitelline coat (Lambert, 2000; Lambert *et al.*, 1997; Rosati, 1985; Rosati *et al.*, 1977). For example, soluble *N*-acetylglucosaminidase activity is associated with the ascidian egg surface and is thought to remove sperm-binding sugar residues to eliminate vitelline coat receptivity (Lambert and Goode, 1992; Lambert *et al.*, 1997; Matsuura *et al.*, 1993). *N*-acetylglucosaminidase activity has also been purified from CGs in *Xenopus* (Prody *et al.*, 1985) and *Mus* (Miller *et al.*, 1993a). The enzyme is active in *Xenopus*, causing a significant loss in ZPC oligosaccharide mass (Vo *et al.*, 2003). The functional eutherian ortholog, however, does not contribute the same degree of modification because the electrophoretic mobility of ZPC is unaltered following CG exocytosis (Bauskin *et al.*, 1999; Miller *et al.*, 1993a). The absence of substrate modification or byproducts associated with its activity suggests that, like the sperm ligand (see Section V.C, earlier in this chapter), either reversible ionic interactions between the *N*-acetylglucosaminidase and its preferred oligosaccharides or another steric modification blocks gamete interactions in eutherians.

C. Modifying the Egg ECM by Addition

The identification of nonenzymatic lectin-like proteins within CGs (see Section III.B, earlier in this chapter) suggests that steric masking of essential sugar moieties may supplement deglycosidase activity or may alone be sufficient to inhibit sperm binding in some animals. Two prominent examples include the oligomerizing XL35/CGL from *Xenopus* (Chamow and Hedrick, 1986; Chang *et al.*, 2004; Nishihara *et al.*, 1986) and potential carbohydrate-binding pocket motifs such as dimerized CUB domains in sea urchins (Bork and Beckmann, 1993; Romero *et al.*, 1997; Varela *et al.*, 1997; Wong and Wessel, 2006). The precipitation of XL35/CGL is dependent on binding of a galactose-rich oligosaccharide found in the *Xenopus* J1 jelly layer, resulting in its local precipitation and the formation of an impenetrable calcium-rich barrier (see Section IX.A, earlier in this chapter) (Arranz-Plaza *et al.*, 2002; Chang *et al.*, 2004; Hedrick and Nishihara, 1991; Nishihara *et al.*, 1986; Quill and Hedrick, 1996; Tseng *et al.*, 2001).

In the sea urchin, the egg bindin receptor EBR1 is retained in the vitelline layer core of the fertilization envelope but is probably masked by the adsorption of non-enzymatic CG proteins (Carroll *et al.*, 1986; Ruiz-Bravo *et al.*, 1986). This mechanism is similar to the loss of lectin-accessible sugars of the vitelline layer immediately following fertilization, including a 50% decrease in wheat germ agglutinin-binding saccharides such as *N*-acetylglucosamine (GlcNAc) and *N*-acetylneuraminic acid (NeuNAc) (Kitamura *et al.*, 2003). In mammals, a lectin-like protein could interfere with sperm–zona binding by blocking sperm-binding sites. Alternatively, proteolysis of ZPA and subsequent conformational changes to the zona (see Section IX.B, earlier in this chapter) may be prerequisite for proper lectin-like epitope masking, as demonstrated by the retention of sperm-binding capacity when ZPA is not cleaved (Rankin *et al.*, 2003).

Although the oligosaccharide-binding molecules are likely derived from CGs, such an adaptation may not be necessary. In marsupials, for example, CG exocytosis does not provide a full block to polyspermy; instead, the CG-derived permanent block is supplemented by the application of a mucoid shell over the modified zona during its travels across secretory epithelium of the oviduct isthmus (Figs. 1 and 2). This coating is believed to mask all unoccupied sperm receptors and to trap any supernumerary, zona-bound sperm prior to membrane binding (Breed and Leigh, 1990, 1992; Jungnickel *et al.*, 1999; Rodger and Bedford, 1982a,b; Selwood, 1992). Such a mechanism may be successful because of the apparent low stoichiometry of sperm-to-ovum within the oviduct (Rodger and Bedford, 1982a) and the low binding frequency of capacitated sperm to zonae *in vitro* (Mate *et al.*, 2000). Not surprisingly, application of such a distal physical block does not prevent the persistence of multiple sperm in the perivitelline space (Jungnickel *et al.*, 1999), a phenomenon consistent with the 5% rate of polyspermy in some species (Breed and Leigh, 1990). Distinct from the oviductal glycoproteins that matriculate into the zona and enhance homologous sperm binding (see Section II.D, earlier in this chapter) (Buhi, 2002; O'Day-Bowman *et al.*, 2002; Rodeheffer and Shur, 2004; Schmidt *et al.*, 1997), these zygotic mucoid coatings contain epitopes antigenically conserved with oviductal glycoproteins that supplement the CG-derived zona modification in all mammals. Their biochemical functions include physically blocking sperm penetration, stabilizing the modified zona, and/or acting as an antimicrobicide or spermicide as the embryo travels toward the uterus (Brown and Cheng, 1986; Hoodbhoy and Talbot, 1994; Roberts *et al.*, 1997; Selwood, 1992; Wang *et al.*, 2003). An analogous protective function is attributed to the coatings applied to *Drosophila* and avian embryos by female reproductive organs. The carbohydrate profile of the zygotic *Drosophila* micropyle is enriched in low sperm-affinity sugars such as α-galactose (Gal) and *N*-acetylneuraminic acid (NeuNAc) (Perotti *et al.*, 1990) while the

remaining chorion is coated with anti-bactericidal ceratoxins (Marchini *et al.*, 1997). Similarly, an outer coat is deposited peripheral to the avian perivitelline layer during the zygote's journey through the infundibulum and magnum of the oviduct (Bellairs, 1993; Bellairs *et al.*, 1963; Harper, 1904) (Figs. 1 and 2). Because fertilization at the germinal disc occurs soon after ovulation, this avian coat does not prevent polyspermy; rather it protects the embryo from microbes by coating the outer layer with avidin, lysozyme, and a 62-kDa anti-microbicidal lectin (Cook *et al.*, 1985; Harper, 1904; Marchini *et al.*, 1997; Okamura and Nishiyama, 1978a).

D. Modifying the Egg ECM by Transmogrification

An alternative to physically masking the egg ECM by protein addition is to toughen the old matrix. This "hardening" process is representative of a change in the physical properties of the matrix, including the acquisition of mechanical resilience, protease insensitivity, and chemical resistance (Harvey, 1909; Lindsay and Hedrick, 2004; Sun *et al.*, 2003; Wong *et al.*, 2004; Zotin, 1958). This could be achieved by simple ECM hydration, as observed in the primitive urodele *Hynobius* (Iwao and Jaffe, 1989), by oviduct contributions, as in *Drosophila* (Bloch Qazi *et al.*, 2003), or by complete reorganization and compaction of the ECM contents, as observed in anurans through ZPA proteolysis (see Section IX.B, earlier in this chapter) (Lindsay and Hedrick, 2004). Yet some animals rely on physically cross-linking adjacent proteins via the creation of covalent bonds between adjacent proteins. The most common enzymes that specifically generate these covalent bonds are peroxidases and transglutaminases. Peroxidases generate dityrosine bonds between neighboring proteins (Deits *et al.*, 1984; Gulyas and Schmell, 1980a; LaFleur *et al.*, 1998; Nomura and Suzuki, 1995), whereas transglutaminases create isopeptide amide bonds between glutamine and lysine (Battaglia and Shapiro, 1988; Chang *et al.*, 2002; Lee *et al.*, 1994; Lorand and Graham, 2003; Nemes *et al.*, 2005; Oppen-Berntsen *et al.*, 1990; Yamagami *et al.*, 1992). Both affect the structural integrity of the matrix by adding covalent, intermolecular braces and by fusing polymers into a unified surface. This is most noticeable in scanning electron micrographs of the sea urchin fertilization envelope, where the glycoprotein fibers remain loose when either 3-aminotriazole or glycine ethyl ester are used to inhibit ovoperoxidase or transglutaminase activities, respectively, but stiffen and align under normal conditions (Battaglia and Shapiro, 1988; Deits *et al.*, 1984; Foerder and Shapiro, 1977; Mozingo and Chandler, 1991; Veron *et al.*, 1977) (Fig. 3).

Bridging of proteins via covalent cross-links has the potential to block sperm-binding sites through conformational changes in the surrounding matrix. The specific enzymatic activity utilized, however, depends on the

organism. Unlike sea urchins, most animals have either peroxidase or transglutaminase. For example, peroxidase activity has been localized to mouse CGs and to the outer surface of the zona (Gulyas and Schmell, 1980a). Although further characterization of this activity has not been reported, such a modification suggests that peroxidase-like enzymes may supplement the zinc-dependent proteolysis of ZPA during ECM hardening (Lindsay and Hedrick, 2004). Similarly, peroxidase activity is found in specific chorionic layers of the teleost *Tribolodon* (Kudo, 1988). More concrete evidence for the participation of peroxidase-dependent cross-linking, comes from studies that identified the *o,o*-dityrosine products of this enzyme within the modified ECM. The typical mechanism of peroxidases involves production of free radicals at the *ortho* position of adjacent tyrosine phenyl rings, followed by collapse of these extra electrons to form a single carbon–carbon bond (Chance, 1949; Ljunggrn, 1966; Marquez and Dunford, 1995; Morrison and Schonbaum, 1976). The covalent linkage changes the physical properties of the tyrosine residues, allowing them to yield more blue light (420 nm) when excited with ultraviolet (325 nm) light compared to their monomeric counterparts (Gross, 1959; Heinecke *et al.*, 1993a,b). This characteristic has led to the hypothesis that peroxidases are also responsible for cross-linking eggshells of the dipterans *Anopheles* (Li *et al.*, 1996) and *Dacus* (Mouzaki *et al.*, 1991).

Transglutaminase cross-linking, on the other hand, occurs in various species of dipterans, echinoderms, and teleosts. For example, products of the *defective chorion* (*dec-1*) gene (see Section II.D, earlier in this chapter) are bound within the *Drosophila* chorion through isopeptide amide bonds (Badciong *et al.*, 2001). These γ-glutamyl-ε-lysine bonds are also reported to be in the chorion of teleosts (Chang *et al.*, 2002; Kudo and Teshima, 1998; Lee *et al.*, 1994; Oppen-Berntsen *et al.*, 1990; Yamagami *et al.*, 1992). In *Oryzias*, this enzyme is thought to partially dehydrate and, hence, compact the chorion (Lee *et al.*, 1994). Such a process may occur through the calcium-dependent, cadaverin-sensitive cross-linking of *Gadus* chorion proteins (Oppen-Berntsen *et al.*, 1990), of *Cyprinus* ZPB and ZPC (Chang *et al.*, 2002), or of *Pseudopleuronectes* ZPB, specifically at its $(PQQ)_4$PKY repeats (Lyons *et al.*, 1993). In echinoderms such as sea urchins, transglutaminase activity organizes and stabilizes self-assembly of CG structural proteins within the vitelline layer scaffold (Battaglia and Shapiro, 1988; Kay and Shapiro, 1985). The isopeptide bonds made by this enzyme are essential for retaining CG content proteins in the fertilization envelope and contribute to the permeability barrier of the mature matrix (Cheng *et al.*, 1991; Kay and Shapiro, 1985; J. L. Wong and G. M. Wessel, unpublished observations).

The selective advantage for one type of cross-linking activity over another may depend on additional factors introduced upon zygotic activation. Such conditions include the availability of catalytic cations such as calcium (see

Section IX.A, earlier in this chapter), signal transduction within the zygote that regulates substrate availability (see Section IX.E, later in this chapter), and modifications to the ECM that would affect the accessibility of target substrates. Yet the final products—covalent cross-links that irreversibly alter the physical attributes of the egg ECM—are the same. Why would an animal select for peroxidases, with the additional requirements of a hydrogen peroxide–generating source (Heinecke and Shapiro, 1989; Takahashi *et al.*, 1989; Wong *et al.*, 2004), over a single enzyme like transglutaminase? Perhaps retention of ovoperoxidase activity is more a result of the presence and calcium-dependent activation of a hydrogen peroxide generating system initially maintained for early cleavage and cell proliferation processes (see Burdon, 1995; Chen *et al.*, 2004; Kamata and Hirata, 1999; Maulik and Das, 2002; Stone and Collins, 2002; Wong and Wessel, 2005), a holdover that facilitates the reduction of otherwise toxic hydrogen peroxide released during the postfertilization calcium wave. The consequential selection for tyrosine-rich structural proteins (Wong and Wessel, 2004, 2006), however, proves more difficult to reconcile, particularly considering the glutamate and lysine residues used by transglutaminase are generally more abundant. Perhaps further work in sea urchins, who require both transglutaminase and ovoperoxidase activity to complete fertilization envelope maturation (Battaglia and Shapiro, 1988; Deits *et al.*, 1984; Foerder and Shapiro, 1977; Veron *et al.*, 1977), will provide an answer.

E. Regulation of ECM-Modifying Enzymes

Regardless of enzyme–substrate specificity, the absence of regulated proteinase, peroxidase, and transglutaminase activity during construction of the physical block could be just as catastrophic to the zygote as not using any modifiers at all. Thus, it should be expected that the individual enzymes possess various methods of regulation, particularly because they are released into the extracellular environment and are expected to perform for a brief period only within the egg ECM.

Most of what is known about the enzymatic regulation of CG-derived proteins relates to sea urchins. CG lumens are acidic, approximately pH 5.5 (Haley and Wessel, 2004b). Because the pH of seawater lies between 7.5 and 8.0, CG contents experience a rapid and dramatic change in their environment upon exocytosis. This pH shift is thought to regulate the activity of the CG serine protease (CGSP1) (Haley and Wessel, 1999, 2004b) and ovoperoxidase (Deits and Shapiro, 1985; Deits and Shapiro, 1986). For CGSP1, the acidic CG environment maintains the enzyme in its pro–form; upon alkalization of its environment, the enzyme autoactivates (Haley and Wessel,

2004b). This conversion occurs in seconds, allowing for the rapid cleavage of proteins that attach the vitelline layer to the egg membrane and that help construct the fertilization envelope (Haley and Wessel, 1999, 2004a,b). Ovoperoxidase undergoes a similar change from an inactive to an active form upon alkalization of its environment, but this enzyme requires about 10 min to complete its conformational hysteresis (Deits and Shapiro, 1985, 1986) and can be suppressed by proteolysis (Haley and Wessel, 2004b). Thus, the egg has two independent methods that remove the potentially toxic activity of ovoperoxidase from its surface before nonspecific cross-linking activity begins: tethered separation from the surface by proteoliaisin (Somers *et al.*, 1989; Weidman *et al.*, 1985; Weidman and Shapiro, 1987) and CGSP1–specific repression (Haley and Wessel, 2004b).

A second means of regulating ovoperoxidase in the sea urchin is to control the source of its primary substrate, hydrogen peroxide (Boldt *et al.*, 1981). This NADPH-dependent oxidase activity, observed first in sea urchins (Warburg, 1908), is the main source of hydrogen peroxide in the zygote (Foerder *et al.*, 1978; Heinecke and Shapiro, 1989, 1992; Wong *et al.*, 2004). It is a member of the dual oxidase family of enzymes, containing an amino-terminal peroxidase domain, a carboxy-terminal NADPH reductase domain, and a cytoplasmic linker with two calcium-binding EF hands (Wong *et al.*, 2004). This sea urchin egg dual oxidase (Udx1) resides at the egg cortex and is sensitive to intracellular calcium concentrations, PKC phosphorylation, and intracellular pH (Foerder *et al.*, 1978; Heinecke and Shapiro, 1989, 1992; Wong *et al.*, 2004). Udx1 regulation complements the hysteretic delay in ovoperoxidase activation (Deits and Shapiro, 1985), where a similar lag time is expected for PKC activation of the reductase component *in vivo* (see Section VIII.B, earlier in this chapter). As cytoplasmic pH rises (Johnson and Epel, 1976, 1981; Shen and Steinhardt, 1978), Udx1 activity is depressed, decreasing hydrogen peroxide production, and consequentially down-regulates ovoperoxidase activity by depleting its major substrate (Wong *et al.*, 2004). The amino-terminal peroxidase of Udx1 is hypothesized to play a protective role against rampant hydrogen peroxide diffusion toward the zygote, similar to the scavenging activity of catalase (Wong *et al.*, 2004) and ovothiol (Turner *et al.*, 1986, 1987, 1988). The identification of ovothiols in many marine invertebrate eggs (Turner *et al.*, 1987) suggests that hydrogen peroxide production, possibly by Udx1 orthologs, is a conserved event in the block to polyspermy.

Is it also critical to shut off ovoperoxidase activity after complete hardening? Prolonged (120-min) exposure to hydrogen peroxide causes the purified enzyme to auto-inactivate (Deits *et al.*, 1984). The corresponding shutdown of Udx1 activity (Wong *et al.*, 2004) prevents this long-term exposure, possibly to retain the anti-microbicidal activity of ovoperoxidase within the fertilization envelope (Klebanoff *et al.*, 1979), whose source of hydrogen

peroxide could be zygotic Udx1 (Wong and Wessel, 2005). Thus, at least in sea urchins, the downstream effects of phospholipase C activity become important in the initiation and completion of the permanent block to polyspermy, as well as in the survival of the early embryo. The IP3-dependent calcium wave is essential for CG exocytosis to release the structural proteins and enzymes necessary for modifying the vitelline layer and for regulation of Udx1, whereas DAG is essential for PKC activity that modulates the NADPH-oxidase activity. Without these three components, the sea urchin fertilization envelope would remain unfinished, leaving the embryo exposed to the whims of its environment.

F. Unusual Suspects

A minor cluster of animal eggs exhibits significant biochemical and morphological changes to their egg ECM that qualify as a true physical block to polyspermy, yet the source of these alterations is not known. Although most of these eggs release the contents of their CGs upon fertilization, the contribution of these vesicles toward the ECM is negligible. For example, marsupials primarily rely on oviductal glycoproteins to coat and mask the zona from sperm rather than promoting the CG-dependent modifications observed in eutherians (see Section IX.C, earlier in this chapter) (Breed *et al.*, 2002). Other animals completely lack CGs, such as mollusks (Hylander and Summers, 1977; Togo *et al.*, 1995), ascidians (Lambert *et al.*, 1997; Rosati *et al.*, 1977), and nematodes (Singson, 2001). Zygotes of mollusks and ascidians depend on subtle changes to avoid supernumerary fusions, including the alteration of plasma membrane conductance and/or topology. One such source of these enzymes may originate from ascidian test cells, who are thought to contribute to postfertilization events that establish the permanent block to polyspermy (Rosati *et al.*, 1977), thus displaying a life cycle similar to that of serum platelets, anucleate blood cells filled with secretory vesicles whose content participate in inflammation (reviewed in King and Reed, 2002). Nematodes, on the other hand, somehow ensure monospermy at the level of the spermatheca. Soon after fertilization, zygotes subsequently acquire a chitinous eggshell as they pass through the uterus, thereby hindering further sperm–egg interactions (Singson, 2001).

Anurans use a variety of methods to establish a mechanically sound block to polyspermy. For example, some anurans simply rely on ECM reorganization and hydroscopic swelling of the outer jelly layers to create a resilient barrier against sperm (Elinson, 1986; Hedrick and Nishihara, 1991; Wolf *et al.*, 1976). In *Discoglossus*, CGs are exocytosed within 5 min of egg activation, because of the initial calcium wave (Nuccitelli *et al.*, 1988), but no changes to the egg ECM are observable until 20 min after egg activation,

when the overlying jelly plug begins to liquefy because of peroxidase-like activity (Campanella *et al.*, 1992; Pitari *et al.*, 1993). Dissolution of the plug by oxidative loss of disulfide bonds ensures that the once-ordered plug structure is destroyed, thereby eliminating access of additional sperm to the dimple (Campanella *et al.*, 1992; Pitari *et al.*, 1993). The enzyme responsible for this liquefaction derives from vacuoles released after the cortical reaction has passed (Campanella *et al.*, 1992). Thus, while all anuran zygotes appear to modify their egg jelly layers to establish the permanent block to polyspermy, they utilize extremely diverse mechanisms.

Physical blocks are also observed in animals whose eggs do not undergo a standard CG release or completely lack CGs, or even secretory granules that remotely resemble such organelles. Examples of these animals include the mollusks chiton (Buckland-Nicks *et al.*, 1988), abalone (Vacquier and Lee, 1993), and bivalves (Togo and Morisawa, 1999), the dipteran *Drosophila* (Mahowald *et al.*, 1983), urodeles (Charbonneau *et al.*, 1983; Iwao, 1989; Jego *et al.*, 1986; Makabe-Kobayashi *et al.*, 2003), ascidians (Rosati *et al.*, 1977), and nematodes (Singson, 2001). Are plasma membrane-associated electrical changes sufficient to prevent supernumerary sperm fusion, as suspected in the primitive urodele *Hynobius* (Iwao, 1989) and mollusks *Crassostrea* (Alliegro and Wright, 1983; Togo and Morisawa, 1999), *Tonicella* (Buckland-Nicks *et al.*, 1988), and *Callochitin* (Buckland-Nicks and Hodgson, 2000)? If not, then how do these externally fertilized eggs cope with the high risk of polyspermy? Do they have nonelectrical mechanisms—such as secretion of modifying enzymes from secondary vacuoles (Campanella *et al.*, 1992; Pitari *et al.*, 1993), supernumerary sperm extrusion (Yu and Wolf, 1981), or female pronuclear choice (Gould and Stephano, 2003)—to prevent or reject additional sperm at the surface or in the cytoplasm? One dramatic change in ascidians is the release of follicle cells upon fertilization (De Santis *et al.*, 1980). During the courting process, sperm must pass through tightly apposed follicle cells attached to the vitelline coat, using a mechanism that may require active participation of these cells (De Santis *et al.*, 1980). Might the follicle cells direct the sperm to a favorable region on the vitelline coat or maintain sperm-receptive tufts of fibers on its outer surface? If so, then their loss after fertilization could be the primary physical alteration that inhibits supernumerary sperm binding (Rosati, 1985; Rosati *et al.*, 1977). The use of glycosidases to abolish sperm receptivity along the vitelline coat surface has been postulated to supplement the loss of follicle cells, thereby enhancing the physical block to polyspermy (Lambert, 2000; Lambert *et al.*, 1997).

Alternatively, a nonelectrical plasma membrane block in many animal zygotes has been documented to be sufficient to block polyspermy. For example, modification of integral components along the zygotic plasma membrane has been proposed to account for membrane blocks in mammals (Hoodbhoy and Talbot, 1994). One model is that the CG-derived glycopro-

teins that form the mammalian CG envelope (Figs. 1 and 2) establish a significantly charged surface that repels sperm still trapped in the perivitelline space. Such a mechanism is consistent with the bias against sperm fusion overlying the mammalian metaphase II spindle and polar body, which colocalizes with prematurely released CGs (Cran and Esper, 1990). Unfortunately, the timing of CG envelope formation may not be early enough to establish an effective block if sperm are abundant in the perivitelline space prior to CG release. An alternative mechanism lies at the plasma membrane itself: Retraction or morphological alteration of all fusogenic microvilli or physical detachment of the old receptive membrane from the zygote have been observed in the annelid *Chaetopterus* (Eckberg and Anderson, 1985), chiton (Buckland-Nicks and Hodgson, 2000; Buckland-Nicks *et al.*, 1988), crustaceans (Brown and Clapper, 1980; Goudeau and Becker, 1982), dipterans (Mahowald *et al.*, 1983), sea urchins (Longo *et al.*, 1974, 1986), anurans (Talevi and Campanella, 1988), marsupials (Breed and Leigh, 1992), and at the membrane subjacent to the micropyle of teleosts *Danio* (Wolenski and Hart, 1987) and *Rhodeus* (Ohta and Iwamatsu, 1983). Could cortical remodeling, perhaps via an integrin-dependent cytoskeletal reorganization mediated at gamete fusion (see Sections VII.B and VII.C, earlier in this chapter), be sufficient to block polyspermy? Mouse and hamster zygotes utilize both plasma membrane and zona blocking methods (Cherr *et al.*, 1988; Tatone *et al.*, 1994; Wolf and Hamada, 1979); rabbit zygotes do not display any significant modifications to their zona or plasma membrane (Overstreet and Bedford, 1974), yet all their eggs remain monospermic. Could there be even more types of blocks to polyspermy used throughout the animal kingdom?

X. Descendents of Requisite Polyspermy?

Physiological polyspermy is the condition when multiple sperm are allowed to fuse with the egg, but subsequently only one male pronucleus is merged with the haploid egg nucleus. This mechanism of fertilization is prevalent in some orders of animals. Occasional physiological polyspermy is observed in domesticated *Drosophila* species, some naturally ovulating between 1 and 10% polyspermic zygotes (Fitch *et al.*, 1998; Snook and Markow, 2002), whereas the marsupial *Sminthopsis* exhibits at least 5% polyspermy in the oviduct (Breed and Leigh, 1990). Urodeles fail to release monospermic zygotes, despite the use of a spermatheca (Elinson, 1986), and birds and reptiles, both with large eggs, almost require physiological polyspermy to ensure the "certainty of fertilization" (Harper, 1904).

Common urodeles such as newts and salamanders (excluding *Hynobius*) produce eggs lacking CGs, and are incapable of generating a fast electrical block to polyspermy (Jego *et al.*, 1986). Although a physical block eventually forms between jelly layers—because of the hydration and consequential precipitation of lectins as the laid eggs age—this is not on a time-scale that favors monospermy because nearly 90–100% of all eggs are polyspermic (Elinson, 1986; Iwao, 1989; Jego *et al.*, 1986). This ultraslow physical block may have evolved to protect the new embryo from a second wave of sperm insemination and/or microbes rather than the second sperm to encounter the egg ECM (Jego *et al.*, 1986). Rather than committing the resources to generate a timely physical block, urodele zygotes use mechanisms that suppress all cytoplasmic sperm nuclei that do not fuse with the egg pronucleus, pushing them away from the animal hemisphere into the vegetal to avoid further developmental complications (Elinson, 1986; Fankhauser, 1932; Iwao, 1989; Iwao and Elinson, 1990). This activity is predicted to involve a local concentration of factors around the egg pronucleus/zygotic nucleus that controls the timing of cell cycle reentry; other amphibians lack cytoplasmic localization of this factor and are thereby thought to be incapable of recovering from polyspermy (Iwao and Elinson, 1990).

As expected for a zygote that undergoes requisite physiological polyspermy, modifications to the intimate avian vitelline membrane and egg plasma membrane do not change immediately after fertilization (Bellairs *et al.*, 1963). Upon ovulation, the perivitelline layer is weakened over the germinal disc where the sperm bore visible tunnels through the fibrillar meshwork (Bramwell and Howarth, 1992; Okamura and Nishiyama, 1978a), a process that promotes supernumerary sperm penetration. These holes are subsequently patched by the oviductal application of an outer layer eggshell coat, but the process of glycoprotein adsorption is not rapid enough to restrict the quantity of sperm fusing (Okamura and Nishiyama, 1978a). Avian zygotes instead limit the number of pronuclear fusion events to unity by expelling the accessory sperm pronuclei and their associated centrioles from the germ disc into extraembryonic regions punctuated by yolk granules (Bellairs, 1993; Harper, 1904; Okamura and Nishiyama, 1978b). There, the pronuclei continue to undergo asynchronous division through the early stages of cleavage, but eventually degenerate (Harper, 1904).

Among vertebrates, the use of physiological polyspermy is generally restricted to more ancient lineages, for example, reptiles, birds, urodeles (Elinson, 1986), and chondroichthyes such as sharks and chimera (Hart, 1990). At face value, this suggests that methods to cope with polyspermy may be older than the complexity associated with establishment of a block to polyspermy. Yet the essential components of the egg ECM between purely monospermic and physiologically polyspermic animals are often homologous, suggesting that only a fine distinction exists between the selection of

ECM components for sperm binding versus their coevolution as postfertilization scaffold proteins necessary to establish a block to polyspermy. Is it really more efficient for an egg to package a self-assembling barrier into granules that must be coordinately released soon after fusion than to partition a select group of cytoplasmic molecules that can "choose" a dominant sperm pronucleus? Broadcast spawners would clearly benefit by retaining self-assembling barriers because such structures provide both a physical block to polyspermy *and* a protective shell for embryogenesis. Internally fertilized animals, particularly ones that use oviductal contributions to create a protective shell for development, benefit as much from such an egg-derived barrier. In fact, the energy expenditure required to synthesize CGs would likely selectively decrease the quantity of eggs produced to favor the survival of the potential zygote. Thus, one major factor influencing the selection of a cytoplasmic (physiologic) or extracellular (monospermic) block to polyspermy could be the type of insemination used (internal or external).

Within a deuterostome order, it is possible to trace phylogenetic paths that map the more ancient mechanisms to cytoplasmic blocks, whereas the most recently diverged taxa use extracellular blocks (see Figs. 4 and 6). For example, amphibians are represented by urodeles and anurans. Most urodeles exhibit internal fertilization, whereas anurans display both internal and external (see Section V.A, earlier in this chapter) (Elinson, 1986; Toro and Michael, 2004). Urodeles also exhibit a cytoplasmic block to polyspermy, despite the rare presence of a fast electrical block in the more primitive members (e.g. *Hynobius*) (Charbonneau *et al.*, 1983; Iwao, 1989; Iwao and Jaffe, 1989). Most urodele species also lack CGs (Elinson, 1986). On the other hand, most anurans possess both a fast electrical block and CGs, regardless of the type of insemination used (Elinson, 1986; Iwao, 1989; Iwao and Jaffe, 1989). Thus, monospermy is favored through extracellular modifications. In *Discoglossus*, monospermy is further enhanced by severe polarization of egg, but relies on both an efficient fast electrical block and a delayed CG-dependent modification of the ECM to ensure monospermy (Campanella *et al.*, 1992; Nuccitelli *et al.*, 1988; Pitari *et al.*, 1993; Talevi and Campanella, 1988). Under the rare circumstances when polyspermy does occur in *Discoglossus*, the zygote can rapidly eliminate the supernumerary sperm before its pronucleus has completely penetrated the cytoplasm (Talevi and Campanella, 1988). Based on the hypothesis that the cytoplasmic block is more ancient than an extracellular block, urodeles would be placed basal to anurans, with *Discoglossus* more basal to the other anurans such as *Xenopus*, *Bufo*, and *Eleutherodactylus*. Unfortunately, this organization cannot be tested against the pedigree because amphibians are thought to be diphylogenetic, where urodeles and anurans may have arisen from different lineages (Elinson, 1986). Within anurans, however, *Discoglossus* is thought to occupy a more basal position than the others listed. Thus, the

transition from a cytoplasmic block to an extracellular block may have required the sequential acquisition of a fast electrical block, followed by a more robust CG contribution to the zygotic ECM in anurans.

A more robust comparison can be made using the phylogeny of fish. Sharks and chimeras utilize spermathecae during internal fertilization, but still require a cytoplasmic block at fertilization (Hart, 1990; Neubaum and Wolfner, 1999). Other orders of spawning fish release highly polarized eggs that exhibit increasingly more sophisticated specializations (see Fig. 4): Petromyzontids use the earliest signs of a localized site for enhanced fusogenicity, namely a tuft of sperm-attractive jelly, extension of an acrosomal process following acrosome exocytosis, as well as both fast electrical and permanent blocks to polyspermy initiated by specialized CGs below the site of sperm fusion (Dabrowski *et al.*, 2004; Kobayashi and Yamamoto, 1994; Kobayashi *et al.*, 1994). Next are the chondrosteans, whose use of a cluster of multiple micropyles and an acrosomal process reduces sperm/egg ratios but does not ensure monospermy as elegantly as the single micropyle found in teleosts (Hart, 1990; Cherr and Clark, 1986). Thus, fish eggs exhibit a gradient of complexity along its pedigree (Fig. 4). Sharks and chimeras are phylogenetically basal to other bony fish, which rank in the order petromyzontids, chondrosteans, to teleosts from most ancient to most recently diverged. Thus, as in anurans, cytoplasmic blocks presumably evolved before extracellular blocks in fish. The rapid specialization in ECM morphology from a polarized site of sperm entry to a single micropyle is also an intriguing transition. Ironically, with the progression toward a single micropyle comes a reduction in the number of CGs contributing to zygotic ECM modifications (Hart, 1990; Hart and Donova, 1983) and the loss of the sperm acrosome and acrosomal process—two features hypothesized to be advantageous for spawning animals (see Section IV, earlier in this chapter). Thus, in fish, extracellular blocks to polyspermy appear to be undergoing minimization in the overall energy expenditure during gametogenesis. Yet selection of this specific method to favor monospermy requires a significant degree of morphological and molecular complexity in the egg ECM. Does this progress, then, truly represent advancement? For spawners, yes; for animals using internal fertilization, not really.

Could the association between cytoplasmic blocks and internal fertilization indicate an overall trend in the progressive evolution of an animal lineage? Nematodes fertilize internally and display requisite monospermy in the absence of any ECM modifications (Singson, 2001). Most dipterans use micropyles to achieve monospermy during internal fertilization (Snook and Markow, 2002). Mammalian eggs are internally fertilized and achieve monospermy with the help of CGs and associated cumulus and/or oviductal cells (Selwood, 1992). Mollusks spawn, but only require a change in membrane and cytoskeletal organization to maintain monospermic conditions

(Dufresne-Dube *et al.*, 1983; Hylander and Summers, 1977). Decapods also spawn, using a fast hyperpolarization and an elaborate hierarchy of CGs to establish monospermic conditions (Gould and Stephano, 2003; Talbot and Goudeau, 1988). Echinoderms spawn, but use the full spectrum of ECM modifications to sustain monospermy (Shapiro *et al.*, 1989). Separating these monospermic animals based on their method of insemination, a similar trend of increasing complexity in the type of extracellular monospermic blocks can be distinguished: Within either internal or external fertilizers lies a gradient of increasing ECM complexity that parallels the predicted phylogenetic position of the taxon. Hence, more ancient animals possess the fewest plasma membrane or ECM modifications, whereas the more recently diverged animals have acquired the most (Fig. 6).

Yet consider all animals originated from a hypothetical "common" ancestral egg that possessed all of the aforementioned mechanisms involved in the permanent block to polyspermy, namely a fast electrical block, abundant CG contributions to the ECM, and contributions from the oviduct if the animal is internally fertilized. Next, consider the amount of time each taxon has had to individually refine its eggs from this common ancestor. In such a hypothetical situation, selection would favor the most efficient mechanism of monospermy, one that requires minimal expenditure of resources while still ensuring a high rate of fertilization success. In such a scenario, the taxa with the least amount of time to refine the process of fertilization would look most like the common ancestor. Conversely, those around longer would have had time to rework the process, eventually losing most of the extraneous parameters in favor of a streamlined process. One result of this time-dependent evolution would be the complete loss of extracellular blocking mechanisms in favor of physiological polyspermy because it represents the most efficient mechanism and requires the least expenditure of energy: namely, the intracellular localization of specific factors that choose the sperm pronucleus to fuse with. With such simplification may come the adoption of an embryonic coat for protection during development. Mapping these criteria based on time of separation from the common ancestor would yield a cladogram of animal phylogeny quite similar to our current working model. Granted, the hypothetical scenario would miscalculate the attributes of a few animals, such as echinoderms and dipterans, but the overall pattern would be strikingly familiar. Therefore, it is quite possible that the process of animal fertilization may have a common ancestor.

XI. Perspective

Dynamics between the egg ECM and sperm are essential to achieve monospermic fertilization. Common structural motifs are retained in egg matrices of many different animal phyla, as well as overlapping enzymatic

contributions from the CGs postfertilization. A common method of ECM modifications was most likely used during fertilization in the most common ancestor to animals, so what were the original requirements? Based on our current understanding, common ultrastructure, molecular motifs, and mechanisms are used during specific stages of fertilization and the block to polyspermy, including (1) the indiscriminate auto-polymerizing ZP domain as a common building block of the egg ECM (Jovine *et al.*, 2002); (2) species-specific sperm receptors composed of signature branched oligosaccharides bound to homologous proteins found in both the egg ECM and the oviductal enhancements; (3) the interaction of at least one transmembrane protein with its complement to initiate sperm–egg fusion; (4) the use of CG–derived proteases, protein–protein cross-linking enzymes, and lectins to render the egg ECM incapable of further sperm binding after fertilization; and (5) exchange of the egg ECM with a more intimate zygotic ECM of different composition than the original that can serve as a substratum for embryonic development and signaling (Bellairs *et al.*, 1963; Matsunaga *et al.*, 2002; Selwood, 1992).

A clear distinction exists, however, between internally and externally fertilizing animals: Internally fertilizing animals have also retained the participation of oviduct epithelial contributions toward sperm retention and deposition of a mechanical mucoid coat onto the surface of the zygote. These anatomical contributions to fertilization may have relaxed the selective pressure for more robust ECM-associated mechanisms that are prominent in externally fertilizing animals. Thus, although further characterization of the proteins composing the egg ECM and CGs must be made, we speculate that an essential set of orthologs has been retained throughout evolution for the purpose of blocking polyspermy. Modifications and adaptations of these original processes were made by each animal pedigree, thus achieving the present diversity in mechanisms of monospermic fertilization.

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Dishevelled: A Mobile Scaffold Catalyzing Development

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I. Introduction
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Wnt proteins are secreted glycoprotein ligands that regulate critical aspects of development, including cell proliferation, apoptosis, and cell fate. For those pathways downstream from the "canonical" Wnt/beta-catenin signaling, from the "non-canonical" or planar cell polarity (PCP), and from the Wnt-Ca^{2+}/cyclic guanosine monophosphate (cGMP) pathway, Wnt activation of its cellular receptor, a member of the superfamily of G-protein–coupled receptor Frizzled family, requires both heterotrimeric G proteins and the phosphoprotein Dishevelled. Our understanding of the roles of Dishevelled proteins in development is evolving and most recent observations suggest that Dishevelled proteins act as scaffolds essential for Wnt signaling, providing docking sites for a diverse and interesting set of protein kinases, phosphatases, adaptor proteins, G proteins, and other scaffolds such as Axin. The protein–protein interactions of Dishevelled are dynamic, as is the spatial localization of this "toolbox" of signaling molecules involved in development. Much excitement awaits the elucidation of the complete set of tools in the toolbox and of the dynamic regulation of Dishevelled proteins and their interacting proteins.
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I. Introduction

Cell signaling takes on a special significance in development. Cell biologists view the expansion in our understanding of cell signaling molecules, pathways, and networks as a central challenge to more fully understanding early development. Layering onto this challenge the task of creating a complex highly-structured multicellular organism composed of 10^2–10^9 cells, all derived from a single cell, provokes a stark appreciation for the task confronting the developmental biologist. Discovered only 20 years ago, the Wnt family of secreted glycoproteins is known to regulate virtually all aspects of development (Cadigan, 2002). Not surprisingly, dysregulation of Wnt pathways results in disruption of normal development in humans, as well as other diseases including cancer (Moon *et al.*, 2004). The study of the biology of Wnt signaling has been filled with surprises. Although features of the canonical or Wnt/beta-catenin pathway controlling expression of genes regulated in a Lef/Tcf-dependent manner have been uncovered by classical genetics, remarkably, new elements continue to be identified, such as their cellular receptors Frizzleds (cloned in 1996 [Wang *et al.*, 1996]), co-receptors LRP5/6 (identified in 2000 [Wehrli *et al.*, 2000]), heterotrimeric G proteins (identified in 2001 [Liu *et al.*, 2001]), and the prenylation of Wnt itself (discovered 2003 [Willert *et al.*, 2003]) solving a 20-plus-year mystery in the challenge of isolating active Wnt ligand.

Whether it is in flies or in mice, the known proximal elements of the Wnt/beta-catenin pathway include Wnt, LRP5/6, Frizzled-1, heterotrimeric G proteins, and an interesting family of phosphoproteins termed *Dishevelled* for the manner in which *Drosophila* present themselves when the gene for the single member of this family in flies (Dsh) is inactivated (Klingensmith *et al.*, 1994). Basic aspects of development, especially planar cell polarity (PCP), are disrupted in the Dishevelled mutant (Theisen *et al.*, 1994). Embryonic lethality results from complete loss of Dishevelled in the fly (Perrimon and Mahowald, 1987), mimicking loss of the fly Wnt wingless (Wg). Incomplete loss of Dishevelled provokes other phenotypes, including disorganization of the ommatidia in the eye, and phenotypes in which structures in neighboring cells, for example, the denticles or wing hairs, are no longer properly organized with the plane of the epithelium, generating a decidedly "disheveled" or unkempt appearance (Krasnow *et al.*, 1995). In mammals there are three members of the Dishevelled (Dvl) family, Dvl-1, -2, and -3, products of three genes whose protein primary sequences differ significantly from one another, although all possess several landmark motifs and docking sites (Wharton, 2003). Results from numerous laboratories identifying numerous Dsh/Dvl-associated proteins, suggest Dsh/Dvl proteins as core to multicomponent signaling complexes, and suggest a dynamic spatial localization for

Dsh/Dvl. Each of these "leads" explored in this chapter requires vigorous follow-up if we are to fully appreciate the multifaceted functions of Dsh/Dvl. To simplify the discussion of current information on the Dsh/Dvl family, the mammalian Dvl-2 will be targeted for detailed analysis, with discussions expanding to paralogues and orthologues in the broader family.

II. Dishevelled, a Scaffold/"Toolbox" for Wnt Signaling

Inspection of primary sequences *in silico* suggests a key role of Dvl (i.e., multivalent docking). Three conserved domains provide the major landmarks of Dvls: a *Di*shevelled and A*x*in (DIX) binding domain at the N-terminus; a *p*ost-synaptic density-95, *d*iscs-large and *Zonula occludens*-1 (PDZ) domain in the midregion of Dvl; and a Dishevelled, Egl-10, Pleckstrin (DEP) domain located about midway between the PDZ domain and the C-terminus of Dvl (Fig. 1). The DIX domain itself enables the possible dimerization of Dvl with other members of the Dvl family and with Axin (Zeng *et al.*, 1997), which itself is a scaffold protein functioning downstream of beta-catenin to organize the multiprotein complex responsible for establishing the intracellular concentration of beta-catenin (Luo and Lin, 2004). The PDZ domain provides a docking site for a large number of proteins that include protein kinases (e.g., casein kinase 1, casein kinase 2, and PAK), phosphatases (e.g., phosphoprotein phosphatase 2C family members), adaptor proteins such as beta-arrestins, and importantly Frizzleds with a C-terminal PDZ ligand structure (Kay and Kehoe, 2004). Two regions of basic residues bracket the PDZ domain and these regions appear to bind the

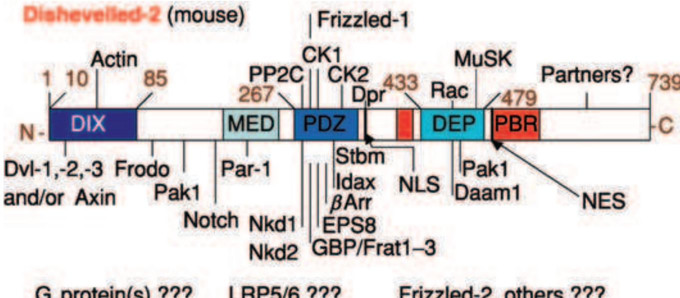

Figure 1 Schematic of mammalian (mouse) Dishevelled-2 (Dvl-2). There are more than 20 suspected Dishevelled-interacting proteins (DiPs) for Dvl-2. Not shown are several additional phosphoprotein phosphatases (PPTases), including PP2A isoform subunits, PPT2A, PTP-BL, and phosphotyrosine phosphatases. A large region of the C-terminus (in which the three mammalian Dvl differ significantly) displays no known docking site for Dvl-interacting proteins, although it seems likely that such proteins have only escaped detection.

Par-1 kinase (Sun *et al.*, 2001) and the product of the naked cuticle gene (Nkd) (Wharton *et al.*, 2001). These regions may also function in association with the inner leaflet of the cell membrane, attracted to the negative charges there. The organization of the N-terminal basic residues has some similarity to the membrane effector domain (MED) of the MARCKS protein (McLaughlin *et al.*, 2002), providing the possibility of a dynamic reversible association with the lipid bilayer. Finally, the DEP domain enables protein–protein interactions between Dvl and Daam1 (Boutros *et al.*, 1998), linking Dvl to the small molecular weight guanosine triphosphatases (GTPases) like RhoA, as well as between Dvl and protein kinases like the muscle-specific kinase, MuSK. Lacking from our understanding of the landscape of Dvl is the region(s) responsible for interaction with heterotrimeric G proteins and perhaps Frizzled/LRP. More than a dozen other known or suspected protein-binding domains can be found in Dvl, creating perhaps a multivalent docking station or "toolbox" to support Wnt signaling in at least Wnt/beta-catenin, PCP, and convergent extension (CE) pathways known to be regulated by the canonical elements.

III. The Contents of the "Toolbox"

The three primary domains of Dvl scaffolds (i.e., DIX, PDZ, and DEP) have been well described and are conserved throughout metazoan evolution. Less well defined are the protein-binding sites outside of these three domains of Dvls and the complete contents of these "toolboxes," with many of the Dvl-associated proteins only suspected of direct functional interactions with Dvl (Table I). The Dvl-associated proteins that make up the contents of the toolbox can be organized into four functional drawers of Dvl (Fig. 2): G-protein–coupled receptor (GPCR) signaling (e.g., Frizzled, co-receptor LRP5/6, Go protein, Gq protein, and the GPCR adaptor beta-arrestin); beta-catenin signaling (e.g., other Dvl proteins, Axin, Nkd, Frat, Idax, GBP/Frat, Frodo, GSK-3β, and β-TrCP); signaling of tissue polarity/PCP (Strabismus, Van Gogh, Trilobite, Daam1, RhoA, Rho-associated protein kinase [ROCK], Rac, c-Jun N-terminal kinase [JNK], and Prickle); and lastly the more generalized "tools" in the signaling toolbox, especially protein kinases/phosphatases (e.g., Ck1, Ck2, GRK2, Par-1, PAK, PP2C, and borrowing from the literature of other such scaffolds perhaps even PKA and PKC). Work has shown PKA signaling to play an important role in myogenesis induced by Wnt ligands (Chen *et al.*, 2005). These tools are assembled into at least three basic pathways (Fig. 2): the Wnt/beta-catenin pathway, controlling the activation of genes whose transcription is regulated by Lef-Tcf–sensitive promoters (Fig. 2A); the Wnt/ROCK/JNK pathway, controlling PCP and cell movements (Fig. 2B); and the Wnt/Ca^{2+}/cyclic

Table I Dishevelled-Interacting Proteins (DiPs)

Pathway	DiP	Interaction site	References (not exhaustive)
Wnt/beta-catenin	Frizzled-1	PDZ/?	Wong *et al.*, 2003
	Go/Gq	Not known	Katanaev *et al.*, 2005; Liu *et al.*, 2001
	Axin	DIX	Wharton, 2003
	β-arrestin	PDZ	Chen *et al.*, 2001
	CK1	PDZ	Peters *et al.*, 1999
	Dvl-1, -2, -3	DIX	See text
	GBP/Frat	PDZ	Li *et al.*, 1999
	Frodo/Dapper	DIX	Cheyette *et al.*, 2002
	Idax	PDZ	Hino *et al.*, 2001
	Nkd	Basic/PDZ	Rousset *et al.*, 2001
Wnt/JNK	Frizzled-1	PDZ/?	Wong *et al.*, 2003
	Go	Not known	Liu *et al.*, 2001
	Daam1	DEP	Habas *et al.*, 2001
	Dvl-1, -2, -3	DIX	See text
	Prickle	PDZ/DEP	Tree *et al.*, 2002
	Rac1	DEP	Fanto *et al.*, 2000
	Smgs	DEP	See text
	Strabismus	PDZ	Park and Moon, 2002
Wnt/Ca^{2+}/cGMP	Frizzled-2	?	?
	Go/Gt	Not known	Ahumada *et al.*, 2002; Liu *et al.*, 1999
	β-arrestin	PDZ	Chen *et al.*, 2003
	Dvl-1, -2, -3	DIX	See text
Wnt/multiple pathways	CK2	PDZ	Willert *et al.*, 1997
	Eps8	PDZ	Inobe *et al.*, 1999
	MuSK	DEP	Luo *et al.*, 2002
	PAK	DIX/PDZ	Luo *et al.*, 2002
	Par-1	Basic	Sun *et al.*, 2001
	PP2C	PDZ	Strovel *et al.*, 2000

guanosine monophosphate (cGMP) pathways, controlling calcium-sensitive transcription factors (e.g., NF-AT), phosphorylation of substrates of protein kinase G, and tissue separation/ventral axis specification (Fig. 2C). The central role of Dvl in virtually all of Wnt signaling is obvious from Fig. 2. For the animal cells expressing three isoforms of Dvl (i.e., Dvl-1, Dvl-2, and Dvl-3), the central question remains as to which Dvl(s) is (are) mediating each of these pathways? How do the three Dvls interact with each other and compete for binding to Axin and other DIX domain–bearing proteins?

Equally intriguing is the early observation that the transmembrane receptor for lateral inhibitory signals in the fly, Notch, is a Dsh-binding protein that may suppress Wg signaling by sequestering Dvl (Zecchini *et al.*, 1999). Notch, like heterotrimeric G proteins and LRP5/6, may provide the basis for

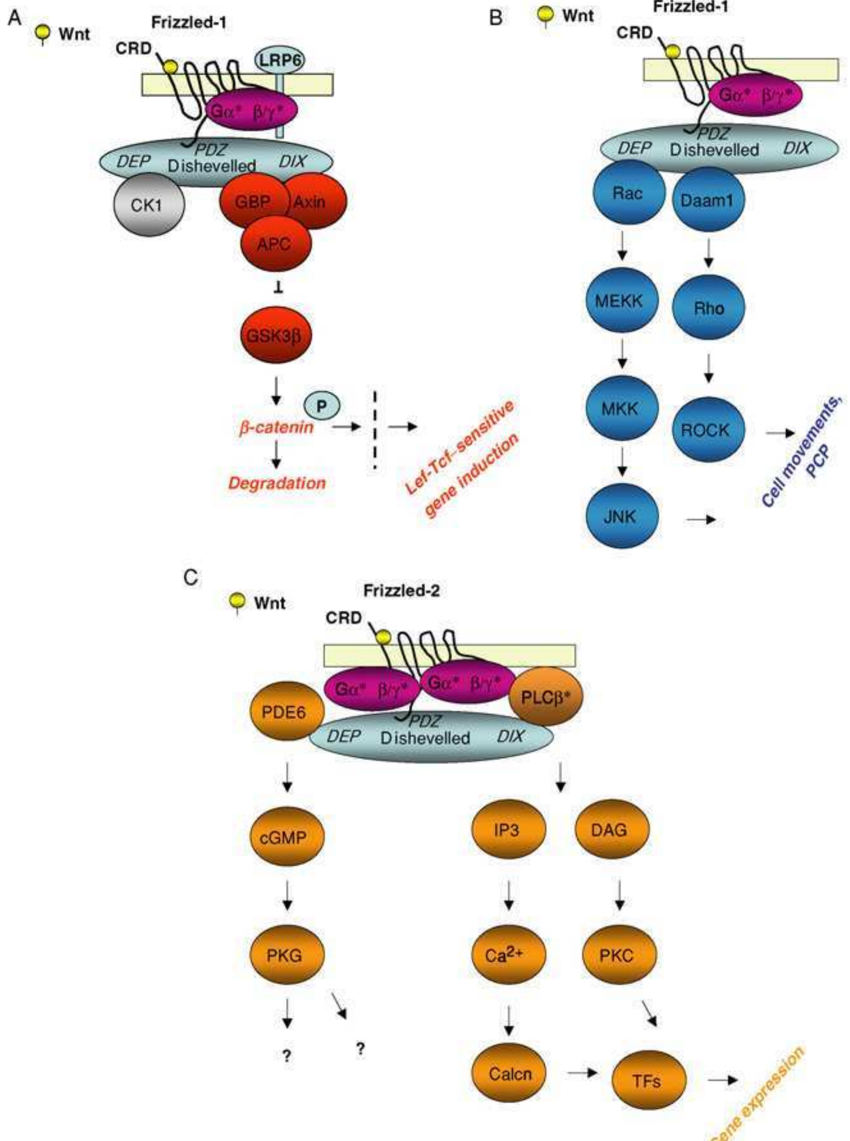

Figure 2 The three Dvl-based signaling pathways of Wnt signaling: the Wnt/beta-catenin pathway regulating the intracellular and nuclear accumulation of beta-catenin and the Lef-Tcf–sensitive genes involved in development (A); the Wnt/ROCK/JNK pathway regulating planar cell polarity and cell movements (B); and the Wnt/Ca^{2+}/cGMP pathways controlling the activation of Ca^{2+}-sensitive responses and suppressing the activation of cGMP-dependent responses (C). For greater details, see review articles cited in the text.

transient membrane association of Dvl, an important spatial aspect of Wnt signaling (see later discussion). A more complete description of the contents of the Dvl toolbox requires detailed analyses using various approaches, both biochemical, cell biological, and functional in nature. Current proteomic mass spectrometry avenues can establish precise sites of posttranslational modification, such as phosphorylation and sumoylation, which may enable docking of partner proteins by Dvl. Defining the phosphorylation sites and motifs required for kinase recognition will assist in identifying those protein kinases for which Dvls are bona fide substrates. In tandem with targeted mutagenesis of these sites, changes in Wnt-stimulated phosphorylation of Dvl can be compared with functional consequences, using a variety of sensitive readouts.

At least in mammals, the presence of three Dvls provides an obstacle using functional readouts that may be overcome by knockdown of competing Dvls. Why mammals express three distinct Dvls remains a fundamental question in development. Equally challenging will be deducing the temporal sequence for the assembly of these multivalent signaling complexes during Wnt-stimulated activation, deactivation, and resensitization/recycling. The best paradigm for achieving this overarching goal is that developed for the successful analysis of key members of the 40-plus members of the [protein kinase] A-kinase anchoring proteins (AKAPs) scaffolds (Malbon *et al.*, 2004b; Wong and Scott, 2004).

IV. Mobile Scaffold?

Several reports provide background about Dvl localization and mobility in cells. In most instances, Dvl appears to be largely confined to the cytoplasm (Habas and Dawid, 2005). Overexpression of Frizzleds appears to provoke a relocalization of a significant amount of Dvl to the inner leaflet of the lipid bilayer (Fig. 3), presumed to reflect the association of Dvl with Frizzled or some Frizzled-interacting protein (G protein [?], LRP5/6 [?]). In some cases, activation of cells by Wnt has led to a greater localization of Dvl at the cell membrane, whereas in others, activation by Wnt leads to an apparent redistribution of Dvl from the cell membrane. Caution must always be applied to the interpretation of studies in which overexpression may well violate the stoichiometry among signaling complexes, leading to interesting but not necessarily relevant observations about the signaling elements. Observations using autofluorescent fusion proteins of Dvl suggest that a functional nuclear export sequence (NES) is present in the C-terminus of Dvl, a motif that enables cycling of proteins out of the nucleus (Itoh *et al.*, 2005). But what is Dvl doing in the nucleus? Loss of this NES had little effect on Wnt/beta-catenin signaling, whereas mutations that prevented nuclear

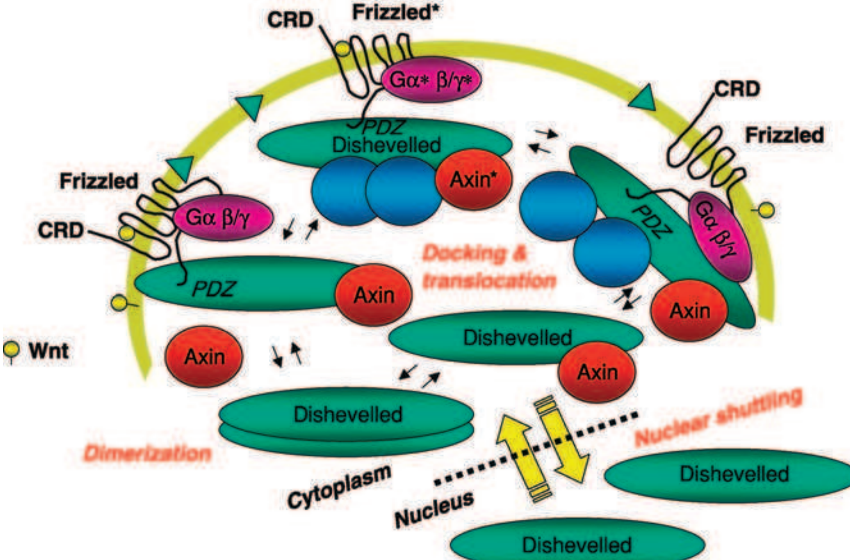

Figure 3 Schematic of Dishevelled (Dvl) localization and trafficking in response to activation of Frizzled by various Wnt proteins. Dvls can be found in several subcellular compartments within the cell, especially in the proximity to the inner leaflet of the cell membrane. Elsewhere, it can generally be found throughout the cytoplasm, although the nature of this localization and the cellular elements to which Dvl might attach transiently (e.g., cytoskeletal elements) remains to be studied. Evidence has been presented for "nuclear shuttling" of Dvl to and from the nucleus, as well as for the presence of both a nuclear localization sequence (NLS) and a sequence that is required for nuclear export (NES). The "docking and translocation" of various Dvl-interacting proteins is highlighted in this cartoon, and there exists more than 20 proteins that are believed to interact with Dvl in Wnt signaling. The presence of a DIX domain in all three forms of Dvl and in the structure of Axin implicates "dimerization" (or oligomerization) of Dvl in homologous assemblies (with another Dvl) or in heterologous assemblies with Axin. The nature and extent to which Dvls form these oligomers is not known.

localization also block this canonical, but not the noncanonical, pathways. Substitution of a well-known nuclear localization sequence in the mutated Dvl molecule restored the nuclear localization and rescued Wnt/beta-catenin signaling (Habas and Dawid, 2005; Weitzman, 2005). If these observations are universal, what is the role of the nuclear-targeted Dvl? Additionally, sumoylation has been shown to confer reversible nuclear targeting upon substrate proteins (Watts, 2004). Is sumoylation playing any role in the biology of Dvl?

Some insights into the trafficking of a Dvl-based "toolbox" may be drawn from the study of the AKAP family, particularly those AKAPs (e.g., AKAP250) that interact with signaling complexes (Wong and Scott, 2004),

which include GPCR (like Frizzleds) and G proteins (Fig. 4). Both AKAP250 (Fig. 4A) and Dvl-2 (Fig. 4B), for example, represent toolboxes that each contain numerous kinases, phosphatases, adaptor molecules, and others that enable proper signaling (Malbon *et al.*, 2004a). The interactions between the toolbox and its contents for both AKAP250 and Dvl appear to be reversible and dynamic in character (Malbon *et al.*, 2004b). Each scaffold not only docks protein kinases but also serves as a substrate for these kinases, including serine/threonine protein kinases (e.g., PKA, PKC, Par-1), as well as for tyrosine kinases. Interestingly, it has been shown that several of the tools in the AKAP toolbox operate strictly in a *cis*-configuration, that is, the PKA phosphorylates only the AKAP to which it is docked (Tao *et al.*, 2003). This startling observation provides some explanation for the extraordinary spatial constraints we anticipate for complex signaling networks to restrict signal propagation to only network members being targeted and not the whole network. For Dvl-based signaling, we can anticipate similar spatial constraints, in which cells expressing all three Dvls can segregate downstream signaling as necessary while providing a central level for integration of signals from the broader network.

V. Major Hurdles Ahead

Many of the key unresolved questions about Dvl biology and the roles of Dvls in development will require new strategies. Mass spectrometric strategies of proteomics are powerful enough strategies with which to provide a detailed map of the Dvl molecules isolated from naive cells compared to Wnt-activated cells. A major and formidable challenge of mass spectrometry–based strategies to analysis of Dvl is the relative low abundance of the molecules. As protein abundance in most cells spans five, six, or more orders of magnitude, analysis of Dvl alone will require extensive purification steps that themselves may have very low yields. Using pull-down assays on immobilized affinity matrices from whole cell lysates, multivalent signaling complexes can be isolated and inspected for known (e.g., via immunoblotting) and novel (e.g., via proteomics) binding partners, illuminating the entire contents of the Dvl toolbox. Unfortunately, there is decidedly little biochemical knowledge of Dvls, only that which is known by the study of the various well-known protein motifs present in the molecules (e.g., DIX, PDZ, and DEP). Our own experience, gained from years of study of relatively low abundance molecules such as GPCRs, G proteins, and AKAPs, is that the more detailed *in vitro* biochemistry available on the molecule at the start, the easier and more feasible the analysis of the molecules *in vivo*. For many such situations, successful proteomic analysis of pull downs from cell lysates is dependent on first developing the fragmentation patterns and sequence of

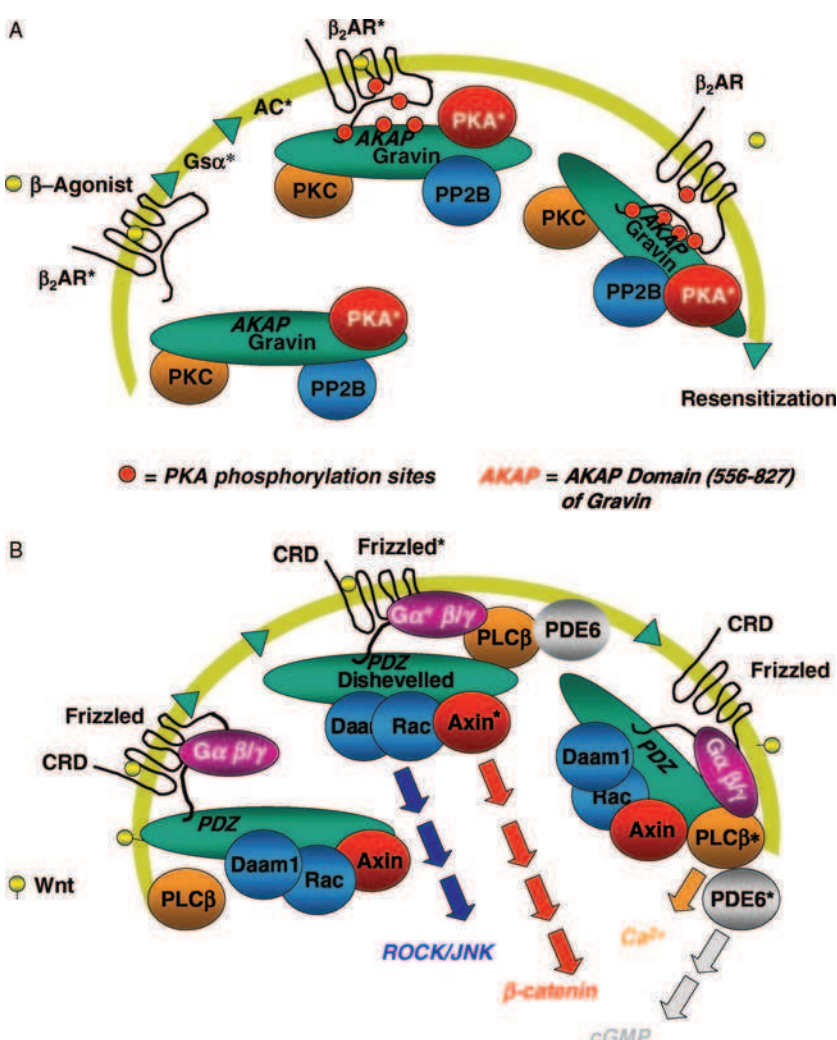

Figure 4 Comparison of the mobile scaffold functions of AKAP250 (A) and Dvl (B). For the AKAP250 situation, β_2-adrenergic receptor (β_2AR) is localized to the cell membrane via its seven transmembrane-spanning segments, providing three intracellular loops and a C-terminal "tail" in the cytoplasmic compartment of the cell. The activation of this G-protein–coupled receptor (GPCR) (β_2AR*) by binding of an agonist stimulates the activation of Gs (Gsα*) and activation of adenylyl cyclase (AC*), leading to the production of cyclic adenosine monophosphate (cAMP) and the activation of protein kinase A (PKA*). In the absence of GPCR activation, AKAP250 (a.k.a., gravin) docks several kinases and at least one phosphatase, namely protein kinase C (PKC), PKA, and PP2B. When activation of the receptor-mediated pathway occurs, activation of PKA leads to phosphorylation of both the receptor and the scaffold gravin. The phosphorylation of the receptor occurs on serine/threonine residues in the intracellular loop and C-terminal tail. Gravin undergoes phosphorylation by PKA, confined

purified targets, in this case a Dvl. With microgram quantities of protein expressed in any number of high-expression systems, one can work out all of the details and fragmentation patterns before operating at the threshold of sensitivity for most mass spectrometers. The good news is that this investment provides a wealth of information on the protein, posttranslational modification, and potential partners necessary for the study of Dvl-based signaling complexes.

Study of the spatial localization and dynamics of Dvl in naive and Wnt-treated cells presents even more formidable challenges at this "meso" scale of biology, a scale too large for x-ray crystallography and too small for detailed inspection by fluorescence microscopy. Large macromolecule complexes, if uniform and abundant, can be probed with atomic force microscopy (AFM) and/or cryoelectron microscopy (CEM). But how to monitor the actual protein–protein interactions of Dvl with potential partners? How to determine whether Dvls actually oligomerize with other Dvl molecules and/or with Axin? Fortunately bioluminescence resonance energy transfer (BRET) provides a strategy in which a sea daisy luciferase is fused with one partner of interest, while the sea daisy green fluorescent protein moiety is fused to another protein. Energy transfer can be monitored in live cells in a sensitive manner, providing compelling data on the ability of Dvl to interact physically with a partner molecule, such as Go protein, Frizzled, Axin, another Dvl, and so on. Theoretically speaking these three approaches appear to be the most promising for unraveling the mysteries of Dvl-associated proteins. Once the full cellular complement of Dvl-associated proteins is established, BRET, AFM, and CEM can be employed to deduce both the proximity and the temporal character of the protein–protein interactions.

VI. Concluding Remarks

Major gaps remain in our understanding of the molecular basis of Wnt action in development. One such high-value target for analysis is the phosphoprotein Dishevelled, shown to play an essential role in development of

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largely to the "AKAP domain," which reversibly interacts dynamically with the GPCR. The PKA-catalyzed phosphorylation enhances the docking of AKAP250/gravin to this GPCR and is essential for the resensitization process which follows, a process dependent on the protein phosphatase PP2B. For Dishevelled, it appears that this phosphoprotein associates with the Frizzled-1 GPCR via the PDZ ligand "tail" of the GPCR and the PDZ domain common to all Dvls. The Dvl provides a docking site for numerous protein kinases, phosphatases, and adaptor molecules and is reported to shuttle to and from the nucleus in response to Wnt stimulation. Thus, both AKAP250 and Dvl appear to be "mobile scaffolds" that act as toolboxes of signaling pathway components that must traffic to various intracellular compartments to complete the biology of cell signaling for the β_2-adrenergic receptor and the GPCR Frizzled, respectively. See the text for details.

the fly, worm, frog, fish, and mammals. Dvl is at the vertex of cell signaling emanated from Wnts downstream to the canonical Wnt/beta-catenin pathway and PCP. Cell proliferation, apoptosis, and cell fate are governed largely by Wnt-derived signals, so the gaps in our knowledge of Dishevelled are worthy of intense investigation. Future experiments focus on three distinct but interrelated efforts: elucidation of the domains by which Dishevelled proteins propagate and integrate signals; completion of a now rudimentary census of Dishevelled-associated proteins, organized in families of GPCR-related proteins, beta-catenin–based signaling, PCP gene products, and protein kinases/phosphatases critical to Wnt signaling; and discovery of the spatial and temporal regulation of Dishevelled within the cell, delving into the order by which protein–protein interactions link Wnt binding to development. Although trends favor Dishevelled proteins' roles in the canonical and PCP pathways, exploring in greater detail whether Dishevelled proteins play similar roles in Wnt signaling through the noncanonical Wnt/Ca^{2+}/cGMP pathway (or others) would seem a prudent investment.

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3

Sensory Organs: Making and Breaking the Pre-Placodal Region

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Sensory placodes are unique domains of thickened ectoderm in the vertebrate head that form important parts of the cranial sensory nervous system, contributing to sense organs and cranial ganglia. They generate many different cell types, ranging from simple lens fibers to neurons and sensory cells. Although progress has been made to identify cell interactions and signaling pathways that induce placodes at precise positions along the neural tube, little is known about how their precursors are specified. Here, we review the evidence that placodes arise from a unique territory, the pre-placodal region, distinct from other ectodermal derivatives. We summarize the cellular and molecular mechanisms that confer pre-placode character and differentiate placode precursors from future neural and neural crest cells. We then examine the events that subdivide the pre-placodal region into

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individual placodes with distinct identity. Finally, we discuss the hypothesis that pre-placodal cells have acquired a state of "placode bias" that is necessary for their progression to mature placodes and how such bias may be established molecularly. © 2006, Elsevier Inc.

I. Introduction

A. Cranial Placodes and Their Derivatives

The "sensory placodes" were first described more than a century ago as localized thickenings in the cranial ectoderm of vertebrates (von Kupffer, 1891). Although placodal structures are also observed during formation of numerous other organs, such as teeth and hair, here we refer only to those that form crucial parts of the sensory nervous system. These sensory placodes contribute to the special sense organs (the olfactory epithelium, eye, and ear) and to the cranial sensory ganglia. Within these sensory structures, placode-derived cells generate a vast array of functionally different cell types, which we describe briefly (extensive descriptions can be found in Baker and Bronner-Fraser, 2001; Graham and Begbie, 2000; Webb and Noden, 1993).

Three of the eight placodes give rise to both specialized receptor cells and the neurons that convey this sensory information to the central nervous system (CNS). The olfactory placode generates odorant- and pheromone-sensing cells that populate the epithelium of the nose, and via the olfactory nerve, these primary sensory neurons directly project to the olfactory bulb of the telencephalon (Buck, 2000; Couly and Le Douarin, 1985). Unlike other neurons, the olfactory receptor neurons in the nasal epithelium regenerate throughout life (Farbman, 1994). The olfactory placode is unique in that it generates glial cells, which ensheathe the olfactory and vomeronasal nerves (Couly and Le Douarin, 1985; Klein and Graziadei, 1983). This placode also produces gonadotropin-releasing hormone (GnRH)–secreting neurons that migrate to different positions in the CNS and ultimately control aspects of reproductive behavior (Dellovade *et al.*, 1998; Muske, 1993; Schwanzel-Fukuda and Pfaff, 1989; Wray *et al.*, 1989).

The otic placode gives rise to the complex chambers of the inner ear and, within these structures, to various cell types including supporting and endolymph-secreting cells, as well as the mechanosensory hair cells that detect acoustic and vestibular stimuli. The sensory neurons that innervate these receptor cells are also derived from the otic placode, their cell bodies being located in the acoustic and vestibular ganglia of the eighth cranial nerve. Unlike glial cells of the olfactory nerve, glial cells of the eighth nerve are of neural crest origin (D'Amico-Martel and Noden, 1983).

The lateral line organs, found along the entire body of fish and aquatic amphibians, arise from two groups of placodes (one pre-otic and one post-otic)

and are responsible for the detection of disturbances in the water, or weak electrical field changes. These organs are important for animal behavior, including prey detection, schooling behavior, and obstacle avoidance. Like the otic placode, lateral line placodes give rise to the sensory and to supporting cells in the ectoderm, as well as to the neurons that connect to the sensory cells (Northcutt and Brandle, 1995).

Two other groups of placodes also give rise to sensory neurons (but not to the actual sensory cells they innervate) and, together with neural crest cells, form cranial ganglia. Neuroblasts that delaminate from the trigeminal placode generate the trigeminal ganglion of the fifth cranial nerve, whose glial cells are of neural crest origin (D'Amico-Martel and Noden, 1983). Axons projecting from this ganglion convey somatosensory information, including temperature, pain, and touch, from regions of the head such as the skin, jaws, and teeth (D'Amico-Martel and Noden, 1983; Schlosser and Northcutt, 2000). At placode stages, the trigeminal primordium is subdivided into two molecularly distinct regions, the ophthalmic and the maxillomandibular placodes, reflecting the different targets that become innervated at a later stage by the trigeminal neurons (Baker *et al.*, 1999; Begbie *et al.*, 2002). The trigeminal ganglion initially comprises two lobes (ophthalmic and maxillomandibular), which in most craniates fuse at later stages (Northcutt and Brandle, 1995; Schlosser and Northcutt, 2000).

The three epibranchial placodes (geniculate, petrosal, and nodose) give rise to the distal ganglia of the seventh, ninth, and tenth cranial nerves, respectively. Axons emanating from these ganglia convey viscerosensory and gustatory information from the oropharyngeal cavity, as well as the heart and other visceral organs. The proximal parts of these ganglia are of neural crest origin, as are the glial cells that envelop their axons (D'Amico-Martel and Noden, 1983).

While all the placodes described so far generate neurons, the two remaining cranial placodes are nonneurogenic. The single adenohypophyseal placode located in the anterior midline gives rise to the endocrine cells of the adenohypophysis, the anterior component of the pituitary gland (Couly and Le Douarin, 1985). Finally, the lens placode forms the lens of the eye, although the simplicity of this structure belies the vital role it plays in eye development and function (Chow and Lang, 2001; Cvekl and Tamm, 2004).

B. A Common Placode Ground State?

As illustrated in this brief summary, the various structures generated by cranial placodes are large and functionally diverse, as are the cell types that arise from them. Despite this they have been grouped into a family of structures with a presumed common developmental history (Baker and

Bronner-Fraser, 2001; Jacobson, 1963; Northcutt and Gans, 1983; Streit, 2004). Indeed, they do share certain similarities, which seem to justify this grouping, including the formation of columnar epithelia, placode invagination (adenohypophysis, olfactory, lens, otic), and epithelial-mesenchymal transition (i.e., delamination of neuroblasts). In addition, all placodes contribute to the cranial nervous system and are, with the exception of the lens and adenohypophysis, neurogenic. However, their structural and functional diversity raises the question of whether it is indeed useful to consider them as a homogeneous group (Begbie and Graham, 2001). If the sensory placodes are to be considered a "family," then all members must share certain developmental traits, though dysfunctionality is allowed, because individual members may go on to adopt unique fates.

Thus, for the concept of the placodal family to prove valuable, two conditions must be met. First, all placode precursors should at some point of their development acquire unique properties—a "placodal ground state"—that distinguishes them from cells with other fates. For example, placodes are ectodermal derivatives, but so is the epidermis and the CNS. Therefore, "ectodermal character" cannot be considered a specific "placodal" trait, although it is an essential part of the "placodal family" program. Second, the "placodal ground state" should be a prerequisite for subsequent differentiation into any placode derivative. In other words, this state should represent a branch point in development through which all placode precursors must pass; thereafter, they may acquire distinct characteristics that identify them as specific placodes, such as otic or lens.

In this chapter, we summarize the evidence that supports the idea of a "pre-placodal region" (PPR). We then review how this PPR is positioned and confined to the head ectoderm, in concert with the establishment of other ectodermal derivatives like the neural plate and the neural crest territory. We then discuss how the continuous pre-placodal territory splits to form individual placodes. Finally, we return to the functional significance of the PPR and discuss the possibility that it represents a common "placode ground state."

II. The "Pre-Placodal Region"

If there is indeed a "placodal ground state" upstream of the development of all placodes, at which stage during embryogenesis is it likely to exist? By the time placode morphology is apparent, placodes are already specified and sometimes even committed to a particular placodal fate. For example, the otic placode chick is committed to form an otic-like vesicle shortly after the placode forms at the 10-somite stage (Groves and Bronner-Fraser, 2000), and similar observations have been made in other species (Gallagher *et al.*,

1996; Waddington, 1937). The olfactory placode is morphologically discernible much later (24 somite stage) and seems to be committed to an olfactory fate at this stage (Carpenter, 1937; Haggis, 1956; Siggia, 1936; Street, 1937; Zwilling, 1940b). Thus, cells in different placodes are not equivalent and this stage cannot represent a "placodal ground state" from which placode fates diversify.

Substantial differences in gene expression and specification are also apparent long before placodal thickenings appear. By the time the neural tube closes, precursors for individual placodes are already confined to distinct locations of the cranial ectoderm, where they are associated with the tissues that are later required for placode patterning or normal placode differentiation (Fig. 1A). For example, otic precursors are located next to rhombomeres 5 and 6, whereas lens precursors concentrate in the ectoderm overlying the optic vesicle (Bhattacharyya *et al.*, 2004; D'Amico-Martel and Noden,

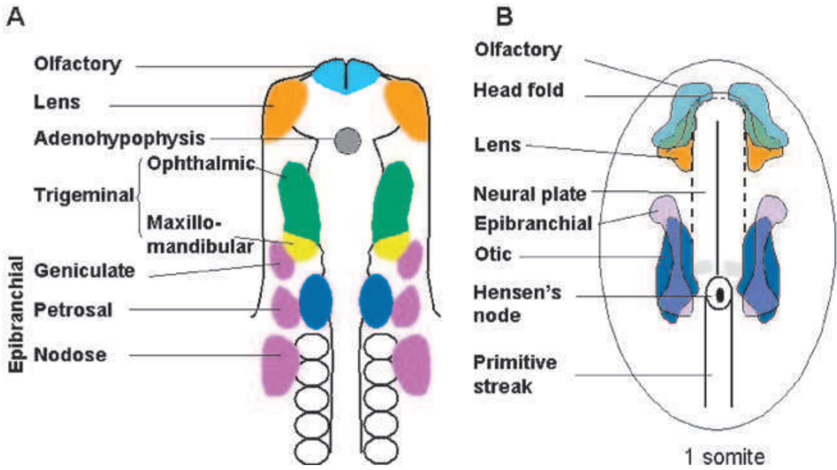


Figure 1 Fate maps for sensory placodes in the chick. (A) At the 10–13 somite stage, precursors for different sensory placodes are separate from each other and occupy distinct positions along the neural tube. Note: The adenohypophyseal placode lies in the ectoderm ventral to the neural tube. Fate maps are modified from Bhattacharyya *et al.* (2004) and D'Amico-Martel and Noden (1983). (B) At the early neurula stage, precursors for different placodes are intermingled and surround the neural plate in a horseshoe-shaped domain from hindbrain to forebrain levels (modified according to Bhattacharyya *et al.* [2004] and Streit [2002]). Note: There are currently no fate maps available for the trigeminal placode, whose precursors presumably lie between future lens and otic/epibranchial cells; the position of epibranchial precursors has not been fully analyzed and they may occupy a larger region than depicted in the diagram. A similar arrangement of placode precursors is found in zebrafish embryos at the late gastrula stage (Kozlowski *et al.*, 1997; Whitlock and Westerfield, 2000).

1983). In addition, these primordia can already be distinguished by unique gene expression profiles; the otic territory starts to express *Pax2* (Groves and Bronner-Fraser, 2000; Heller and Brandli, 1997; Pfeffer *et al*., 1998), whereas the prospective lens ectoderm upregulates *Pax6* (Amirthalingam *et al*., 1995; Bhattacharyya *et al*., 2004; Li *et al*., 1994; Puschel *et al*., 1992; Walther and Gruss, 1991). Concomitant with differential gene expression, these regions begin to be specified (i.e., develop according to their normal fate when cultured in isolation) (Barabanov and Fedtsova, 1982; Gallagher *et al*., 1996; Groves and Bronner-Fraser, 2000; Sullivan *et al*., 2004; Zwilling, 1940a). Thus, even before placode formation, precursors for different placodes have acquired some unique characteristics.

However, these placode precursors are not yet committed and can still be diverted along different placodal lineages when exposed to relevant signals. For example, tissue that is likely to have a trigeminal placode fate can be diverted to an otic or epibranchial fate if given the appropriate signals (Begbie *et al*., 1999; Ladher *et al*., 2000). Likewise, the presumptive lens ectoderm can adopt adenohypophyseal character (Gleiberman *et al*., 1999). Together, these findings argue that by the late neurula stage, the "placode family" has already begun to diverge, although the differences are not yet irreversible and fates are still plastic. Therefore, this stage is also likely to be downstream of any putative "placodal ground state."

In contrast, at early neurula stages, precursors for different placodes are intermingled in a horseshoe-shaped domain that abuts the neural plate (Bhattacharyya *et al*., 2004; Kozlowski *et al*., 1997; Streit, 2002). This domain is characterized by a unique gene expression profile that distinguishes it from neural plate and neural crest cells. We term this territory the *PPR* because it is the only region of the embryonic ectoderm that contains placode precursors. To address how the PPR is established at neurula stages, it is important to consider earlier patterning events in the ectoderm. In the following section, we summarize how changes in cell fate and gene expression, from blastula to early neurula stages, lead to PPR formation.

A. Location of Precursors and Gene Expression

1. Neural and Epidermal Bias at Blastula Stages

The three germ layers, endoderm, mesoderm, and ectoderm, are generated from the blastula stage embryo by the process of gastrulation. Before gastrulation, precursors for all three layers show considerable overlap, as do precursors for the neural and nonneural ectoderm (Dale and Slack, 1987; Hatada and Stern, 1994; Kimmel *et al*., 1990). In contrast, *in vitro* studies from chick and *Xenopus* suggest that different regions of the blastula are

already specified as epidermal or neural tissue; when cultured in isolation, only medial epiblast (chick) or dorsal animal caps (*Xenopus*) express neural markers, whereas more lateral or ventral regions adopt an epidermal fate (Kuroda *et al.*, 2004; Wilson *et al.*, 2000).

Similar regional differences are also revealed by differential gene expression. Genes such as *Gata2* and *Gata3* (Sheng and Stern, 1999), Dlx5 (Pera *et al.*, 1999), *Bmp7* (Streit *et al.*, 1998), and Epi-1 (Zhang and Jacobson, 1993) are expressed in the epidermally specified domain, whereas genes that are later confined to the neural territory like *Sox3* (Rex *et al.*, 1997), *SoxD* (Mizuseki *et al.*, 1998b), *Otx2* (Bally-Cuif *et al.*, 1995), Geminin (Kroll *et al.*, 1998), and *ERNI* (Streit *et al.*, 2000) are expressed over a large region of the ectoderm (Fig. 2A). Therefore, at pregastrulation stages, fate maps demonstrate extensive intermingling of ectodermal precursors, but gene expression and specification studies suggest that ectodermal patterning events are already underway.

2. Refinement of Neural and Epidermal Regions at Gastrula Stages

During gastrulation, the three germ layers are separated, and within the ectoderm, a fairly clear division between neural and epidermal precursors is established. This approximate segregation is apparent by the onset of gastrulation in zebrafish (Kimmel *et al.*, 1990) and by early gastrulation stages in *Xenopus* (Keller, 1975, 1976). In chick and mouse, this distinction is not apparent before the late gastrula stage (Fernandez-Garre *et al.*, 2002; Garcia-Martinez *et al.*, 1993; Hatada and Stern, 1994; Lawson, 1999; Tam, 1989).

The location of future placode cells at these stages has been assessed in some species. In zebrafish, placode precursors have been mapped at 50% epiboly (early gastrula) and occupy a continuous domain at the border of the cranial neural plate (Kozlowski *et al.*, 1997). Therefore, in zebrafish the PPR is apparent by early gastrula stages. In the chick, however, placode precursors are still widely dispersed during early and late gastrulation, although they are excluded from the most medial region of the epiblast, the future neural plate (Garcia-Martinez *et al.*, 1993; Hatada and Stern, 1994; Streit, unpublished observations). To the best of our knowledge, equivalent fate maps for placode precursors are unavailable in *Xenopus*.

The gene expression domains established prior to gastrulation are broadly maintained during this process, although the boundaries become sharper, reflecting the progressive segregation of cell fates. For example, in the chick, *Sox3* expression continues to concentrate medially in the forming of the neural plate and gradually decreases toward lateral regions (Rex *et al.*, 1997). Conversely, presumptive epidermal markers such as *Gata2*, *Gata3*, *Dlx5*, and *BMP4* show strong lateral expression, which rapidly declines

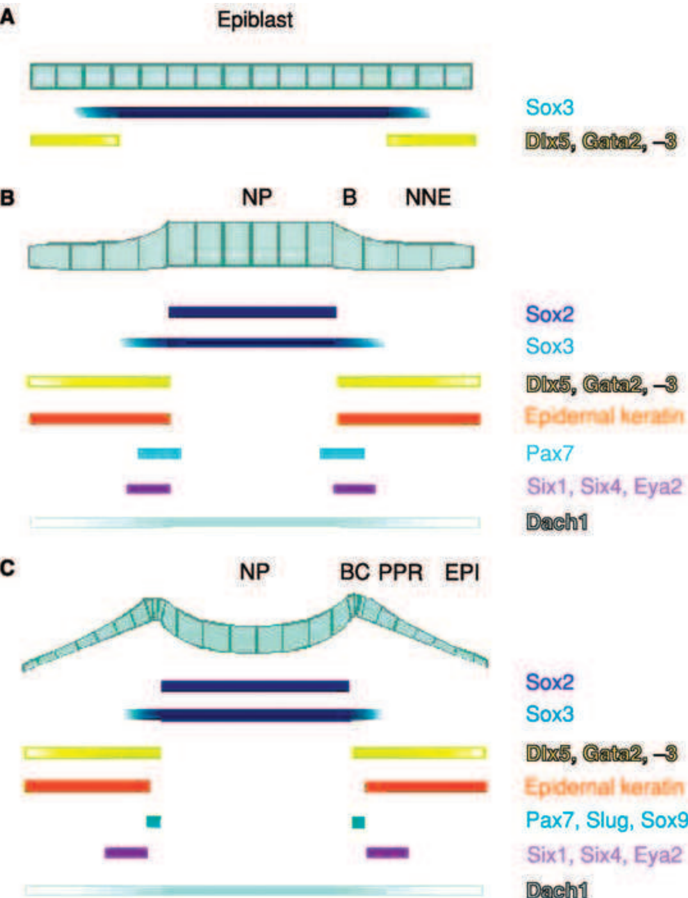


Figure 2 Changes in gene expression reflect subdivision of the ectoderm. Diagrams show cross sections through blastula (A), early neurula (B), and late neurula (C) stages using the chick as an example. Similar changes in gene expression are observed in other species. (A) At blastula stages, the blastoderm is subdivided into a central domain specified as neural (*Sox3*$^{+}$) and a peripheral region specified as epidermal (*Dlx5*/*Gata2*/*Gata3*$^{+}$). (B) Gene expression domains sharpen as the neural plate forms, and early neural crest and PPR markers begin to be expressed in partially overlapping domains at its border. (C) When the neural folds form, neural crest and PPR-specific genes segregate, as do cell fates at the border of the neural plate.

medially (Pera *et al.*, 1999; Sheng and Stern, 1999; Streit *et al.*, 1998). Similar complementary expression patterns are observed in frog and fish. Genes expressed in the future neural territory include *Geminin* (Kroll *et al.*, 1998), *Sox3* (Kudoh *et al.*, 2004), and *SoxD* (Mizuseki *et al.*, 1998b), whereas *Msx1* (Suzuki *et al.*, 1997b), *Foxi1* (Solomon *et al.*, 2003a), and *Np63* (Bakkers

et al., 2002) are expressed in the future epidermal territory. However, PPR-specific gene expression has not been reported at this stage in any species, including zebrafish. Rather, gastrula stage gene expression domains seem to reflect a subdivision of the ectoderm into broad neural and non-neural precursor territories.

3. Emergence of the Pre-Placodal Domain at Neurula Stages

The end of gastrulation overlaps with the formation of the definitive neural plate. Thus, by early neurula stages, fate maps reveal a well-defined neural domain, which shows very little overlap with the nonneural territory (Bhattacharyya *et al.*, 2004; Keller, 1976; Streit, 2002; Woo and Fraser, 1998). At this stage, the PPR can for the first time be discerned both by fate maps and by gene expression patterns. In the chick, placode precursors have been mapped to a broad band of ectoderm that surrounds the neural plate from hindbrain levels toward its most rostral edge (Bhattacharyya *et al.*, 2004; Streit, 2002), reminiscent of their location in zebrafish at gastrula stages (Kozlowski *et al.*, 1997). Within this domain, precursors for different placodes are interspersed, although there is a rough subdivision along the rostrocaudal axis into precursors that contribute to the more anterior and more posterior placodes, respectively (Bhattacharyya *et al.*, 2004; Streit, 2002).

In *Xenopus*, no fate maps are available for comparison, although fate maps of *Ambystoma* (Carpenter, 1937) and ablation studies in *Taricha* (Jacobson, 1963) suggest that placode precursors in amphibians are situated in a similar position. In support of this notion, a study of *Xenopus* shows that tissue next to the anterior neural plate is specified as pre-placodal (Ahrens and Schlosser, 2005).

Gene expression at the early neurula stage reflects the segregation of cell fates described earlier (Fig. 2B). Expression of the definitive neural markers *Sox2* and *NCAM* (Kintner and Melton, 1987; Mizuseki *et al.*, 1998a; Rex *et al.*, 1997) is clearly distinct from genes expressed in the nonneural ectoderm like *Gata2*, *Gata3* (Sheng and Stern, 1999), *Dlx3* (Schlosser and Ahrens, 2004), *Dlx5* (Luo *et al.*, 2001; Pera *et al.*, 1999; Yang *et al.*, 1998), *BMP4* (Fainsod *et al.*, 1994; Nikaido *et al.*, 1997; Streit *et al.*, 1998; Watanabe and Le Douarin, 1996), *Keratin-19* (Sato and Yasugi, 1997), and *XK81* (Jonas *et al.*, 1985; Tribulo *et al.*, 2003). More importantly, a novel set of genes is strongly upregulated in the ectoderm encircling the anterior neural plate.

In chick, *Six1* (Litsiou *et al.*, 2005), *Six4* (Esteve and Bovolenta, 1999; McLarren *et al.*, 2003), and *Eya2* (Mishima and Tomarev, 1998) are expressed in a horseshoe-shaped domain that matches the distribution of placodal precursors described earlier in this chapter. In *Xenopus*, a similar distribution of *Six1* (Bessarab *et al.*, 2004; Glavic *et al.*, 2004; Pandur and

Moody, 2000), *Six4* (Glavic *et al.*, 2004; Kobayashi *et al.*, 2000), *Eya1*, and *Foxi1c* (Schlosser and Ahrens, 2004) is observed, and the same is true in zebrafish for *Eya2* (Sahly *et al.*, 1999), *Six1* (Bessarab *et al.*, 2004), and *Six4* (Kobayashi *et al.*, 2000). Because these genes overlap with the position of placode precursors at neurula stages, they are considered pre-placodal markers (although they are also expressed in other germ layers). Although a large number of other genes show overlapping expression with PPR markers, they are not restricted to the PPR but show broader expression, being detected, for example, in the neural plate (Xiro1, Ziro1) (Glavic *et al.*, 2004) or more generally in the nonneural ectoderm (see previous discussion).

Placode precursors at diencephalic to hindbrain levels lie close to neural crest cells, which arise at the interface of neural and nonneural tissue. Unlike placodes, neural crest cells extend along the entire body axis, although the most anterior neural plate border does not generate neural crest (Couly and Le Douarin, 1985, 1987; Whitlock and Westerfield, 2000; Whitlock *et al.*, 2003). In *Xenopus*, a clear gap is observed between the expression domains of PPR and neural markers at neurula stages, where neural crest–specific transcripts are found (*Slug, FoxD3* [Sasai *et al.*, 2001; Schlosser and Ahrens, 2004]). In chick, a similar segregation of expression domains is observed only when the neural folds form and *Slug* expression is initiated (Litsiou *et al.*, 2005; Nieto *et al.*, 1994) (Fig. 2C). However, there is no evidence that in any species these gene expression domains represent true lineage-restricted compartments.

In summary, comparison of gene expression patterns and fates over time suggests that patterning of the ectoderm begins well before gastrulation. The first step seems to encompass a rough subdivision into neural and nonneural tissue, which is subsequently refined further to generate domains with specific characteristics. A coherent pre-placodal domain, identified by cell fate and a unique combination of genes, can be identified shortly after the neural plate forms.

B. Tissue Interactions

The previous discussion suggests that induction and positioning of the PPR is intimately linked with neural plate and neural crest induction. In the following sections, we discuss the tissue interactions and molecules involved in patterning the ectoderm during early gastrulation and neurulation.

1. Relationship of Neural and PPR Induction

When considering ectodermal patterning, much research has concentrated on factors that promote neural fate, and hence subdivide the ectoderm into neural and nonneural territories. The classic experiments by Spemann and Mangold first demonstrated the role of the amphibian "organizer"—the dorsal blastopore lip—in this process. When grafted near presumptive belly

ectoderm, the gastrula stage organizer generates a secondary embryonic axis that includes a well-patterned neural tube and associated sensory placodes (Nieuwkoop, 1952; Spemann and Mangold, 1924; Waddington and Needham, 1936; reviewed in De Robertis and Arechaga, 2001). Importantly, within this axis, the neural tissue is induced from host ectoderm, which normally never contributes to the nervous system, whereas mesodermal and endodermal derivatives arise mostly from the graft. Organizers with similar properties have been identified in most vertebrates including teleost fish (shield), birds, and mammals (Hensen's node), and the importance of organizer-derived signals and tissues in neural induction and ectodermal patterning has been demonstrated in many studies (reviewed in Bainter *et al.*, 2001; De Robertis and Arechaga, 2001; Stern, 2005).

Experiments have addressed a potential role of the organizer in PPR induction in relation to its neural-inducing activity. As noted earlier, organizer grafts can induce placodal and neural structures. It is, therefore, not surprising that the organizer also induces early PPR genes in avian and amphibian transplantation experiments (Ahrens and Schlosser, 2005; Litsiou *et al.*, 2005). In the chick, analysis of the timing of neural and PPR induction by Hensen's node reveals that induction of neural tissue precedes the formation of the placode territory. Only a few hours after grafting, the node induces the preneural markers *ERNI* and *Sox3* (Streit and Stern, 1999; Streit *et al.*, 2000), and after 9 hr, a *Sox2*-positive neural plate is induced (Streit and Stern, 1999). However, another 5–6 hr are required for robust induction of nonneural (*Dlx5*, *Gata3*, *BMP4*, *Msx1*) and PPR markers (*Six1*, *Six4*, *Eya2*) (Litsiou *et al.*, 2005; Pera *et al.*, 1999; Streit and Stern, 1999). These genes are expressed in the expected horseshoe-shaped domain surrounding the induced neural plate. Therefore, ectopic induction of different markers by the organizer parallels the events during normal development both in time and in space and suggests that the formation of neural tissue precedes PPR induction.

2. Role of the Neural Plate, Mesoderm, and Endoderm

These observations suggest that the neural plate may induce the PPR and that the activity of the organizer is indirect. Experiments in chick and *Xenopus* provide some support for this idea: neural plate grafts induce *Six1* and *Xiro1* expression in competent ectoderm (Ahrens and Schlosser, 2005; Glavic *et al.*, 2004; Litsiou *et al.*, 2005). The same interaction also generates the expression of non-neural (*Dlx5* and *Msx1* [Pera *et al.*, 1999; Streit and Stern, 1999]) and neural crest markers (*Slug*, *Sox9*, *HNK1* [Litsiou *et al.*, 2005; Mancilla and Mayor, 1996; Nieto *et al.*, 1994; Selleck and Bronner-Fraser, 1995]). However, apposition of neural and non-neural ectoderm it is not sufficient to generate the entire set of pre-placodal markers. These observations suggest that although signals from the neural plate contribute to PPR induction,

additional signals are necessary to confer "complete" PPR character on ectodermal cells.

A good candidate tissue to emit such signals is the mesoderm (including heart precursors), which underlies the PPR when PPR-specific genes are first expressed (Colas *et al.*, 2000; Jacobson and Sater, 1988; Redkar *et al.*, 2001; Stainier *et al.*, 1993; Wilens, 1955). Indeed, ablation experiments in amphibian and avian embryos show that lateral head mesoderm is required for the continued expression of PPR markers (Ahrens and Schlosser, 2005; Litsiou *et al.*, 2005). Although in *Xenopus*, this mesoderm is not sufficient to elicit ectopic expression of *Six1* (Ahrens and Schlosser, 2005), in chick it can induce a complete set of PPR markers in cells that normally never form placodes. This inductive ability is restricted to the mesoderm, which normally underlies the PPR. Furthermore, ectopic PPR territory is induced in the absence of neural and neural crest markers, suggesting that PPR induction can be uncoupled from neural crest and neural plate induction (Litsiou *et al.*, 2005).

Although these observations demonstrate the importance of mesoderm in PPR induction, this tissue is not the only source of signals to cooperate with neural plate–derived factors. At the onset of PPR-specific gene expression, the anterior portion of the PPR (the region containing lens, olfactory, and adenohypophysis precursors) is in close contact with the underlying endoderm and prechordal plate only (Colas *et al.*, 2000; Seifert *et al.*, 1993). Indeed, classic embryological experiments indicate a role of signals derived from both tissues in promoting the formation of the olfactory and, to a lesser extent, the lens placode (Jacobson, 1963; Orts-Llorca and Ferrol, 1961; Zwilling, 1940b). Thus, although mesoderm-derived signals are able to induce an ectopic PPR, during normal development this tissue cannot be solely responsible for all aspects of PPR formation.

Thus, the relative contribution of different tissues to PPR induction has not been resolved. However, it is clear that specification of the PPR is a multistep process, which is tightly integrated with the induction of other ectodermal derivatives. Generation of placode precursors seems to require multiple signals that converge from different tissues in the head: the neural plate, mesoderm, and endoderm. Studies have shed some light on the signaling molecules derived from these tissues that induce the PPR in relation to neural and neural crest territories.

C. Signaling Pathways

1. BMP Signaling

The discovery of the organizer prompted a prolonged search for the molecules that recapitulate its neural-inducing ability (reviewed in Bainter *et al.*, 2001; De Robertis and Kuroda, 2004; Munoz-Sanjuan and Brivanlou, 2002;

Stern, 2005; Wilson and Edlund, 2001). In the 1990s, experiments in *Xenopus* led to the proposal that signaling via the bone morphogenetic protein (BMP) pathway plays a key role in ectodermal patterning and that neural plate formation requires BMP inhibition. If animal caps isolated from blastula stage embryos are first dissociated, neural tissue forms after reaggregation (Godsave and Slack, 1989; Grunz and Tacke, 1989; Sato and Sargent, 1989); therefore, the "default" state of these cells has been considered to be "neural." In the same assay, activation of the BMP pathway leads to upregulation of epidermal markers, but loss of neural markers (Suzuki *et al*., 1997a,b; Wilson and Hemmati-Brivanlou, 1995; Wilson *et al*., 1997). Conversely, blocking the BMP pathway, from the two- or four-cell stage, promotes neural marker expression at the expense of epidermis in intact animal caps (Hemmati-Brivanlou *et al*., 1994; Lamb *et al*., 1993; Sasai *et al*., 1995). These observations are at the heart of the "default model" for neural induction, which proposes that BMP inhibition is sufficient to generate neural tissue and that epidermal tissue is actively induced by the BMP pathway (reviewed in Hemmati-Brivanlou and Melton, 1997; Munoz-Sanjuan and Brivanlou, 2002). This model has been extended to argue that a "mediolateral" gradient of BMP signaling patterns the whole ectoderm to generate specific cell fates (Fig. 3). Supporting this idea, animal caps treated with increasing levels of BMP antagonists adopt progressively more "medial" fates. Thus, high levels of BMP activity promote epidermis, intermediate levels induce neural crest and the PPR marker *Six1*, and low or no BMP signaling is required for the formation of neural tissue (Brugmann *et al*., 2004; Tribulo *et al*., 2003; Wilson *et al*., 1997). *In vivo* modulation of the

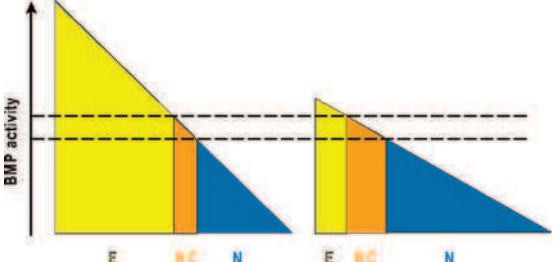


Figure 3 Model for ectodermal patterning through a gradient of BMP activity. This model proposes that different ectodermal fates are specified at different levels of BMP activity (reviewed in Aybar and Mayor, 2002). Although formation of epidermis requires high BMP levels, neural crest cells form at the intermediate and neural plate at low or no levels of activity. When BMP activity is reduced overall (e.g., in zebrafish mutants swirl or somitabun; Barth *et al*., 1999; Nguyen *et al*., 1998) both neural and neural crest domains are enlarged.

BMP pathway provides further support for this idea. Zebrafish mutants that have partially reduced BMP signaling show an expansion of the neural plate but a much larger relative expansion of the neural crest domain (Barth *et al.*, 1999; Nguyen *et al.*, 1998). The PPR domain is likewise expanded by reducing BMP signaling, through localized or global application of BMP antagonists, in both frog and fish (Glavic *et al.*, 2004). In chick, application of BMPs or BMP antagonists close to the neural plate border at gastrula stages causes the neural plate to narrow or widen, respectively, shifting the position of the border (Streit and Stern, 1999). As in the frog and fish, inhibiting BMP signaling expands the PPR in the chick (Litsiou *et al.*, 2005).

Although these data lend some support to the simple BMP gradient model for ectodermal patterning, increasing evidence argues for a more complicated process in which BMP signaling is only one of several components. As described earlier, reducing BMP levels promotes expansion of the neural plate and the PPR, in apparent agreement with the "default" model. However, this model also predicts that specific levels of BMP signaling should induce neural, neural crest, or PPR tissue in any region of competent ectoderm even far from the endogenous domains. This is not the case, because BMP antagonists are insufficient to induce isolated patches of neural tissue in either chick or frog (Delaune *et al.*, 2005; Linker and Stern, 2004). Likewise, PPR markers are not induced at a distance from the endogenous PPR following misexpression of *Smad6* in the chick (Litsiou *et al.*, 2005) or of *Noggin* in *Xenopus* (Ahrens and Schlosser, 2005). In contrast, one report in *Xenopus* suggests that indeed a low dose of *Smad6*, an inhibitor of the BMP pathway, can induce ectopic expression of the neural crest marker *Slug* (Delaune *et al.*, 2005).

Thus, although modulation of the BMP pathway clearly plays a role in allocating different fates to ectodermal cells, this alone does not seem to account for all aspects of ectodermal patterning. Accordingly, other pathways have been proposed as important modulators of neural and PPR induction.

2. FGF Signaling

An increasing body of evidence argues that fibroblast growth factor (FGF) signaling may play a positive role in neural induction and ectodermal patterning (reviewed in Stern, 2005; Wilson and Edlund, 2001). The involvement of FGF in amphibian neural induction has been a matter of controversy for many years. Although some reports support this idea (Barnett *et al.*, 1998; Hardcastle *et al.*, 2000; Hongo *et al.*, 1999; Ishimura *et al.*, 2000; Kuroda *et al.*, 2005; Lamb and Harland, 1995; Launay *et al.*, 1996; Pera *et al.*, 2003; Sasai *et al.*, 1996), others have questioned it (Amaya *et al.*, 1991; Cox and Hemmati-Brivanlou, 1995; Holowacz and Sokol,

1999; Kroll and Amaya, 1996; Pownall *et al.*, 2003; Ribisi *et al.*, 2000). These apparent contradictions may partly stem from the multiple roles of FGF during early development and the different functions of different FGF receptors (FGFRs); for example, mesoderm induction may require FGFR1 activity, whereas neural induction may be mediated through FGFR4 (Hardcastle *et al.*, 2000; Umbhauer *et al.*, 2000).

Analysis of the timing of FGF involvement has provided crucial insight into the sequence of events leading to neural induction. In ascidians, frog, fish, and chick, FGF seems to be required before gastrulation to initiate this process (Bertrand *et al.*, 2003; Delaune *et al.*, 2005; Grifone *et al.*, 2005; Hudson *et al.*, 2003; Kim and Nishida, 2001; Londin *et al.*, 2005; Streit *et al.*, 2000; Wilson *et al.*, 2000). However, FGF alone is not sufficient to induce either *Sox2* or an ectopic neural plate in either chick or frog, although application of FGF8 in the chick rapidly induces the "preneural" genes *ERNI* and *Sox3* (Linker and Stern, 2004; Streit *et al.*, 2000) and even the later-expressed neural marker *Churchill* (Sheng *et al.*, 2003).

Studies in chick suggest that FGF signaling may also be involved in certain aspects of PPR induction. Although FGF signaling can induce the PPR marker *Eya2*, it is not sufficient to elicit expression of *Six1* or *Six4* in competent ectoderm. However, PPR markers are not induced by mesoderm grafts in the presence of the FGF inhibitor SU5402, suggesting that an active FGF pathway is necessary for PPR induction (Litsiou *et al.*, 2005). Likewise, the formation of neural crest cells depends on active FGF signaling, but FGF alone cannot induce neural crest cells from naive ectoderm (LaBonne and Bronner-Fraser, 1998; Mayor *et al.*, 1997; Monsoro-Burq *et al.*, 2003).

3. Integration of BMP and FGF Pathways

As discussed earlier, both BMP and FGF activity control some aspects of ectodermal patterning. However, BMP inhibition is not sufficient to induce ectopic neural or PPR marker genes, whereas FGF signaling only induces certain pre-neural, non-neural, and PPR markers. One possibility is that combined modulation of both the BMP and the FGF pathway is required to generate a complete neural plate and/or PPR. Indeed, ectopic FGF signaling combined with BMP inhibition at blastula stages can induce neural markers in the ventral ectoderm of *Xenopus*, at a distance from the neural plate (Delaune *et al.*, 2005; Linker and Stern, 2004). Interestingly, similar treatment at late gastrula stages leads to ectopic expression of the PPR marker *Six1* (Ahrens and Schlosser, 2005), suggesting that although both neural plate and PPR may have similar requirements for their induction, the competence of the responding tissue may change over time. In contrast, in chick, misexpression of FGF together with *Smad6* is insufficient to induce either neural (Linker and Stern, 2004) or placodal (Litsiou *et al.*, 2005) tissue in the

extraembryonic region at gastrula stages. The reason for the discrepancy between chick and frog is not entirely clear (Linker and Stern, 2004), but a major difference lies in the test tissues used: future epidermis in *Xenopus* and extraembryonic ectoderm in chick. Although both tissues are competent to respond to neural- and PPR-inducing signals, their intrinsic properties will certainly alter the experimental outcome. In addition, the stages at which the experiments are performed—blastula versus gastrula—must be considered when comparing results from different species.

Nonetheless, combined increase of FGF and decrease of BMP signaling can induce markers that are not induced by either treatment alone, suggesting that cells integrate these signaling inputs. It has been suggested that such integration may occur at the level of the BMP effector Smad1 (Pera *et al.*, 2003). BMP signaling causes activation of Smad1 through phosphorylation of its C-terminus, which allows a complex of Smad1 and Smad4 to enter the nucleus and activate downstream target genes. In contrast, MAP-kinase, a mediator of FGF signaling, phosphorylates the linker region of Smad1 to inhibit its action. In this model, the balance of C-terminus and linker region phosphorylated Smad1 determines the dominating pathway and the main activity of FGF would be regulation of BMP signaling (De Robertis and Kuroda, 2004; Pera *et al.*, 2003). Although this provides an attractive mechanism for BMP and FGF signal integration, the situation is likely to be more complex, because FGF signaling can promote neural induction even in the presence of active BMP signaling (Delaune *et al.*, 2005; Kudoh *et al.*, 2004).

The temporal sequence of signaling inputs is also likely to be important. In the experiments described earlier, FGF and BMP treatments are typically administered simultaneously and often very early during development (e.g., at the two- to four-cell stage), which is unlikely to recapitulate the normal patterns of signaling activity. For example, FGF signaling is important for neural induction at blastula stages (see previous discussion), whereas BMP signaling activity (as assessed by phosphorylated *Smad1* levels) is not lost from the neural plate until late gastrula stages (Faure *et al.*, 2002). In agreement with this, overexpression of BMP only blocks the formation of the neural plate at late gastrula stages but is unable to block preneural marker expression (Linker and Stern, 2004; Streit *et al.*, 1998). Therefore, the simultaneous FGF activation/BMP inhibition scenario described earlier is likely to be an oversimplification of the normal signaling events involved in neural and PPR induction.

4. Wnt Signaling

As discussed already, modulation of the FGF and BMP signaling pathways affects various aspects of ectodermal patterning and PPR induction. However, even the combination of BMP inhibition with FGF activation is

insufficient to induce a complete PPR in competent extraembryonic ectoderm of the chick. Therefore, additional pathways, including Wnt signaling, are likely to be involved.

At blastula stages, the medial epiblast of chick is specified as neural tissue in an FGF-dependent manner, whereas the lateral epiblast is specified as epidermis (Wilson *et al.*, 2000). It has been proposed that Wnt signaling in the lateral domain attenuates the response of these cells to FGF and, therefore, promotes epidermal specification (Wilson and Edlund, 2001; Wilson *et al.*, 2001). At blastula stages, canonical Wnt signaling components are expressed in the extraembryonic ectoderm (Schmidt *et al.*, 2004; Skromne and Stern, 2001). Could Wnt signaling also inhibit ectopic PPR induction by FGF activation/BMP inhibition? This does seem to be the case; a combination of FGF activity/BMP inhibition with Wnt antagonists induces ectopic expression of PPR genes (Litsiou *et al.*, 2005). Importantly, this same combination of factors is not sufficient for neural induction in the same tissue (Linker and Stern, 2004). Therefore, as with the mesoderm grafts described earlier, PPR induction can be artificially uncoupled from neural induction.

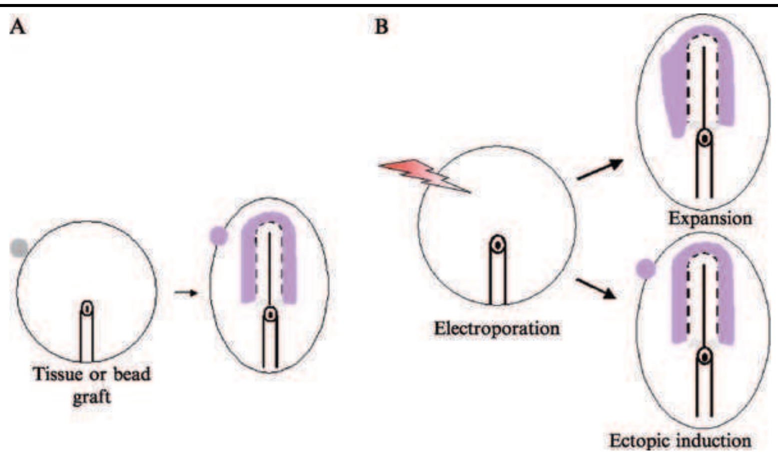
But is Wnt antagonism required for normal formation of the PPR? In chick, Wnt inhibition expands the endogenous PPR both laterally into the future epidermis and posteriorly into the trunk ectoderm (Litsiou *et al.*, 2005). A similar expansion of the PPR has also been reported in *Xenopus* (Brugmann *et al.*, 2004). Conversely, activation of the canonical Wnt pathway in chick inhibits PPR gene expression. Thus, Wnt activity seems to restrict the extent of the PPR, consistent with the expression of Wnt ligands at its lateral and posterior edges (Hume and Dodd, 1993; Litsiou *et al.*, 2005; Schubert *et al.*, 2002).

The canonical Wnt pathway is also involved in the specification of neural crest cells, which abut the medial edge of the PPR (reviewed in Aybar and Mayor, 2002; Knecht and Bronner-Fraser, 2002; Meulemans and Bronner-Fraser, 2004). Accordingly, activation of the canonical Wnt pathway expands the crest territory at the expense of placodal fates, whereas attenuation of Wnt signaling leads to the loss of neural crest (Litsiou *et al.*, 2005). Therefore, Wnt signaling may play an important role in segregating neural crest and PPR precursors.

D. Integrating Tissues and Signaling Pathways

To summarize, induction of an ectopic PPR occurs independently of other ectodermal tissues and can be experimentally uncoupled from neural and neural crest induction (for summary, see Table I). However, during normal development, similar tissues and molecules are involved in the induction of PPR, neural crest, and the neural plate. Now, we present a working

Table I Summary of PPR-Inducing Tissues and Signals



Diagrams in A and B show the experimental paradigm in the chick, used to assess the ability of different tissues or factors to induce ectopic PPR gene expression (data summarized from Litsiou *et al.* [2005]). (A) Tissues or factor-coated beads are grafted into the extraembryonic region at primitive streak stages; PPR gene expression is analyzed at early somite stages. (B) Misexpression of intracellular or secreted factors by electroporation at primitive streak stages. PPR gene expression at neurula stages can be expanded into the adjacent ectoderm or induced in an ectopic patch. A few of these experiments have also been performed in *Xenopus* with similar results: *Six1* is induced by neural plate grafts (Glavic *et al.*, 2004; Ahrens and Schlosser, 2005). Modulation of BMP signaling results in changes of PPR gene expression (Ahrens and Schlosser, 2005; Brugmann *et al.*, 2004; Glavic *et al.*, 2004), whereas the combined injection of BMP antagonists and dnFGFR or Wnt inhibitors leads to a posterior expansion of PPR genes along the induced secondary neural plate (Brugman *et al.*, 2004). HM: head mesoderm; NP: neural plate; nt: not tested; ↓: loss of expression. (See Color Insert.)

	Six4		Eya2		Six1	
Factor/tissue	Expansion	Ectopic induction	Expansion	Ectopic induction	Expansion	Ectopic induction
Neural plate	**nt**	–	**nt**	–	**nt**	+
Head mesoderm	**nt**	+	**nt**	+	**nt**	+
NP/HM	**nt**	**nt**	**nt**	++	**nt**	**nt**
Trunk mesoderm	**nt**	–	**nt**	–	**nt**	–
Anti-BMP	+	–	+	–	–	–
Anti-Wnt	+	–	+	–	+	–
FGF	**nt**	–	**nt**	+	**nt**	–
Anti-Wnt, anti-BMP	+	–	+	–	+	–
FGF, anti-Wnt	**nt**	–	**nt**	–	**nt**	–
FGF, anti-BMP	**nt**	–	**nt**	–	**nt**	–
FGF, anti-BMP, anti-Wnt	**nt**	+	**nt**	+	**nt**	–

Table I (*Continued*)

	Endogenous PPR	Ectopic induction by HM	Endogenous PPR	Ectopic induction by HM	Endogenous PPR	Ectopic induction by HM
FGF inhibition	↓	–	**nt**	**nt**	**nt**	**nt**
Wnt activation	↓	**nt**	↓	**nt**	↓	**nt**
BMP activation	**nt**	**nt**	**nt**	**nt**	**nt**	**nt**

hypothesis to integrate PPR induction with the induction of these other ectodermal derivatives.

In the chick, precursors for all three derivatives express "preneural" markers (*ERNI* and *Sox3*) in an FGF-dependent manner. As described earlier, FGF is also involved in the early phase of neural induction in other organisms. Interestingly, ectopic induction of one of the PPR markers, *Six4*, by FGF activity/BMP inhibition/Wnt inhibition, only requires an early pulse of FGF signaling (Litsiou *et al.*, 2005), suggesting that an early FGF signal is common to PPR and neural induction.

During early gastrulation, the ectoderm is roughly divided into future neural and nonneural domains. Where these domains meet a "border region" is established toward the end of gastrulation, which contains neural, neural crest, and PPR precursors. This border region is characterized not only by expression of "preneural" markers *ERNI* and *Sox3*, but also by expression of nonneural markers like *Dlx5*. Maintenance of this domain may require prolonged FGF signaling (LaBonne and Bronner-Fraser, 1998; Streit and Stern, 1999).

Segregation of cell fates within the border begins at neurula stages when the neural plate is firmly established and PPR-specific genes are expressed; neural crest genes are then expressed at the interface between these domains (Figs. 2C and 4). This "sharpening" of the border region is likely to involve signals from the underlying mesoderm and endoderm. The mesoderm underlying the PPR provides signals, including BMP and WNT antagonists (Chapman *et al.*, 2002; Ogita *et al.*, 2001; Rodriguez Esteban *et al.*, 1999), that permit the upregulation of PPR markers *Six1* and *Six4*. This mesoderm may also be a source of FGF signals, required for the induction of *Eya2*. Inhibitory Wnt signals from neighboring tissues may ultimately set the medial, lateral, and posterior limits of the PPR, as well as promote neural crest formation in the neural folds.

Although this model provides a framework for future investigations, numerous issues are still outstanding. These include the roles of the neural

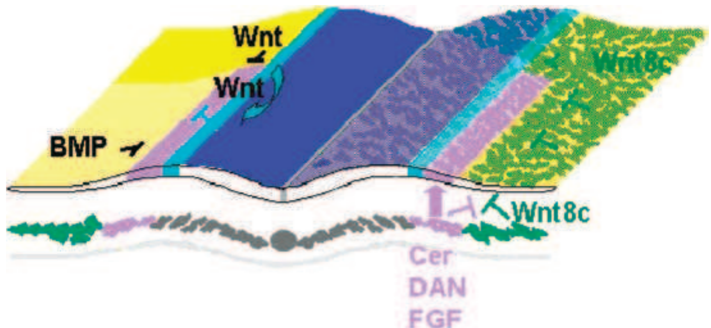


Figure 4 A model for PPR induction. The diagram shows a cross section through an embryo around the three to five somite stage viewed from anterior to posterior (modified after Litsiou *et al.*, 2005). Mesoderm-derived signals that position the PPR (purple) are depicted on the right and signaling events in the ectoderm on the left. Mesoderm lateral and posterior to the PPR (dark green) contains high levels of Wnt signaling and is proposed to restrict PPR gene expression to a narrow band in the head ectoderm. In contrast, mesoderm underlying the PPR (purple) expresses FGF, as well as BMP and Wnt antagonists, protecting the PPR against high levels of Wnt and BMP. The mesoderm is necessary and sufficient to induce the PPR. Wnt expression in the neural folds (turquoise) promotes the formation of neural crest cells but suppresses PPR-specific genes. BMP signaling from the lateral ectoderm may cooperate with mesoderm-derived Wnts to limit the lateral extent of the PPR.

plate and the anterior definitive endoderm and the signals derived from these tissues.

III. Subdivision of the Pre-Placodal Region into Primordia with Distinct Identity

In the previous discussion, we defined the PPR as a common domain that can be identified by gene expression and fate maps at early neurula stages, at the border of the cranial neural plate. However, within a few hours, distinct placodal primordia are apparent from fate mapping, specification, and gene expression studies. In the following section, we consider the processes that control this subdivision of the PPR. We first address anteroposterior (A-P) patterning within the PPR and then discuss potential mechanisms by which intermingled placode precursors are segregated.

A. Early Anterior-Posterior Patterning within the PPR

At neurula stages, precursors for individual placodes are intermingled in the PPR, although an approximate separation of different populations along the A-P axis is already apparent. Thus, anterior placode precursors

(adenohypophyseal, olfactory, lens) are found in the rostral region of the PPR, whereas precursors for the remaining placodes (trigeminal, otic, epibranchial, and in fish and aquatic amphibians, lateral line) are located more caudally.

The generic PPR markers *Six1*, *Six4*, and *Eya2* encircle the cranial neural plate from hindbrain levels toward its most rostral tip, but within this domain, A-P differences are already evident by differential gene expression. From early neurula stages onwards, genes including *Pax6* (Li *et al.*, 1994; Schlosser and Ahrens, 2004), *lens1* (Kenyon *et al.*, 1999), *Pitx3* (Dutta *et al.*, 2005; Zilinski *et al.*, 2005), and *neurogenin-related1* (Schlosser and Ahrens, 2004) are restricted to the anterior PPR. However, so far no molecular markers have been identified that are confined posteriorly. These observations suggest that PPR induction and patterning are intertwined and may to a certain extent occur simultaneously, as has been described for the induction and patterning of the anterior neural plate (reviewed in Stern, 2001; Wilson and Houart, 2004).

Little is known about the control of genes specific for the anterior PPR, although signals from the underlying endoderm are likely to be involved. Classic transplantation and tissue recombination studies suggest that the endoderm supports the formation of the olfactory and lens placodes (Jacobson, 1963). Newer experiments show that the endoderm is also required for the specification of forebrain territory (Withington *et al.*, 2001) and this may be mediated through protection against caudalizing signals including Wnt (Heisenberg *et al.*, 2001; Houart *et al.*, 1998, 2002; Nordstrom *et al.*, 2002; reviewed in Wilson and Houart, 2004). It is possible, though untested, that particular levels of Wnt activity fine-tune the identity of cells in the PPR.

B. Onset of Placode-Specific Gene Expression

Although the PPR shows some A-P patterning at early neurula stages, differences become much more pronounced as neurulation proceeds. Precursors for different placodes congregate in specific locations, although they are yet to acquire placode morphology. The formation of these placode primordia is associated with numerous changes in gene expression. In some cases, genes that were expressed throughout the PPR become restricted to specific placode primordia. For example, *Six1* and *Eya2* are downregulated in the lens primordium but are maintained in the olfactory, adenohypophyseal, and otic primordia (Schlosser and Ahrens, 2004). Likewise, *Dlx5*, which is initially expressed throughout the PPR and surrounding non-placodal ectoderm, becomes restricted to the olfactory and adenohypophyseal region (Pera *et al.*, 1999). In addition, *de novo* expression of a number of genes is observed. For example, *Pax2* becomes expressed in the otic/epibranchial/

lateral line territory (Groves and Bronner-Fraser, 2000; Schlosser and Ahrens, 2004) and *Pax3* in the presumptive trigeminal epithelium (Schlosser and Ahrens, 2004; Stark *et al.*, 1997). In the chick, *Pax6* expression is strongly upregulated in the future lens region and lost from the olfactory and adenohypophyseal primordia (Bhattacharyya *et al.*, 2004; Li *et al.*, 1994), whereas in *Xenopus*, *Pax6* is maintained in all three territories (Schlosser and Ahrens, 2004). A large number of transcription factors are subsequently expressed in particular subsets of cranial placodes, raising the possibility that a "transcription factor code" determines particular placodal identity (see also Torres and Giraldez, 1998). Thus, from a fairly uniform territory, distinct placode primordia are rapidly generated. What are the molecular and cellular mechanisms that control this subdivision of the PPR?

C. Localized Induction of Placode Primordia

Future lens and adenohypophysis cells occupy distinct regions in the PPR, with adenohypophysis precursors found most anteriorly (Cobos *et al.*, 2001; Couly and Le Douarin, 1985; Dutta *et al.*, 2005). Although this segregation is maintained during neurulation, it does not reflect the specification of both tissues. Both primordia are initially specified as lens tissue, but under the influence of ventral midline signals (likely to include sonic hedgehog), the adenohypophyseal region adopts its proper fate (Barabanov and Fedtsova, 1982; Sullivan *et al.*, 2004). In fact, overexpression of Shh in zebrafish induces ectopic adenohypophyseal tissue within the PPR (Herzog *et al.*, 2004), while loss of *Shh* signaling can produce an ectopic lens in place of the adenohypophysis (Kondoh *et al.*, 2000; Zilinski *et al.*, 2005). It is, therefore, likely that localized inducing signals play a major role in determining the identity of adenohypophyseal cells.

D. Separation of Placode Precursors: Large-Scale Morphogenetic Movements

In contrast to the lens/adenohypophysis scenario, precursors for other placodes are intermingled in the PPR. Therefore, exposure of individual precursors to distinct signals requires their prior separation. One possibility is that neighboring PPR cells may be forced apart by large-scale morphogenetic movements that occur during neurulation. By analogy, two spots drawn close together on a balloon may end up far apart when the balloon is inflated. If cells within the PPR undergo random local mixing, their particular position "as the balloon inflates" would cause intermingled precursors to segregate to distinct regions.

There is some support for this model from time-lapse analysis of lens and olfactory precursors in chick. Small groups of cells in the PPR split, so cells that were initially neighbors ultimately come to lie near the anterior neuropore (olfactory placode precursors) or overlie the optic vesicle (lens precursors) (Bhattacharyya *et al.*, 2004). Once separated, these precursors may then be subject to distinct inducing signals. The olfactory-inducing signals may derive from the forebrain (Haggis, 1956; Holtfreter, 1936; Jacobson, 1963; Orts-Llorca and Ferrol, 1961; Raven, 1933), although specific signals have not yet been identified. At later stages, signals from the frontal-nasal mass are required for olfactory placode development and patterning (Bhasin *et al.*, 2003; LaMantia *et al.*, 2000). The signals that promote lens placode formation from the lens primordium are better understood and are generally thought to derive from the optic vesicle (reviewed in Chow and Lang, 2001; Lang, 2004). These signals include RA, FGF, and BMP molecules (Dudley *et al.*, 1995; Enwright and Grainger, 2000; Faber *et al.*, 2001; Furuta and Hogan, 1998; Gopal-Srivastava *et al.*, 1998; Kastner *et al.*, 1994; Vogel-Hopker *et al.*, 2000; Wawersik *et al.*, 1999).

E. Separation of Placode Precursors: Directed Cell Movements

Like lens and olfactory cells anteriorly, otic and epibranchial precursors are interspersed in the posterior PPR (Streit, 2002), and the same "inflating balloon" analogy could explain their separation. From a location close to the neural plate, epibranchial cells seem to be displaced laterally to come into contact with the endoderm of the branchial clefts. Localized signals from the endoderm, among them *BMP7*, then promote the formation of epibranchial placode neurons (Begbie *et al.*, 1999). However, time-lapse analysis reveals that otic placode precursors converge to their final position, often against the direction of general morphogenetic movements (Streit, 2002). This suggests that precursor segregation in the PPR may not be an entirely passive process. It is possible that otic precursors separate from their neighbors because of specific adhesive properties and may move actively toward the otic territory. Future experiments will need to explore these possibilities. After segregation, local reenforcing or inducing signals firmly establish otic identify. Among these, FGF signaling from the hindbrain and underlying mesoderm plays a major role (reviewed in Barald and Kelley, 2004; Riley and Phillips, 2003; Whitfield, 2002). Interestingly, FGFs have been implicated in chemoattraction or repulsion of specific cell populations during development (reviewed in Blelloch *et al.*, 1999; Montell, 1994, 1999; Slack *et al.*, 1996; Szebenyi and Fallon, 1999) and may be good candidates to direct otic precursor movements.

IV. Functional Relevance of the Pre-Placodal Region

At the beginning of this chapter, we outlined two conditions that must be met if cranial placodes can be considered as a family of related structures with a common developmental history. First, the "placode family state" should be unique to placodes, and second, this state should be a prerequisite for further differentiation into placodes with different identities.

The first condition is clearly met. The PPR is a special region of the ectoderm that contains precursors for all cranial placodes and is identified by the expression of a unique set of genes. Ectodermal cells that normally never contribute to placodes can acquire PPR characteristics when exposed to a specific combination of tissues or signals: activation of FGF signaling in combination with BMP and Wnt antagonists (see previous discussion). Both inducing tissues and signals differ from those that induce other ectodermal derivatives, the neural plate, and neural crest cells. Together, these findings provide strong support for the idea that cells within the PPR possess unique properties that distinguish them from epidermal, neural crest, and CNS precursors. What are these properties?

A. A Domain of Placode Competence?

One possibility is that the PPR is the only region of the ectoderm competent to form placodes. However, at neurula stages, ventral ectoderm very distant from the endogenous PPR is still competent to form placodes in amphibians when exposed to appropriate signals (Haggis, 1956; Henry and Grainger, 1987, 1990; Jacobson, 1963; Yntema, 1933; Zwilling, 1940b). Likewise, in the chick, trunk ectoderm can respond to otic inducing signals at fairly late stages (Groves and Bronner-Fraser, 2000), while head ectoderm remains competent to form trigeminal or adenohypophyseal tissue until at least the three- to four-somite stage (Baker *et al.*, 1999; Gleiberman *et al.*, 1999; Stark *et al.*, 1997). Thus, the PPR does not represent a unique state of competence.

B. A Domain of Placode Bias?

A second possibility is that PPR cells possess a predisposition, or bias, toward placodal fate. The concept of bias has been introduced in the context of lens induction. Tissue with a lens bias is not specified as lens. It is, however, able to respond to "weak" lens inducers, which are insufficient to induce lens in tissue that is merely competent. In other words, in a state of bias, cells have received some signals that divert them toward a certain fate

but are not able to execute this fate autonomously (Grainger, 1992; Grainger *et al.*, 1992, 1997).

If the PPR is biased toward generic placodal fate, a "weak" placode inducer should only act within this territory. Numerous transcription factor–encoding genes are essential for normal development of different placodes at early stages, among them *Six3*, *Pax6*, and *Foxi1* (Carl *et al.*, 2002; Collinson *et al.*, 2001; Grindley *et al.*, 1995; Solomon *et al.*, 2003a). Misexpression of these factors results in the formation of ectopic placodes, but only in a restricted region of the ectoderm near other placodes and/or neural tissue (Altmann *et al.*, 1997; Chow *et al.*, 1999; Lagutin *et al.*, 2001; Nissen *et al.*, 2003; Oliver *et al.*, 1996; Solomon *et al.*, 2003b; Zuber *et al.*, 2003). These observations seem to support the idea that cells in the PPR are biased toward placode fate, but at a molecular level, how could such a bias be achieved?

C. Does the *Six/Eya/Dach* Network Confer Placode Bias?

Members of the Six and Eya families are good candidates to play such a role. In vertebrates, six members of the Six family (Six1–6) and four Eya genes (*Eya1–4*) have been identified (Borsani *et al.*, 1999; Xu *et al.*, 1997; reviewed in Kawakami *et al.*, 2000). Genes of these families are found not only in the PPR, but also in all cranial placodes and derivatives thereof. Loss- and gain-of-function approaches reveal that Six and Eya proteins play important roles in sensory organ formation in many phyla from cnidarians to vertebrates, including humans.

Six and Eya genes were first identified in *Drosophila* as sine oculis (so) and eyes absent (eya): mutants for either gene show severe eye phenotypes, whereas their combined overexpression induces ectopic eyes in imaginal discs other than the eye disc (Bonini *et al.*, 1993; Pignoni *et al.*, 1997). Together with dachshund (dac) (Mardon *et al.*, 1994), they are thought to work in a non-linear network to control proliferation and cell fate specification in the compound eye (Chen *et al.*, 1997; Pignoni *et al.*, 1997; Shen and Mardon, 1997; reviewed in Kumar and Moses, 2001). In this context, all three proteins act in a positive feedback loop to regulate their own expression, but also as a transcription factor complex to activate downstream target genes. *In vitro* studies using mammalian homologs reveal that the *Six–Dach* complex represses target genes, whereas the triprotein complex including *Eya* recruits transcriptional activators to activate downstream genes. The phosphatase activity of Eya proteins is essential for this function (Li *et al.*, 2003; Rayapureddi *et al.*, 2003; Tootle *et al.*, 2003; reviewed in Rebay *et al.*, 2005).

In vertebrates, coexpression of all network components in sensory organs including placode derivatives suggests a regulatory relationship between Six,

Eya, and Dach proteins similar to the fly. However, not all aspects of this genetic network are conserved, and the situation is rather complex due to the presence of multiple functional homologs and non-orthologous genes (for detailed discussion, see Donner and Maas, 2004). Nevertheless, conservation of the network has been demonstrated in other systems like kidney and somites (Heanue *et al.*, 1999; Torres *et al.*, 1996; Xu *et al.*, 2003). The ability of vertebrate *Six*, *Eya*, and *Dach* genes to rescue mutant phenotypes in *Drosophila* provides further evidence for their functional similarity (Bonini *et al.*, 1997; Heanue *et al.*, 1999; reviewed in Donner and Maas, 2004; Hanson, 2001).

The PPR coexpresses *Six*, *Eya*, and *Dach* genes, although the precise family member varies in different species. If, as proposed earlier, these genes confer placode bias to ectodermal cells, one might expect that mice lacking their function show early defects that affect all placodes. This is not the case for mutants in Six1, Six4, Eya1, or Dach1, but there is the possibility of functional redundancy between family members (Azuma *et al.*, 2000; Davis *et al.*, 2001; Laclef *et al.*, 2003; Lagutin *et al.*, 2003; Li *et al.*, 2002; Ozaki *et al.*, 2001, 2004; Xu *et al.*, 1999). Unfortunately, detailed analysis of *Six*, *Eya*, and *Dach* gene expression in the mouse PPR has not been reported. Without this information, even the Six1/Six4 compound mice (which have no obvious pan-placode phenotype) may not directly address the question of whether these genes confer placode bias (Grifone *et al.*, 2005). However, experiments in *Xenopus* do support a key role for *Six* genes in PPR formation and ectodermal patterning. Overexpression of *Six1* leads to an expansion of the PPR at the expense of neural, neural crest, and epidermal cells, while its absence results in the loss of other PPR markers (Brugmann *et al.*, 2004). These data suggest that the Six/Eya/Dach network may indeed be relevant to placode formation at an early stage.

V. Conclusions

Sensory placodes give rise to a fascinating diversity of structures and cell types; however during early development, their precursors arise from a unique territory. The mechanisms that induce this PPR are distinct from those that induce neural and neural crest cells, and we have argued that it comprises a region of placode bias. The PPR is a transient territory that is rapidly subdivided, through active or passive cell movements, into distinct placode primordia. Ultimately, distinct fates are reinforced by local inducing signals from adjacent tissues. So, does the PPR represent a "placodal ground state" that is upstream of all placode development or is the "placode family" irrevocably dysfunctional? The jury is still out.

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4

Regulation of Hepatocyte Cell Cycle Progression and Differentiation by Type I Collagen Structure

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Cell behavior is strongly influenced by the extracellular matrix (ECM) to which cells adhere. Both chemical determinants within ECM molecules and mechanical properties of the ECM network regulate cellular response, including proliferation, differentiation, and apoptosis. Type I collagen is the most abundant ECM protein in the body with a complex structure that can be altered *in vivo* by proteolysis, cross-linking, and other processes. Because of collagen's complex and dynamic nature, it is important to define the changes in cell response to different collagen structures and its underlying mechanisms. This chapter reviews current knowledge of potential mechanisms by which type I collagen affects cell behavior, and it presents data that elucidate specific intracellular signaling pathways by which changes in type I collagen structure differentially regulate hepatocyte cell cycle progression and differentiation. A network of polymerized fibrillar type I collagen (collagen gel) induces a highly differentiated but growth-arrested phenotype in primary hepatocytes, whereas a film of monomeric collagen adsorbed to a rigid dish promotes cell cycle progression and dedifferentiation. Studies

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presented here demonstrate that protein kinase A (PKA) activity is significantly elevated in hepatocytes on type I collagen gel relative to collagen film, and inhibition of this elevated PKA activity can promote hepatocyte cell cycle progression on collagen gel. Additional studies are presented that examine changes in hepatocyte cell cycle progression and differentiation in response to increased rigidity of polymerized collagen gel by fiber cross-linking. Potential mechanisms underlying these cellular responses and their implications are discussed. © 2006, Elsevier Inc.

I. Introduction

Cell function in the tissue microenvironment is tightly regulated by a complex interplay of signals from the extracellular matrix, cell–cell contact, and soluble factors. This environment *in vivo* is highly dynamic, maintaining a fine balance between opposing signals, such as stimulatory and inhibitory factors, and synthetic and degradative processes. The extracellular matrix (ECM), composed of insoluble proteins and proteoglycans, is itself a complex network that can undergo changes in both composition and structure during development and disease. Defining changes in cell response to different ECM structures is critical for understanding cell and tissue function.

Hepatocytes, the main epithelial cell type in the liver, perform most of the biochemical functions of the liver, including nutrient metabolism, xenobiotic biotransformation, and synthesis of soluble blood factors. In addition to these important differentiated functions, adult hepatocytes also retain a remarkable regenerative capacity, unlike many adult cells that have lost their proliferative capacity upon differentiation. In response to liver injury, hepatocytes are induced to proliferate until the damaged liver mass is replaced. Although undifferentiated stem cells are present in the liver, which can give rise to hepatocytes particularly during severe injury, the adult hepatocytes are responsible for much of the regenerative capacity of the liver. In fact, it is estimated that a single adult mouse hepatocyte possesses enough clonogenic potential to perform a minimal number of 69 cell doublings, or a 7.3×10^{20}-fold expansion (Overturf *et al.*, 1997). Remarkably, the critical differentiated liver functions are not lost during regeneration, indicating that hepatocytes *in vivo* can coexpress proliferation- and differentiation-specific functions (Michalopoulos and DeFrances, 1997).

The *in situ* environment in the liver facilitates the hepatocytes' important functions. Like most epithelial cells, hepatocytes possess extensive cell–cell contact with each other. However, unlike typical epithelial tissue, the hepatocytes do not sit on a traditional basement membrane. Instead, hepatocytes exist in cordlike structures throughout the liver, separated by sinusoids,

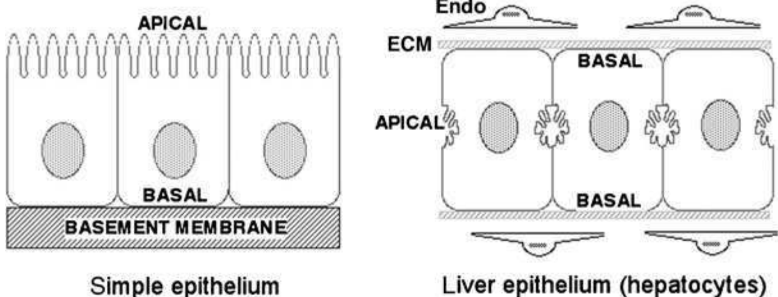

Figure 1 Comparison of typical and hepatocyte epithelium structure. Typical simple epithelial structure consists of cells sitting on a thick basement membrane with opposing basal and apical domains. In contrast, hepatocytes interact with a loose extracellular matrix (ECM) on two or more sides, with apical domains limited to the small bile canalicular regions within the lateral membranes. Highly fenestrated endothelial cells (Endo) loosely separate the hepatocytes from the sinusoidal bloodstream.

specialized capillaries that carry oxygen-rich blood from both the heart and the gut. Because of this unusual architecture, hepatocytes possess a unique polarity, in which its basal (ECM-interacting) surface can occupy more than one side of the cell, and the apical domain is restricted to the bile canalicular region for bile acid excretion into the bile ducts (Fig. 1).

The hepatocytes are separated from the sinusoids by highly fenestrated sinusoidal epithelial cells. The high degree of fenestration provides extensive contact between the blood and the hepatocytes. This region between the endothelial cells and the hepatocytes is called the *space of Disse*. Within this space of Disse lies a loose ECM with which the hepatocytes interact along their "basal" surfaces. In the adult liver, this diffuse matrix is composed mostly of collagen type I and fibronectin, with some collagen types III, IV, and VI, and tenascin (Martinez-Hernandez, 1984; Martinez-Hernandez *et al.*, 1991; Van Eyken *et al.*, 1990). Data to support laminin's presence have been conflicting, but there is evidence of its presence, particularly during liver regeneration (Kato *et al.*, 1992) and development (see later discussion), and thus, laminin is postulated to guide reorganization of newly formed vascular tissue. This diffuse ECM layer, rather than the thicker more typical basement membrane, is believed to facilitate diffusion of macromolecules between the plasma and hepatocytes. Also present within this space is a specialized mesenchymal cell, the stellate cell. This cell is responsible for much of the synthesis and deposition of matrix, and it becomes activated during injury, leading to an increase in type I and III collagen synthesis, deposition, and fibrillogenesis. In chronic or severe cases, extensive fibrosis can develop throughout the liver tissue.

II. ECM Expression During Liver Development and Regeneration

ECM composition in the developing liver is dynamic and distinct from that of the adult. The liver is one of the first organs to develop in the embryo, developing from the prospective ventral endoderm of the foregut (Zaret, 2001). The early hepatic cells migrate into the septum transversum mesenchyme, where they encounter a very different ECM (Cascio and Zaret, 1991). Developmental changes in both the ECM and adhesion receptor expression (Couvelard *et al.*, 1998) by the developing hepatocytes themselves likely play important roles in directing hepatic development. Fibronectin and type IV collagen increase during development in fetal mouse livers, whereas types I and III collagen and laminin decrease (Reif *et al.*, 1900). Both laminin and type IV collagen messenger RNA (mRNA) then increase in neonatal liver but fall to undetectable levels in the developed adult liver (Loreal *et al.*, 1992; Reif *et al.*, 1992). Expression of matrix metalloproteinases (MMPs) is also detectable and dynamic during liver development, with the expression of MMP1 (collagen type I) and MMP2 (collagen type IV) detectable as early as the sixth and seventh week of gestation in human livers (Quondamatteo *et al.*, 1999).

Early in development, the developing vessels in the liver are surrounded by typical basement membrane, but as the endothelial cells develop the high degree of fenestrations seen in adult liver, the basement membrane disappears and a more attenuated matrix is apparent (Martinez-Hernandez and Amenta, 1993). Similarly, during liver regeneration, hepatocyte proliferation leads to clusters of hepatocytes lacking the typical cordlike structure. At this time, laminin synthesis and deposition increase until sinusoids are formed within the new clusters, at which time laminin expression subsides (Martinez-Hernandez and Amenta, 1993). Laminin's presence around hepatic vasculature during both development and regeneration has led to the hypothesis that it is necessary as a scaffold for developing vascular tissue, but its presence is not required in the resting adult tissue (Baloch *et al.*, 1992).

Alteration of the ECM is believed to play an integral role in the initiation of liver regeneration *in vivo*. The mitogenic effects of growth factors such as hepatocyte growth factor (HGF) are greatly enhanced when the liver is pretreated with collagenase (Liu *et al.*, 1994). In addition, one of the earliest events following partial hepatectomy is activation of urokinase-type plasminogen activator (uPA) (Mars *et al.*, 1995), a key initiator of the MMP cascade involved in ECM degradation (Steler-Stevenson, 1996). uPA is involved in the activation of HGF from its inactive single-chain form, sequestered in the liver ECM, to its active two-chain form (Mars *et al.*,

1993; Naldini *et al.*, 1992). Taken by itself, these data may imply that the role of uPA in liver regeneration is restricted to activation of HGF, but even the active form of HGF is unable to promote robust hepatocyte proliferation *in vivo* in the absence of ECM degradation (Mars *et al.*, 1995). Additional studies demonstrate that in mice expressing a mutant type I collagen that is resistant to collagenase degradation, hepatocyte proliferation is impaired during recovery from CCl_4 injury (Issa *et al.*, 2003).

Thus, the alteration of ECM molecules and the expression of MMPs early in liver development and regeneration strongly suggest an important role for ECM in regulating hepatocyte function. It is clear that the composition of the matrix in the sinusoidal area changes during these processes. Additional evidence suggests that not only the composition but also the structure of ECM proteins may change during tissue development and repair. This is particularly evident in the process of liver fibrosis, discussed in the next section.

III. ECM Alterations in Liver Disease

Regeneration is the normal compensatory response to liver injury, leading to restoration of functional liver tissue. However, when the injury is chronic or excessive (e.g., from viral hepatitis, alcohol abuse, drug overdose, metabolic diseases, autoimmune attack, or congenital abnormalities), the insults result in prolonged activation of hepatic stellate cells and resulting fibrotic response (Friedman, 2000). In cases of mild or acute fibrotic response, cells of the liver may be able to produce sufficient extracellular proteases to degrade and resolve the excess collagen, and hepatocyte proliferation is sufficient to replace lost cells. However, in severe and/or chronic cases, several factors may contribute to the tissue's inability to remove the fibrotic tissue, leading to increased replacement of functional tissue with fibrotic components. Cirrhosis develops when fibrosis has become generally irreversible. The fibrotic process is generally characterized by activation of hepatic stellate cells, which degrade the ECM of the space of Disse and replace it with an interstitial matrix rich in fibrillar collagen. This matrix is characterized by a change in both the composition and the amount of ECM, typically leading to a three to fivefold increase in collagen and other ECMs, particularly the fiber-forming collagens type I and III and fibronectin (Okazaki *et al.*, 2003). In addition to increased amounts of fibrillar collagen comes an increase in the degree of interfiber cross-links, which makes the substrate more resistant to proteases (Brenner *et al.*, 2000). During fibrosis, activated stellate cells also exhibit proliferative, motile, secretory/inflammatory, and contractile behavior. Altered cell–ECM contacts and the altered mechanical nature of the microenvironment, through differing matrix composition and

HSC contractile behavior, are two significant factors that potentially lead to the phenotypic changes seen in hepatocytes during fibrosis and cirrhosis.

The accumulated fibrotic tissue has many deleterious effects, including increased resistance to blood flow, leading to portal hypertension. Furthermore, several studies document the reduced capacity of hepatocytes to proliferate in fibrotic liver. A reduction in regenerative potential in DMN-induced fibrosis in rats has been documented, with the degree of hepatic fibrosis inversely correlated with proliferation in a model of rat liver regeneration (MacIntosh *et al.*, 1993). Cyclin D1 expression is inhibited after partial hepatectomy in fibrotic livers (Zhao *et al.*, 2002), but the mechanisms of diminished cyclin D1 expression and the resulting reduced proliferative capacity, however, have not been established.

IV. Hepatocyte Response to Collagen Structure *In Vitro*

A. *In Vitro* Hepatocyte Cultures

Primary hepatocyte culture provides an excellent *in vitro* model for studying cell signaling and cell cycle regulation. Hepatocytes *in vivo* are in a naturally quiescent state, allowing for collection of a fairly homogenous population of resting G0 cells useful for investigating cell cycle progression *in vitro*. Also, they have not undergone the gene expression changes associated with immortalized cells lines and, thus, are more likely to respond to stimuli in a similar manner as cells found *in vivo*. Furthermore, primary hepatocytes can be maintained in a serum-free medium with defined growth factors such as epidermal growth factor (EGF) (Hansen and Albrecht, 1999; Hansen *et al.*, 1994) and can respond to growth factor stimulation by expressing many of the same cell cycle regulatory proteins that are expressed in the liver and that drive liver regeneration *in vivo*.

Among the cell cycle regulatory gene products expressed in response to growth factor stimulation are the cyclins, cell cycle regulatory proteins that associate with cyclin-dependent kinases (cdks) to form active kinase complexes that regulate progression through cell cycle checkpoints. The G1- and S-phase–specific cyclins, cyclin D1, cyclin E, and cyclin A, are expressed during both liver regeneration and hepatocyte proliferation *in vitro* (Albrecht *et al.*, 1993; Ehrenfried *et al.*, 1997). Overexpression of cyclin D1 in cultured hepatocytes or in intact liver *in vivo* can drive entry into the S phase independent of growth factors (Albrecht and Hansen, 1999; Nelsen *et al.*, 2001). Cell cycle–inhibitory proteins such as p27 and p21 form complexes with cdk/cyclins, inhibiting their activity or their ability to associate with each other (Albrecht *et al.*, 1998; Koff and Polyak, 1995; Vidal and Koff, 2000). Activation of cyclin D1 is dependent on the upstream

Ras-Raf-MEK-ERK pathway. Ras, a member of the small guanosine triphosphate (GTP)–binding family of proteins, is activated after both growth factor receptor activation and adhesion. Once active, it can operate through three downstream effector molecules, Raf, PI3K, and Ral/GDS, with each effector inducing a specific signaling pathway (Marshall, 1995). For example, activated Ras recruits Raf, a protein kinase, to the membrane where it is phosphorylated and thus activated. Raf itself then phosphorylates and activates MEK, which is the upstream activator of the mitogen-activated protein (MAP) kinase family. Different MAP kinase pathways have been identified, including the p42/44 *e*xtracellular-*r*egulated *k*inases (ERK1 and ERK2), involved in cell cycle regulation. Each MAP kinase is activated by phosphorylation on serine/threonine residues by upstream kinases (i.e., MEKs, also called *MKKs*). Once activated, these MAP kinases are responsible for phosphorylating numerous intracellular proteins, including transcription factors that then become transcriptionally active (for reviews, see Denhardt, 1996; Roovers and Assoian, 2000).

Hepatocytes are typically studied as primary cells freshly isolated from rat or mouse livers by collagenase perfusion of the tissue. Cell lines such as those derived from hepatomas generally have greatly decreased differentiated function, though enhanced proliferative capabilities. Primary hepatocytes in culture, like many cells, rapidly lose differentiated function upon placement in traditional culture conditions. Hepatocytes will readily adhere to and spread on ECM-coated dishes, and in the presence of sufficient growth factors (i.e., EGF), they can progress through the cell cycle and divide, as described earlier. However, under these conditions, they have limited lifespan *in vitro* (5–7 days) and usually die after one round of the cell cycle. Many studies describe culture conditions in which both viability and differentiated function are retained, such as culturing on a malleable hydrated type I collagen gel or Matrigel, a basement membrane matrix derived from mouse sarcoma cells. Reducing the ECM coating density from 1 μg/cm^2 to 1 ng/cm^2 also switches hepatocytes from a proliferative to a differentiated phenotype (Mooney *et al.*, 1992). Under such conditions, the proliferative capacity is lost. Thus, it appears that proliferative and differentiated functions in hepatocytes *in vitro* are uncoupled, and the hepatocyte must instead "choose" one fate or the other. The mechanism by which that fate is induced by changes in the ECM is not well understood.

The differences in cell cycle progression and differentiated function on different ECM substrates correlate with a distinct difference in cell shape. It has long been recognized that cell shape plays a critical role in cell cycle progression (Folkman and Moscona, 1978). In most cases, cells that are able to flatten and spread on the substrate progress through the cell cycle, while those that remain round are quiescent or undergo apoptosis. This has been described in hepatocytes cultured either on a thin monomeric film of type I

collagen, on which they spread and proliferate or on a malleable polymerized gel of the same type I collagen, on which they are growth arrested and highly differentiated (Hansen and Albrecht, 1999). The correlation between a flattened cell shape and cell cycle progression in hepatocytes is not limited to type I collagen substrates. Cell cycle progression is also blocked by adhesion to Matrigel (Nagaki *et al.*, 2000), which prevents spreading, as well as adhesion to low-density collagen or fibronectin-coated dishes, in which spreading is limited by availability of ECM ligands (Mooney *et al.*, 1992). Experiments using precise amounts of fibronectin printed over a defined area demonstrate that even when ECM ligand availability is equal, but the area over which cells are allowed to spread is limited, cell cycle progression is blocked (Huang *et al.*, 1998; Singhvi *et al.*, 1994). Short peptides containing the integrin-binding RGD amino acid sequence promotes adhesion without spreading or DNA synthesis, whereas longer peptides presenting RGD in a different conformation promote adhesion, spreading, and cell cycle progression (Bhadriraju and Hansen, 2000). Thus, a critical factor in determining response to growth factors *in vitro* is the ECM's ability to promote cell spreading.

Cell spreading appears to provide a cytoskeletal structure that effectively couples growth factor signaling with integrin signaling to drive mitogenesis (Assoian and Zhu, 1997; Ingber, 2003; Mammoto *et al.*, 2004). This pathway, typically studied in fibroblasts, involves activation of focal adhesion kinase (FAK) and rho-dependent formation of large focal adhesions. These types of complexes, however, are rarely found *in vivo*, where complexes called *fibrillar adhesions* involving $\alpha_5\beta_1$ integrin adhesion to fibronectin are more prevalent (Cukierman *et al.*, 2001). These fibrillar adhesions also form in pliable three-dimensional (3D) cultures, suggesting the malleability of the ECM regulates the formation of different integrin-mediated signaling complexes. It is likely that focal adhesions typically found in two-dimensional cultures on rigid plastic *in vitro* will not accurately represent the same type of interactions between growth factor receptor and integrin signaling pathways found *in vivo*. In hepatocytes, an epithelial cell type that does not form large focal adhesion complexes, a flattened cell shape disrupts differentiated functions while promoting progression into the S phase in response to EGF or other growth factors (Hansen and Albrecht, 1999; Hansen *et al.*, 1994; Mooney *et al.*, 1992).

B. Effects of Collagen Structure on Cell Cycle Signaling

Type I collagen has a complex macromolecular structure, as do most other ECM proteins, and changes in this structure can determine the cellular response. Type I collagen molecules are composed of three protein subunits wound together to form a triple helical structure. Noncollagenous domains

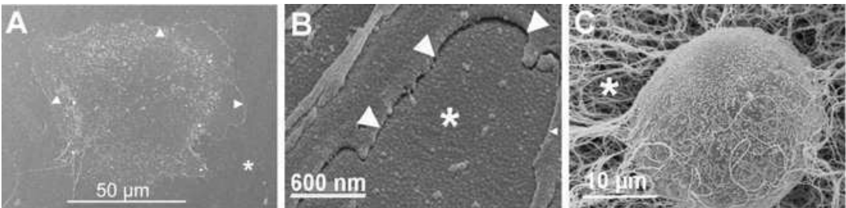

Figure 2 Field emission scanning electron microscopy (FESEM) of hepatocytes adherent on collagen film (A, B) or gel (C) for 24 hr. Collagen film was prepared by coating dishes with collagen diluted in a basic carbonate buffer (pH 9.4) as described elsewhere (Hansen and Albrecht, 1999). To produce a collagen gel substrate, 50 μl NaOH was added to each milliliter of 5× Williams Media E. One part 5× Williams Media E (+NaOH) were mixed with four parts Vitrogen 100 (final concentration ~2.4 mg/ml), added to a Petri dish at approximately 1 ml/10-cm dish and incubated at 37° for 1 hr. After coating, all plates were washed twice in phosphate-buffered saline (PBS) and incubated at least 20 min in 1% bovine serum albumin (BSA) in Williams E medium to block any noncoated sites. Arrowheads represent edges of adherent cells on film, and asterisks (*) represent collagen substrate.

(i.e., non–triple helical structure) at either end of the molecule are cleaved off by specialized enzymes after secretion into the extracellular space. Multiple type I collagen molecules can then bundle together to form fibrils and thicker fibers, held together by hydrogen bonding under physiological conditions. Additional chemical cross-linking between the fibrils can also occur, thus strengthening the fiber network. This native fibrillar network can be re-created *in vitro* by neutralizing commercial collagen preparations so that a fibrillar network forms, leading to a hydrated collagen gel. Heretofore in this chapter, this fibrillar network is referred to as a "gel." In contrast, type I collagen can also be adsorbed onto a dish under denaturing conditions, in which it is presumed to retain a nonnative monomeric structure. This thin adsorbed conformation is referred to as a "film." Effects of collagen structure on cell morphology are demonstrated in Fig. 2, along with detailed descriptions of the collagen preparations (Hansen and Albrecht, 1999; Hansen *et al.*, 1994; Mooney *et al.*, 1992).

As stated earlier, hepatocytes cultured on or within collagen gel or Matrigel exhibit high levels of differentiated function, such as albumin secretion, urea production, and expression of liver-specific genes (DiPersio *et al.*, 1991; Kono *et al.*, 1997; Lazar *et al.*, 1995; Moghe *et al.*, 1996; Parsons-Wingerter and Saltzman, 1993), but have much diminished levels of DNA synthesis compared to hepatocytes cultured on a collagen film (Fassett *et al.*, 2003; Hansen and Albrecht, 1999). Cells on the film have increased mitogenic signaling pathway activation, higher levels of cell cycle protein expression (cyclins), and increased DNA synthesis (Fassett *et al.*, 2003; Hansen and Albrecht, 1999), but reduced differentiated functions. However, despite increased growth factor signaling and cell cycle progression, hepatocytes adherent to

collagen film have limited survival compared to cells adherent to polymerized collagen gels (De Smet *et al.*, 2001). Thus, type I collagen provides a homogeneous defined substrate in which signaling pathways driving hepatocyte cell growth, differentiation, and cell death can be investigated.

In some cell types, fibrillar collagen gel increases expression of p27 (Henriet *et al.*, 2000; Schocklmann *et al.*, 2000) and/or p21 (Koyama *et al.*, 1996) to block DNA synthesis, with little effect on cyclin expression. In hepatocytes, type I collagen substrate configuration regulates the expression of cyclins in response to growth factors and, thus, alters cell cycle progression (Fassett *et al.*, 2003; Hansen and Albrecht, 1999). Cyclin D1, cyclin E, and cyclin A expression are all induced in response to EGF in cells adherent to plastic dishes coated with collagen film (Hansen and Albrecht, 1999; Fassett, *et al.*, 2006). However, growth factor–induced expression of cyclin D1 and cyclin A is inhibited in cells adherent to collagen gel, and likewise, DNA synthesis is inhibited (Fassett *et al.*, 2003; Hansen and Albrecht, 1999; Fassett *et al.*, 2006). Notably, overexpression of cyclin D1 in hepatocytes restores DNA synthesis in cells adherent to collagen gel (Hansen and Albrecht, 1999), demonstrating that the defect in G1–S progression in cells adherent to collagen gel may be in part due to lack of cyclin D1 expression. Cyclin E expression, unlike cyclins A and D1, is not blocked by adhesion to collagen gel (Fassett *et al.*, 2006). Thus, certain growth factor–dependent genes such as cyclin D1 and A are repressed by adhesion to fibrillar type I collagen gel, whereas others (cyclin E) are induced independent of collagen structure. This differential induction of growth factor–dependent genes by adhesion to different forms of type I collagen can be used to further dissect the signaling pathways that might be important for activation of particular cell cycle proteins.

As collagen structure appears to regulate the ability of EGF to drive expression of cell cycle proteins and cell cycle progression, it seems likely that signaling pathways related to integrin and/or the EGF receptor (EGFr) are involved. The Ras/ERK pathway, described earlier, is inducible by both growth factors and adhesion (Chen *et al.*, 1994; Morino *et al.*, 1995; Zhu and Assoian, 1995) and is critical to hepatocyte cell cycle progression (Fassett *et al.*, 2003; Rescan *et al.*, 2001; Talarmin *et al.*, 1999). To determine whether collagen structure affects EGF-dependent signaling pathways involved in cyclin D1 induction, the ras/raf/MEK/ERK signaling cascade was examined in hepatocytes adherent to collagen film or gel. In response to EGF, ERK activity is induced to similar levels on both film and gel during the first few hours of stimulation, after which levels of ERK activity decreases on both film and gel. However, under continual presence of EGF, ERK activation in cells adherent to collagen film increases again prior to transition into the S phase (Fig. 3A), and this increase correlates with increased expression of cyclin D1 (Fig. 3B) and cyclin A expression (Fassett *et al.*, 2003). This second

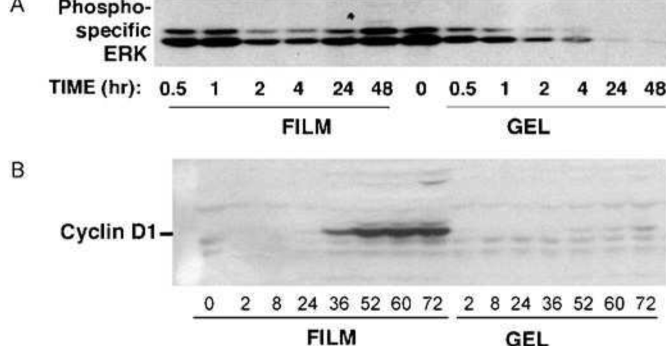

Figure 3 Phosphorylated ERK and cyclin D1 are inhibited on collagen gel. Western blot analysis using antibodies against phosphorylated ERK (A) and cyclin D1 (B) from primary hepatocytes cultured on type I collagen film or fibrillar collagen gel for the indicated hours after plating demonstrates a reduction in the late G1 ERK phosphorylation and expression of cyclin D1 on gel.

peak of ERK activation is much diminished in cells adherent to collagen gel, and this peak of ERK activity appears important for cyclin D1 expression, because a chemical inhibitor of MEK (U0126) added before the second peak strongly inhibited cyclin D1 expression and DNA synthesis (Fassett *et al.*, 2003). Likewise, inhibition of Ras or Raf using dominant negative mutants blocked cyclin D1 expression on collagen film (Fassett *et al.*, 2003). Importantly, overexpression of constitutively active mutants of Ras, Raf, or MEK in hepatocytes on collagen gel overcame DNA synthesis inhibition, demonstrating that inhibition of the Ras/ERK pathway is responsible, at least in part, for gel-dependent cell cycle inhibition (Fassett *et al.*, 2003).

Furthermore, constitutively active Ras can significantly increase cell spreading on collagen gel (Fassett *et al.*, 2003). These data suggest that the lack of spreading on gel is not due just to the malleability of the substrate failing to provide sufficient resistance to cell-generated tension. Rather, hepatocytes possess the ability to spread on this malleable substrate but are prohibited from doing so in the absence of activated Ras, likely because of effects on the cytoskeleton. Thus, it appears that fibrillar collagen gel either actively initiates Ras-inhibitory signals upon hepatocyte adhesion or fails to sufficiently generate positive signals required to activate Ras.

C. Mechanism of EGF Signaling Inhibition by Fibrillar Collagen Gel

Because adhesion to collagen film inhibits EGF activation of ERK and cell spreading, and because activated Ras can restore spreading, cyclin D1 expression, and G1–S progression, there may be a signal initiated by

collagen gel that represses EGF signaling at or upstream of Ras activation. This possibility was further supported by the finding that overlay of collagen gel upon hepatocytes already adherent to collagen film was able to block active cell cycle progression into the S phase (Fassett *et al.*, 2003). This suggests that the mechanism of cell cycle inhibition on collagen gel may involve initiation of an inhibitory signal that usurps EGF signaling, rather than simply an inability to induce sufficient stimulatory signals. One potential candidate for such an inhibitory signal is cyclic adenosine monophosphate (cAMP)–dependent protein kinase A (PKA), because it is known to inhibit several cell cycle signaling components and be induced upon loss of adhesion. The activation of PKA has been demonstrated to occur in response to loss of adhesion in fibroblasts (Howe and Juliano, 2000). PKA has also been shown to block EGF signaling through inhibition of the EGFr itself (Barbier *et al.*, 1999), inhibition of raf (Dumaz and Marais, 2003; Hafner *et al.*, 1994; Schramm *et al.*, 1994), inhibition of PAK, which activates raf (Howe and Juliano, 2000), and inhibition of src (Schmitt and Stork, 2002). Analysis of primary hepatocytes attached to collagen gel versus film demonstrated that PKA activity was indeed higher in cells adherent to collagen gel (data not shown; Fassett *et al.*, 2006). PKA activity was also increased in response to collagen gel overlay in hepatocytes already progressing through G1 on collagen film, suggesting that the formation of new adhesions to collagen gel induces PKA in adherent cells. When PKA activity was inhibited using the specific PKA inhibitor H-89, cell spreading on collagen gel increased, as did late G1 ERK activation, cyclin D1 and cyclin A expression (Fig. 4A), and DNA synthesis (Fassett *et al.*, submitted). However, cyclin E expression was not affected by adhesion to fibrillar collagen (data not shown; Fassett *et al.*, 2006). These results demonstrate that activation of PKA by adhesion to collagen gel, but not collagen film, contributes to the gel-dependent inhibition of EGF signaling and cell cycle progression in hepatocytes.

To further investigate the mechanism by which PKA blocks EGF signaling in collagen gel, tyrosine phosphorylation of the EGFr, a required event in its activation, was analyzed in lysates of EGF-stimulated cells adherent to either substrate. A number of reports have demonstrated that overall phosphotyrosine levels of the EGFr either decreased (Iwashita *et al.*, 1990) or increased (Budillon *et al.*, 1999; Ghosh-Dastidar and Fox, 1984) in response to cAMP or cAMP-inducing agents. It has also been reported that EGFr phosphorylation is not blocked by adhesion to compliant laminin or collagen gels (Gardner *et al.*, 1996), even though downstream signaling to DNA synthesis is inhibited. Similar to these latter results on laminin or collagen gels, there was little difference in early total phosphotyrosine levels of the EGFr in hepatocytes adherent to either fibrillar or monomeric collagen.

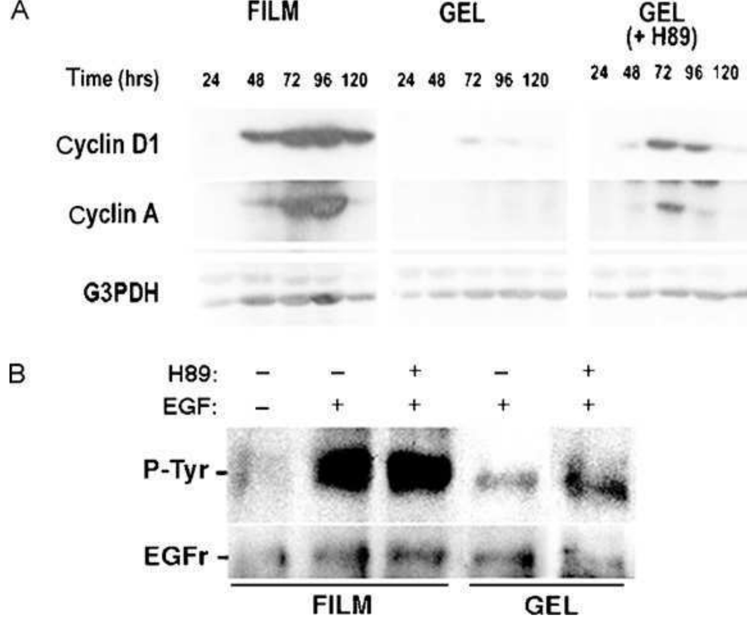

Figure 4 Inhibition of PKA activity restores cyclin D1 and A expression, and EGF receptor phosphorylation on collagen gel. (A) Western blot analysis of cyclin D1 and A protein expression in hepatocytes cultured on film, gel, or gel plus 2 μM H89 for the indicated times after plating. G3PDH is a housekeeping gene measured as a protein loading control. (B) Epidermal growth factor receptor (EGFr) phosphorylation was measured by immunoprecipitating EGFr from hepatocyte lysates after 9-hr cultures on film, gel, or gel plus 2 μM H89, followed by Western blot analysis using antibody against phosphotyrosine antibody (P-Tyr) and EGFr.

Overall levels of EGFr phosphorylation, however, were higher on collagen film after 9 hr of stimulation, suggesting that adhesion to collagen gel was unable to sustain EGFr activation and adhesion to collagen film (Fig. 4B; Fassett *et al.*, 2006). Inhibition of PKA activity partially overcomes the later inhibition of EGFr tyrosine phosphorylation (Fig. 4B; Fassett *et al.*, 2006). Therefore, it appears that adhesion to collagen film particularly promotes sustained EGFr function that involves EGFr phosphorylation, while adhesion to collagen gel diminishes this response through a PKA-dependent mechanism. The ability of the EGFr to drive some EGF-dependent events, such as expression of cyclin E, while signaling to other gene targets such as cyclin D1 is inhibited possibly because of the selective regulation of specific EGFr tyrosine residues by adhesion to collagen gel (Fassett *et al.*, 2006).

D. Adhesion Receptor Specificity

Differential growth induction by different substrates or substrate conformations could be explained in part by different adhesion receptors binding to each substrate, resulting in induction of different signaling pathways. The most common and well-defined family of adhesion receptors is the integrin receptor, a transmembrane heterodimeric receptor composed of an α- and a β-subunit. Upon binding to its ECM ligand, the cytoplasmic tail of integrins recruits several intracellular proteins, including vinculin, paxillin, talin, and signaling molecules such as focal adhesion kinase, which form a link to the actin cytoskeleton. These complexes at the cytoplasmic face of adhesion are referred to as *focal adhesions*. Two integrins, $\alpha_1\beta_1$ and $\alpha_2\beta_1$, have been shown to bind to distinct regions within type I collagen in fibroblasts (Gullberg *et al.*, 1990, 1992). Could differences in receptor binding between collagen film and gel explain the difference in hepatocyte growth activation? Previous studies in this and other laboratories fail to find the presence of $\alpha_2\beta_1$ integrins on primary hepatocytes, except following cytokine activation (data not shown; Volpes *et al.*, 1991). Adhesion blocking studies in this laboratory demonstrate almost complete inhibition of hepatocyte binding to collagen film with either α_1- or β_1-blocking antibodies, whereas α_1 antibody only inhibited adhesion to fibrillar collagen gel by 50% (Fig. 5), suggesting the presence of another collagen receptor. Because hepatocytes in culture express fibronectin (Stamatoglou *et al.*, 1987), which can bind to

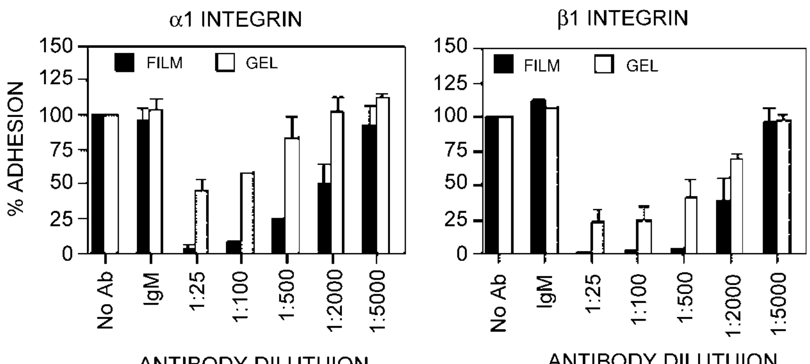

Figure 5 Inhibition of hepatocyte adhesion by anti-integrin receptors. Freshly isolated hepatocytes were preincubated at 4° for 30 min in the presence of anti-integrin blocking antibody, immunoglobulin M (IgM) control, or no antibody, diluted in serum-free defined medium, then plated in 96-well plates coated with the indicated substrate. After 90 min, wells were gently washed 4× and adherent cells were determined using PH268 fluorescent dye quantitated in a fluorescent plate reader. Data represent average plus or minus the standard deviation of two to three experiments.

type I collagen, as well as its receptor $\alpha_5\beta_1$ integrin (Stamatoglou *et al.*, 1990), it has been proposed that fibronectin may act as a bridge to which hepatocytes bind, and the fibronectin in turn binds to the collagen (Gullberg *et al.*, 1990). Yet even this scenario does not fully explain the difference in proliferative capacity on film and gel, as hepatocytes proliferate on a film of fibronectin, to which they bind via the $\alpha_5\beta_1$ receptor, and they proliferate on type I collagen film (Mooney *et al.*, 1992), demonstrating that binding the $\alpha_5\beta_1$ receptor does not induce growth-inhibitory signals.

A family of non-integrin collagen receptors have been identified, called the discoidin domain receptors (DDRs), named for their homology to a region of the discoidin protein found in *Dictyostelium discoideum*. At least two family members have been identified thus far, DDR1 and DDR2, with additional isoforms due to alternative splicing (Vogel, 1999). These receptors possess intrinsic tyrosine kinase activity in their cytoplasmic tails, suggesting an ability to transmit signals into the cell in response to adhesion to the ECM. Upon collagen binding, DDR1 becomes phosphorylated and recruits Shc, but it does not induce ERK activation. This signaling appears to be independent of EGFr and β_1-integrin signaling (Vogel *et al.*, 2000). Interestingly, the DDR2 receptor binds only collagen gel, not collagen film (Shrivastava *et al.*, 1997; Vogel *et al.*, 1997), presenting the possibility of differential signaling on different conformations of collagen. The role of this or other potential adhesion receptors in the differential regulation of cell cycle by type I collagen structure has not yet been determined.

E. Mechanical Signaling by Type I Collagen

Mechanical differences between collagen film and gel could also contribute to differences in cell signaling, because the collagen film–coated plastic forms a more rigid substrate than collagen gel. Mechanical differences can affect the ability of substrates to promote specific cytoskeletal arrangements that may provide vital docking sites, or scaffolds, for signaling molecules (Harris *et al.*, 2001). Cells on malleable gel may lack the ability to form such signaling scaffolds because of reduced mechanical property, or rigidity, of the substrate. Several studies have shown that application of a mechanical force to integrins stimulates several intracellular signaling pathways compared to integrin binding without force application (Choquet *et al.*, 1997; Meyer *et al.*, 2000; Rosenfeldt and Grinnell, 2000; Wang *et al.*, 1993). Once bound to its ligand, integrins link to the intracellular contractile actomyosin cytoskeleton and apply tension to the matrix. A rigid matrix capable of resisting that tension will generate force on the integrin. In contrast, less force will be generated on a malleable substrate, such as a gel, which does not resist tension, and this lack of resistance to cellular tension can lead to

intracellular changes in biochemical signaling. In other systems, pliability of the ECM can disrupt growth factor signaling and cell cycle progression. Non-transformed cells adherent to collagen-coated polyacrylamide gels of different flexibility did not spread and were growth inhibited when the gels were pliable (Wang *et al.*, 2000). Likewise, fibroblasts embedded in a collagen gel proliferated when the gel remained attached to the culture plate and, thus, provided tension, but when the gel was released ("floating" gel) from the edges of the well, cell signaling to ERK was inhibited and cell cycle arrest occurred (Fringer and Grinnell, 2001; Rosenfeldt and Grinnell, 2000). Epithelial cells also demonstrate phenotypic changes in response to collagen rigidity. Breast epithelial cells differentiate into tubules on floating collagen gels but not on attached gels. This tubulogenesis required cell-generated contraction of the gel, which was reduced on attached or higher density gels (Wozniak *et al.*, 2003). Increased tension at the site of cell adhesion also alters both the conformation of the substrate (Krammer *et al.*, 1999), potentially altering receptor specificity, and the expression of integrins in adherent cells (Jenkins *et al.*, 1999). In addition, cells binding to a fibrillar substrate develop qualitatively and quantitatively different adhesion complexes (Katz *et al.*, 2000; Zamir *et al.*, 1999, 2000). The proteins recruited to the cytoplasmic face of the fibrillar adhesion complex have reduced phosphorylation, with more tensin and less paxillin than the conventional focal contact formed on a coated dish. This difference may be due in part to differing rigidity, as immobilization of the fibrillar substrate increases paxillin, vinculin, and phosphotyrosine (Katz *et al.*, 2000). Although few studies have addressed a role for such forces in noncontractile nonmotile cells such as hepatocytes, studies from this laboratory clearly demonstrate a requirement for actomyosin contractile forces in hepatocyte cell cycle progression (Bhadriraju and Hansen, 2004). A potential role of substrate rigidity on hepatocyte cell cycle response to type I collagen rigidity is summarized in Fig. 6.

To investigate whether mechanical characteristics of fibrillar collagen are responsible for PKA-dependent disruption of EGF signaling in hepatocytes, type I collagen gels were cross-linked to varying degrees using glutaraldehyde. Analysis using atomic force microscopy demonstrated that increased glutaraldehyde concentrations increased rigidity of collagen gels (data not shown; Wilhelm, Fassett, and Hansen, manuscript in preparation), similar to that seen with cross-linked gelatin (Dimitriadis and al., e., 2002; Radmacher *et al.*, 1995). Cross-linking Matrigel, another common substrate for hepatocyte culture, results in a similar increase in stiffness (Semler *et al.*, 2000). As rigidity of the fibrillar collagen gel increased, cell spreading also increased (Fig. 7A), accompanied by increased ERK activation, cyclin D1 expression, and DNA synthesis (Fig. 7B). Thus, cross-linking the fibers, which increases substrate rigidity, restored EGF signaling and cell cycle progression.

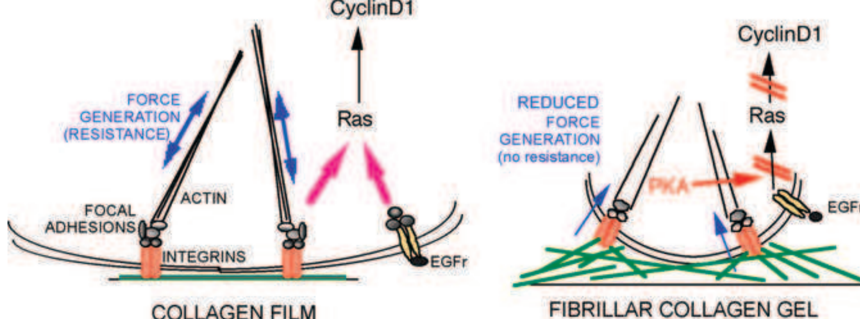

Figure 6 Schematic summary of proposed effects of substrate rigidity on adhesion structure and signaling. Rigid collagen film resists actomyosin contractility and thus likely promotes full focal adhesion formation, epidermal growth factor receptor (EGFr) activation, and subsequent Ras/ERK/cyclin D1 signaling. In contrast, the malleable collagen gel does not resist actomyosin contractility, leading to reduced focal contact formation, inability to fully activate EGFr, and inhibition of Ras and downstream pathways.

F. Regulation of Hepatocyte Differentiation and Survival by ECM

Hepatocytes adherent to collagen gel or Matrigel, embedded within gels, or attached to a substrate that blocks spreading retain higher levels of differentiated functions (Ben-Ze'ev *et al.*, 1988; Hansen and Albrecht, 1999; Mooney *et al.*, 1992; Rana *et al.*, 1994). The signaling mechanisms behind the cell shape modulation of differentiated function are unknown. However, a number of changes in expression of genes involved in growth versus differentiation have been identified that may play a role in driving these differences. One common marker of differentiated function in hepatocytes is albumin, a serum protein synthesized and secreted abundantly by hepatocytes. Another set of differentiation markers in hepatocytes are liver-enriched transcription factors that play important roles in regulating hepatocyte-specific gene expression. Among these is CCAAT-enhancer binding protein (C/EBPα), a transcription factor whose consensus sequence is found in the promoter of several liver-specific genes, including albumin (Nerlov and Ziff, 1994). Its stable expression is associated with transition from proliferative to differentiated phenotype (Runge *et al.*, 1997), while its inhibition leads to increased DNA synthesis (Soriano *et al.*, 1998). C/EBPα expression is regulated *in vitro* by ECM, with expression of C/EBPα decreasing shortly after adhesion to plastic dishes and stabilized in cells adherent to Matrigel (Runge *et al.*, 1997).

To assess the level of differentiation on the different type I collagen substrates, both albumin secretion into the culture medium and C/EBPα protein expression were assessed on film, gel, and glutaraldehyde cross-linked gel

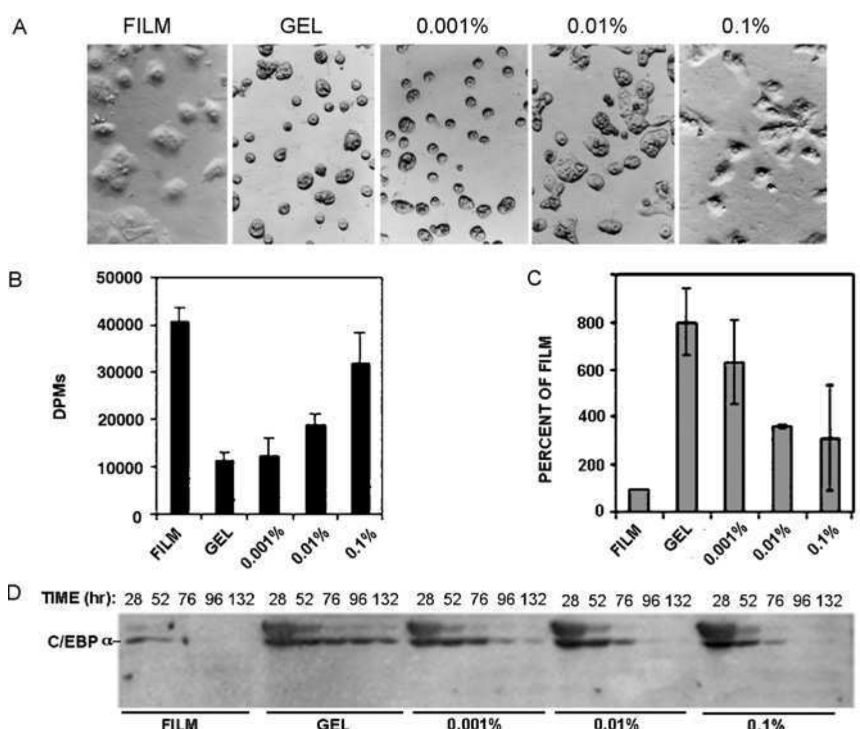

Figure 7 Cross-linked collagen gel promotes cell cycle progression and dedifferentiation. Freshly isolated hepatocytes were cultured on film, gel, or gel cross-linked for 10 min with the indicated concentrations of glutaraldehyde, followed by extensive washes with NH_4Cl and phosphate-buffered saline (PBS). The following parameters were measured: (A) phase contrast microscopy with Varel optics; (B) ^{3}H-thymidine uptake as a measurement of DNA synthesis; (C) albumin secretion measured in media samples collected from 72 to 96 hr after plating; (D) Western blot analysis of hepatocyte lysates using anti-C/EBPα antibody.

(Fig. 7C). Albumin secretion was highest on gel, and levels of secretion diminished with increased glutaraldehyde concentration, with secretion being lowest on collagen film. Albumin secretion is detectable to the eighth day of culture in all gel conditions except 0.1% but is always lower on stiffer substrates at all time points tested. Similarly, on gel, C/EBPα expression is maintained to at least 132 hr, diminishing only slightly over time (Fig. 7D). On film, it is expressed at a low level early in culture and is not detected after 52 hr in culture. In response to cross-linking, the level of C/EBPα expression increases, relative to film although it does not reach the level seen on gel. Interestingly, the time at which expression diminishes on gel appears to be correlated with substrate stiffness, and even the lightest cross-linking results in reduced duration of expression.

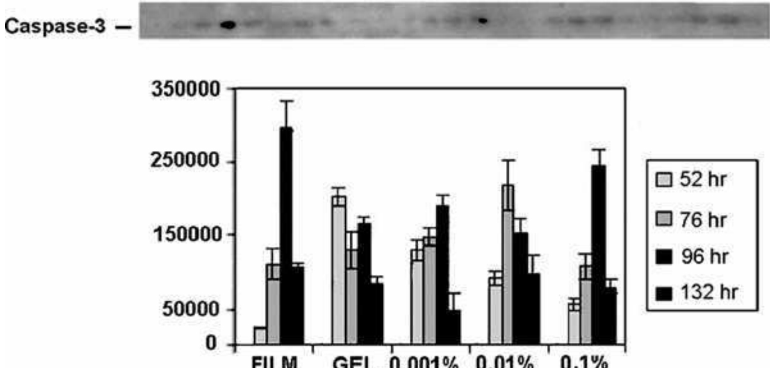

Figure 8 Apoptosis markers are increased on collagen film. Western blot analysis (top panel) using antibody against the cleaved (activated) caspase-3 of hepatocytes cultured on collagen film, gel, or cross-linked gel. Gels were cross-linked with the indicated concentrations of glutaraldehyde for 10 min, followed by extensive washes with NH_4Cl and phosphate-buffered saline. Bottom panel is densitometric quantitation of the top blot.

Several reports indicate prolonged survival of hepatocytes along with enhanced differentiated function observed on malleable substrates such as collagen gel or Matrigel. The effects of type I collagen structure and malleability on apoptosis were examined by measuring the presence of caspase-3 protein, a common marker of apoptosis, in hepatocyte lysates from the different substrates (Fig. 8). Caspase-3 expression was generally higher on film than all gel substrates, peaking at 96 hr. On gels, this peak was not as definitive, with the exception of the stiffest substrate, which showed similar timing but was not as robust. This suggests that a malleable substrate provides some protective effect against apoptosis in primary hepatocyte culture. These results are intriguing in light of several studies in fibroblasts and other cell types in which apoptosis increases in malleable (floating gels) versus attached collagen gels (Grinnell *et al.*, 1999). The mechanism for this apoptotic response to substrate compliance is not clear.

Interestingly, inhibition of PKA activity by H-89 treatment does not appear to adversely affect hepatocyte differentiation using albumin secretion and C/EBPα as markers. Albumin secretion is sustained with H-89 treatment on day 6 (Fig. 9A). Levels were still significantly higher than film at this time point and only slightly reduced from secretion rates of gel and 0.001% samples during the first 96 hr in culture. Albumin secretion diminishes in correlation with substrate stiffness, as described previously. But even at the heaviest cross-linking density, both control and H-89–treated cultures still demonstrate albumin secretion at the latest time points, where film cultures do not. Similarly, the expression of C/EBPα is sustained, regardless of H-89

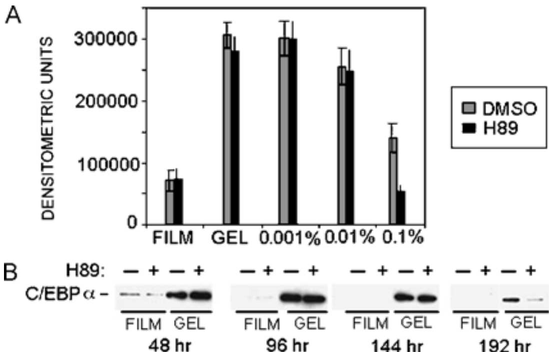

Figure 9 Inhibition of PKA has minimal effect on differentiated function. (A) Hepatocytes were cultured on collagen film, gel, or gel cross-linked for 10 min with the indicated concentration of glutaraldehyde. Albumin secreted into the medium between days 5 and 6 was measured using anti-albumin antibody and dot-blot analysis, followed by densitometry. (B) C/EBPα protein expression was analyzed by Western blot analysis in hepatocytes cultured on gel or film in the presence or absence of protein kinase A (PKA) inhibitor, H89.

treatment until day 8 (Fig. 9B) in gel cultures. Only at later time points of H-89 exposure (e.g., 8 days) was C/EBPα decreased, and this reduction may reflect toxicity from prolonged exposure to drug (data not shown). On film, H-89 does not appear to affect C/EBPα expression, as it is down-regulated quickly with or without drug. These data suggest that culture conditions can be established in which hepatocyte cell cycle progression is promoted by manipulating intracellular signaling pathways without loss of differentiated function.

G. Interplay of Cytokines and ECM in Hepatocyte Cell Cycle Regulation

The results of comparing monomeric to fibrillar collagen and manipulation of collagen rigidity by cross-linking suggest that rigidity of the ECM is a critical driving force of cell cycle progression and differentiated function *in vitro*. As stated earlier, this effect of rigidity and the requirement for cell spreading is not limited to type I collagen but is also demonstrated in a number of other models, including Matrigel, polyacrylamide gels, as well as rigid substrates with defined patterns or density of ECM that limit spreading. This should cause one to wonder, if rigidity and the cell spreading response play such a critical role *in vitro*, how does the liver overcome this lack of rigidity *in vivo*? While malleability of the liver has not been quantitated during quiescent and regenerating conditions, the obvious compliance of the liver during dissection suggests it would not provide a rigid substrate to drive cell spreading and cell cycle progression. Thus, the process of

hepatocyte proliferation during liver regeneration must involve activation of signaling pathways that avoid dependence upon rigidity. A number of studies *in vivo* suggest that the liver must be "primed" by cytokines in order to respond to growth factors HGF or EGF (Webber *et al.*, 1998). Upon initiation of regeneration, hepatocytes *in vivo* are exposed to an early burst of the cytokines tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6). Although HGF is believed to play a critical role in hepatocyte proliferation during liver regeneration, it is not sufficient to induce hepatocyte proliferation in the absence of IL-6 (Cressman *et al.*, 1996) or TNF-α (Akerman *et al.*, 1992). This priming corresponds with the competence/progression model of cell cycle progression (Stiles *et al.*, 1979), in which resting cells must receive a specific signal that initiates the exit from G0 and promotes responsiveness to subsequent growth factors.

To determine whether exposure to these cytokines would have any effect on hepatocyte proliferation on collagen film or gel, hepatocytes were plated on the two substrates in medium containing TNF-α (20 ng/ml) and IL-6 (20 ng/ml) for the first 24 hr. The medium was then replaced with medium containing EGF without the cytokines. Control cultures had EGF present the entire culture period without cytokines. Both DNA synthesis and cyclin D1 expression were enhanced in hepatocytes on collagen gel first cultured in the presence of TNF-α/IL-6 (Fig. 10). Although the level of each response on gel with cytokines does not reach that seen on collagen film, it is possible that the cytokine concentration and/or time of exposure could be optimized to induce a response similar to that on collagen film. These data suggest that cytokines present during liver regeneration *in vivo* may provide a set of "priming" signals similar to that provided *in vitro* by rigid substrates such as collagen film–coated plates or cross-linked gels.

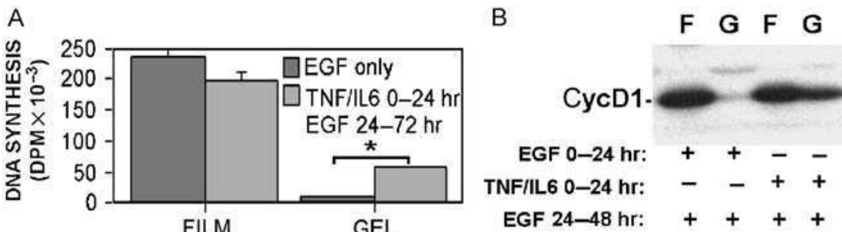

Figure 10 Initial stimulation with TNF-α/IL-6 allows hepatocyte cell cycle progression on fibrillar collagen. Hepatocytes were cultured on collagen film or gel in the presence of tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) without epidermal growth factor (EGF), followed by EGF alone. (A) DNA synthesis was assessed by [^{3}H]thymidine incorporation from 52 to 72 hr and compared to cultures containing EGF without cytokines for the entire culture period. (B) Cyclin D1 protein expression was assessed using Western blot analysis and anti-cyclin D1 antibody.

V. Summary and Implications

Elucidating the mechanisms by which type I collagen substrates regulate hepatocyte proliferation and differentiation will be of great benefit to understanding hepatocyte response to ECM alterations *in vivo*, as well as optimizing hepatocyte cultures *in vitro*. The studies performed in this laboratory and others indicate that changes in the structure of type I collagen alter hepatocyte proliferative response by changing intracellular signaling pathways governing cell cycle progression. We have found that enhanced PKA activity induced by fibrillar collagen gel plays an important role in inhibiting cell cycle events downstream of EGF (Fassett *et al.*, 2006). Many questions remain, however, such as the mechanism by which PKA becomes elevated on collagen gel, as well as the mechanism by which differentiated functions are enhanced on gel.

A critical factor in determining the cellular response to the ECM appears to be the rigidity of the matrix. Increasing rigidity leads to dedifferentiation and increased cell cycle progression. This relationship is observed in many cell types, including fibroblasts, endothelial cells, breast epithelial cells, and hepatocytes. Interestingly, this relationship is lost in transformed cells (Wang *et al.*, 2000), suggesting that it is a critical factor in maintaining normal growth control. The ability of substrate rigidity to regulate proliferative and differentiated functions does not preclude a role for receptor specificity in this regulation. As discussed earlier, different receptors, both integrin and non-integrin, recognize type I collagen, and each of these receptors has defined intracellular domains that interact either with cytoskeleton and specific signaling molecules (integrins) or possess intrinsic tyrosine kinase activity (DDRs), indicating the potential for differential signal transduction. Receptor signaling may be involved in fine-tuning the ECM response or perhaps takes the place of compliance-dependent signaling during ECM composition changes *in vivo* if compliance is not significantly altered.

As discussed previously, the liver is a soft tissue, and thus, *in vivo* ECM malleability of the liver is likely more comparable to collagen gel. Although the collagen film–coated plate is an important *in vitro* tool to promote cell cycle progression in cell cycle–regulation studies, the physiological significance of the hepatocyte response to rigid ECM-coated plates is not clear. Likewise, while a consistent relationship between *in vitro* cell spreading and proliferation has been demonstrated in many cell types including hepatocytes, the high cell density and tissue architecture of the liver make such a spread of morphology *in vivo* highly unlikely, in contrast to cells such as fibroblasts whose low cell density in interstitial matrix allows for significant cell extension. The "spreading requirement" *in vitro* must, thus, be

manifested differently *in vivo* in hepatocytes, perhaps by more subtle changes in cytoskeletal structure or by different signaling pathways that bypass the requirement for changes in cytoskeletal structure. Indeed, studies from this laboratory demonstrate the ability of non-spread hepatocytes to proceed into the S phase of the cell cycle if intracellular signaling pathways are altered, such as overexpression of cyclin D1, suggesting that spreading per se is not required if sufficient regulatory events can be supplied to a rounded cell (Hansen and Albrecht, 1999). Clues as to what the *in vivo* stimulus might be, if not ECM rigidity and cell spreading, are provided by the preliminary studies utilizing TNF-α/IL-6 stimulation (Fig. 7). In these studies, hepatocyte cell cycle progression is promoted on collagen gel if hepatocytes are first exposed to TNF-α/IL-6, which are required *in vivo* for hepatocyte proliferation during liver regeneration (Akerman *et al.*, 1992; Cressman *et al.*, 1996). Hepatocytes appear to be governed by the competence/progression model of cell cycle progression in which cells must first be primed or stimulated by a "competence" factor stimulating exit from the G0 resting phase into G1 cell cycle phase, in order to respond to subsequent "progression" factors that stimulate further progression through G1 into S phase (Fausto, 2000; Mead *et al.*, 1990; Stiles *et al.*, 1979). It, thus, appears that signals transduced by the rigid collagen film substrate may provide the competence necessary to respond to mitogens such as EGF. Fibrillar type I collagen gel lacks the ability to provide this competence factor, and hepatocytes, thus, remain quiescent on this substrate in spite of the presence of EGF. TNF-α/IL-6 appears to be able to provide sufficient competence signal to switch the hepatocytes to a competent state, allowing responsiveness to EGF.

In viewing a summary of the hepatocyte response to different type I collagen substrates (Fig. 11), several points regarding the relationship between ECM-induced proliferation and differentiation become clear. A rigid type I substrate provided by either collagen-coated dish or cross-linked fibers induces dedifferentiation and cell cycle progression. Stimulation of PKA activity using a PKA agonist leads to cell rounding and growth arrest (data not shown; Fassett *et al.*, 2006). The effects of PKA agonist on differentiated function has not been tested in this model; however, PKA does induce certain hepatocyte-specific metabolic pathways responsive to cAMP (Lazennec *et al.*, 2000; Viitalia *et al.*, 2001). No studies to our knowledge demonstrate any ability to promote differentiated function on a rigid substrate, suggesting that this type of substrate is incompatible with a differentiated phenotype. On a malleable collagen substrate (i.e., gel), the opposite phenotype is induced, with high differentiated function and growth arrest. Inhibition of PKA activity can promote cell cycle progression, but in contrast to all the other conditions in which growth and differentiated functions are inversely correlated, cell cycle stimulation by PKA inhibition on collagen gel does not appear to result in loss of differentiated function. Effects of PKA on apoptosis or

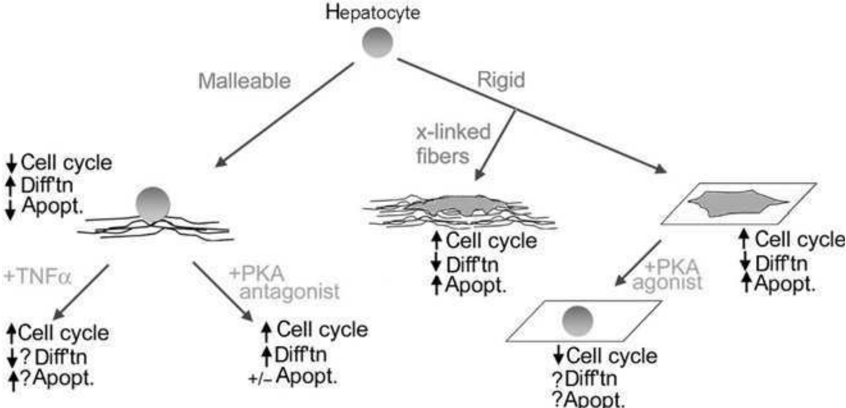

Figure 11 Summary of hepatocyte response on collagen substrates. Question mark (?), data not determined; +/−, conflicting reports in literature.

hepatocyte longevity in culture suggest that prolonged exposure to the PKA-inhibiting drug may lead to toxicity and cell death by 8 days in culture. However, this effect may be minimized by manipulating the drug dose and/or duration of exposure. While the effects of TNF-α on differentiated function and apoptosis have not been directly tested in this system, studies in the literature suggest that TNF-α does have a dedifferentiating effect *in vitro* (Dahn *et al.*, 1994; Kowalski-Saunders *et al.*, 1992; Muntane-Relat *et al.*, 1995). However, it is possible that differences in culture conditions may lead to different results than those reported in the literature. Indeed, it has been shown that TNF-α can induce either proliferation or apoptosis in hepatocytes depending on the existing level of intracellular antioxidants (Pierce *et al.*, 2000). Taken together, these data demonstrate that type I collagen structure determines the switch between proliferative and differentiated phenotype by regulating specific signaling pathways and suggest that it may be possible to promote both proliferation and differentiation *in vivo* by manipulating intracellular signaling pathways on collagen gel and/or providing specific cytokines to provide the necessary competence signals. Notably, it has been shown that multiple rounds of hepatocyte proliferation can be induced without loss of differentiation in co-cultures with liver biliary cells and alternating periods of TNF-α and EGF (Serandour *et al.*, 2005). It is significant that ECM degradation was required for initiating any new hepatocyte division cycle in response to TNF-α, indicating that the appropriate combination of matrix structure and TNF-α may be sufficient for the simultaneous maintenance of proliferative and differentiated phenotypes (Serandour *et al.*, 2005).

Identifying factors that allow hepatocytes to overcome the growth-inhibitory properties of a malleable substrate such as fibrillar collagen gel

while retaining differentiated functions would be extremely useful for various applications. First, it could provide important insight into both cell cycle and differentiation regulatory mechanisms that may be applicable to multiple cell types. In addition, from a clinical perspective, hepatocytes have great potential in cell-based therapies for the treatment of liver disease. Liver failure is a significant cause of mortality, and because of the complex nature of liver function, few treatment options are available. Although liver transplantation is effective, its use is limited by donor organ availability. For this reason, treatments such as bioartificial liver devices incorporating living hepatocytes have been pursued as viable treatment options. Furthermore, hepatocyte cultures are critical for drug testing and metabolism studies, but this application is limited by the difficulty maintaining differentiated function and viability and the inability to promote repeated hepatocyte propagation *in vitro*. Thus, the ability to maintain and propagate differentiated hepatocytes in culture will further the success of such cell-based treatments and testing. Finally, understanding hepatocyte response to changes in type I collagen structure will likely lead to a better understanding of hepatocyte function and regenerative capability during liver fibrosis, in which an accumulation of fibrillar type I collagen is associated with a life-threatening reduction in hepatocyte proliferation, leading ultimately to loss of functional liver tissue and organ failure.

In conclusion, it is clear that the mechanical and biochemical nature of collagen structure greatly affects the hepatocyte differentiation–proliferation balance. Much progress has been made in this laboratory and others to elucidate regulatory mechanisms and identify conditions that can enhance collagen-dependent hepatocyte proliferative and/or differentiated functions *in vitro*. Understanding this regulatory control will contribute to designing scaffolds and biomaterials for tissue engineering and bioartificial liver devices, for understanding aberrant signaling during disease processes such as fibrosis, and for discovering molecular targets for research or therapy. The requirement for ECM remodeling in liver regeneration and its ability to switch hepatocyte phenotypes demonstrates a central role of type I collagen in liver structure and function. The ability to use combinations of defined collagen structures, cytokine/growth factors combinations, and possible manipulation of intracellular signaling pathways may ultimately allow the researcher or clinician to fine-tune hepatocyte response for specific functions.

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5

Engineering Stem Cells into Organs: Topobiological Transformations Demonstrated by Beak, Feather, and Other Ectodermal Organ Morphogenesis

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To accomplish regenerative medicine, several critical issues in stem cell biology have to be solved, including the identification of sources, the expanding population, building them into organs, and assimilating them to the host. Although many stem cells can now differentiate along certain lineages, knowledge on how to use them to build organs lags behind. Here we focus on topobiological events that bridge this gap, for example, the regulation of number, size, axes, shape, arrangement, and architecture during organogenesis. Rather than reviewing detail molecular pathways known to disrupt organogenesis when perturbed, we highlight conceptual questions at the topobiological level and ask how cellular and molecular mechanisms can work to explain these phenomena. The avian integument is used as the Rosetta stone because the molecular activities are linked to organ forms that are visually apparent and have functional consequences

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during evolution with fossil records and extant diversity. For example, we show that feather pattern formation is the equilibrium of stochastic interactions among multiple activators and inhibitors. Although morphogens and receptors are coded by the genome, the result is based on the summed physical-chemical properties on the whole cell's surface and is self-organizing. For another example, we show that developing chicken and duck beaks contain differently configured localized growth zones (LoGZs) and can modulate chicken beaks to phenocopy diverse avian beaks in nature by altering the position, number, size, and duration of LoGZs. Different organs have their unique topology and we also discuss shaping mechanisms of liver and different ways of branching morphogenesis. Multi-primordium organs (e.g., feathers, hairs, and teeth) have additional topographic specificities across the body surface, an appendage field, or within an appendage. Promises and problems in reconstitute feather/hair follicles and other organs are discussed. Finally, simple modification at the topobiological level may lead to novel morphology for natural selection at the evolution level. © 2006, Elsevier Inc.

I. Introduction

One of the most fundamental questions in biology is how the single dimension genomic codes are transformed into three-dimensional forms that are even able to morph temporally. As the genomics of different organisms are gradually completed, in the post-genomic age, we need to learn more about how the molecular events are translated to biological structures and how cells are arranged in time and space to build an organ. In the last decade, many secreted regulatory pathways (e.g., sonic hedgehog [Shh], bone morphogenic protein [BMP], and Wnt) were identified and developmental biologists gained a lot of new understanding and insight into the morphogenetic processes in development and diseases (Hogan and Kolodziej, 2002; Moon *et al.*, 2004; Scott, 2000; Tickle, 2003). However, as we all analyzed molecular pathways more, we gradually grew less satisfied that we could disrupt organ formation by misexpressing certain molecular pathways but did not know how the molecular pathways work together to build an organ. We have the ability to dissect molecular pathways and we know certain molecular pathways are essential, yet we do not know enough to assemble them into organs (Fig. 1).

Maybe we should also look at a more global level in order to strive for integration of multiple molecular and cellular pathways. Maybe it is time to revisit the topobiology concept. As Dr. Gerald M. Edelman (1988a) muses, "While the triumph of molecular biology answers the question on the chemical nature of genes and how hereditary traits are transmitted, it does not fully

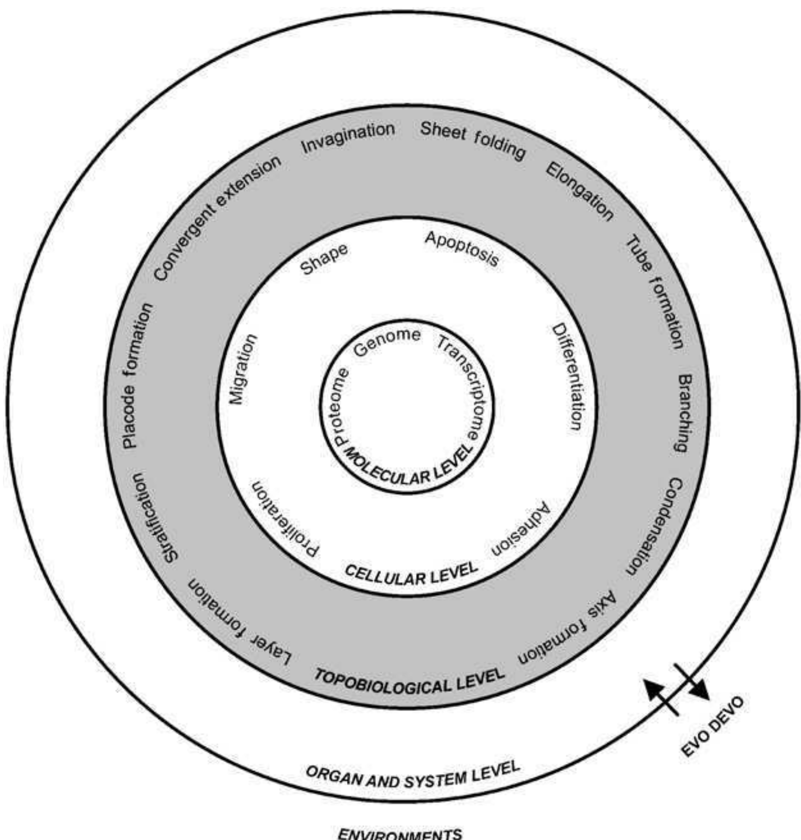


Figure 1 Levels of organ formation. From molecules to the organism, there are different levels of interactions. Each level is important and interdependent but also operates with different principles.

answer the question on how genes determine traits." He felt that "it is very difficult to account for the forms, patterns or shapes of complex animals simply by extrapolating from the rules governing the shape of proteins," and therefore turned to "the other side of biology," hence the birth of "topobiology." He defined *topobiology* as the "place dependent molecular interactions at the cell surface" (Edelman, 1988a). He emphasized the fundamental importance of cell proliferation, adhesion, migration, death, and differentiation, and particularly the links of cell collectives by cell adhesion molecules and the regulation of these links. A single cell is capable of proliferation, migration, shape changes, apoptosis, and differentiation, but cell adhesion, epithelial sheet morphogenesis, and tissue interactions require cell

collectives. The topobiology concept focuses on multicellular activities to examine how multipotential stem cells are organized into tissues and organs, with particular architectures, sizes, and shapes.

The advent of genomics provides a "dictionary" of molecules, but we still lack the syntax of how this information is used. New understanding has been gained for studying molecular interactions, enhancer regulations, and pathway activities. These molecular events are integrated at the cellular level (Fig. 1). The basic information is genetically determined because the numbers of adhesion molecules or morphogen receptors on the cell membrane are predetermined by the genome; however, the interaction among these cells is a physicochemical phenomenon. Tissue and organ organization and structure reflect an equilibrium of thousands of chemical reactions within a particular physical constraint. The importance of physicochemical phenomena at this level has been pointed out previously (Kiskowski *et al.*, 2004; Newman and Frisch, 1979; Oster *et al.*, 1985). However, major research efforts and hence progress has been at the molecular and cellular level. The concept of topobiology did not get the attention it deserves and the parameters for topobiology remain mostly elusive. This knowledge is even more urgent now as we start to work on stem cells and hope to build an organ for regenerative medicine.

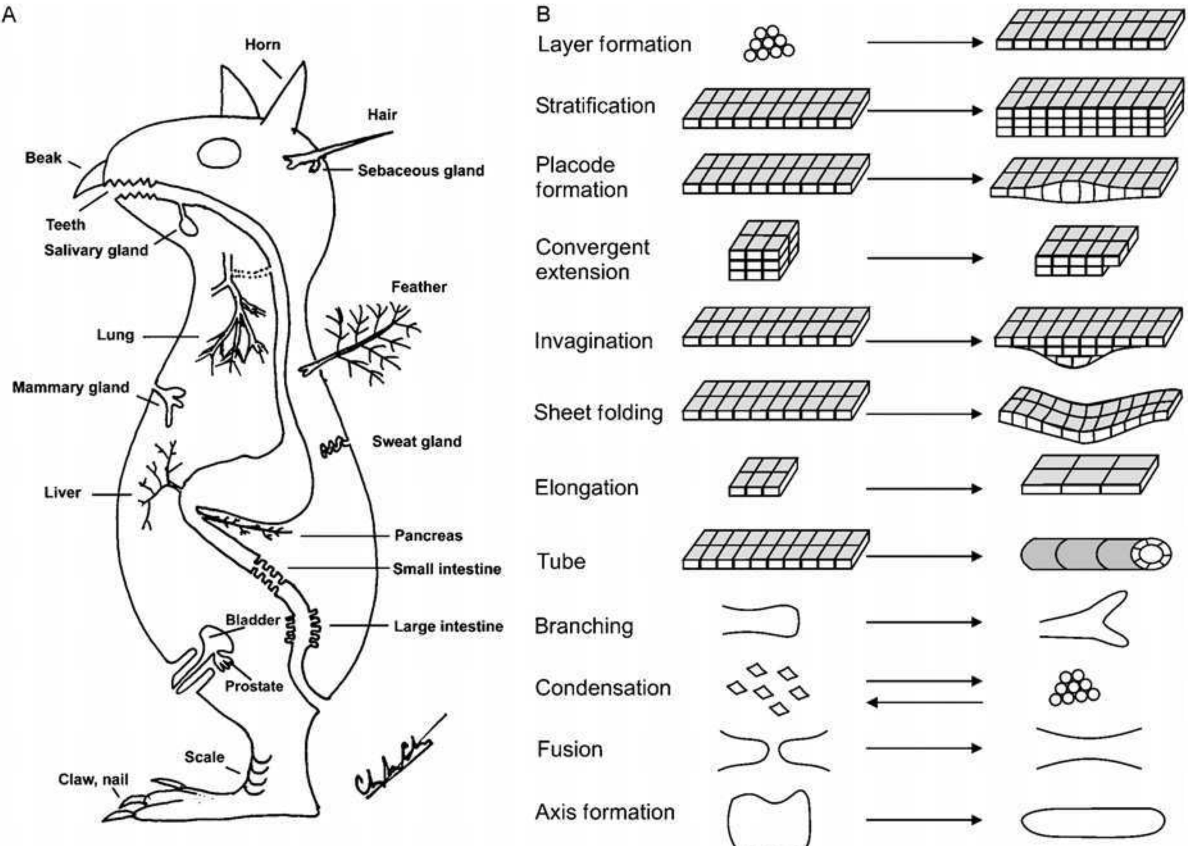
To understand how an organ is built, our laboratory has been using the avian integument as the Rosetta stone. Avian feathers and beaks are good models because the end points show distinct morphologies with functional consequences. Their evolution occurs through a series of novel topobiological events, which add evolutionary novelties that can be selected out by the environment. The accessibility of avian embryos and regenerating feather follicles provides excellent opportunities for tackling cellular and molecular events experimentally (Brown *et al.*, 2003). Thus, they are excellent models to further develop the concept of topobiology. In this chapter, we first identify gaps that need to be bridged in stem cell biology and introduce progress that has been made in the topobiology of epithelial organs. The work on feather organogenesis has been of intense interest because of the many newly excavated feather-related fossils from northern China, and our effort to link molecular findings with these intermediate "proto-feather" morphologies (reviewed in Prum and Brush, 2002; Chuong *et al.*, 2003; Sawyer and Knapp, 2003). The beak is used because the diverse beak shapes in Galapagos finches inspired Darwin's Evolution Theory. The breakthrough by Tabin's and our group (Abzhanov *et al.*, 2004; Wu *et al.*, 2004a) was praised in the accompanying *Science* commentary, which said "Darwin will be pleased" (Pennisi, 2004). These works are examples demonstrating how natural selection engineers organ forms on a grand scale of hundreds of millions of years in the context of "Evo-Devo." We then briefly apply the topobiology concept to mammalian ectodermal organogenesis,

liver shaping, lung branching, etc. We also discuss the regional specificity issue that we must face in engineering organs. At the end of this chapter, we reflect on how understanding these principles may contribute to the engineering of stem cells. With this progress, we can further develop the topobiology concept to mean "bioinformation generated by topology-dependent molecular expression and cellular behavior."

II. Between Stem Cells and Organs

Stem cell biology has emerged as an important new discipline of translational research in the context of regenerative medicine. Several issues are important in stem cell biology research. They are (A) identifying sources of stem cells, (B) expanding stem cell populations while maintaining their properties, (C) engineering stem cells to form the tissue/organ desired, and (D) having the engineered tissues/organs assimilate into the host. For the first issue, the research at this stage has been on embryonic stem cells and identifying possible sources of adult stem cells (Fuchs and Segre, 2000; Lako *et al.*, 2002; Li and Xie, 2005; Toma *et al.*, 2005). Somatic nuclei transfer technology has allowed the progress of therapeutic cloning. For the second issue, scientists have worked on culture conditions and found some promising clues. For instance, Wnt has been found to help expand hematopoietic stem cells (Reya *et al.*, 2003).

The third issue is how to engineer these cells to organ-like structures and be useful for the host. This has proven to be of different difficulty levels for different types of organs. For hematopoietic cells, multiple blood cell types float in the bloodstream without being organized into a particular form and can function in response to cytokines. This lack of structural organization makes blood a relatively easy organ to work with, and as a result, hematopoietic stem cells have already been used successfully in clinical practice. The next level is to have engineered tissues that secrete needed extracellular factors required to alleviate disease conditions, such as insulin from pancreatic beta cells for diabetes (Efrat, 2004; Lumelsky *et al.*, 2001) or dopamine-secreted neurons for Parkinson's disease (Snydeer and Olanow, 2005). The next challenging level is to be able to produce certain shapes suitable for functional morphology. For example, it is now possible to induce chondro-differentiation from mesenchymal cells in culture, but it is still very difficult to have these cells form the right contours on a cartilage or bone element. The use of a biodegradable polymer scaffold to generate auricular-shaped cartilage (Shieh *et al.*, 2004) can facilitate the process when a better solution is not available. It would be best to find out how nature performs morphogenesis in development, but even nature "forgets" how to do it during regeneration in the adult; during the body's effort to regenerate in response


A
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Teeth
Salivary gland
Feather
Lung
Mammary gland
Sweat gland
Liver
Pancreas
Small intestine
Bladder
Large intestine
Prostate
Scale
Claw, nail
B
Layer formation
Stratification
Placode formation
Convergent extension
Invagination
Sheet folding
Elongation
Tube
Branching
Condensation
Fusion
Axis formation

to osteoarthritis, bone spurs form, which cause more damage. Even if we can have a functional tissue/organ entity, we still have to learn how to make them connect with the host. For example, a group of beating cardiomyocytes have to coordinate the motion of the whole myocardium and a group of transplanted neurons has to be connected with other parts of the brain. Finally, stem cell–derived organs have to survive without being rejected by the host immune system or competed out by the native cells. Therefore, while stem cell engineering holds promise, there are many challenges before the knowledge is translated to clinical applications.

The focus of this chapter is on the third issue: how to engineer stem cells to form the tissue/organ desired. Suppose current stem cell research reaches a stage at which we have enough stem cells that can be induced to form different differentiated phenotypes. How do we direct them to form organs? We need to position ourselves to answer these questions. Developmental biology used to be considered a basic science operating in an ivory tower. Now scientists appreciate that tissue engineering and developmental biology are two sides of the same coin: When nature does it, it is developmental biology; when humans do it, it is stem cell engineering. The best way to engineer stem cells is to learn how to guide them in nature's way.

III. Topobiological Transformation Events in Epithelial Organ Formation

Here we use *topological transformation* to mean the conversion from one cell collective configuration to the other. It does not entirely fit the definition in mathematics, but we use the term to emphasize the geometric aspect of tissue morphogenesis: the forming and dissolution of cell groups, the shifting arrangement, the making and elimination of boundaries, the orientations, etc. In fact, the creation or removal of boundaries or breaking of epithelial sheet makes them topologically nonequivalent. The formation of epithelial organs involves topological transformation of a two-dimensional (2D) epithelial sheet into different structures (Fig. 2A). In ectodermal organ formation, they can evaginate out to form bumplike configurations (e.g., scale), some with elaborate surface (e.g., molar), protrusions (e.g., canine, claw),

Figure 2 Topobiological transformation events during epithelial organ formation. (A) A prototype animal with ectodermal and endodermal organs. Although these epithelial organs appear diverse, they share similar morphogenesis-related signaling pathways and topobiological principles (modified from Chuong, 1998). The molecular basis of epithelial appendage morphogenesis. (B) Types of topobiological transformation events. These events are meaningful only at the level of cell groups (epithelial sheet, mesenchymal condensations), not at the single cell level. We need to learn more about how molecular mechanisms contribute to these events.

elongated filaments (e.g., hair), some with hierarchical branches (e.g., feathers), etc. They can also invaginate to form tubes (e.g., sweat glands), some with branching (e.g., salivary glands, mammary glands), follicles (e.g., hair, feather), etc. (Chuong, 1998). In the endoderm, similar topological transformations occur in the gut. Regional specialization of epithelia leads to the formation of the stomach, intestines, lungs, liver, and pancreas, which form by budding from the gastrointestinal tract during embryonic development. These apparently different epithelial organs actually share similar topobiological transformation events (i.e., an event that changes the topological configuration of cells before and after it happens). The involved molecular mechanisms have begun to be understood. Some examples are given (Fig. 2B).

Layer formation: In this event, randomly arranged epithelial cells start to join with each other. The progeny of cell proliferation remains in the same sheet as the axial orientation of mitosis within the 2D plane. Epithelial cell adhesion molecules such as E-cadherin were first shown to have this function (Nagafuchi *et al.*, 1987).

Stratification: Some mitosis becomes asymmetric with a mitotic axis becoming perpendicular to the epithelial sheet. The daughter cells remaining in the basal layer can still proliferate (the beginning of stem cells), while the other daughter cells, now postmitotic, start to pile up, forming multiple layers. Stratification enables the epithelia to form a multilayered barrier, protecting the organism from its environment, and allows functional diversification. Activation of the p63 pathway is involved in the stratification process (Koster *et al.*, 2004; Koster and Roop, 2004). p63 is expressed early in the epidermal lineage when cells are still forming a single layer (Green *et al.*, 2003; Koster *et al.*, 2004). p63-null mice fail to form stratified epithelial derivatives (Mills *et al.*, 1999).

Convergent extension: Convergent extension allows a change of shape of epithelial sheets by cell rearrangements. Lateral and medial cells become polarized and then the lateral cells intercalate between the medial cells, causing an extension along the anteroposterior axis (Keller, 2002). This process was originally shown to be responsible for gastrulation in *Xenopus* and zebrafish (Keller, 1986), gut elongation in sea urchins (Ettensohn, 1985; Hardin and Cheng, 1986), the formation of the avian primitive streak (Wei and Mikawa, 2000), and shaping of the avian neural plate (Schoenwolf, 1991; Schoenwolf and Alvarez, 1989). It is likely to be a fundamental topological transformation process involved in other organ formation. Signaling along the noncanonical Wnt pathway is likely to be involved.

Invagination: Invagination of epithelial tissues is seen in the organization of the neuroepithelium in *Xenopus* (Schoenwolf and Alvarez, 1989). It also

plays a critical role in tooth formation (Jernvall and Thesleff, 2000). The activation of Wnt/β-catenin and the suppression of BMP by *noggin* leads to an invagination of the epithelial placode to initiate hair follicle formation (Jamora *et al.*, 2003).

Tube formation: Tube formation can occur through rearrangements of epithelial cells to form a lumen within an elongated cell cord. Tubular structures can form in many ways. An epithelial sheet can curl and seal itself to form a tube. This occurs during neural tube formation (Colas and Schoenwolf, 2001). This involves cell shape changes forming a narrow apical region and a broad basal region. Tubes can also form by budding out from an epithelial surface. The lung is thought to branch out in this manner (Hogan and Kolodziej, 2002; Metzger and Krasnow, 1999). A mass of cells can invaginate to form a central cavity, as occurs during salivary gland formation (Melnick and Jaskoll, 2000). Apoptosis may play a role in this mechanism (Coucouvanis and Martin, 1995). In angiogenesis, hemangioblasts form an aggregate called *blood islands*. The inner cells become hematopoietic stem cells while the outer cells become angioblasts, which go on to multiply and differentiate into endothelial cells forming the blood vessels. So cords of hemangioblasts hollow out to form a tube (reviewed in Baron, 2003).

Branching: Branching is used to increase the surface area for interactions with the environment, be it internal or external. Branching involves the splitting of the long axis into two. While the end results can be quite similar, they can be generated from very different mechanisms. It can be generated by differential growth or death. The process is seen in lung and mammary gland morphogenesis (see Section VI, later in this chapter), as well as in feather barb branching.

Condensations and decondensations: This involves increased cell adhesion that brings out a group of highly compacted cells, or the reverse of this process. Not only physically does a cell collective form or dissolve, but there are also changes of cell properties due to signaling initiated by cell contacts. The formation of dermal condensations is a very early step in feather formation (Chuong and Edelman, 1985a; Jiang and Chuong, 1992). The regulation of this process leads to periodic pattern formation (see Section VII, later in this chapter). The migration of neural crest cells is a good physiological example of epithelial–mesenchymal transformation (Kang and Svoboda, 2005).

Fusion: When two cell collectives meet, the epithelial can remain as two entities with a surface boundary in between, or the boundary disappears and two cell collectives fuse into one. This may occur through epithelial–mesenchymal transformation (Kang and Svoboda, 2005) or may involve apoptosis.

IV. Feather Morphogenesis

Feathers on the bird body show hierarchical branch patterns (Prum and Dyck, 2003). The major types of avian feathers include contour feathers, remiges, rectrices, downy feathers, etc. (Lucas and Stettenheim, 1972). A typical avian feather consists of a shaft (rachis) and barbs. The barbs are composed of a shaft (ramus) and many smaller branches (barbules) (Fig. 3A). Different feathers show variations in symmetry. Down feathers are radially symmetric. Their rachis is absent or very short. Contour feathers have a weak bilateral symmetry. Flight feathers are bilaterally symmetric and some become bilaterally asymmetric (see later discussion) (Fig. 5). A contour feather can have a distal pennaceous region and a proximal plumulaceous region, so the feather can help the integument function for contour/communication display with the distal portion but maintain warmth with its proximal plumulaceous portion (Fig. 9C). The plumulaceous regions are made of similarly shaped barbules both proximal and distal to the ramus. They are loose and fluffy. The pennaceous regions are made of groove-shaped proximal barbules and hook-shaped distal barbules. Therefore, the distal barbules of a barb interlock with the proximal barbules of the barb above, forming a feather vane using a Velcro-like mechanism.

A. Development

During avian embryonic development, feather formation starts with a placode, which is composed of elongated epithelia accompanied with dermal condensations (Sengel, 1976; Wu *et al.*, 2004b). These feather primordia elongate and protrude out to form feather buds, topologically transforming a 2D flat epidermis into a three-dimensional (3D) structure (Chuong and Edelman, 1985b) (Fig. 3C). Feather buds are originally radially symmetric but soon acquire anteroposterior polarity through interactions with the epithelium. Feathers then start to elongate and develop a proximal-distal axis. Feathers form follicles that offer advantages over skin appendages that do not, such as scales. The follicular structure protects the epithelial stem cells and dermal papillae. Localization of the stem cells within a protected environment enables regeneration through natural feather molting cycles and induction by plucking. New cell proliferation at the follicle base pushes the more differentiated portions of the feather filament to the distal end. Feather filaments go through epithelial invaginations and evaginations to form the barb ridges, which precede the formation of the barbs and barbules. The barb ridges further differentiate into the barbule plates, axial plates, and marginal plates. Barbule plate cells later keratinize to become the feather structure, while marginal plate and axial plate cells undergo apoptosis, die,

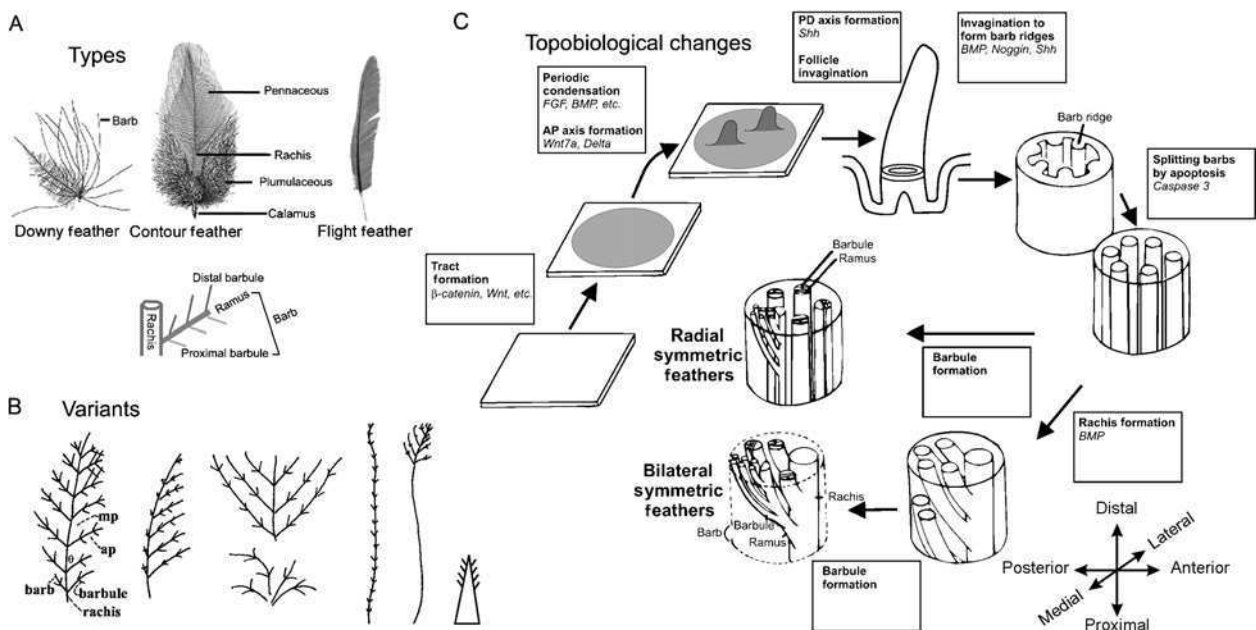


Figure 3 Feather types (A), variants (B), and topobiological events in development (C). Panel A is adopted from Lucas and Stettenheim, 1972. Panel B is modified from Chuong, 1998. Panel C is modified from Chuong and Edelman, 1985b.

and become spaces (Chang *et al.*, 2004) (Fig. 4). The central pulp undergoes apoptosis, allowing the feathers to unfold and assume their characteristic flat shapes, transforming a 3D cylinder back to a 2D plane. Topobiological transformation events are listed in the boxes in Fig. 3C. In each process, signaling molecules are used in different ways (reviewed in Widelitz *et al.*, 2003; Jiang *et al.*, 2004; Wu *et al.*, 2004b, and references within), and some

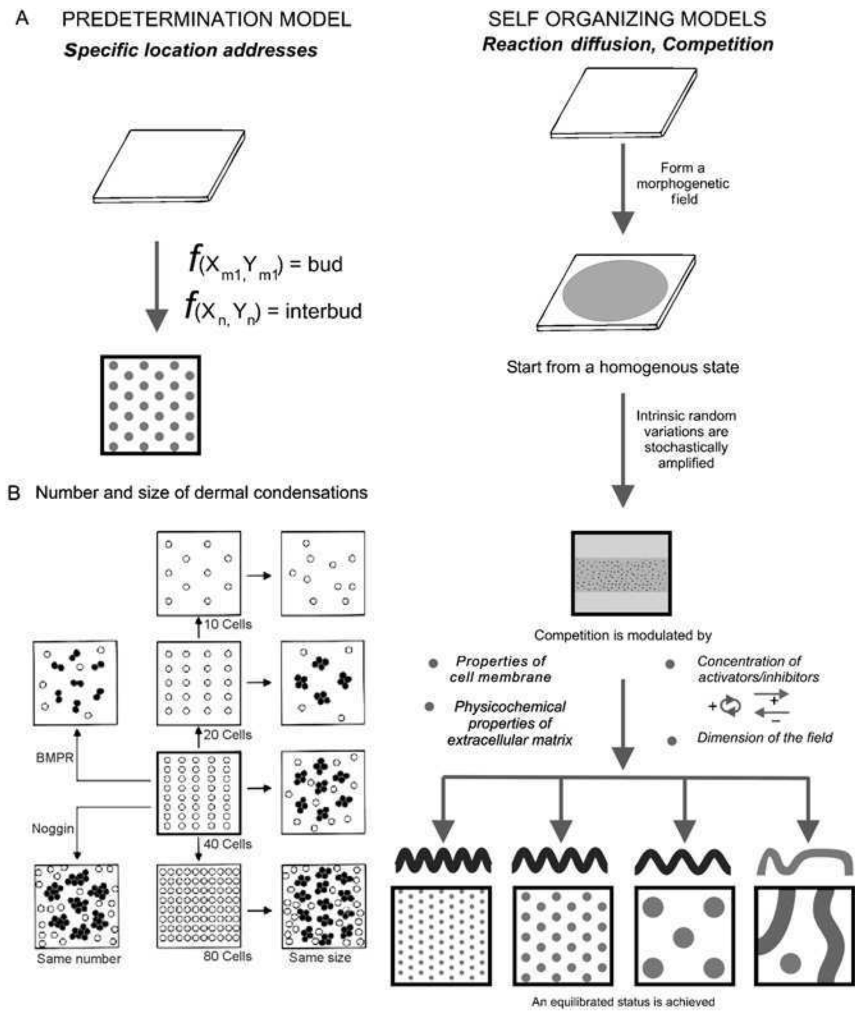


Figure 4 Pattern-forming processes that regulate the number and size of multiple primordia within a field. Panel B is from Jiang *et al.*, 1999. Part of it is from Jiang *et al.*, 1994, 2004.

(e.g., BMP, Shh) are used repetitively in different contexts in the so called *co-optive* use of signaling modules (Harris *et al.*, 2002).

With so many topological parameters involved, tuning of some of these parameters can lead to different feather shapes (Prum and Williamson, 2001), generating the diverse feather shapes in nature. The range of feather variants can be appreciated in Bartels (2003) and the interesting photos in *Extraordinary Chickens* (Green-Armytage, 2000). Schematic examples of these variants can be seen in Fig. 3B. To obtain different feather shapes, one can simply change the relative length of the rachis, barbs, and barbules. For example, in Fig. 3A, the middle one represents the fluffy contour feathers of an ostrich, the right one is a strong flight feather of an eagle, and the left represents the contour feathers on the trunk of pheasants and the natal down. The one on the right represents the scalelike feathers of a penguin in which the rachis is enlarged while barbs and barbules are miniaturized. There are also the spectacular peacock tail contour feathers, and the many unusual decorative feathers found on birds of paradise.

An interesting point is that they are all keratinocytes built into different architectures. The variations do not just exist among different avian species but can exist in the same individual. Furthermore, the epidermal stem cells can be guided by the dermal papilla to form different feather types in different skin regions (Cohen and Espinasse, 1961; our unpublished data).

B. Topobiology of Multiprimordium Organs

Some organs are made of multiple primordia. Each primordium can be considered as one organ, but they work together as a functional unit. This can be seen often in integument organs such as teeth, hairs, feathers, etc. All teeth have to work together to serve the function of breaking up food. Feathers in a tract also have to work together. A single feather does not permit flight, but together multiple pennaceous feathers can connect to form a feather vane, as discussed earlier. While cells differentiate, the topology (i.e., the number, shape, size, and arrangement of individual primordium) is crucial for the way that particular organs work and provides a new level of functional integration and variation.

Feathers are laid out in exquisite patterns on the surface of the chicken embryo. These regular patterns have inspired scientists to think about how such regular patterns arise (Held, 1992). In general, one category of model considers that the fates of cells are predetermined by their position, whether the molecular coordinates exist in the form of specific enhancer sequences or as a morphogen gradient (Fig. 4). The other category considers the major driving force is based on physicochemical phenomena. The reaction–diffusion mechanism has been used to describe periodic patterning in

inanimate objects and in living systems (Gierer and Meinhardt, 1972; Jung *et al.*, 1998; Moore *et al.*, 1998; Nagorcka and Mooney, 1985; Turing, 1952). In reaction–diffusion, random fluctuations in molecular expression become amplified to form peaks and valleys. These, however, are unstable. The peaks and valleys were later postulated to be maintained and propagated through chemical interactions or mechanical forces. Meinhardt and Gierer (1974, 2000) proposed that some molecules distributed by a reaction–diffusion mechanism might stimulate the production of the periodic structures (activators) while some suppress their synthesis (inhibitors) through autocatalysis and cross-catalysis. Activators also have the ability to further stimulate the production of activators and induce the production of inhibitors. Based on these models and our experimental results (Jiang *et al.*, 1999, 2004; Jung *et al.*, 1998), we propose a model for feather pattern formation. It consists of the following events. (1) Competent cells without specific identity are distributed in the field and move randomly. (2) Extracellular activators and inhibitors governed by a reaction–diffusion mechanism diffuse in the field. (3) Cells respond to activators and inhibitors stochastically and the results are manifested in changes of cell adhesion. (4) Cell cluster formations (dermal condensations) are reversible initially, then become committed once a threshold is reached. (5) The pattern reached is the result of competitive equilibrium. If the system is reset without changing any parameter, the pattern with similar topology will reappear, but it will not be identical to the original pattern.

If feather patterns are predetermined, scrambling the cells should not change their fates. The feather reconstitution model (Jiang *et al.*, 1999) offered an opportunity to test this, because it allowed us to recombine a fixed-sized epithelium with different numbers of mesenchymal cells. When increasing numbers of mesenchymal cells were used, we could expect either the same number of primordia with increased size or the same size of primordia with increased numbers of primordia (Fig. 4B). Experimental results show that for mesenchymal cells derived from the same region, the feather primordia were always the same size. When mesenchymal cell density was below the threshold, no primordia formed. At lower mesenchymal cell density, primordia appeared in random positions, not as aborted rows of a hexagonal lattice. As more cells were added, the number of primordia increased until they reached a maximal packing density, and feathers appeared to be arranged in a hexagonal pattern. However, this hexagonal pattern is a result of maximal packaging, not a consequence of preset molecular codes or positional values.

Thus, the feather precursor cells at this stage are truly stem cells; they can become either bud or interbud cells. The size, number, and spacing of feather primordia can be regulated by altering the properties of cells or the microenvironment (Jiang *et al.*, 1999; Shen *et al.*, 2004). To help patients,

dermatologists can implant hair follicles one by one into the alopecic scalp. We can foresee if all these parameters can be set right, the delivered stem cells should be able to self-organize into multiple hair follicles as they do during embryonic morphogenesis.

C. Evolution

During the morphological transformation from reptiles to birds, new challenges were imposed on early birds to reengineer themselves from a tetrapod form mainly living on the land to a smaller bipedal animal with wings to live in the sky. The Jehol Biota spreading in northern China is unique because it contains unique features and many plants and animals are preserved in outstanding condition (Zhou *et al.*, 2003). It is particularly valuable for the analysis of the evolution of birds because birds evolved from reptiles during this period (Chatterjee, 1997; Chiappe, 1995; Feduccia, 1999). Early research suggested that feathers evolved from an elongation of scales enlisted for protection. It was then subdivided over time to form pennaceous and then plumulaceous feather types (Regal, 1975) (Fig. 5, Model 1). Thus, the order of formation is scales → elongated scales → the vanelike scale plates → partial pennaceous vanes with an rachis like central axis → bilaterally symmetric feathers → plumulaceous barbs → radially symmetric downy feathers (also see Wu *et al.*, 2004b). From the developmental and molecular studies, Prum (1999), Prum and Brush (2002), and us (Chuong *et al.*, 2000; Yu *et al.*, 2002) propose that the order of formation is buds → follicle → cylindrical feather filaments → splitting to form radially symmetrically arranged barbs → radially symmetric downy feathers with plumulaceous barbules. By topologically changing the slanting angles of barb ridge organization, a rachis is created and the other lineage can lead to bilaterally symmetric plumulaceous feathers → bilaterally symmetric pennaceous vanes → bilaterally asymmetric vanes (Fig. 5, Model 2). This is also the order observed in development. In a broad sense of ontogeny repeating phylogeny, this probably occurred in evolution too. Indeed, a series of fossils were discovered representing intermediate forms of feathers or featherlike appendages from the Jehol Biota of China.

Furthermore, considering the topology of epithelium and mesenchyme, the scale is different from feathers (Chuong *et al.*, 2003; Prum, 1999) (Fig. 6). The scale dermis remains in the adult, and both anterior and posterior sides of scales are equivalent to the suprabasal side of the epidermis (Fig. 5, Model 1a). In contrast, in the developing feather follicles, the cylindrical feather filament surrounds the mesenchymal pulp with the basement membrane facing inside. Upon maturation, apoptosis of the pulp epithelium and shedding of the feather sheath allows the feathers to open. Thus, the anterior and

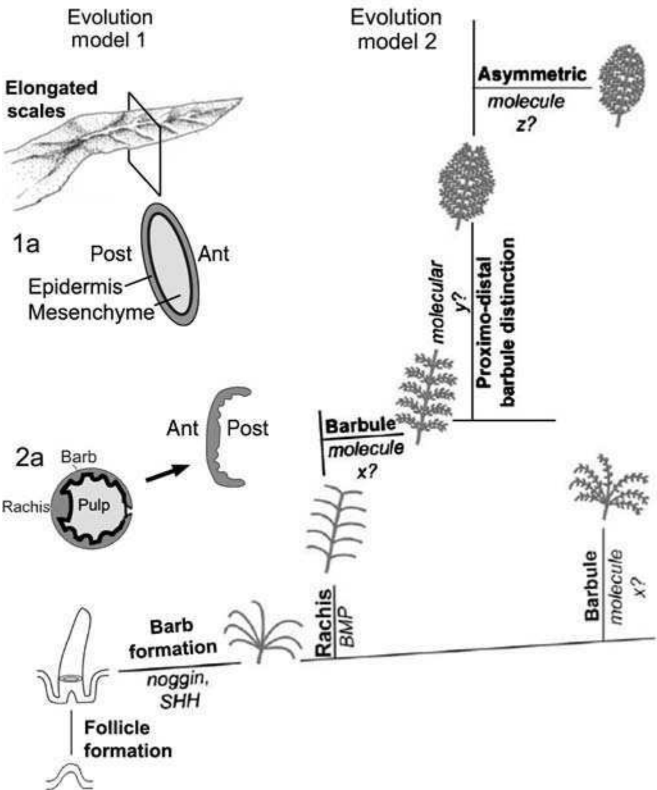


Figure 5 Models on feather evolution. Model 1 proposes elongated scales as the origin of the feather (modified from Regal., 1975). Model 2 proposes that a series of novel topobiological transformation events, as evolution novelties, transform epidermal buds into complex feathers. Panels 1a and 2a are cross-sections.

posterior side of the feather vane originally faces the suprabasal and basal layer, respectively (Chang *et al.*, 2003) (Fig. 5, Model 1). An elongated scale may show branches and may be called a "non-avian feather" (Jones *et al.*, 2000) but is not an avian feather.

From these results, a set of criteria have been developed to define the true avian feathers (Chuong *et al.*, 2003). It includes (1) possessing actively proliferating cells in the proximal follicle for a proximodistal growth mode; (2) forming hierarchical branches of rachis, barbs, and barbules, with barbs that can be bilaterally or radially symmetric, formed by differential cell death; (3) having a follicle structure, with a mesenchyme core during development; (4) when this matures, it consists of epithelia without a mesenchyme core with two sides of the vane facing the previous basal and suprabasal

layers, respectively; and (5) having epithelial stem cells and the dermal papilla in the follicle, which maintains the ability to molt and regenerate.

Work in molecular biology laboratories has allowed us to start to identify molecular pathways involved in each of these processes (Harris *et al.*, 2002; Yu *et al.*, 2004) (Fig. 6). We have developed a novel feather plucking/regeneration model to misexpress genes in the regenerating feather stem cells (Yu *et al.*, 2002). This allows us to gauge the contribution of each molecular pathway. We showed that BMP promotes rachis formation while *noggin* promotes barb branch formation. Shh is important to set up the spacing between barbs (Chang *et al.*, 2004). Harris *et al.* (2002) also showed that BMP2 and Shh mediate barb ridge formation and have developed an activator/inhibition model to explain the branch patterning (Harris *et al.*, 2005).

Recently, we identified feather stem cells and found they assume a ring configuration in the collar region. Interestingly, the ring is horizontally placed in radial symmetric downy but tilted anterior-posteriorly (A-P) in bilaterally symmetric flight feathers (Yue *et al.*, 2005). Furthermore, an A-P Wnt 3a gradient was identified, and flattening out the Wnt gradient experimentally caused bilaterally symmetric feathers to become radially symmetric (Yue *et al.*, 2006). These results provide supports for the hypothesis that diverse feather forms can be generated by topobiological modulation of stem cells, rather than specific molecular blueprints. Putting previous works together (Prum, 1999; Chuong *et al.*, 2000), we can summarize Evo-Devo of feathers as the following. First, the formation of feather follicles made stem cells and growth zone cells shift proximally to a protected environment and also allowed continuous growth and molting. Second, the feather filament branch became barbs, forming downys which are efficient in thermal regulation. Third, topological alterations of stem cell configuration allowed the formation of rachis and bilateral symmetry. Fourth, asymmetric barbules formed that interweaved barbs into a vane, enabling the birds to develop flight. Thus, a series of topobiological transformation events opened the entire sky for the Aves class. In a way, the sky niche is the best ‘‘patent award’’ given to birds for their successful evolutionary novelties.

V. Beak Morphogenesis

The recruitment of forelimbs as wings allowed a newly found mobility resulting from flight and opened vast ecomorphological possibilities. However, this came at a cost because animals now needed to develop a new feeding mechanism without the use of arms. This exerted selection pressures on the evolving structure of the face; a strong, lightweight, and effective feeding apparatus had to evolve. Furthermore, the beak had to show an ability to evolve through adaptive radiation to different ecoenvironments.

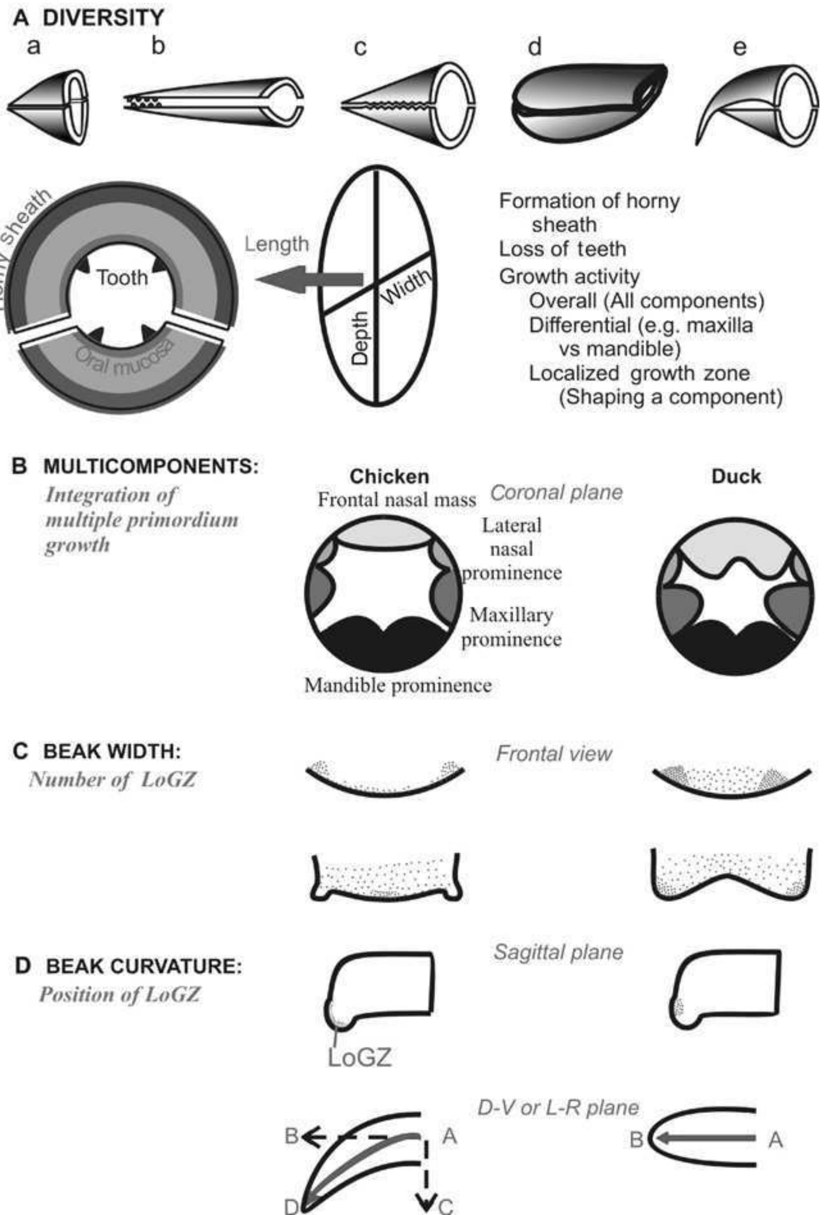


Figure 6 Molecular shaping of the beak. (A) Diverse beak shapes and the basic design of beaks. By positioning localized growth zone in different numbers and positions, the beak can become different shapes.

The results are the amazing transformation of the snout into a large range of beak topologies adapted to different ecological niches (Zweers *et al.*, 1997). At the global scale, it involves a reptile snout–bird beak transformation. At the finer scale, it involves the fine-tuning of Galapagos finches that inspired Darwin's Evolution Theory (Grant, 1986). At the developmental level, how are the different shapes of beaks produced (Fig. 6A)?

A. Development

The embryonic chick face is composed of multiple facial prominences (reviewed by Francis-West, *et al.*, 1998, 2003; Helms and Schneider, 2003) (Fig. 6B). Mesenchymal processes covered by epithelium surround the developing mouth. These prominences grow out together to form the face. The upper beak is formed from the frontal nasal mass (FNM) and MXP on the side. Lateral nasal masses have only smaller contributions and are not emphasized here. The lower beak is derived from the paired mandibular prominences (MDPs), which contain the two Meckel's cartilages. Cellular fate tracing with DiI labeling illustrates that cell populations centered around the nasal pits, the midline of the paired MDPs, and at sites of fusion contribute most to the overall expansion (McGonnell *et al.*, 1998). These data suggest that there are specific localized growth zones in these originally nearly round prominences. When the beak forms, FNM and MDPs assume an elongated shape, while MXPs remain short and ball-like. These developing facial prominences change shape substantially in developing stages, leading to the formation of primary and secondary palates. Therefore, the final shape and size of each prominence is the combination of the diffuse random growth and the directed localized growth in that prominence. Growth and morphogenesis of the prominences must be tightly coordinated to obtain the final distinct configuration of the face.

Experiments show that the identity of facial prominences are specified early in the neural crest stage (Couly *et al.*, 2002; Noden, 1983) and are coordinated by signaling molecules (Francis-West *et al.*, 2003). An elegant experiment by transplanting duck crest into quail embryos (forming duail) and quail crest into duck embryos (quack) shows the beak morphology is in accord to the origin of the cephalic neural crest (Schneider and Helms, 2003). The identity of an MXP can be respecified to an FNM by a combination of noggin and retinoic acid (Lee *et al.*, 2001). BMP, fibroblast growth factor (FGF), Shh, and Hox are involved in the formation of these prominences (Ashique *et al.*, 2002a,b; Barlow and Francis-West, 1997; Creuzet *et al.*, 2002; Helms and Schneider, 2003; Hu *et al.*, 2003; Hu and Helms, 1999; Richman *et al.*, 1997; Wilke *et al.*, 1997). An epithelial region in the FNM with juxtaposed FGF8/Shh was shown to induce beak outgrowth (Hu *et al.*,

2003). Indeed FGF8/Shh were shown to induce cranial chondrogenesis *in vitro* and *in vivo* (Abzhanov and Tabin, 2004).

Although the facial morphology is determined by the crest cells (Schneider and Helms, 2003), we are interested in how chicken and duck faces develop differently in the late stages of morphogenesis. We showed that there are localized mesenchymal cell proliferative zones (LoGZ) in the FNM. In both chickens and ducks, there were two LoGZ at lateral FNM at (chicken H&H) stage 26. They converged into one in the chicken but remained as two in the duck. We showed that this region is enriched with BMP4 and further showed that BMP4 is involved in mediating LoGZ activity (Wu *et al.*, 2004a) (Fig. 6C). Independently, Dr. Tabin's group pursued Galapagos Island finch beaks directly. Using cDNA library subtraction, they also found the main candidate for beak diversity is BMP4. They went on to use chickens to show that BMP4 is functionally involved (Abzhanov *et al.*, 2004). The concept is that a special activity may not be based on the presence or absence of a signaling molecule. Rather, the configuration of signaling molecule expressing cell clusters is important. This is further demonstrated in the cleft primary palate chicken mutant in which the abnormality is due to the failure of FGF8 to become restrictively expressed, not the absence or mutation of FGF8 (MacDonald *et al.*, 2004). Therefore, BMP is likely to be the major mediator of beak growth, while other morphoregulatory molecules can act on the BMP pathway and in this way adjust its activity and, therefore, the shape of the beak. How the messages in the chicken or duck neural crest cells are translated into the topological differences of localized growth zones in the FNM remains to be investigated.

B. Topology of Multicomponent Organs

One unique aspect of the beak is that it represents a paradigm of "complex morphogenesis" in which an organ is made from multiple components, in contrast to "simple morphogenesis" in which the whole organ is sculpted from one primordium. Comparing the limb bud with facial morphogenesis, the limb bud is a paradigm of "simple morphogenesis." Developmental biologists have learned a lot of the molecular mechanisms of limb morphogenesis in the last decade (Capdevila and Izpisua Belmonte, 2001; Dudley and Tabin, 2000; Niswander, 2003; Tickle, 2003). Through careful analyses of many laboratories, we now learned how molecular pathways (FGF, Shh, Hox, Wnt, etc.) are involved in apical ectodermal ridge (AER), zone of polarizing activity (ZPA), and dorsal-ventral patterning that work together to shape the limb from a single primordium.

In contrast, the beak is made from the coordinated growth of multiple facial prominences. We try to define the following three categories of growth

activities during beak morphogenesis: (1) Concerted "overall growth activities" are responsible for the global expansion of the face, (2) "diffuse growth zone," the dispersed mesenchymal growth in each prominence contributes to different dimensions of the face, (3) the "localized growth zone" (LoGZ), which focuses on the temporospatial growth activities within individual prominences, molding specific shapes out of one prominence (Fig. 6). There appears to be a global overall growth activity in all facial prominences, and yet each facial prominence has its distinct localized growth zone. Some facial prominences have multiple LoGZs. Thus, for the beak of each bird, a unique facial configuration emerges from the undulating landscape of global growth activities with peaks and valleys fine-tuned by LoGZs and localized apoptotic zones.

Complex morphogenesis offers more opportunities to generate morphological diversity (Fig. 6A), but the complex process is also prone to errors, as seen in the high incidences of cleft palate/lips due to lack of coordination of cellular events (MacDonald *et al.*, 2004). We can speculate a giant beak as seen in the Toucan may be produced when the "overall growth activity" is high. By increasing the "diffuse growth activity" in the maxilla or mandible alone, asymmetrically bigger upper/lower beaks may be generated, as seen in parrots and pelicans. By adjusting the configurations of "LoGZs," flat beaks like those in ducks or vertical beaks like those seen in the seagulls may be produced. By positioning the LoGZ in a horizontal or oblique angle, beaks may grow straight as in the duck or curved as in the eagle. By sustaining the activity of a focused LoGZ, a long sharp beak as seen in the crane can be produced. The molecular bases of these interesting beak designs remain to be investigated.

C. Evolution

How do we define an "avian beak?" An avian beak requires the formation of a horny sheath, loss of teeth, and the modification of the maxilla and mandibles into unique shapes. From the reptile to bird, the toothed jaws were gradually transformed into beaks. Indeed, in reptiles, beaks were seen in *Psittacosaurus* (a beaked dinosaur) and even in today's turtles. During the evolution of the beak, the trend is the gradual reduction and eventual loss of teeth, coupled with the formation of the horny sheath by thickened epidermal differentiation (Feduccia, 1999). Some Mesozoic birds existed representing intermediate stages (Fig. 7).

Archaeopteryx had uniform reptilian teeth in both its upper and its lower jaw. Longirostravis (125 million years ago) had a very long and slender rostrum and signs of the presence of a horny sheath (Hou *et al.*, 2004). Ten small and conical-shaped teeth are arranged in pairs and preserved in

Animal	Classification	Head	Horny sheath	Teeth	Beak shaping	Reference
Sinosauropteryx	Theropod		–	+	Reptile-like	Chen *et al.*, 1998
Archaeopteryx	Archaeornithes (most primitive birds)		–	+	Reptile-like	Feduccia, 1999
Longirostriornis	Archaeornithes		+	+ (number reduced)	Elongated	Hou *et al.*, 2004.
Confuciusornis	Archaeornithes		+	–	Conical	Hou *et al.*, 1995
Gallus (Chicken)	Ornithurae (modern birds)		+	–	Conical	
Anas (Duck)	Ornithurae		+	–	Wide and flat	
Psittacines (Parrot)	Ornithurae		+	–	Big and curved upper beak	
Geospiza (Finch)	Ornithurae		+	–	Various shapes	Grant, 1986

Figure 7 Evolution of beaks. Different shapes of snout from reptiles, Mesozoic birds, and today's birds are represented.

the distal snout. As this is the earliest wading bird, the preservation of teeth in the anterior snout may have facilitated securing its prey. The arboreal Confuciusornis is likely to be among the early birds that have formed a real beak with a complete loss of teeth in both of the upper and the lower beak (Hou *et al.*, 1996). The diversity of beaks is shaped by diet and reflects adaptive radiation (Feduccia, 1999; Lucas and Stettenheim, 1972). Darwin's finches in the Galapagos Islands are derived from a common ancestor and have evolved different sizes and shapes of beaks. The variation is subject to natural selection and environmental changes (Grant, 1986). In other birds, seed eaters such as chickens, quails, and pigeons have conical beaks. Ducks have soft, leathery, and flattened beaks for filtering food from the mud and water (Lucas and Stettenheim, 1972). Hawks have curved upper beaks for raptorial tearing.

To summarize beak morphogenesis, we have learned that beaks are made of the same differentiation materials (bone, horny sheath), but they form diverse shapes in different species. The different shapes are based on different

topobiologically arranged cellular activities. By varying the proportion of the width, depth, and length, different dimensions, and their angles, the architecture of the beak is laid down. By modulating the number, size, and positions of LoGZs, the beak can be further shaped (Fig. 6). We have learned that BMP pathway members, agonists and antagonists, may work as molecular candidates mediating the formation of a spectrum of morphologies for selection. Our experimental study with chickens showed that we can indeed produce beaks phenocopying those in nature by modulating different developmental steps (Wu *et al.*, 2004a). It is likely that the diversification of beak shapes was achieved by modulating prototypical molecular modules during the evolution of the beak. We now know that the BMP4 pathway is involved and can start by studying molecules related to this pathway.

VI. Topobiology of Other Organs

Similar topobiological events take place in other organs as well. To continue the discussion of the integument, we have applied this concept to analyze the effect of tilting the balance of BMP activity on the formation of various integument organs. We used K14 to drive the expression of noggin in the basal layer of the integument. Ectodermal organ formation shares induction, morphogenesis, differentiation, and regenerative phases. Because K14-induced expression of noggin suppressed BMP activity at different stages of integument organ formation, the consequences are different (Plikus *et al.*, 2004). When BMP is suppressed at the induction stage, the number of hair follicles increases. When BMP is suppressed at the morphogenesis stage, the size of the genitals is increased. Suppressing BMP also causes conversion of sweat glands and meibomian glands into hairs. Moderate reduction of BMP activity in claw morphogenesis causes splitting of claw growth zone into multiple small growth zones and hence multiple nail plates. Complete suppression converts claw regions into epidermis. In addition, molar teeth change cusp shapes and sizes (Plikus *et al.*, 2005). Thus, the change of phenotypes can be appreciated in the context of morphoregulation (Edelman, 1988b). Since the changes of number, size, and shape here are relatively minor, we also asked whether these should be considered true pathology (pathology only if it is nonfunctional) or if they may be phenotypic variations that may be useful someday if the environment changes (Plikus *et al.*, 2004). Topobiological analyses also have been used to analyze the change of cell adhesion during hair follicle morphogenesis (Muller-Rover *et al.*, 1999). Invagination of hair placodes also has been successfully explained by increased expression of noggin and β-catenin (Jamora *et al.*, 2003).

Among the visceral organs, the liver has a unique morphology with an asymmetric apex growing out from the liver lobes. We showed that initially there are diffuse growth activities and that BrdU-labeled cells are distributed all over the developing liver primordia in embryonic day 4 (E4) chicken embryos. At E7, proliferating cells become limited to the outermost layer of the developing liver primordia. The duration of this stage determines the overall size of the liver. At E8, the proliferative zones become localized to the apex and a few regions in the outer margin to allow expansion in those specific regions, producing unique liver shapes (Suksaweang *et al.*, 2004) (Fig. 8). β-Catenin mediates growth zone activity, and different liver morphologies are produced when β-catenin is overexpressed or suppressed (Suksaweang *et al.*, 2004). As the liver primordia become mature toward the center, the hepatoblasts start to organize into a unique hepatic architecture, from layers to clusters, acini configuration, and hepatic cords.

In the lung, formation of branches increases the surface area for air sac/endothelial contact and is essential for its function. Branching occurs at the growing tips. Retinoic acid induces the expression of FGF10 (Desai *et al.*, 2004). Epithelial Shh helps to restrict the expression of mesenchymal FGF10. FGF10 defects lead to tracheobronchial truncations. BMP4 further restricts FGF10 expression along the proximal-distal axis (Affolter *et al.*, 2003; Bellusci *et al.*, 1996). Through a feedback loop, FGF10 increases BMP4 expression levels. It is thought that Shh present at the growing tip down-regulates FGF10 in the center, effectively splitting the field and inducing lung branching. Transforming growth factor-β1 (TGF-β1) is also expressed at branch sites and proximal regions of the branches. It promotes the deposition of extracellular matrix molecules and is believed to inhibit branching.

In the mammary gland, branching is largely dependent on matrix metalloproteinases. Branching occurs at the terminal end-buds but also can occur along the side of the ducts by budding. As in the lung, branching of mouse mammary glands 1, 2, 3, and 5 appears to be dependent upon FGF10 expression (Mailleux *et al.*, 2002). The epithelial ducts are surrounded by myoepithelial cells and a dense stroma containing connective tissues and fibroblasts. Hormonal stimulation during estrous cycles leads to expanded growth and branching followed by regression during involution. Levels of Msx1 and possibly Msx2 drop during lactation and return during involution (Phippard *et al.*, 1996), showing their possible regulation by hormones.

In contrast, branching of feather barbs occurs via a different mechanism. The feather filament cylinder forms first, and then cells between barb ridges go through apoptosis to sculpt out the spaces (Chang *et al.*, 2004) (Fig. 3). This is similar to digit separation in the limb. Thus, similar organ morphologies may be achieved through totally different topobiological mechanisms.

It should also be pointed out that in some organs, the end points of organogenesis can be chemical reactions (e.g., liver) or electric activities

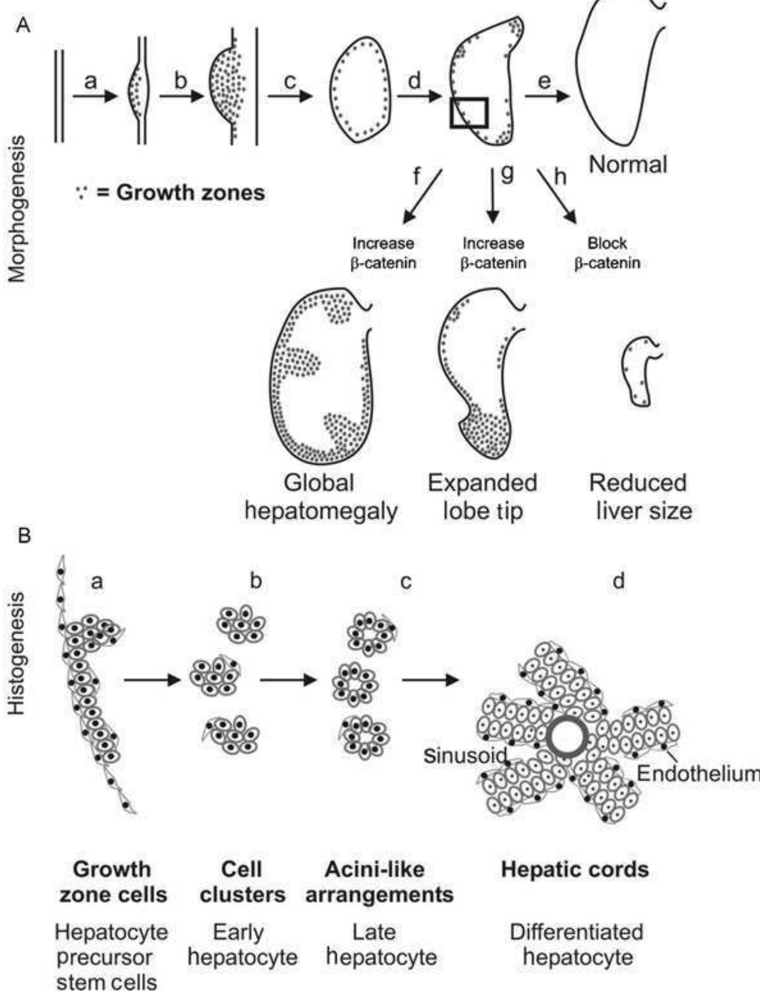


Figure 8 Topobiological events in liver development. Stippled region: growth zone. The growth zone is changed from diffuse, to outer layer of developing primordia, to selected region of growing liver (from Suksaweang *et al.*, 2004).

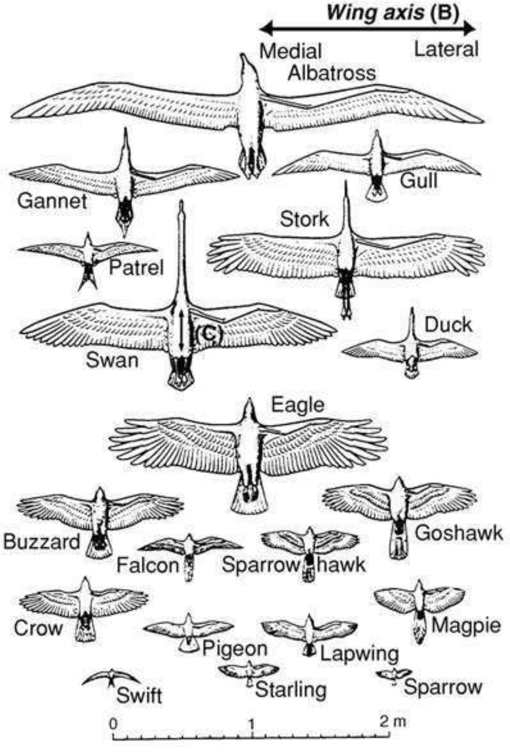
(e.g., brain). The topobiology concept was originally applied to brain function (Edelman, 1988a). For these, the topological arrangements are also important because they provide the essential anatomical constraints for cell groups to interact and connect. We chose integument organs because the consequence is obvious and helpful for us to decipher the topobiological principles.

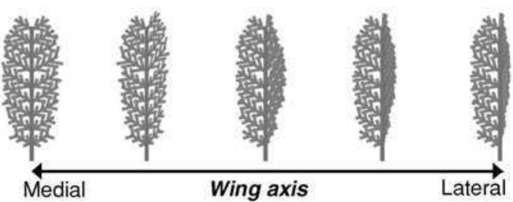
VII. Topographic Specificity of Multiprimordia Organs

The multiplicity of certain ectodermal organs allows regional specification for diverse functions. The regional specificity can be considered at different hierarchical levels: (1) across the whole body surface, (2) across an appendage field, and (3) within one appendage organ.

The *regional specificity across the body surface* can be appreciated clearly in humans. In our facial skin, eyebrows, lips, palms, soles, nails, etc., different skin regions have fundamentally similar skin and skin appendage structures, but with topological variations for specialized functions (Chuong, 1998; Chuong *et al.*, 2002). The mouse appears furry and the regional differences do not appear to be as apparent. We can see clear differences in vibrissae, tail skin, footpads, claws (Plikus *et al.*, 2004; Plikus *et al.*, 2006; Sundberg, 1994). Though not very obvious, there are also dorsal-ventral differences (Candille *et al.*, 2004) and primary/secondary hair differences (Botchkarev *et al.*, 2002). In other mammals, these differences can be exaggerated and different hair follicles respond differently to seasonal changes. The regional specificity is very clear in birds. There are downy feathers, contour feathers, flight feathers, tail feathers, scales, claws, beaks, combs, etc. (Lucas and Stettenheim, 1972) (Fig. 9A). Every small region is specialized to make the best use of the skin. Yet these regional diversifications are the results of evolutionary novelty and natural selection. The ''proto-feathered'' dinosaurs, *Sinornithosaurus*, about 120 million years ago had similar ''proto-feathers'' all over the body without much appreciable regional specificity (Chen *et al.*, 1998; Xu *et al.*, 2001).

What are the molecular bases of these regional specificities? Classic tissue recombination experiments implied that the determinants are in the mesenchyme, if the epidermal cells maintain ''stem cell'' properties, competent in its multipotentiality and not irreversibly committed (Fig. 10, the bidirectional arrows in the epidermal cell column). Differences in dorsal and ventral dermal progenitors have been defined (Fliniaux *et al.*, 2004a), yet the molecular basis remains elusive. We have earlier observed Hox proteins expressed differently in different body regions of the developing feather buds and have suggested the Hox code hypothesis for the regional specificity of the skin (Chuong *et al.*, 1990). The different Hox expression patterns observed in human dermal fibroblasts derived from different body regions are consistent with this hypothesis (Chang *et al.*, 2002). The involvement of Tbx15 in the dorsal/ventral mouse coat is another exciting advance (Candille *et al.*, 2004). With genome availability and microarray technology, a topographical mapping of skin regions over the body surface will provide insight to help zoom in on the molecular basis of regional specificity. This control of the specificity is also critical to regulating the type of ectodermal organs one may obtain from stem cells (Fig. 10).





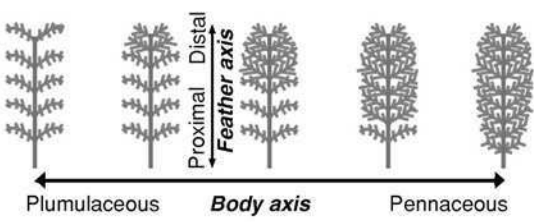


Figure 9 Topographic regional specificities. (A) Regional specificity across the body surface is illustrated in different species of birds. They also fly in different modes with different wing shapes. (B) Regional specificity across an appendage field is best demonstrated by the array of primary remiges on the wing. (C) Intraappendage regional specificity is best demonstrated by contour feathers on the trunk. (Panel A is from Feduccia, 1999.)

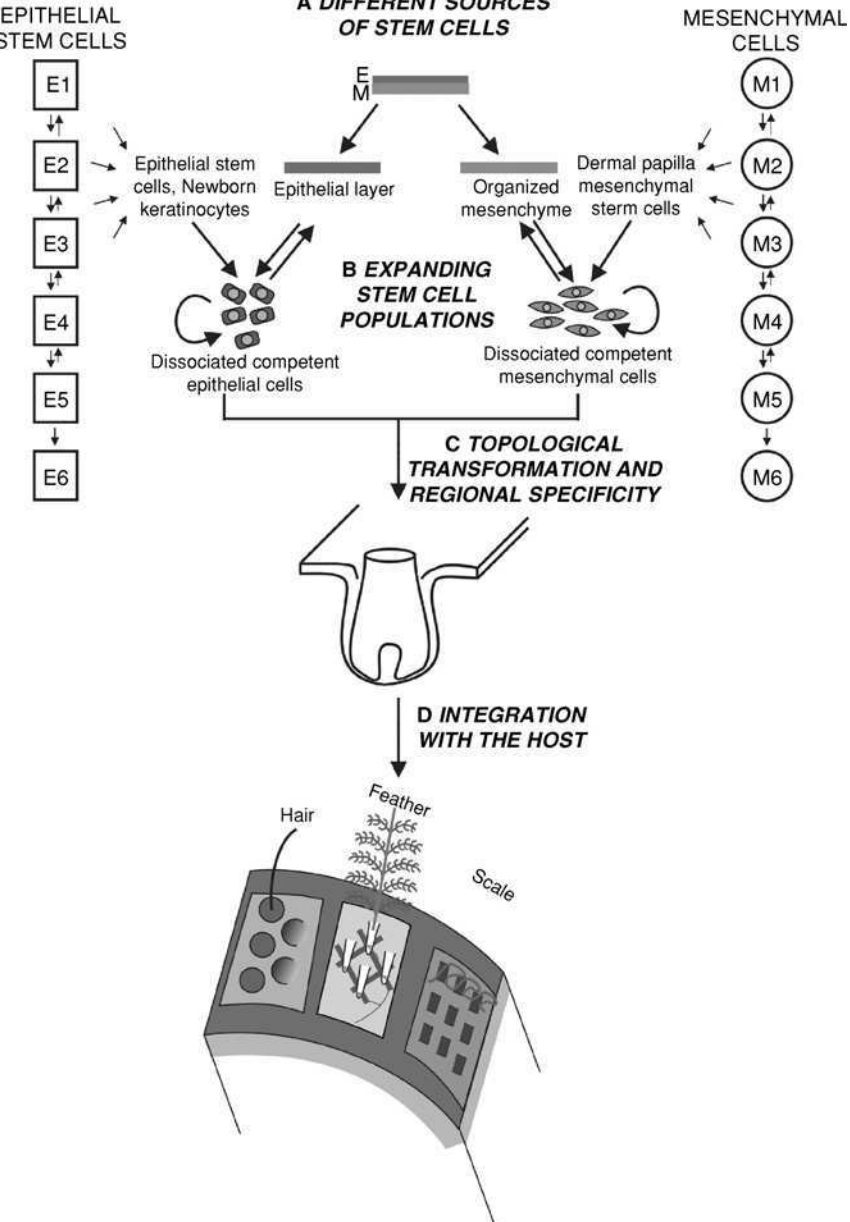


Figure 10 Epithelial and mesenchymal cell recombination to generate new organs. The four issues in stem cell biology (A–D) are highlighted, and ectodermal organ formation is used for illustration. (A) Sources of stem cells can be from embryonic stem cells, adult stem cells, or somatic nucleus transplantation. Cells on the lateral columns indicate different stages during

In the bird, the body regions are established by dividing the body surface into different fields or tracts during development (Dhouailly *et al.*, 2004; Jiang *et al.*, 2004; Sengel, 1976). By having multiple feathers in one feather tract, another level of *topobiological specificity is possible across the feather tract*. There are different modes of flight based on different wing shapes (Feduccia, 1999) (Fig. 9A). The shape of the wing is made by the combination of the 20–30 flight feathers (remiges). Their relative lengths form the contour of the wing. Because the length of the feather shaft is a function of the duration of the growth phase (like the anagen phase of the hair cycle), the shape of the wing becomes the spatial layout of multiple flight feathers from the medial to the lateral regions of the wing (in which the midline of the body is the medial; one can also consider this as the proximal-distal axis of the limb bud), each with its own temporal cycle regulation, but together add up to form a distinct shape of the wing. Another level of complexity is imposed on top of this array of flight feathers: the medial/lateral bilateral asymmetry (again, here we use the body axis, not the feather rachis as the reference point). According to aerodynamic engineering, the feather in the most lateral wing is most bilateral asymmetric, with the lateral vane much narrower than the medial vane (Fig. 9B). This feature was used to judge whether a fossil bird is a good flyer (Feduccia, 1999). Birds that give up flight (e.g., on isolated islands) soon lose this level of asymmetry over several generations. Two aspects of interest pertain to the molecular basis of this process: one is by what topobiological mechanism lateral/medial asymmetry is produced from the bilaterally symmetric flight feathers; the other is how this molecular activity can be displayed in a graduated medial-lateral fashion.

In mammals, the differences of hair follicles within a domain are not clearcut. There are hair whorls on human scalp, which indicate a relationship among hair follicles during development (Plikus and Chuong, 2004). In frizzled-6–null mice, there are also disoriented hair follicles leading to variable whorls and tufts, suggesting a role of frizzled-6 in hair follicle orientations (Guo *et al.*, 2004). In some adult mutant mice, clear and shifting alopecic domains are observed on the surface of mouse body (Ma *et al.*,

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progression of stem cells. The downward arrows mean differentiation. The reverse arrows mean de-differentiation, which eventually disappears, meaning that cells are fully committed and their fates cannot be reversed anymore. (B) Cell populations are expanded with the idea that the stem cell properties, self-renewal and pluripotentiality, will not be lost or deregulated to become tumors. (C) Competent epithelial stem cells and regional specific mesenchymal cells are combined in the proper environment to generate organs. If everything is set right, they can self-organize in normal morphogenesis. In tissue engineering, we need to learn these principles and the regulation of specificity. (D) A single feather follicle would not be too useful if it is not connected to other parts of the body and coordinated as part of the system (Fig. 1, 10D). Ectodermal organs have to be connected with other systems via angiogenesis, myogenesis, and neurogenesis to be fully integrated with the organism.

2003; Suzuki *et al.*, 2003). However, these are due to problems of cyclic alopecia in which hair filaments are dislodged from the follicle at a specific time of hair cycle (Ma *et al.*, 2003). These are problems of hair cycling (Sten and Paus, 2001), not regional specificity. Tooth fields have similar types of topological modulations to generate different sizes and shapes of incisors, canines, and molars (Jernvall and Thesleff, 2000; Plikus *et al.*, 2005). These specializations do not exist in most reptiles or Mesozoic birds (Hou *et al.*, 2003, 2004).

There are further *regional variations within a single appendage organ*. For example, the graded topological modulation of feathers can be seen in contour feathers. In the trunk, the functions of each feather are further divided along the proximal-distal axis. The distal region is made of pennaceous barbs (for contouring or communication), and the proximal domain is made of plumulaceous barbs (for thermal insulation) (Fig. 9C). Furthermore, the ratio of plumulaceous versus pennaceous regions changes gradually among adjacent feathers in the same feather tract, reflecting the need of different body parts to make the best balance between preserving body temperatures and streamlining body shapes. Such regional specific modulation of organ morphology makes the most effective use of every keratinocyte. In other organs, this type of sophisticated modification among cell groups may also exist (e.g., different brain regions, cortex laminations, neuronal circuits) (Edelman, 1988b). Yet the feather is a good model because it lays out all topological arrangements clearly: The barbule represents a row of 10–20 keratinocytes connected in a head-to-tail fashion.

VIII. Integration of Stem Cells and Organs to Reach the Level of System Biology

We now come back to the stem cell issue. In the beginning, we emphasized that there are four types of issues that stem cell biology have to solve to achieve the goal of regenerative medicine (Fig. 10A–D). Using the skin as an example, progress has led to new understanding in the interfollicular epidermal stem cells (Watt, 2002) and hair bulge stem cells (Morris *et al.*, 2004; Tumbar *et al.*, 2004) (Fig. 10A). We have learned the importance of the niche in regulating stem cell homeostasis (Fig. 10B). We also have learned that, to a limit, these epidermal progenitors can be dedifferentiated and transdifferentiated. Indeed it is most interesting to observe the conversion of part of the scales into feathers, amniotic membranes into feathers and hairs (Fliniaux *et al.*, 2004b), sweat glands/meibomian glands into hairs (Plikus *et al.*, 2004), and even adult cornea epithelium into hairs (Pearton *et al.*, 2005). Research in genetic and epigenetic regulation should shed more light on the control of cellular phenotypes.

Suppose this research bears fruit and we are able to form an organ; how then do we direct it to become part of the host and function in a useful manner? One ideal situation is to have competent epidermal stem cells and induce mesenchymal cells incubated in a microenvironment with proper chemical signaling and topological setting, and then let them self-organize (Fig. 10). This type of approach was pioneered in Moscona's cell aggregate approaches to form feathers, retina, lentoid, livers, etc. (e.g., Garber *et al.*, 1968; Vardimon *et al.*, 1988). In these aggregates, a quite remarkable degree of histogenesis and chemical differentiation was achieved in the 3D aggregates, yet their topological relationships are random. We constrained dissociated feather mesenchymal cells into a 2D configuration and put on top a competent epithelia sheet. With this topological arrangement, we were able to obtain a reconstituted skin with an array of evenly spaced and oriented feather follicles (Jiang *et al.*, 1999; our unpublished data). In the mouse, Lichti *et al.* (1995) mixed a population of competent epidermal and dermal cells in a chamber that was transplanted on a nude mouse. The cells sort out to form hair follicles. This procedure was simplified and improved to generate exogenous hair organs that are supported by the host can can cycle (Zheng *et al.*, 2005). This is very good progress, albeit the hair filaments point to the center of the aggregates, forming a cyst. We still have to make the topobiological events right before stem cell engineering can be applied to humans.

Stem cell biology is just at its dawn. There are many critical issues to be solved and knowledge from multiple disciplines to be integrated. Assuming we could have access to sources of stem cells and know, to a certain level, how to induce their differentiations someday soon, here we focus on the issue of guiding stem cells into organs. We identify the fundamental and practical importance of topobiological events in building the architecture or an organ. We turn to Nature to learn how she solves the simple to complex designs of ectodermal organs. Using feather and beak morphogenesis to decipher the principles, we observe a succession of topobiological transformation events, taking the epithelia from a flat sheet to more and more complex structures.

Some of these topobiological principles are likely to be in operation in other organogeneses as well. These processes are important in development and morphological evolution and have to be considered in tissue engineering. There may be a long way to go, but the process is exciting and the best is yet to come.

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6

Fur Seal Adaptations to Lactation: Insights into Mammary Gland Function

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The fur seal (*Arctocephalus* spp. and *Callorhinus* spp., members of the pinniped family) is a mammal with the unusual capability to modulate its lactation cycle by turning milk production on and off without the typical mammalian regression and involution of the mammary gland. Lactation has evolved from constraints arising from the spatial and temporal separation of infant nursing and maternal foraging as the mother gives birth and feeds the pup on land while acquisition of nutrients for milk production occurs at sea. The lactation cycle begins with the female fur seal undergoing a perinatal fast of approximately 1 wk, after which time she departs the breeding colony to forage at sea. For the remainder of the long lactation period (116–540 days), the mother alternates between short periods ashore suckling the young with longer periods of up to 4 wk of foraging at sea. Milk production

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continues while foraging at sea, but at less than 20% the rate of production on land. Fur seals produce one of the richest milk reported, with a very high lipid content contributing up to 85% of total energy. This feature serves as an adaptation to the young's need to produce an insulating blubber layer against heat loss and to serve as an energy store when the mother is away foraging at sea. This atypical pattern of lactation means mothers have long periods with no suckling stimulus and can transfer high-energy milk rapidly while on land to minimize time away from foraging grounds. The absence of suckling stimulus and milk removal during foraging does not result in the onset of involution with associated apoptosis of mammary secretory cells and a subsequent progressive breakdown of the cellular structure of the mammary gland. The mechanisms controlling lactation in the fur seal mammary gland have been investigated using molecular and cellular techniques. These findings have shed light on the processes by which the unique features of lactation in the fur seal are regulated. © 2006, Elsevier Inc.

I. Introduction

A. Pinniped Lactation Strategies

1. Evolution and Classification of Pinnipeds

During the evolutionary history of the Class Mammalia, several lineages secondarily invaded the aquatic environment. The fossil record suggests that this transition occurred several times and would have involved morphological, physiological, and behavioral modifications from the terrestrial ancestors (Barnes *et al.*, 1985; Demere *et al.*, 2003). The degree of these modifications is evident in the extent to which the particular lineages have adapted to the aquatic environment (Barnes *et al.*, 1985). Unlike cetaceans (whales and dolphins), which evolved a totally aquatic existence, pinnipeds (seals, sea lions, fur seals, and walrus) must still spend time on land to breed (Bonner, 1984).

The three families within the suborder Pinnipedia (order Carnivora) include: Phocidae (true seals), Odobenidae (walrus), and Otariidae (eared seals: sea lions, fur seals). It is believed that all three families evolved from a common carnivorous ancestor (bearlike or doglike) around 25 million years ago and diverged sometime during the middle Miocene (10 million years ago) (Berta *et al.*, 1989; Fordyce, 2002).

2. Maternal Investment Strategies

All pinnipeds must return to land to give birth. As marine mammals, their adaptations have reduced their agility on land and they are potentially vulnerable to terrestrial predators during the period of dependence of the

young. Seals appear to have adapted to these risks by adopting various strategies, which relate to the location of secure breeding sites (e.g., on remote islands or ice flows), social breeding assemblies, which provide protection from predators for the individuals within the group, and the duration of lactation (abbreviated or extended) (Bonner, 1984). The differing lengths of lactation periods between species offer advantages to mother and pup with respect to specific environmental conditions and resources. Pinnipeds give birth to only one pup and females rear only their own offspring. Nursing is the only postpartum investment required by the mother. Pinniped milk is considered among the most nutritious mammalian milk and is composed of 30–60% fat content and 5–15% or more protein content (Oftedal and Iverson, 1995). This allows for a short and rapid period of energy transfer from mother to pup. There are different approaches to lactation and energy transfer between the three families.

a. Odobenids (Walrus) "Aquatic Nursing" Strategy. Walruses (*Odobenus rosmarus*), which predominantly forage in sheltered shallow coastal regions, use an aquatic nursing strategy whereby a pup accompanies its mother and suckles at sea upon leaving the natal site (Oftedal *et al.*, 1987). Female Odobenids build up their blubber stores before giving birth on ice flows. After a short fasting period on land following birthing, they return to sea to forage with their pup (Oftedal *et al.*, 1987). Lactation continues for 2–3 yr, but at approximately 5 mo of age, walrus pups are foraging and nursing (Bonner, 1984). Walrus milk is relatively low in fat compared to other pinniped species, and consequently, pups grow at a slower rate and investment during lactation is greatly extended.

b. Phocids (True Seals) "Fasting" Strategy. The majority of phocid seal species are large bodied and have adopted a strategy of reducing the lactation period, using a "fasting strategy" (Trillmich, 1996). These animals use body reserves of nutrients stored during previous foraging trips for continuous milk production (Costa, 1991; Trillmich, 1996). Generally, the larger bodied phocid seals fast on land during the majority of the lactation period, which depending on the species lasts 4–42 days until the pup is weaned (Bowen *et al.*, 1985; Thomas and Master, 1983). Most of the smaller species (<100 kg) will feed only during the later part of lactation because maternal stores are insufficient to cover the entire lactation period (Boness and Bowen, 1996; Bowen *et al.*, 1992; Oftedal *et al.*, 1987). In order for sufficient nutrients and energy to be transferred, a rich milk is secreted. This can result in average daily weight gains throughout lactation of about 23% of neonatal weight. Phocid pups grow rapidly because of the exceptionally high levels of fat and energy in their mother's milk; however, growth is primarily in fat stores and there is little increase in lean body mass. Southern elephant seal

(*Mirounga leonina*) pups are born weighing approximately 20 kg and wean at 21 days old, weighing 120 kg, and the maternal mass change during lactation is around a 200-kg loss. Elephant seal pups fast after weaning and can spend 23 mo *de novo* synthesizing lean tissue through the mobilization of existing protein stores and developing the diving skills necessary for survival at sea (Thorson and Boeuf, 1994). This energy-rich and rapid transfer approach allows for a very abrupt period of investment by the mother.

c. Otariidae (Fur Seal, Sea Lion) "Foraging" Strategy. The small-bodied otariid seals demonstrate one of the more extreme adaptations to lactation, utilizing a "foraging cycle" or "attendance strategy" (Trillmich, 1996), whereby females accumulate moderate blubber stores, come ashore to give birth, and following a postpartum fast of approximately 1 wk, they return to sea leaving their pup on land while they forage (Trillmich, 1996). Otariids are much smaller animals than phocids and, as such, are required to replenish their body stores in order to continue lactating. The remainder of lactation (4 to more than 12 mo depending on species) is characterized by the alternation of periods of several days ashore suckling the young and extended periods at sea (Bonner, 1984; Oftedal *et al.*, 1987; Trillmich, 1996) (Fig. 1). Foraging trips are variable and depend highly on the season, age of pup, and resource availability (Beauplet *et al.*, 2003; David and Rand, 1986; Gentry and Holt, 1986). After body reserves have been adequately resupplied, females return to land to nurse their pup. Time spent by females on land nursing are usually short, lasting only 1–3 days before the mother's body reserves have again been depleted, requiring her to return to sea and feed.

Within the Otariidae, variation in foraging trip duration is observed among species. Sea lions tend to have short foraging trips (1–4 days), whereas fur seals generally have longer period of maternal absence (Bonner, 1984). Most

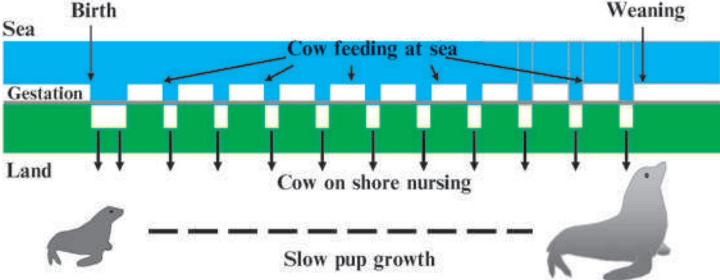

Figure 1 The "foraging" lactation strategy of the fur seal. The pregnant female arrives at shore to give birth and remains with the pup for approximately 1 wk. For the remainder of lactation, females alternate trips to sea, with short trips ashore to suckle their pup (adapted from Bonner, 1984).

fur seal species have maternal foraging trip durations of between 4 and 8 days (Trillmich and Lechner, 1986), but some extreme exceptions do occur. For example, the sub-Antarctic fur seal (*Arctocephalus tropicalis*) is known to make some of the longest foraging trips of all fur seals (Beauplet *et al.*, 2003; Georges and Guinet, 2000), with trips lasting an average of 23 days (Georges and Guinet, 2000) to 28 days (Kirkman *et al.*, 2002) in late lactation. Foraging trips of up to 25 days have also been reported for the Juan Fernandez fur seal (*Arctocephalus philippii* [Francis *et al.*, 1998]) and Cape fur seal (*Arctocephalus pusillus pusillus* [Gamel *et al.*, 2005]). In a number of species, the duration of maternal foraging trips has also been shown to increase as pups get older (David and Rand, 1986; Gentry and Holt, 1986).

During time at sea the mammary gland does not involute but produces less milk compared to that of the onshore lactating female (Arnould and Boyd, 1995b). For example, milk production in Antarctic fur seals (*Arctocephalus gazella*) has been shown to continue while the female is foraging at sea, but at only one-fifth the rate of production on land (Arnould and Boyd, 1995b). Milk protein gene expression is also reduced during the foraging trip. Expression of the β-casein, αS2-casein, and β-lactoglobulin gene transcripts has been found to be downregulated in the mammary gland of the foraging Cape fur seal (Sharp *et al.*, 2005), correlating with decreased milk volume. The fur seal mammary gland, therefore, undergoes repeated cycling of high milk production while the pup is suckling and low milk production in the absence of suckling. This type of lactation is unique among mammals because of the extreme duration of intersuckling bouts and the rapid rate of energy transfer while suckling and results in a slower growth rate than phocids (Oftedal *et al.*, 1987).

3. Reproduction

With the exception of one species, all pinnipeds are annual seasonal breeders. Estrus and mating are synchronized by the timing of parturition and may begin as early as 4 days postpartum in Hooded seals (*Cystophora cristata*). Estrus and mating for otariids usually occur within 2 wk after giving birth and for phocids occurs at the end of lactation. For this family estrus and mating occur within 6 wk of giving birth, except for Mediterranean monk seals (*Monachus monachus*), in which it is greater than 2 mo. For all pinnipeds, mating is followed by an embryonic diapause (delayed implantation), which lasts from 2.0 to 4.5 mo for most pinniped species (Atkinson, 1997) and up to 14 mo in the Australian sea lion (*Neophoca cinerea*) (Gales *et al.*, 1997). Embryonic diapause occurs in many other mammalian species (Lopes *et al.*, 2004). In pinnipeds, blastocyst reactivation and implantation is activated by photoperiod and a corresponding increase in estradiol-17β and

progesterone (Boyd, 1991; Daniel, 1974, 1981; Temte, 1985). Placental gestation (implantation to parturition) follows for 7–8 mo, allowing for annual breeding. For the Australian sea lion, the longer interbirth interval causes the breeding season to be nonannual and aseasonal. Factors responsible for this prolonged gestation are unknown, but it is believed that it has evolved as an adaptation to patchy and low prey abundance (Gales and Costa, 1997). These breeding cycles occur every 17.6 mo, creating a seasonal drift with each breeding.

II. Fur Seal Mammary Gland

A. Morphology of the Lactating Mammary Gland during Suckling and Foraging

The mammary gland of the fur seal, though not visible externally, is voluminous and follows the contours of the body, spreading over the lower thorax, abdomen, and sides of the body (King, 1983; Scheffer, 1962). Fur seals have four ventrally located teats on the abdominal wall, which turn outward under the influence of a suckling pup (King, 1983). Studies of the ductal system of the phocid mammary gland demonstrate that it resembles those of other eutherians with adaptations to the compressed nature of the gland, seen as radiations of large canals running parallel to the skin surface, draining into a small gland cistern under the teat (Tedman and Bryden, 1981). There is little comparable information for the fur seal mammary gland.

Like that of all mammals, the fur seal mammary gland undergoes an intense period of lobulo-alveolar development during pregnancy, and at lactation, the gland is almost entirely composed of secretory epithelium (Fig. 2A and B). Myoepithelial cells encase the luminal epithelial cells in the ducts and are in contact with a laminin and collagen IV–rich basement membrane. Surrounding the ductal network and accounting for more than 80% of the mammary volume is a highly compartmentalized stroma. During lactation on shore, the alveoli are engorged with milk containing a large amount of lipid (Fig. 2B). During the mother's extended foraging trip, the alveoli appear less distended, epithelial cells surrounding the alveoli appear more cuboidal, and the lipid component is decreased within the milk (Fig. 2C). The reduction of milk protein secretion correlates with decreased milk volume (Arnould and Boyd, 1995b) and histological data suggest that reduction of milk volume must occur quickly, as the mammary gland does not appear full or engorged while foraging.

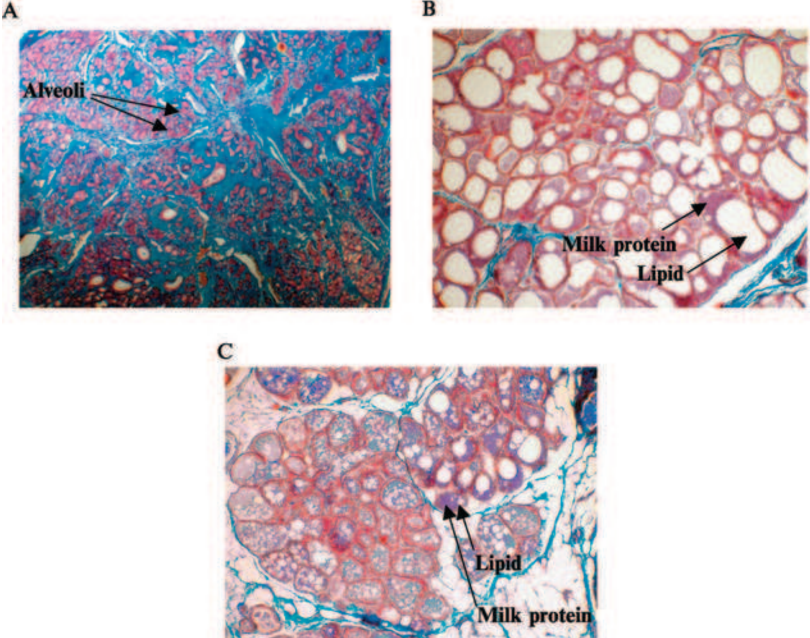

Figure 2 Mammary gland morphology. Histological sections of the mammary gland from (A) pregnant (placental gestation), (B) lactating while nursing on shore (in embryonic diapause), and (C) lactating while foraging at sea (in embryonic diapause) Cape fur seals. Sections are stained with hematoxylin and eosin. Immature alveoli in the pregnant gland are indicated; lipid (white) and milk protein (pink) are indicated in the onshore and offshore mammary glands. Magnification ×100.

In most mammals during natural weaning, as alveoli fill with milk due to cessation of suckling, the mammary epithelial cells start to downregulate milk protein gene expression and the epithelium regresses and enters involution (Li *et al.*, 1997). This process is characterized by apoptotic cell loss and gland remodeling in readiness for a subsequent pregnancy (Lund *et al.*, 1996; Metcalfe *et al.*, 1999; Strange *et al.*, 1992; Walker *et al.*, 1989). Studies of these processes has revealed that involution occurs in two distinct phases (Lund *et al.*, 1996). When experimentally induced by forced weaning, the mouse mammary gland initiates the first phase of involution within a few hours of pup removal and is initially morphologically characterized by an accumulation of milk in the alveoli and limited apoptosis of epithelial cells (Strange *et al.*, 1992; Walker *et al.*, 1989). This can be reversed up to 1.5 days after weaning when epithelial cell apoptosis starts to dominate the process (Jaggi *et al.*, 1996). Three to five days after weaning, the second phase of involution is initiated. It is characterized morphologically by the degradation

of the basement membrane, a collapse of alveoli, infiltration of macrophages, and restructuring of the gland to a virgin-like state. During the second phase, apoptosis of epithelial cells continues until 50–80% of the epithelial cells have been cleared from the gland (Walker *et al.*, 1989).

The extent of apoptosis associated with cessation of suckling in the mammary gland of the foraging Cape fur seal has been analyzed using Apotag (Chemicon) and has been shown to be barely detectable even after a probable extended period where there is no sucking stimulus. Therefore, it appears that the gland does not regress in the absence of suckling (Sharp, Hornby, and Nicholas, unpublished data).

III. Composition of Fur Seal Milk

Mammals produce milk that differs greatly in composition among taxa and throughout the course of lactation (Jenness, 1974; Jenness and Sloan, 1970). The major milk constituents are water, fat, protein, and carbohydrates, although the amounts and proportions differ between species, with fat varying from less than 10g/kg (lemur) to more than 400g/kg (fur seal) (Table I).

The main function of milk fat and carbohydrates is to supply energy to the suckling young, while protein is a source of amino acids for incorporation into protein synthesized by the young (Jenness, 1986). Fatty acids in milk are also important in development of the neonatal retina and brain (Regester *et al.*, 1997). The production of high-fat-content milks has evolved several times, both to offset heat loss by the young in arctic and aquatic species and to avoid excessive water loss by female desert species (Jenness, 1986; Jenness and Sloan, 1970). Furthermore, species that nurse infrequently also have a tendency to produce high-fat milks to increase milk energy (Jenness, 1986).

Evolution has clearly played a role in altering the substrate demands of mammary glands by alteration of milk composition. When coupled with the substantial taxonomic variation in lactation intensity, as reflected in litter size, peak energy output, and lactation length, mammary glands pose greatly different demands on the body in different mammals.

A. Fat

The milk produced by all marine mammals is extremely rich in fat, meeting the requirements for rapid growth and sustainability within extreme environments of offspring (Oftedal *et al.*, 1987; Riedman, 1990). Fur seal milk has one of the highest lipid content of all milks, with up to 42% lipid during peak

Table I Milk Composition at Peak Lactation of Various Mammals

	Fat (%)	Protein (%)	Carbohydrate (%)	Ash (%)	Total solids	Reference
Antelope	1.3	6.9	4	1.3	25.2	Dill *et al.*, 1972
Bat	15.7	8.5	3.9		27	Kunz *et al.*, 1995
Bear, Polar	31	10.2	0.5	1.2	42.9	Cook *et al.*, 1970
Camel	3.2	2.8	4.1	0.8	10.9	Elamin and Wilcox, 1992
Cat	9.0	11.1	4.0	—	25.4	Adkins *et al.*, 1997
Cow, Jersey	5.5	3.9	4.9	0.7	15.0	Cerbulis and Farrell, 1975
Deer	19.7	10.4	2.6	1.4	34.1	Bruggemann *et al.*, 1973
Dog	8.3	9.5	3.7	1.2	20.7	Luick *et al.*, 1960; Oftedal, 1984
Dolphin	14.1	10.4	5.9	—	30.4	Pervaiz and Brew, 1986
Elephant	15.1	4.9	3.4	0.76	26.9	Peters *et al.*, 1972
Goat	3.5	3.1	4.6	0.79	12	Lauer *et al.*, 1969
Guinea Pig	3.9	8.1	3	0.82	15.8	Nelson *et al.*, 1951
Horse	1.6	2.7	6.1	0.51	11	Oftedal *et al.*, 1983
Human	5.0	0.9	7.2	0.2	12.6	Jenness, 1979
Kangaroo	10.3	7	2		26	Muths, 1996
Mink	8	7	6.9	0.7	22.6	Fink *et al.*, 2001
Monkey, owl	2.1	2.1	7.2	0.36	14.5	Cicmanec and Campbell, 1977
Monkey, lemur	0.9	1.2	8.4		9.9	Tilden and Oftedal, 1997
Opossum	6.1	9.2	3.2	1.6	24.5	Green *et al.*, 1996
Pig	8.2	5.8	4.8	0.63	19.9	Heidebrecht *et al.*, 1951
Rabbit	14.4	15.8	2.7	2.1	35.2	Anderson *et al.*, 1975
Reindeer	22.5	10.3	2.5	1.4	36.7	Luick *et al.*, 1974
Seal, Australian fur	42.1	10.4	—	0.7	67.7	Arnould and Hindell, 1999
Seal, grey	50	12	0.7	0.8	67.7	Baker, 1990
Seal, northern fur	45.6		—		61	Dosako *et al.*, 1983
Sheep	5.3	5.5	4.6	0.9	16.3	Williams *et al.*, 1976
Tasmanian bettong	18	10			45	Rose *et al.*, 2003
Wallaby	24	13	0.8		40	Green *et al.*, 1980, 1983
Whale	34.8	13.6	1.8	1.6	51.2	Lauer and Baker, 1969

lactation, which is considerably more than that of terrestrial mammals (Arnould and Hindell, 1999; Baker, 1990; Bonner, 1984; Jenness and Sloan, 1970; Oftedal, 1984). For the fur seal, lipid-rich energy-dense milk is an important adaptation to repeated fasting periods of pups while females are away at sea, during which time a large proportion of the pup's nutrition is used for maintenance rather than growth (Trillmich and Lechner, 1986).

Milk lipid content in fur seals has also been shown to be related to maternal body condition (Arnould and Boyd, 1995b; Arnould and Hindell, 1999). Consequently, as milk production while the female is on land is derived from stored body reserves, milk lipid content has been shown to decrease during maternal attendance periods (Arnould and Boyd, 1995b; Costa and Gentry, 1986; Georges *et al.*, 2001; Goldsworthy and Crowley, 1999).

The fatty acid profiles of milk lipid have been determined for a number of seal species, revealing changes associated with either diet or stage of fasting (Georges *et al.*, 2001; Ochoa-Acuña *et al.*, 1999; Trillmich *et al.*, 1988; Van Horn and Baker, 1971). In most mammals, a proportion of lipids present in milk are synthesized by the mammary gland (Stein and Stein, 1967); however, fatty acid profiles of pinniped milk suggest that most fatty acids are transferred from prey components and maternal stores to milk triglycerides with minimal modification of the fatty acid composition (Georges *et al.*, 2001; Iverson *et al.*, 1995, 1997). Studies using gene expression analysis of 10,000 expressed sequence tags (ESTs) from a Cape fur seal mammary gland cDNA library have failed to detect expression of genes involved in lipid synthesis (Sharp, Lefevre, and Nicholas, unpublished data), providing further evidence that the lipid content of fur seal milk results from the transfer of fatty acids from the mother's blood directly into the milk. With the suggestion that *de novo* fatty acid synthesis is minimal in the mammary gland (Oftedal, 1993), combined with the knowledge of the virtual absence of milk sugars, comes the realization that the fur seal mammary gland largely synthesizes milk protein. This makes the fur seal an ideal model for the study of milk protein synthesis.

B. Protein

The protein concentration of fur seal milk ranges from 10 to 22% and is among the highest of any mammal (Arnould and Boyd, 1995b; Arnould and Hindell, 1999; Davis *et al.*, 1995; Georges *et al.*, 2001; Goldsworthy and Crowley, 1999; Trillmich and Lechner, 1986). The proportions of casein to whey protein vary considerably between species (Anderson *et al.*, 1985), including the pinnipeds (Ashworth *et al.*, 1966; Trillmich *et al.*, 1988), and may reflect differences in diet between species. While the main function of milk proteins may be to provide amino acids, these proteins have a diverse array of additional functions, including the transport of minerals such as

calcium and phosphate by caseins, and trace elements such as iron by lactoferrin, supply of immunoglobulins, and further undefined roles (e.g., β-lactoglobulin).

1. β-Lactoglobulin

Although the biological function of β-lactoglobulin remains to be determined, its presence in the milk of several mammals has suggested a nutritional role, supported also by its ability to interact with a great variety of hydrophobic ligands, such as retinol (Cho *et al.*, 1994; Dufour and Haertle, 1991; Dufour *et al.*, 1990; Futterman and Heller, 1972; Lange *et al.*, 1998; Narayan and Berliner, 1997), fatty acids, and triglycerides (Dufour *et al.*, 1990; Frapin *et al.*, 1993; Narayan and Berliner, 1997; Qin *et al.*, 1998; Wang *et al.*, 1997; Wu *et al.*, 1999; Zsila *et al.*, 2002). This leads to speculation that β-lactoglobulins may enhance vitamin A uptake in suckling offspring (Kushibiki *et al.*, 2001; Said *et al.*, 1989). The β-lactoglobulin–retinol complex is known to bind receptors in the intestine of suckling calves (Perez *et al.*, 1989) and may be involved in lipid metabolism, possibly by enhancing lipase activity (Perez *et al.*, 1992). This proposed function of β-lactoglobulin would be significant with regard to fur seal metabolism, given that milk lipid accounts for up to 42% or more of the total composition of fur seal milk (Arnould and Hindell, 1999; Oftedal, 1993).

Cane *et al.* (2005) reported 10 distinct whey bands from the milk of the Australian fur seal (*Arctocephalus pusillus doriferus*) following electrophoretic separation, and N-terminal sequencing showed the majority of the proteins to be β-lactoglobulin, similar to that reported for ruminant, canine, and dolphin milks (Bell *et al.*, 1981; Hambling *et al.*, 1992; Kushibiki *et al.*, 2001). At least three isoforms of this protein appear to be secreted in the milk. In addition, screening of cDNA libraries from Cape and sub-Antarctic fur seal lactating on shore and offshore mammary glands revealed the presence of at least two β-lactoglobulin variants (Cane *et al.*, 2005; Sharp *et al.*, 2005) with significant homology to canine and feline β-LG amino acid sequences (Halliday *et al.*, 1990; Pervaiz and Brew, 1986).

2. Lysozyme

Lysozyme is a known antimicrobial agent in milk (Maga *et al.*, 1998; Priyadarshini and Kansal, 2002). This protein is of unusually high concentration in the milks of carnivorous species (Brew and Grobler, 1992), although the biological significance of this is not clear. Lysozyme concentrations are known to increase in bovine and human milk during mastitis (Harmon *et al.*, 1976; Prentice *et al.*, 1985), with a protective function against mammary infection and inflammation (Semba *et al.*, 1999). Cloning

of the sub-Antarctic fur seal lysozyme gene (Cane, 2005) shows that the gene appears to have evolutionary conservation with canine milk lysozyme, sharing similar residues known for calcium-binding activity in other species (Koshiba *et al.*, 2001). Calcium-binding lysozymes have previously been determined to be major components of the whey fraction of milk of Carnivores and Perissodactyls (Brew and Grobler, 1992). The results of Pervaiz and Brew (unpublished) and Cane (2005) show that calcium-binding lysozymes are also common to two superfamilies of the Pinnipedia, the phocids and otariids.

3. Caseins

Caseins account for 40–75% of the total protein in the milks of fur seals (Ashworth *et al.*, 1966; Trillmich *et al.*, 1988). The Cape fur seal expresses β-casein (Sharp *et al.*, 2005), αS1-casein (Cane, 2005), αS2A-casein and αS2B-casein (Sharp *et al.*, unpublished) genes during lactation. Recent studies (Cane *et al.*, 2005) have shown the casein micelle of the Australian fur seal is composed of five caseins, whereas Ronayne de Ferrer *et al.* (1996) observed four casein bands after sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE) of milk proteins from the Southern elephant seal. Their study also described five distinct whey proteins, but only serum albumin was identified according to its mobility in the gel (Ronayne de Ferrer *et al.*, 1996).

In the lactating mammary gland of Cape fur seals foraging at sea, the most abundant casein transcript was observed to be αS1-casein, followed by αS2-, β-, and κ-caseins (Sharp, Lefevre, and Nicholas unpublished data), which follows the trend for casein expression in most species where α- and β-caseins are most abundantly expressed (Gaye and Houdebine, 1975; Thepot *et al.*, 1991). Human is one of the exceptions, where casein is composed mostly of the β- and κ-casein species (Hansson *et al.*, 1994).

Caseins evolve exceptionally rapidly at the amino acid level (Wolfe and Sharpe, 1993). This pattern is assumed to result from the relaxed evolutionary constraints on nutritional milk protein that lack enzymatic function. In Cape and sub-Antarctic fur seals, consistent with observations of other mammals studied, β-casein and κ-casein are more highly evolutionarily conserved with sequences of other species (Cane, 2005; Lenasi *et al.*, 2003) than the α-caseins, which are more variable. Alignments of Cape fur seal casein protein sequences have been analyzed cladistically to assess the phylogenetic placement of the fur seal relative to the major lineages. Independent cladistic analyses of the β-casein and κ-casein genes produced broadly congruent results and indicated that the fur seal is placed within the Carnivore group (Fig. 3). For both genes, the implication is that the fur seal is more closely related to the panda and dog (Carnivores) than to Ruminants

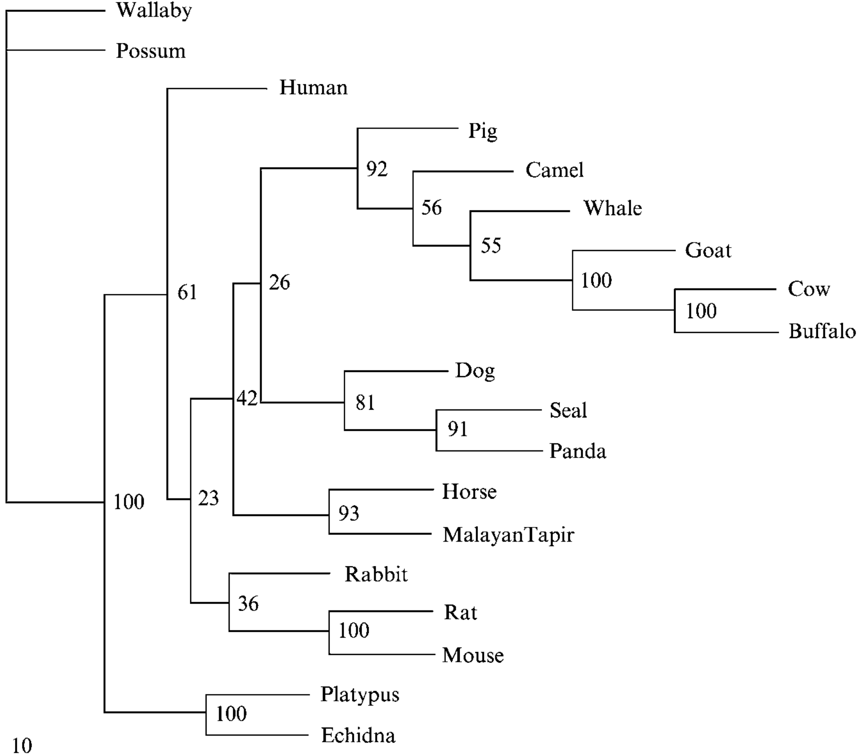

Figure 3 Phylogenetic relationship of the β-casein proteins. Lengths of the lines from the nodes indicate evolutionary distances. Bootstrap values are shown. (Acc. nos. Bovine P02666; Buffalo Q9TSI0; Camel Q9TVD0; Canine Q9N2G8; Goat P33048; Horse Q9GKK3; Human P05814; Mouse P10598; Panda P79092; Pig P39037; Possum Q9XSE4; Rabbit P09116; Rat P02665; Sheep P11839; Mayalan Tapir Q29136; Whale P79231; Platypus JA. Sharp unpublished; Echidna, JA. Sharp unpublished).

(camel, pig, tapir, goat, cow, buffalo, horse), Rodents (mouse, rat), Cetacean (whale), Lagomorph (rabbit), Monotremes (platypus, echidna), Marsupials (wallaby, possum), and Primate (human). A number of osteological features also link the fur seal with the dog (reviewed by King, 1983).

C. Carbohydrate

The milk of pinnipeds, and other marine mammals, contains little or no sugar (Oftedal, 1993; Riedman, 1990). Lactose, the predominant carbohydrate in the milk of most mammals, appears to be present in very low concentrations in the milk of phocids (Kerry and Messer, 1968; Messer *et al.*, 1988) but

completely absent in the milk of the otariids (Urashima *et al.*, 2001). In addition, α-lactalbumin, a whey protein required for lactose synthesis, appears to be entirely absent from the mammary glands of California sea lions (*Zalophus californianus*) (Johnson *et al.*, 1972) and Northern fur seals (*Callorhinus ursinus*) (Dosako *et al.*, 1983).

In most species, lactose is the major osmole of milk (Oftedal *et al.*, 1987) and its virtual absence from the milk of pinnipeds suggests that secretion of the aqueous phase of the milk is controlled by different mechanisms in these species (Oftedal *et al.*, 1987). Typically, in milk that is low in lactose and other sugars, osmolality is maintained by comparatively higher concentrations of sodium, potassium, and chloride (Oftedal, 1993). The sodium and potassium concentrations in the milk of Australian and Antarctic seals are comparatively higher (Cane *et al.*, 2005) than the concentrations in the milk of terrestrial species such as humans and cows (Green *et al.*, 1980; Nicholas and Hartmann, 1991; Schryver *et al.*, 1986). It appears that the reduction in lactose synthesis, which limits the glucose demand of the mammary gland and allows for high-fat, low-water milk (Oftedal, 1993), has evolved as a mechanism in the fur seal to produce high-energy milk with reduced volume, which is necessary for the maintenance of their adopted foraging lactation strategy.

It is interesting that although the milk of the Tammar wallaby (*Macropus eugenii*) and other animals of extreme adaptation of lactation where the milk contains less than 1% (w/v) carbohydrate in late lactation, the carbohydrate moiety is exclusively monosaccharides (Green *et al.*, 1980). It is also at this late stage of lactation that milk volume increases and significant changes in sodium and potassium concentrations occur, whereby an increase in their concentrations is evident as carbohydrate declines (Green, 1984). This is consistent with fur seal milk collected on shore throughout lactation, where carbohydrate concentrations are low, sodium and potassium levels in the milk are higher than observed for other species, and the volume of milk produced is greater than that at sea (Arnould and Boyd, 1995a). Although sodium and potassium levels in the milk are high, a major osmole in milk that regulates milk volume in the fur seal and Tammar wallaby is yet to be identified.

IV. Changes in Milk Protein in the Fur Seal Lactation Cycle

As discussed previously, the composition of milk produced by pinnipeds varies not only between the phocids and otariids, but also between species within the otariid family. However, unlike the changing milk protein composition of many terrestrial mammals, gross milk protein composition during the lactation cycle of the otariids does not appear to alter over the

length of lactation (Arnould and Boyd, 1995b; Costa and Gentry, 1986; Donohue *et al.*, 2002; Georges *et al.*, 2001). In addition, a study (Cane *et al.*, 2005) analysis of the milk of the Australian fur seal and the Antarctic fur seal throughout the lactation cycle showed no significant change in either the total milk protein or the individual proteins.

Several studies of otariid lactation have reported changes in the concentration of milk components during a suckling period ashore (Arnould and Boyd, 1995a,b; Costa and Gentry, 1986; Georges *et al.*, 2001; Goldsworthy and Crowley, 1999; Ochoa-Acuña *et al.*, 1999). Arnould and Boyd (1995b) reported a decline in both milk lipid and protein content during 1–2 day nursing periods of the Antarctic fur seal. Milk protein content declined after 16–24 hr ashore, and yet the amount of protein in the milk initially was found to be correlated with the duration of the previous foraging trip (Arnould and Boyd, 1995b). Similar declines in milk protein content for the sub-Antarctic fur seal were reported by Goldsworthy and Crowley (1999) and the Juan Fernandez fur seal (Ochoa-Acuña *et al.*, 1999) after resumption of nursing following arrival back on land.

Although the protein composition of milk does not alter over the lactation period of otariids, Arnould and Boyd (1995b) have reported that during a foraging trip at sea, milk production in Antarctic fur seals was reduced to only 19% of that observed for onshore animals. This decline in milk production has been shown to correlate with downregulation of milk protein gene expression in Cape fur seals (Fig. 4). The levels of expression of β-lactoglobulin and casein genes have been analyzed from onshore lactating, at-sea lactating (animals in embryonic diapause), and late pregnant (placental gestation and nonlactating) fur seal mammary tissue by both Northern blot (Sharp *et al.*, 2005) and reverse transcriptase–polymerase chain reaction (RT-PCR) techniques (Sharp and Nicholas, unpublished data). Both analyses indicate that these genes are expressed at low levels in the mammary gland of late pregnant (placental gestation and nonlactating) Cape fur seals. The mammary gland of fur seals lactating on shore show high levels of expression with a reduction in gene expression in the mammary gland of the at-sea fur seal.

It may be expected that lysozyme expression levels would increase in the mammary tissue and milk of female fur seals foraging at sea for extended periods, when the frequency of mammary infection is likely to be greatest (Nickerson, 1989). However, increased expression of the lysozyme gene in mammary tissue of females foraging at sea has not been observed by either Northern blot (Cane, 2005) or RT-PCR analyses (Fig. 4). Lactoferrin, another whey protein that possesses antimicrobial activity, has been observed to be transcriptionally upregulated during involution of ruminant mammary tissue where the gene product acts to prevent bacterial invasion and infection (Molenaar *et al.*, 1996). However, in Cape fur seals the lactoferrin gene is

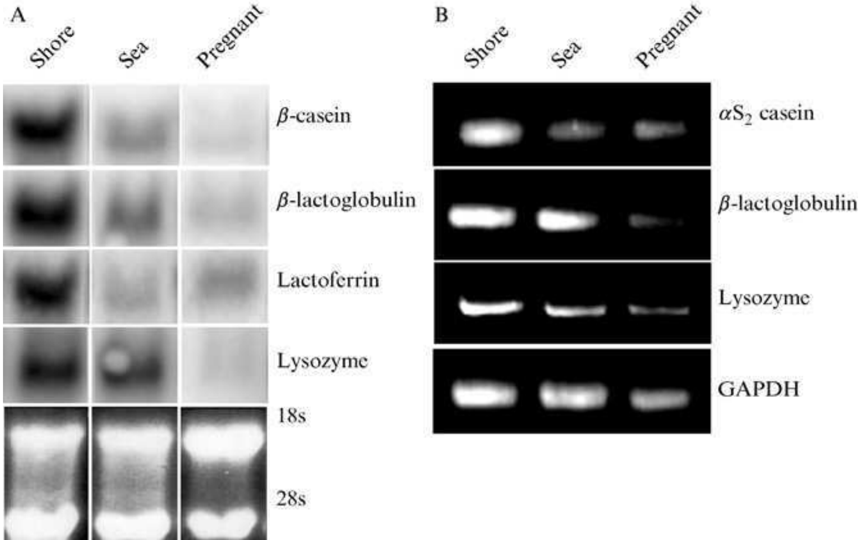

Figure 4 Expression analysis of milk protein genes. (A) Northern blot analysis of milk protein gene expression in the mammary gland of pregnant (embryonic diapause and nonlactating) and lactating on shore and lactating at sea (animals in embryonic diapause) Cape fur seals. Lower panel shows ethidium bromide–stained gel prior to Northern blot for comparison of RNA loading and integrity. The 18s and 28s RNA bands are indicated. (B) Reverse-transcriptase–polymerase chain reaction (RT-PCR) analysis showing milk protein gene expression of αS2-casein, β-lactoglobulin, and lysozyme genes in the different fur seal mammary tissues. GAPDH was used as a control gene for equal expression.

transcriptionally upregulated in the onshore lactating mammary gland and downregulated in the at-sea mammary gland (Cane, 2005) (Fig. 4A). The absence of an upregulation of both lysozyme and lactoferrin while at sea suggests that either local protection of the fur seal mammary gland may not be required at this time or an alternative mechanism is in place.

The specific factors that control lactation in the fur seal to reduce milk production and avoid entering apoptosis while foraging are unknown. However, it is clear that through the process of environmental selection, the fur seal appears to have uncoupled the mechanism of milk reduction from involution completely in order to undertake its lactation cycle.

The use of microarray technology has become instrumental in comprehensive studies of gene expression, providing valuable insights into the molecular mechanisms of biological processes (Ji *et al.*, 2004). This powerful gene expression profiling technique allows the simultaneous analysis of the expression levels of thousands of genes (Celis *et al.*, 2000). However, largely due to a lack of sequence information, this technology has been restricted primarily to a few model species and human (Ji *et al.*, 2004). The close

evolutionary relationship between the fur seals and other members of the Carnivore group (Flynn *et al.*, 2005), in particular the dog, has enabled the opportunity to utilize Affymetrix GeneChip Canine Genome Arrays. This array, representing 21,700 gene transcripts derived from sequences in cDNA libraries for 11 canine tissues and public content from GenBank, has been used to analyze gene expression profiles between mammary tissue of lactating onshore, lactating offshore (animals in embryonic diapause), and pregnant (animals in placental gestation and nonlactating) Cape fur seals. Sequence analysis of 10,000 ESTs from the offshore lactating Cape fur seal has enabled cross-species comparisons of DNA similarity to other species (Sharp, Lefevre, and Nicholas, unpublished data). High-sequence conservation between the Cape fur seal and dog, 95% similarity at the DNA level (Fig. 5), permits a significant detection rate of measurable hybridization signals between seal cDNA and the Canine microarray. With between 20 and 26% present calls detected on the chip, significant expression of mammary genes could be identified and confirmed by other methods. For example, the expression profiles of the β-casein gene were observed to decline when females were at sea foraging compared to expression levels while on shore (Fig. 6), confirming results previously observed by Northern blot and RT-PCR (Fig. 4), validating the data derived from Affymetrix arrays. Annotation of 10,000 EST sequences, representing approximately 4000 genes

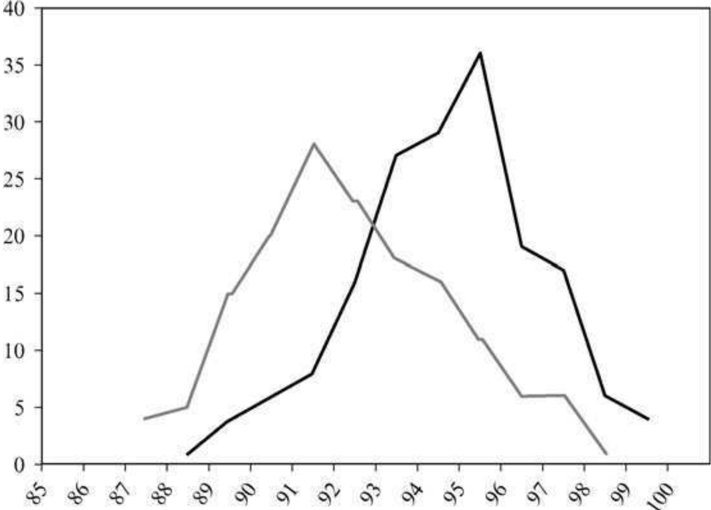

Figure 5 Distribution of similarity (percent identity) between assembled seal expressed sequence tag (EST) sequences and dog (black) or human (gray) Unigene representative sequences. Only blast alignments with a high score (>700) were selected. Gene sequence similarity peaks at 95% between the Cape fur seal and the dog and 91% between the Cape fur seal and humans.

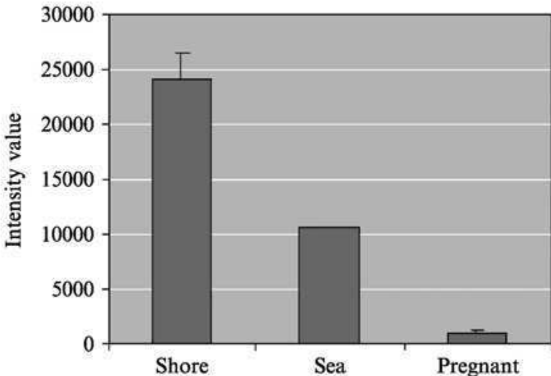

Figure 6 Expression analysis of milk protein genes. Analysis of β-casein expression using canine Affymetrix chips hybridized to complementary DNA (cDNA) probes generated from RNA from pregnant (placental gestation and nonlactating, n = 2) and lactating on shore (n = 2) and lactating at sea (n = 1) (animals in embryonic diapause) Cape fur seals. Standard errors are shown.

expressed in the lactating offshore Cape fur seal mammary gland, allows for identification of sequences detected by Affymetrix analysis (Sharp, Lefevre, and Nicholas, unpublished data). Cluster analysis of expression profiles from these data has revealed that the overall expression profile of lactating mammary gland of the foraging Cape fur seal (in embryonic diapause) is more closely related to the profile of pregnant nonlactating animals (placental gestation) than the profile obtained from onshore lactating animals (in embryonic diapause) (Fig. 7). This suggests that the interruption of lactation in foraging animals involves a major reprogramming of mammary gland expression.

V. Mechanisms that Regulate Lactation

In most mammals the mammary gland is largely controlled at two levels: endocrine control by numerous steroid and peptide hormones and autocrine control through local factors produced by secretory epithelial cells of the mammary gland.

A. Local Factors

In most mammals the consequence of reduced nursing results in local milk accumulation where putative factors have the ability to act on the mammary epithelium to elicit such processes as apoptosis and anoikis, causing mammary gland involution and remodeling. The process of apoptosis plays

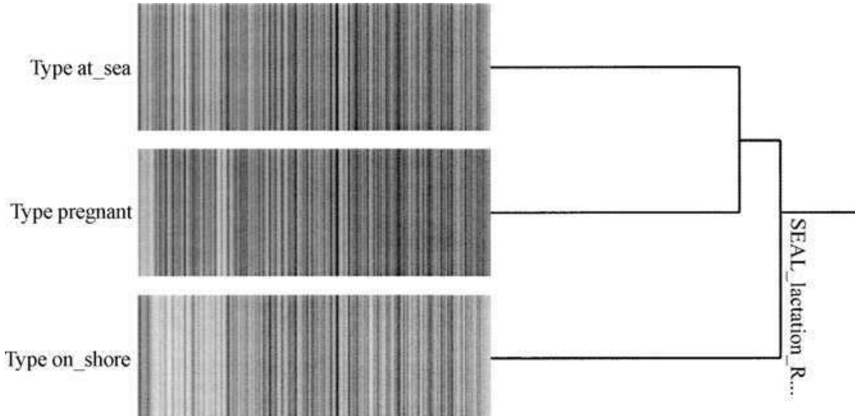

Figure 7 Cluster analysis of gene expression profiles from the pregnant, lactating while foraging, and lactating on shore mammary gland. For this analysis, we selected 1020 Cape fur seal mammary messenger RNA (mRNA) expression signals hybridized to an Affymetrix canine microarray with RNA expression more than 250 in any sample type. Hierarchical clustering was conducted using Euclidian distance. Pregnant (placental gestation and nonlactating) and onshore lactating (in embryonic diapause) data represent an average of two animals. Offshore data represent a single sample. The offshore lactating (in embryonic diapause) profile is closer to the pregnant type than to the onshore lactating profile.

an important part in the removal of the differentiated mammary epithelia during weaning-induced involution (Strange *et al.*, 1992; Walker *et al.*, 1989). It has been shown in mice that closure of a single mammary gland provokes the accumulation of milk, which results in changes in gene expression and apoptosis within the closed gland but not of the remaining glands of the same animal (Li *et al.*, 1997; Marti *et al.*, 1997). Involution occurs through a process of programmed cell death, but the molecular events that initiate or regulate apoptosis of the secretory epithelial cells remain unclear.

1. α-Lactalbumin

The α-lactalbumin protein, secreted in most mammalian milk and involved in the synthesis of lactose, has been implicated in the process of mammary gland involution (Baltzer *et al.*, 2004; Hakansson *et al.*, 1995, 1999). In particular, a multimeric, partially unfolded form of α-lactalbumin known as *HAMLET*, has been shown to induce changes typical of apoptosis in local areas in the mammary gland of lactating mice where the protein was released from plastic pellet inserts (Baltzer *et al.*, 2004). The HAMLET form has also been shown to have broad activity against cells of both human and animal origin, inducing apoptosis within 6 hr in several human and murine cell lines such as the Jurkat and L1210 leukemia cell lines, the A549 lung cancer line,

and the A498 kidney cancer line (Hakansson *et al.*, 1995; Svensson *et al.*, 2000). Low concentrations of monomeric bovine α-lactalbumin have also been shown to initiate apoptosis after a 4-day delay (Sternhagen and Allen, 2001), suggesting that the monomeric bovine α-lactalbumin may act to initiate involution after the withdrawal of suckling and accumulation of milk in the mammary gland after several days.

As discussed previously, it has been suggested that milk from otariid pinnipeds contains little or no lactose (Dosako *et al.*, 1983; Messer *et al.*, 1988; Schmidt *et al.*, 1971; Urashima *et al.*, 2001), prompting the proposal that α-lactalbumin, which is involved in lactose synthesis, may be absent in the fur seal. Analyses of milk from the Northern fur seals (Dosako *et al.*, 1983) and California sea lions (Johnson *et al.*, 1972) also suggest the α-lactalbumin protein is absent in milk. This, combined with the new evidence of involvement of α-lactalbumin in the involution process, prompts the idea that the absence of this protein in otariids may play some role in the evolution of their adopted lactation strategy.

Studies using Affymetrix GeneChip Canine Genome Arrays have indicated that the α-lactalbumin gene in the mammary gland of the Cape fur seal is expressed while females are on shore and expression declines while females are at sea (Sharp, Lefevre, and Nicholas, unpublished data). Subsequent analyses in our laboratory have suggested this to be a modified α-lactalbumin transcript (Reich, Sharp, Lefevre, Arnould, and Nicholas, unpublished data). It is not yet known whether the modified fur seal α-lactalbumin protein is secreted, but molecular modeling (Lefevre, unpublished data) indicates it would have impaired ability to form an active lactose–synthase complex (Pike *et al.*, 1996). Additional research using *in vitro* models indicates that Cape fur seal mammary epithelial cells are sensitive to the apoptotic effects of monomeric bovine α-lactalbumin at a range of concentrations, causing cells to decline in cell number after exposure for 4 days (Hornby, Sharp, and Nicholas, unpublished data). These new results suggest that the modified fur seal α-lactalbumin may have lost its ability to exert a negative effect on cell proliferation and survival. As a consequence of this, the otariids may be able to elude apoptosis of mammary secretory cells, allowing them to make extended foraging trips. In contrast, the continuous milk production and regular suckling by the young in phocids may prevent the local effects of α-lactalbumin in the milk from having an impact on cell survival/apoptosis of the mammary epithelial cells until weaning. It is interesting to speculate that the absence of biologically active α-lactalbumin in otariid milk may be consistent with the absence of apoptosis in the mammary gland of lactating fur seals during foraging and that loss of this protein has been due to evolutionary pressure to alter the lactational strategy of the otariid family of seals.

2. Feedback Inhibitor of Lactation

Studies in lactating animals from various species indicate that a regulatory mechanism of milk secretion involves a chemical inhibitor (Knight *et al.*, 1994; Peaker *et al.*, 1998; Wilde *et al.*, 1987, 1988, 1989). Experiments using *in vitro* models have identified a small whey protein, feedback inhibitor of lactation (FIL), that fulfills this role (Blatchford *et al.*, 1998; Rennison *et al.*, 1993; Wilde *et al.*, 1987, 1988). FIL is synthesized by the secretory epithelial cells of the mammary gland, is secreted into the alveolar lumen along with other milk constituents, and acts on the synthesis and secretory pathway by binding a putative receptor on the apical surface of the epithelial cells (Blatchford *et al.*, 1998; Rennison *et al.*, 1993). It is proposed that FIL blocks translation of milk protein transcripts (Rennison *et al.*, 1993) and inhibits secretion of milk constituents. An inhibitory effect of FIL on milk protein secretion has been demonstrated in both tissue culture (Blatchford *et al.*, 1998) and *in vivo* models. Secretion of milk protein has been shown to temporarily decrease when introduced into the mammary gland of lactating goats (Wilde *et al.*, 1995). Therefore, it is conceivable that FIL may play a role in downregulation of secretion of milk proteins in the fur seal mammary gland during foraging. This proposal would support evidence in the Antarctic fur seal that shows that the mammary gland has 80% less milk production while the fur seal is foraging (Arnould and Boyd, 1995b) and the histological examination that shows alveoli are not distended with secretory products in lactating mammary tissue offshore. Preliminary experiments (Cane *et al.*, 2005) have demonstrated a FIL-like activity in fractionated Antarctic fur seal milk. However, the level of inhibitory activity measured was similar to that reported for other species (Blatchford *et al.*, 1998) and did not differ in the milk from fur seals arriving onshore after foraging at sea or departing the colony after they had been on shore suckling their pups. This does not preclude a major role for FIL in the lactation strategy of the fur seal, but further work is required to evaluate this regulatory mechanism.

3. Unidentified Factors

Unlike any other mammal studied thus far, the fur seal appears to have a unique ability to reversibly downregulate expression of milk protein genes in the absence of involution. As discussed earlier, downregulation of milk protein gene transcripts has been observed in the foraging Cape fur seal. This suggests that another unknown mechanism, acting at the transcriptional level of milk protein production, appears to be involved in the fur seal lactation strategy. It is tempting to speculate that perhaps it is this mechanism, combined with low levels of fur seal variant α-lactalbumin, that enables fur seal to avoid the local effects of milk accumulation that would otherwise lead to involution in the mammary gland of the foraging animal.

B. Systemic Factors

In other mammals the presence of a suckling stimulus causes the release of the lactogenic hormone, prolactin, from the pituitary gland (Meites, 1959; Schmidt *et al.*, 1971). The absence of this stimulus causes prolactin levels in the body to decline and milk to accumulate within the gland, leading to distension of alveoli and milk compositional changes. Finally, involution and remodeling of the mammary tissue ensue (Oftedal *et al.*, 1987; Schmidt *et al.*, 1971).

The mechanisms that control reduction of milk production and involution of the mammary gland have been studied widely in murine systems and have shown that both systemic and local signals, derived from the accumulation of milk within alveoli, are involved in these processes (Li *et al.*, 1997; Peaker *et al.*, 1998; Quarrie *et al.*, 1995; Travers *et al.*, 1996). One scenario hypothesizes that after weaning, the systemic downregulation of either prolactin or glucocorticoid levels results in the inhibition of intracellular signaling cascades (Hennighausen *et al.*, 1997; Travers *et al.*, 1996). This has been supported by findings that show prolactin treatment following litter removal in mice delays mammary apoptosis (Feng *et al.*, 1995) and exogenously administered glucocorticoids can suppress mammary apoptosis when nursing ceases (Feng *et al.*, 1995; Lund *et al.*, 1996).

Many species have elevated prolactin levels during lactation, such as rabbits (203 ng/ml) (Kermabon *et al.*, 1994), horses (25 ng/ml) (Neuschaefer *et al.*, 1991), and sows (43 ng/ml) (Dusza and Krzymowska, 1981). Published values for pinnipeds during lactation tend to be much lower; for example, the circulating levels in the Elephant seal are 6 ng/ml (Boyd, personal observation) and 5 ng/ml in the Antarctic fur seal (Boyd, 1991). Plasma prolactin in the Antarctic fur seal has been observed to be elevated from 1 to 2 days before parturition and peaks during the perinatal period at 0–3 days postpartum (Boyd, 1991). Prolactin then declined slowly throughout the remainder of the perinatal period and remained at a low level. There was also no significant change in the prolactin levels throughout lactation (Boyd, 1991). Therefore, the positive feedback from nipple stimulation during suckling observed in other mammals does not appear to be necessary for maintenance of prolactin levels in otariids (Oftedal *et al.*, 1987). To date, no studies have investigated how prolactin levels vary between foraging and suckling fur seals. We propose that if prolactin levels are either maintained or increased in the foraging fur seal, a prolactin survival cascade may be protecting the epithelial cell of the mammary gland in the foraging fur seal, preventing the gland from entering involution, while a local mechanism may act to downregulate milk protein gene expression.

Other possible mediators of survival signals in the mammary gland, such as glucocorticoids and insulin-like growth factors (IGFs) (Farrelly *et al.*,

1999; LeRoith *et al.*, 1995; Lund *et al.*, 1996), have not been examined in the fur seal but may provide a mechanistic role in protecting epithelial cells of the mammary gland from involution (Feng *et al.*, 1995; Hadsell *et al.*, 1996) as milk production decreases.

The study of mammary gland differentiation and lactation *in vivo* is difficult in species such as the fur seal where access to mammary tissue is limited. Mammary cells cultured in 3D to form alveoli-like mammospheres (Barcellos-Hoff *et al.*, 1989; Blatchford *et al.*, 1999) offer an attractive system in which to identify local and systemic factors that control lactation and the susceptibility of lactating mammary epithelial cells to apoptosis. Mammary epithelial cells have been prepared from the gland of a late pregnant (placental gestation) Cape fur seal (Cane, 2005). These cells initially grow as a monolayer when introduced to either plastic or a suspended pliable membrane. After several days in culture, the cells deposit an extracellular matrix (ECM), which subsequently initiates formation of mammospheres (Fig. 8). In contrast, mammary cells of other species such as the cow, human, and mouse do not deposit significant amounts of matrix within the same time period and require exogenous Matrigel for mammosphere formation (Ackland *et al.*, 2001; Barcellos-Hoff *et al.*, 1989; Li *et al.*, 1987; Stoker *et al.*, 1990).

Over a period of 14 days, fur seal mammospheres develop and undergo cavitation to form a lumen by initiating regulated apoptosis of the cells within the structure (Blatchford *et al.*, 1999), leaving a thin layer of epithelial cells on the surface to resemble the normal mammary alveolus (Sharp and Nicholas, unpublished observations) (Fig. 8A and B). The basolateral polarity of the cells is maintained within mammospheres, and like mammospheres derived from other species, fur seal mammospheres are capable of mammary gland–specific function such as expression of the BLG-I and αS2-casein genes in response to lactogenic hormones (Fig. 8D), confirming that prolactin is essential for milk protein gene expression. The use of the mammosphere model will allow characterization of local factors to further elucidate the mechanism of uncoupling milk production and involution in the lactating mammary gland of the fur seal.

C. Mechanical Stress

Engorgement, due to lack of suckling and accumulation of milk within alveoli, causes mechanical stresses between the ECM and alveoli epithelial cells. This stress initiates new and independent signaling cascades that activate the apoptotic program during the first phase of involution (Boudreau *et al.*, 1995; Clark and Brugge, 1995). In order to overcome the consequences of mechanical stress on alveoli, it appears likely the fur seal rapidly reduces

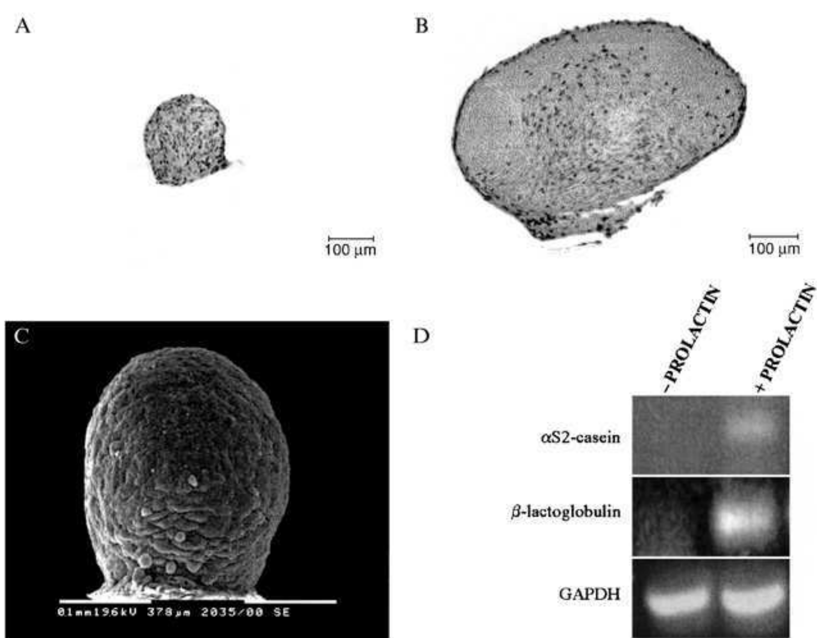

Figure 8 Fur seal mammospheres. Hematoxylin and eosin–stained 5-μm sections of (A) an immature mammosphere grown for 7 days without a central lumen and (B) a mature mammosphere at day 14 showing the formation of the lumen void of cells. (C) Electron micrograph revealing mammosphere structure. (D) Prolactin-responsive expression of the β-lactoglobulin and αS2-casein gene in fur seal mammospheres. Reverse-transcriptase–polymerase chain reaction (RT-PCR) analysis of total RNA from mammospheres was used for detection of expression of the milk protein genes, BLG-I and αS2-casein after 48-hr treatment $\pm$prolactin (PRL; 1 μg/ml).

its milk production by downregulation of milk protein genes while at sea foraging, reducing the event of alveoli engorgement and limiting mechanical stress. This phenomenon is particularly evident from our studies on the morphology of the Cape fur seal mammary gland, which show the mammary gland of the foraging seal is not engorged with milk and alveoli are less filled with milk than those of the onshore lactating mammary gland (Fig. 2).

It is postulated that if the basement membrane (BM) surrounding alveoli stretches due to engorgement with milk, the molecular interactions between the BM and adhesion receptors become altered and may lead to reduced ligand-binding interacting sites (Banes *et al.*, 1995). For example, the levels of ligand-bound β1-integrin are significantly decreased during the transition from lactation to involution in mice (McMahon *et al.*, 2004) and direct attachment of epithelial cells to the ECM occurs through basally located integrins (Alford and Taylor-Papadimitriou, 1996; Weaver *et al.*, 1997). The affinity modulation of integrin activity and, therefore, a potential inability to

respond to survival signals from the BM may contribute to the induction of apoptosis at the onset of involution. We have found evidence that β1-integrin is upregulated in the foraging Cape fur seal mammary gland compared to the onshore nursing mammary gland, and we predict this would assist to counteract any effects of the loss of the β1-integrin/epithelial cell interaction if the gland was under any form of mechanical stress during foraging (Sharp and Nicholas, unpublished data). Indeed a candidate mechanism for avoiding alveoli collapse and cell death is by upregulation of ECM components, thus avoiding degradation of the ECM, preventing the transduction of apoptotic signals (Blatchford *et al.*, 1999). In this context, it is interesting that fur seal mammary epithelial cells when grown in culture have the unique capacity to secrete significant amounts of ECM, which in turn leads to formation of hormone-responsive mammospheres.

VI. Conclusion

The study of fur seal milk and mechanisms controlling lactation in the fur seal has revealed these animals to share a number of characteristics with other lactating mammals. Fur seal mammary epithelial cells are capable of responding *in vitro* to prolactin to express milk protein genes, suggesting that the fur seal mammary gland uses systemic control mechanisms similar to other mammals to control milk production. Molecular analyses of fur seal milk protein genes reveal high protein similarity to milk proteins from other species. Milk of the fur seal is high in fat and protein and low in water content, a characteristic of marine mammals that rely on the fast transfer of energy and nutrients to combat cold climates and availability of water. However, unlike most other mammals, the fur seal is able to inhibit apoptosis and maintain mammary epithelial cells in a viable and hormone-responsive state while at sea and possibly to stimulate mammary growth to increase milk production. New platforms have been developed and enabled elucidation of mechanisms involved in fur seal lactation. Utilization of transcription profiling, canine Affymetrix analysis, cDNA libraries, and *in vitro* cell culture has aided in discovery of the processes by which the unique features of lactation in the fur seal are regulated. These platforms will continue to facilitate the attempt to solve unanswered questions about lactation cycles in these animals.

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