Current Topics in Developmental Biology

Volume 74

Edited by
Gerald P. Schatten

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Developmental Biology

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525 B Street, Suite 1900, San Diego, California 92101-4495, USA
84 Theobald's Road, London WC1X 8RR, UK

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ISBN-13: 978-0-12-153174-4
ISBN-10: 0-12-153174-0

PRINTED IN THE UNITED STATES OF AMERICA
06 07 08 09 9 8 7 6 5 4 3 2 1

Contents

4

Stem and Progenitor Cells in the Formation of the Pulmonary Vasculature

Kimberly A. Fisher and Ross S. Summer

5

Mechanisms of Disordered Granulopoiesis in Congenital Neutropenia

David S. Grenda and Daniel C. Link

6

7

8

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Numbers in parentheses indicate the pages on which the authors' contributions begin.

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1

Membrane Origin for Autophagy

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Autophagy is a degradative transport route conserved among all eukaryotic organisms. During starvation, cytoplasmic components are randomly sequestered into large double-membrane vesicles called autophagosomes and delivered into the lysosome/vacuole where they are destroyed. Cells are able to modulate autophagy in response to their needs, and under certain circumstances, cargoes, such as aberrant protein aggregates, organelles, and bacteria can be selectively and exclusively incorporated into autophagosomes. As a result, this pathway plays an active role in many physiological processes, and it is induced in numerous pathological situations because of its ability to rapidly eliminate unwanted structures. Despite the advances in understanding the functions of autophagy and the identification of several factors, named Atg proteins that mediate it, the mechanism that leads to autophagosome formation is still a mystery. A major challenge in unveiling this process arises from the fact that the origin and the transport mode of the lipids employed to compose these structures is unknown. This compendium will review and analyze the current data about the possible

Current Topics in Developmental Biology, Vol. 74

0070-2153/06 $35.00
DOI: 10.1016/S0070-2153(06)74001-7

membrane source(s) with a particular emphasis on the yeast *Saccharomyces cerevisiae*, the leading model organism for the study of autophagosome biogenesis, and on mammalian cells. The information acquired investigating the pathogens that subvert autophagy in order to replicate in the host cells will also be discussed because it could provide important hints for solving this mystery.

I. Introduction

In eukaryotic cells, the principal locations where protein catabolism occurs are the proteasome and the lysosome. The proteasome mostly recognizes and degrades cytosolic factors that have been specifically marked with polyubiquitin chains (Roos-Mattjus and Sistonen, 2004). The lysosome in contrast, requires active transport in order for the different substrates destined for elimination to reach its interior where the proteases are located. Four different pathways can deliver intracellular proteins into the lysosome lumen: endosomal transport routes, chaperone-mediated autophagy (CMA), microautophagy, and macroautophagy, the latter generally referred to as autophagy (Dunn *et al.*, 2005; Katzmann *et al.*, 2002; Klionsky, 2004; Majeski and Dice, 2004). The endosomal transport routes and CMA are mostly devoted to the transport of polypeptides, whereas microautophagy and autophagy deliver other cellular constituents because these pathways are the only ones able to internalize entire organelles and bacteria. Eukaryotes, in particular fungi, can use microautophagy to eliminate peroxisomes and is the only cellular function that has indisputably been assigned to this pathway (Dunn *et al.*, 2005). Autophagy, on the other hand, can deliver various cargoes to the lysosome interior and has multiple physiological roles.

A. Molecular Mechanism for Autophagy

The hallmark of this catabolic pathway is the sequestration of cargoes by large cytosolic double-membrane vesicles called autophagosomes (Reggiori and Klionsky, 2005). The autophagosomes successively dock and fuse with mammalian lysosomes or the yeast and plant vacuoles releasing the inner vesicles into the lumen of these organelles (Reggiori and Klionsky, 2005). The biogenesis and consumption of these structures can be divided into six discrete steps: induction, expansion, vesicle completion, docking, fusion, and breakdown (Fig. 1).

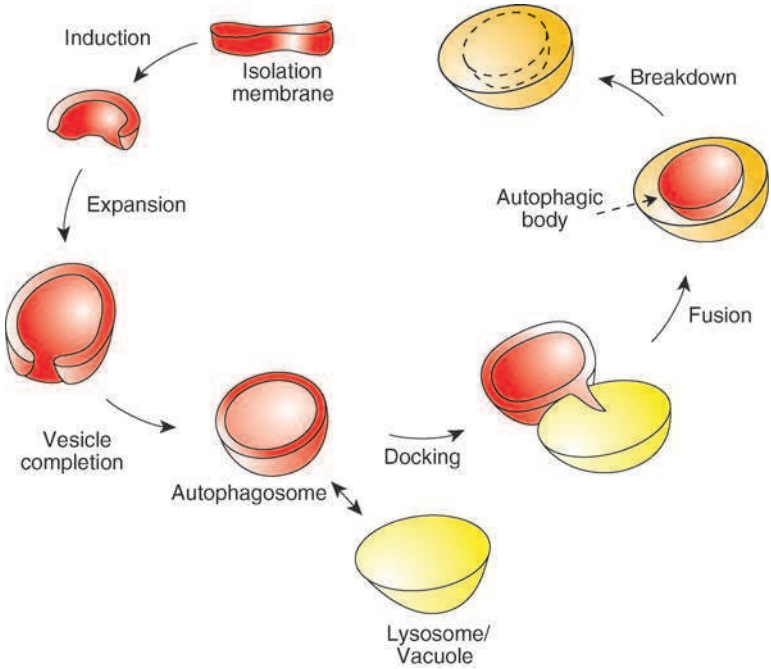

Figure 1 Conceptual model for autophagy. The basic mechanism of autophagy is the sequestration of the cargo material (bulk cytoplasm, protein aggregates, organelles, or pathogens) by a cytosolic double-membrane vesicle named an autophagosome. Extracellular stimuli or the recognition of a specific intracellular cargo induce the expansion of the isolation membrane. Upon vesicle completion, the autophagosome docks with the lysosome/vacuole and successively fuses with it. In this way the inner vesicle is liberated inside the vacuole where it is finally consumed together with the cargo by resident hydrolases. This schematic represents nonspecific autophagy and does not show specific types of autophagy including the Cvt pathway.

1. Induction

Autophagosomes are generated by the elongation of a small template membrane, termed the isolation membrane or phagophore (Fengsrud *et al.*, 2004; Mizushima *et al.*, 2001; Noda *et al.*, 2002; Reggiori and Klionsky, 2005). There are several of these structures per cell but it still remains unknown where they are derived from. The surface of this small compartment is decorated with Atg5 and Atg16, and its formation requires phosphatidylinositol (PtdIns)-3-kinase activity (Mizushima *et al.*, 2001, 2003). There are two ways of triggering the expansion of the isolation membrane, and they differ depending if the process of autophagy is selective or nonselective (Section I.B) (Reggiori and Klionsky, 2005). When this

pathway is selective, the binding to the isolation membrane of the cargo that has to be specifically eliminated (or in the case of resident hydrolases, activated) leads to the expansion of this structure (Ogawa *et al.*, 2005; Shintani and Klionsky, 2004b). In contrast to selective autophagy, which is induced by intracellular components, the nonselective process is governed by extracellular stimuli such as nutrients or cytokines (Gutierrez *et al.*, 2004; Lum *et al.*, 2005; Shintani and Klionsky, 2004a). In both cases, covalent conjugation of the ubiquitin-like Atg12–Atg5 seems to be the step that initiates the expansion of the isolation membrane (Mizushima *et al.*, 2001).

2. Expansion

The expansion of the isolation membrane is basically the simultaneous elongation and nucleation of this little cisterna (Fig. 1). It is not known how the Atg12–Atg5 complex recruits additional membranes, but the crescent autophagosome acquires more Atg12–Atg5 and Atg16 along with a second ubiquitin-like molecule, Atg8/LC3, that is unconventionally linked to phosphatidylethanolamine (PE), and probably the rest of the Atg proteins (Mizushima *et al.*, 2001, 2003). Two expansion mechanisms are possible, one that relies on delivery of lipid bilayer by vesicular traffic (vesicular expansion) and one based on the fusion of small compartments (cisternal expansion) (Reggiori and Klionsky, 2005). In addition, it has been suggested that retrograde traffic balances double-membrane vesicle biogenesis by recycling some Atg proteins, such as Atg9, and also recovering from the forming autophagosome membrane components specific to the compartment(s) of origin (Meiling-Wesse *et al.*, 2005; Nazarko *et al.*, 2005; Nice *et al.*, 2002; Reggiori *et al.*, 2003, 2004a,b).

3. Vesicle Completion

When the two extremities of the forming autophagosomes reach each other, they fuse together sealing the vesicle (Fig. 1). This fusion event, at least in yeast, appears to be SNARE-independent and triggers an uncoating reaction where the externally localized components dissociate from the vesicle surface (Ishihara *et al.*, 2001; Reggiori and Klionsky, 2005; Reggiori *et al.*, 2004b). In particular, the ubiquitin-like protein Atg8–PE is proteolytically released from its lipid moiety by the Atg4 protease, whereas the transmembrane protein Atg9 is completely retrieved (Kirisako *et al.*, 1999; Reggiori *et al.*, 2004a). It is still unknown which factor senses completion of the double-membrane vesicle and initiates this disassembly.

4. Docking and Fusion

Once uncoated, the double-membrane vesicle docks with the lysosomes/vacuoles (Fig. 1). In mammalian cells, this association is facilitated by microtubules and seems to require dynein whereas in yeast it is independent of these structures (Aplin *et al.*, 1992; Fengsrud *et al.*, 1995; Kirisako *et al.*, 1999; Punnonen and Reunanen, 1990; Ravikumar *et al.*, 2005; Webb *et al.*, 2004). The fusion between the autophagosome and the lysosome/vacuole occurs as soon as these organelles dock and it is mediated by a set of proteins also used for other fusion reactions with the lysosome/vacuole (Section I.C). During this event, the external membrane of the autophagosome becomes part of the lysosome/vacuole surface whereas the inner autophagosomal vesicle is liberated in the interior of this organelle and now called an autophagic body (Fig. 1).

5. Breakdown

The limiting membrane of the autophagic body is immediately attacked and consumed by resident lysosomal/vacuolar hydrolases allowing these enzymes to gain access to the content of this vesicle. As a result, the cargoes are also degraded into their basic constituents (or in the case of certain resident hydrolases, processed to their active form; Fig. 1).

B. A Multitask Pathway

Autophagy has been known for a long time as an adaptation response to starvation and as the major factor in the turnover of long-lived proteins. But in recent years, it has become evident that autophagy plays an active role in several other physiological tasks highlighting its versatility and adaptability. We now know that this catabolic pathway participates in cellular processes such as development, cellular differentiation and rearrangement, elimination of aberrant structures, lifespan extension, MHC class II presentation of cytoplasmic antigens, and type II programmed cell death, as well as protecting against pathogens (both viruses and bacteria) and tumors (Cuervo *et al.*, 2005; Debnath *et al.*, 2005; Deretic, 2005; Edinger and Thompson, 2003, 2004; Kirkegaard *et al.*, 2004; Komatsu *et al.*, 2005; Kondo *et al.*, 2005; Levine and Klionsky, 2004; Paludan *et al.*, 2005; Rubinsztein *et al.*, 2005; Shintani and Klionsky, 2004a). As a result, this degradative transport route plays a relevant role in the pathophysiology of neurodegenerative, cardiovascular, muscular, and autoimmune diseases, and some malignancies (Edinger and Thompson, 2003, 2004; Kondo *et al.*, 2005; Rubinsztein *et al.*, 2005; Shintani and Klionsky, 2004a; Towns *et al.*, 2005).

Autophagy provides one effective way to adjust and cope with these various situations by rapidly delivering large fractions of the cytoplasm, aberrant protein aggregates, superfluous or damaged organelles, and invading pathogens into the lysosome/vacuole interior where they are destroyed by resident hydrolases (Reggiori and Klionsky, 2005).

The adaptability of this pathway is due to its ability to select specific cargoes when forced by circumstances. It has been believed for a long time that autophagy was a nonspecific process because when induced by starvation, cytoplasmic components and organelles were randomly sequestered into autophagosomes; however, this pathway can also be selective (Table I) (Reggiori and Klionsky, 2005). In the yeast *Saccharomyces cerevisiae,* for example, aminopeptidase I (Ape1) and α-mannosidase (Ams1) form a large oligomer that is unconventionally delivered from the cytoplasm directly to the vacuole interior through a process known as the cytoplasm to vacuole targeting (Cvt) pathway (Kim *et al.*, 1997; Shintani *et al.*, 2002). This transport route is specific and biosynthetic. Precursor Ape1 (prApe1) is packed into double-membrane vesicles called Cvt vesicles, which are four to eight times smaller in surface area than autophagosomes (Baba *et al.*, 1997; Scott *et al.*, 1997). In the same organism, dysfunctional mitochondria are preferentially eliminated by autophagy (mitophagy) as well as superfluous peroxisomes (pexophagy) (Table I) (Hutchins *et al.*, 1999; Priault *et al.*, 2005). The specific sequestration of peroxisomes into double-membrane vesicles and their subsequent degradation has also been very well described in other fungi such as *Pichia pastoris*, *Hansenula polymorpha*, and *Yarrowia lipolytica* (Dunn *et al.*, 2005). Mammalian cells on the other hand, seem not to possess a transport route similar to the Cvt pathway, but there are indications that mitophagy could occur (Bota and Davies, 2001; Elmore *et al.*, 2001; Rodriguez-Enriquez *et al.*, 2006). Pexophagy has also been reported (Luiken *et al.*, 1992; Yokota, 1993; Yokota *et al.*, 1994). It has lately been shown that autophagy can be selective in mammalian cells as well, as evidenced by the

Table I Types of Selective Autophagy

Name	Cargo	Organism
Cvt pathway	prApe1, prAms1	*S. cerevisiae*, *P. pastoris*
Pexophagy	Peroxisomes	*S. cerevisiae*, *P. pastoris*, *H. polymorpha*, *Y. lipolytica*, and mammals
Mitophagy	Mitochondria	*S. cerevisiae* and mammals
Xenophagy	Bacteria and virus	Plants and mammals

The different types of selective autophagy, their specific cargoes, and the organisms that have been described are indicated.

specific recognition and disposal of invading bacteria and potentially also of intracellular viruses (Table I) (Deretic, 2005; Kirkegaard *et al.*, 2004; Levine, 2005). In addition, a study analyzing conditional knock-out mice defective for autophagy has revealed that the mutant animal accumulates numerous ubiquitinated aggregates in the cytosol, suggesting that this covalent protein modification could serve to specifically target to autophagosomes large structures that have to be eliminated (Komatsu *et al.*, 2005).

C. Autophagy-Related Genes

The process of autophagy has been known for at least 40 years, but because none of the specific components involved in this pathway were known, the studies about this degradative transport route were limited to morphological and phenomenological observations. In the last 15 years, genetic screens, mostly in the yeast *S. cerevisiae* and fungi such as *P. pastoris* and *H. polymorpha*, have lead to the isolation of 18 genes termed *AuTophaGy-related* (*ATG*) genes whose products are specifically involved in this catabolic pathway (Table II) (Klionsky, 2004; Klionsky *et al.*, 2003).

The extent of the conservation of this pathway between eukaryotes was first revealed by comparing the genomes once various sequencing projects were completed (Reggiori and Klionsky, 2002). It became immediately evident that most of the *ATG* genes had one or more homologs in higher eukaryotic organisms. The cellular role of some of them has now been explored and in all the analyzed cases, it has been demonstrated that the homologs function as orthologs (Table II) (Levine and Klionsky, 2004; Reggiori and Klionsky, 2002).

The same genetic approaches have also led to the discovery of nine *ATG* genes dispensable for bulk autophagy but essential for the Cvt pathway and/or pexophagy (Table III). Their products are mostly involved in cargo selection and the final sealing of the double-membrane vesicle, indicating that additional components are required for the autophagosomes to be able to enwrap specific cargoes. It is important to note that these genes involved in specific types of autophagy do not have clear homologs in higher eukaryotes sustaining the idea that the Cvt pathway and pexophagy are probably only present in fungi (Reggiori and Klionsky, 2002).

In addition to the Atg proteins, the genetic screens in yeast have also permitted the identification of additional components required for the normal progression of autophagy that are shared with other intracellular transport routes (Table IV). The function of several of these factors in the other pathways was already known and that has helped in clarifying the mechanism of autophagy. For example, yeast vacuoles can fuse with late endosomes [multivesicular bodies (MVB) pathway] or possibly with vesicles

Table II Yeast *S. cerevisiae* Genes Specifically Involved in Autophagy, Cvt Pathway, and Pexophagy

Protein	Step	Role	Interactions	Orthologs
Atg1	Formation/ expansion	Serine/ threonine kinase	Atg13, Atg11, Atg17	D.d., C.e., P.p., H.p.
Atg2	Formation/ expansion	Atg9 recycling	Atg9, Atg18	P.p.
Atg3	Formation/ expansion	Atg8 conjugation system (E2)	Atg7, Atg8, Atg12	H.s., D.m., P.p.
Atg4	Formation/ expansion	Cysteine protease	Atg8	H.s., M.m., D.m., P.p.
Atg5	Formation/ expansion	Atg12 conjugation system	Atg12, Atg16	H.s., M.m., D.d.
Atg6[a]	Formation/ expansion	PtsIns-3-P synthesis	Atg14, Vps15, Vps34	H.s., M.m., D.d., C.e.
Atg7	Formation/ expansion	Atg8 and Atg12 conjugation systems (E1)	Atg3, Atg8, Atg12	H.s., M.m., D.d., C.e., A.t., P.p.
Atg8	Formation/ expansion	Ubiquitin-like protein	Atg3, Atg4, Atg7, Atg19	H.s., M.m., R.n., D.d., C.e., A.t., P.p., H.p.
Atg9	Formation/ expansion	Transmembrane protein	Atg2, Atg18, Atg23	H.s., M.m., A.t., P.p.
Atg10	Formation/ expansion	Atg12 conjugation system (E2)	Atg12	H.s., M.m.
Atg12	Formation/ expansion	Ubiquitin-like protein	Atg3, Atg5, Atg7, Atg10, Atg16, Atg17	H.s., M.m., D.d.
Atg13	Formation/ expansion	Modulates Atg1 activity	Atg1, Atg17, Vac8	–
Atg14	Formation/ expansion	PtsIns-3-P synthesis	Atg6, Vps15, Vps34	–
Atg16	Formation/ expansion	Associates with the Atg12–Atg5 complex	Atg5, Atg12, Atg16	H.s., M.m., P.p.
Atg17[b]	Formation/ expansion	Modulates Atg1 activity	Atg1, Atg13, Atg11, Atg12, Atg24	–
Atg18	Formation/ expansion	PtsIns-3-P binding protein	Atg2, Atg9	H.s., A.t., P.p.
Atg22[c]	Vesicle breakdown	Transmembrane protein	–	–
Atg23	Formation/ expansion	Cycling factor	Atg9	–

H.s. = *Homo sapiens*; M.m. = *Mus musculus*; R.n. = *Rattus norvegicus*; D.m. = *Drosophila melanogaster*; D.d. = *Dictyostelium discoideum*; C.e. = *Caenorhabditis elegans*; A.t. = *Arabidopsis thaliana*; P.p. = *Pichia pastoris*; H.p. = *Hansenula polymorpha*; Y.l. = *Yarrowia lipolytica*.

[a] In yeast, Atg6 plays an important role in endosomal trafficking.

[b] Atg17 is required for autophagy and pexophagy but not Cvt pathway.

[c] Atg22 is not necessary for both pexophagy and the Cvt pathway.

Table III Yeast Genes Specifically Involved in the Cvt Pathway and/or Pexophagy

Protein	Cvt	Pexophagy	Step	Role	Organism
Atg11	+	+	Formation/ expansion	Cargo receptor/ adaptor	S.c., P.p., H.p.
Atg19	+	−	Formation/ expansion	Cargo receptor	S.c.
Atg20[a]	+	+	Formation/ expansion	PtdIns-3-P binding protein	S.c.
Atg21[b]	+	?	Formation/ expansion	PtdIns-3-P binding protein	S.c., H.p.
Atg24[a]	+	+	Formation/ expansion	PtdIns-3-P binding protein	S.c., P.p.
Atg25[c]	−	+	Fusion	Coiled-coil protein	H.p.
Atg26[c]	−	+	Vesicle completion	UDP-glucose:sterol glucosyltransferase	P.p.
Atg27	+	N.D.	Formation/ expansion	PtdIns-3-P binding protein	S.c.
Atg28[c]	−	+	Vesiculation	Coiled-coil protein	P.p.
Tlg1[a]	+	N.D.	Formation/ expansion	vSNARE	S.c.
Tlg2[a]	+	N.D.	Formation/ expansion	tSNARE	S.c.
Vps45[a]	+	N.D.	Formation/ expansion	Sec1 homolog	S.c.

N.D. = not determined. A plus or a minus mark indicates whether the protein is required for a pathway. S.c. = *Saccharomyces cerevisiae*; P.p. = *Pichia pastoris*; H.p. = *Hansenula polymorpha*.

[a] In *S. cerevisiae*, these proteins also catalyze the retrieval transport from early endosomes.

[b] Atg21 is not required for pexophagy in *S. cerevisiae* but is essential for the same process in *H. polymorpha*.

[c] These factors have no counterparts in *S. cerevisiae* or the homologs do not have a role in pexophagy.

[?] One report has indicated that Atg21 is essential for pexophagy, another affirms that Atg21 is not required for this process.

derived from the endosome [carboxypeptidase Y (CPY) pathway], Golgi-derived vesicles [alkaline phosphatase (ALP) pathway], and with themselves (homotypic fusion). In all these cases, cells use an identical fusion machinery, which consists of SNARE proteins, Sec18 (NSF), Sec17 (α-SNAP), a Rab-GTPase, and the class C Vps protein complex also known as the HOPS complex. The same components have also been found to be exploited for the fusion of double-membrane vesicles (Table IV) (Reggiori and Klionsky, 2002; Wang *et al.*, 2003). Similarly, it is also now evident that the dissolution of autophagic bodies is mediated by the same hydrolases that degrades the

Table IV Yeast *S. cerevisiae* Genes Involved in Autophagy, Cvt Pathway, and Pexophagy but Also in Other Endosomal Transport Routes

Protein/complex	Step	Role
Atg15	Vesicle breakdown	Lipase
Ccz1–Mon1 complex (Ccz1, Mon1)	Docking/fusion	Tethering/docking factor
HOPS complex/class C Vps protein complex (Vps11, Vps16, Vps18, Vps33, Vps39, Vps41)	Docking/fusion	Tethering factor/Rab effector
Pep4	Vesicle breakdown	Vacuolar protease
Prb1	Vesicle breakdown	Vacuolar protease
PtsIns-3-kinase complex (Vps15, Vps34)	Formation/expansion	PtsIns-3-P synthesis
Trs85	Formation/expansion	Tethering factor
Vac8	Formation/expansion	Vacuole landmark
Vam3	Docking/fusion	tSNARE
Vam7	Docking/fusion	vSNARE
VFT complex (Vps51, Vps52, Vps53, Vps54)	Formation/expansion	Tethering factor
Ykt6	Docking/fusion	vSNARE
Vti1	Docking/fusion	vSNARE
Ypt7	Docking/fusion	Rab-GTPase

MVB internal vesicles once these are released into the vacuole lumen (Table IV) (Epple *et al.*, 2003; Reggiori and Klionsky, 2002).

II. Membrane Source in the Yeast *S. cerevisiae*

A. Pre-autophagosomal Structure

Most of the Atg components are peripheral membrane proteins that transiently associate with the nascent autophagosomes. In contrast to mammalian cells where several isolation membranes can be simultaneously activated, a single perivacuolar site of organization for double-membrane vesicle formation (named the pre-autophagosomal structure, PAS) is observed in the yeast *S. cerevisiae* (Kim *et al.*, 2002; Suzuki *et al.*, 2001). The PAS is believed to be the yeast counterpart of a mammalian isolation membrane and in this unicellular eukaryote, most of the Atg proteins appear to be primarily restricted to this location. This unique site seems also to be present in *H. polymorpha* (Monastyrska *et al.*, 2005a,b). In *P. pastoris*, however, several Atg components are distributed to more than one punctate structure

(Ano *et al.*, 2005; Chang *et al.*, 2005; Kim *et al.*, 2001b; Mukaiyama *et al.*, 2004; Stromhaug *et al.*, 2001). It is unclear if this represents a difference between organisms or is due to different growth conditions. *P. pastoris* is mostly used for the study of pexophagy and therefore grown in special media containing carbon sources that induce peroxisome proliferation.

It is unclear where the PAS is derived from and at which point it becomes membranous. The study of the Cvt pathway has provided insights into how this structure is generated. After synthesis, prApe1 forms a large oligomer that first associates with the Atg19 cargo receptor and then with the Atg11 adaptor to form the Cvt complex (Shintani *et al.*, 2002). This large cytosolic protein aggregate then moves in close proximity to the vacuole surface, where it induces the recruitment of the rest of the Atg factors, triggering the formation of the Cvt vesicle (Shintani and Klionsky, 2004b; Yorimitsu and Klionsky, 2005). Neither the PAS nor the vesicles are efficiently formed in the absence of any of the Cvt complex components, indicating that the cargo stimulates the biogenesis of these structures (Shintani and Klionsky, 2004b). This requirement is overcome when cells are nitrogen-starved (Kim *et al.*, 2001b; Shintani and Klionsky, 2004b).

Because of its dynamic properties, the PAS should not be seen as a static or defined organelle but more as a structure in constant remodeling. It remains unclear at which stage and how membranes are transported at the PAS, but because of their association with lipid bilayers, two proteins, Atg8 and Atg9, could be important for dissecting this event.

B. Atg8

Atg8 is a soluble ubiquitin-like protein and its carboxy-terminal arginine is removed by the Atg4 cysteine protease leaving a glycine residue at the new carboxy terminus (Kim *et al.*, 2001a; Kirisako *et al.*, 2000). Atg8 is activated by the E1 enzyme Atg7 through a thioester bond between its carboxy-terminal glycine and cysteine 507 of Atg7 (Kim *et al.*, 1999; Kirisako *et al.*, 2000; Komatsu *et al.*, 2001). Atg8 is subsequently transferred to the E2 enzyme Atg3 via a new thioester bond between these two proteins (Ichimura *et al.*, 2000; Kim *et al.*, 2001a). Atg8 is finally covalently conjugated to a PE molecule, becoming tightly membrane associated (Ichimura *et al.*, 2000). This linkage is reversible because Atg8 can be proteolitically released from its lipid moiety by Atg4, an event that takes place once the double-membrane vesicles are completed (Section I.A.3) (Kirisako *et al.*, 1999; Reggiori *et al.*, 2004a).

It is unclear where the Atg8 conjugation to PE occurs. This protein is normally lipidated in mutants unable to form the PAS indicating that this modification takes place at a different subcellular location (Suzuki *et al.*,

2001). This is supported by the fact that Atg8–PE localizes to the PAS but also to tiny cytosolic vesicles (Kirisako *et al.*, 1999). These data, however, do not exclude the possibility that Atg8–PE conjugates are formed at the PAS as well. The association of Atg8–PE with membranes prior to getting concentrated at the PAS suggests that the Atg8–PE structures could be at least in part the source of autophagosome lipid bilayers. This idea is supported by the observation that in the absence of Atg8, membranes fail to be delivered to the PAS and therefore the size of autophagosomes is strongly reduced (Abeliovich *et al.*, 2000; Kirisako *et al.*, 1999; Lang *et al.*, 1998).

It remains a mystery where the tiny Atg8–PE containing vesicles are derived from, but one possibility is that that they originate from early compartments of the secretory pathway, for example, the endoplasmic reticulum (ER) and/or the Golgi apparatus. This hypothesis is based on two experimental findings. First, Atg8 binds two vSNAREs required for both anterograde and retrograde transport between the ER and Golgi apparatus (Legesse-Miller *et al.*, 2000). Second, this ubiquitin homolog has been detected on autophagosome-like structures derived from the Golgi complex and/or ER (Section II.D.2) (Reggiori *et al.*, 2004b).

C. Atg9

Atg9 is the only integral membrane protein essential for double-membrane vesicle formation (Noda *et al.*, 2000). This protein is probably transported to the PAS with at least part of the lipids or lipid bilayers required to create this structure. This notion is corroborated by the fact that the totality of Atg9 is associated with membranes (Noda *et al.*, 2000; Reggiori *et al.*, 2005b). Atg9 cycles between the PAS and several unknown punctate structures dispersed in the cytosol supporting the idea that it could partially supply the forming autophagosomes with membranes (Reggiori *et al.*, 2004a). A fraction of these punctate structures are Atg9 aggregates residing on the mitochondria surface (Reggiori *et al.*, 2005b). This suggests that this organelle could provide the nascent autophagosomes with at least part of its lipid bilayers.

However, it cannot be excluded that Atg9 trafficking carries out other functions. Under certain conditions, autophagy becomes one of the principal sources of energy for the cell (Kuma *et al.*, 2004; Lum *et al.*, 2005). Because the mitochondria provide the other primary supply of energy, one could imagine that Atg9 is used to coordinate the two sources.

The sorting mechanism for Atg9 transit from mitochondria to the PAS is unknown, but under growing conditions this event is induced by Cvt complex assembly and requires actin (Reggiori *et al.*, 2005a; Shintani and Klionsky, 2004b). In contrast, the retrieval transport of this transmembrane protein from the PAS has been characterized in more detail and shown to be

regulated by the Atg1–Atg13 signaling complex and requires Atg2, Atg18, and the PtdIns-3-phosphate generated by the Atg14-containing PtdIns 3-kinase complex (Reggiori *et al.*, 2004a). This recycling event, however, seems to be to some extent differently organized in *P. pastoris* during micropexophagy, possibly because other membranous structures are used and assembled in a different way during this invagination process (Chang *et al.*, 2005).

D. Yeast Organelles and Autophagy

1. Endoplasmic Reticulum

An initial analysis concerning the role of yeast early secretion (*sec*) mutants in autophagy has revealed that several of them are essential for autophagosome formation (Ishihara *et al.*, 2001). This class of genes is involved in transport out of the ER (Kaiser and Schekman, 1990). Successive studies, however, have shown that these mutants have an indirect negative effect on both the Cvt pathway and autophagy (Hamasaki *et al.*, 2003; Reggiori *et al.*, 2004b). One possible explanation of their phenotype is that they alter the ER morphology and consequently impair several functions of this organelle, including the putative one to supply membranes for double-membrane vesicle formation. For example, the ER is structurally connected with the mitochondria and the disruption of the ER organization in the early *sec* mutants causes the fragmentation of the mitochondrial reticulum (Prinz *et al.*, 2000). As mentioned, Atg9 partially localizes to mitochondria, and in this class of mutants its trafficking out of this compartment is severely impaired (Reggiori and Klionsky, submitted).

2. Golgi Apparatus

Atg20, Atg24, Tlg1, Tlg2, Trs85, Vps45, and the subunits of the Vps-fifty-three (VFT) complex are part of retrieval transport routes from endosomal compartments back to the Golgi apparatus, and consequently they are important in maintaining certain functions of this organelle (Hettema *et al.*, 2003; Holthuis *et al.*, 1998; Sacher *et al.*, 2000, 2001; Siniossoglou and Pelham, 2001). These proteins have also been shown to be required for the Cvt pathway and some of them also play an important role in double-membrane vesicle biogenesis during pexophagy and autophagy (Tables III and IV) (Abeliovich *et al.*, 1999; Meiling-Wesse *et al.*, 2005; Nazarko *et al.*, 2005; Nice *et al.*, 2002; Reggiori *et al.*, 2003). It is unclear, however, why these pathways are impaired in the absence of these factors. One possibility is that retrograde traffic from the forming double-membrane vesicles is

essential for the expansion and/or completion of these structures (Meiling-Wesse *et al.*, 2005; Reggiori *et al.*, 2004a,b). A second hypothesis is that similarly to what was predicted for early *sec* mutants, an alteration of the Golgi apparatus functions could interfere with the lipid bilayer delivery essential for the creation of these large vesicles.

The major difficulty in investigating the contribution to autophagy of both the ER and the Golgi apparatus is that these two organelles depend on each other for their proper function. Mutations that affect one of these two compartments indirectly perturb the other one. Along these lines, the interpretation of the block of both the Cvt pathway and autophagy in the *sec7* mutant is not simple (Reggiori *et al.*, 2004b). Nevertheless, the analysis of this strain has led to important information. Sec7 is a GDP/GTP exchange factor required for trafficking through the Golgi complex (Franzusoff and Schekman, 1989; Jackson and Casanova, 2000). The inactivation of this protein provokes the accumulation of unsealed, autophagosome-like structures that are decorated with Atg8 (Reggiori *et al.*, 2004b). These membranous arrangements enwrap ribosomes and cytosol and have been previously named Berkeley bodies (Esmon *et al.*, 1981; Novick *et al.*, 1980). This surprising result indicates that potentially, double-membrane vesicles can be created in large part by altering the activity of a single enzyme; however, it cannot be excluded that this is an indirect phenomenon.

3. Endosomes

Vps4 is a protein essential for the invagination of the late endosome limiting membranes and therefore MVB biogenesis (Babst *et al.*, 1998; Katzmann *et al.*, 2002). A unique *VPS4* allele was isolated in a screen for mutations that result in autophagy induction even in the presence of nutrients (Shirahama *et al.*, 1997). This led to an initial interpretation that endosomes play a relevant role in autophagosomes biogenesis. However, reports where the functions of these compartments have been severely impaired by specific gene deletions have revealed that the integrity of the endosomal system is not essential for either the Cvt pathway or autophagy (Epple *et al.*, 2003; Reggiori *et al.*, 2004b).

III. Lipid Bilayer Origin in Mammalian Cells

A. Uncertain Origin of the Isolation Membrane

In contrast to the late stages of the autophagosome biogenesis where lipid bilayers are derived from endosomal compartments, the origin of the mammalian isolation membrane or phagophore remains uncertain. It is still

unknown if this small sequestering cisterna is formed *de novo* or derived from a preexisting organelle (Fengsrud *et al.*, 2004). A major problem in trying to investigate its origin is that these structures and autophagosomes are mostly composed of lipids and depleted in transmembrane proteins making particularly difficult the detection of specific organelle markers (Fengsrud *et al.*, 2000; Hirsimaki *et al.*, 1982; Punnonen *et al.*, 1989; Reunanen *et al.*, 1985; Stromhaug *et al.*, 1998). This unique characteristic is one line of evidence that the isolation membranes and autophagosomes differ structurally from the other subcellular organelles. This observation also implies that whatever the origin of the lipid bilayers used to form autophagosomes, integral membrane components are segregated away from them.

Two models could explain how protein-depleted membranes are obtained. In the first, isolation membranes are derived from a specialized organelle subdomain where autochthonous proteins are gradually excluded. A similar process has been shown to occur during peroxisome biogenesis from the ER (Geuze *et al.*, 2003; Tabak *et al.*, 2003; Tam *et al.*, 2005). In the second model, the same cisterna is progressively emptied of integral membrane factors by retrieval transport— a phenomenon hypothesized to occur during double-membrane vesicle formation in yeast (Reggiori *et al.*, 2004a,b). It is also possible that both mechanisms coexist.

Numerous studies have been published investigating the source for autophagosome lipid bilayers in mammalian cells but their conclusions often contrast. Thus various organelles, such as the ER, the Golgi complex, and the plasma membrane, have been suggested to be the origin of double-membrane vesicles. Because of the heterogeneity in the results, no unanimous agreement in the field has been reached. For example, several studies have reported the presence of ER-marker proteins in the isolation membranes and autophagosomes but others have shown that these structures lack ER-resident factors (Arstila and Trump, 1968; Dunn, 1990a; Furuno *et al.*, 1990; Reunanen *et al.*, 1985; Stromhaug *et al.*, 1998; Yamamoto *et al.*, 1990; Yokota *et al.*, 1994).

As with the ER, the role of the Golgi complex as a lipid donor for the early autophagosome intermediates remains ambiguous. Some studies have shown the presence of Golgi protein markers in these structures whereas others have failed to detect them (Arstila and Trump, 1968; Dunn, 1990a; Frank and Christensen, 1968; Locke and Sykes, 1975; Yang and Chiang, 1997; Yokota *et al.*, 1994). The membranes of the *cis*-Golgi network have been shown to possess the same compositional characteristics of the isolation membrane (Fengsrud *et al.*, 2004; Locke and Sykes, 1975; Reunanen *et al.*, 1988; Yamamoto *et al.*, 1990).

Only a few reports have indicated that the autophagic cisternae are derived from the plasma membrane and their conclusions have been challenged when other investigators have failed to detect plasma membrane protein markers in

these structures (Araki *et al.*, 1995; Arstila and Trump, 1968; Bosabalidis, 1994; Ericsson, 1969; Fengsrud *et al.*, 2004; Oledzka-Slotwinska and Desmet, 1969; Reunanen *et al.*, 1988). Importantly, autophagosomes have a low cholesterol content validating the idea that their membranes are not derived from the plasma membrane (Reunanen *et al.*, 1985).

The discrepancy between all these analyses could be due, in part, to different experimental approaches and techniques used in the various laboratories. But one possibility that should not be discarded a priori is that autophagosomes could be a mosaic of membranes derived from more than one organelle. For example, the isolation membrane could originate from one compartment and the additional lipid bilayers required for its expansion be acquired from other sources. In addition, the different contributions could vary depending on the tissues with cells able to derive the membranes from the most suitable reservoirs.

B. Atg8

The Atg8 conjugation system is highly conserved in higher eukaryotic cells (Table II) (Ohsumi and Mizushima, 2004; Tanida *et al.*, 2004). In mammals, there are at least three Atg8 homologs: the microtubule-associated protein 1 (MAP1) light chain 3 (LC3), the Golgi-associated ATPase enhancer of 16 kDa (GATE-16), and the γ-aminobutyric acid $(GABA)_A$-receptor-associated protein (GABARAP) (Mann and Hammarback, 1994; Sagiv *et al.*, 2000; Wang *et al.*, 1999). It should be noted that these three proteins were first isolated because of their involvement in other trafficking pathways. The mammalian counterparts of Atg4 process these three Atg8 homologs by exposing their conserved C-terminal glycine which then interacts with mammalian Atg7 and Atg3 homologs before being covalently linked to a lipid (Hemelaar *et al.*, 2003; Scherz-Shouval *et al.*, 2003; Tanida *et al.*, 2001, 2002). The target phospholipid has not yet been unequivocally identified, but strong evidence suggests that it is PE (Kabeya *et al.*, 2004; Tanida *et al.*, 2004).

Of the three homologs, LC3 has been best characterized as an autophagosomal marker in mammalian autophagy. The newly synthesized LC3 precursor is processed cotranslationally to generate a soluble LC3 form (LC3-I) that, upon starvation, becomes membrane-bound and has greater mobility than LC3-I when resolved by SDS-PAGE (Kabeya *et al.*, 2000). The lipidated protein, called LC3-II, localizes on both autophagosomes and autolysosomes (Kabeya *et al.*, 2000). These *in vitro* results have been confirmed using transgenic mice expressing GFP-LC3 (Mizushima *et al.*, 2004). Unfortunately, the small amount of LC3-II generated prior to induction of autophagy is already associated with the double-membrane vesicles formed

by the basal activity of this pathway and LC3-I is not clearly associated with a distinct membranous structure (Kabeya *et al.*, 2004). Therefore, the subcellular localization of these molecules has not furnished insights about the lipid bilayer source.

Both GATE-16 and GABARAP possess a form II and localize to LC3-positive autophagosomes that are induced by starvation (Kabeya *et al.*, 2004). Thus, it remains a possibility that they participate in autophagy in addition to, or instead of, their originally described functions. Because the three mammalian Atg8 homologs are differently expressed in various tissues (Tanida *et al.*, 2004), another intriguing option is that these proteins are involved in supplying the autophagosome with membranes derived from different compartments depending on the cell type; for example, GATE-16 from the Golgi complex and GABARAP from the same organelle as well as the synaptic cisternae (Kittler *et al.*, 2001; Kneussel *et al.*, 2000; Sagiv *et al.*, 2000).

C. Atg9

A report has demonstrated that the two human proteins with high homology to Atg9, HsAtg9L1 and HsAtg9L2, are its orthologs (Yamada *et al.*, 2005). In human adult tissues, HsATG9L1 is ubiquitously expressed, whereas HsATG9L2 is highly expressed in placenta and pituitary gland. Importantly, the authors have also shown that these two factors are not distributed on mitochondria. Instead they localize to a perinuclear region, suggesting that in higher eukaryotes Atg9 could supply autophagy with membranes by deriving lipid bilayers from a different reservoir. This observation could also explain why HsAtg9L1 and HsAtg9L2 cannot substitute for the yeast Atg9 (Reggiori *et al.*, 2005b; Yamada *et al.*, 2005). However, HsAtg9L2 possesses a nonfunctional mitochondrial targeting sequence that is also present in its closest higher eukaryote homologs (Yamada *et al.*, 2005). This characteristic raises the possibility that this is an ancient localization signal. Because the subcellular distribution of HsAtg9L1 and HsAtg9L2 have not been carefully examined and the preliminary localization analysis was performed with overexpressed proteins, the identification of the precise localization of these two proteins could provide insights into membrane dynamics during autophagosome biogenesis in mammals.

D. Autophagosome Maturation

In mammalian cells, autophagosomes, also called initial autophagic vacuoles (AVi), undergo a stepwise maturation process that can be followed ultrastructurally by monitoring the disintegration status of their internal lipid

bilayer and cargoes (Fig. 2) (Dunn, 1990b; Eskelinen, 2005; Fengsrud *et al.*, 2004; Rabouille *et al.*, 1993). These morphological changes correlate with the increasing acquisition of lysosomal makers (Berg *et al.*, 1998; Dunn, 1990b; Liou *et al.*, 1997; Tanaka *et al.*, 2000). Autophagosomes, which contain intact cytosol and organelles, fuse first with endosomal vesicles and MVB turning into early degradative autophagic vacuoles (AVd) or amphisomes. These structures successively fuse together or with lysosomes becoming late AVd or autolysosomes. The degradation of the internal material starts in the early AVd and continues in the late AVd until completion.

In contrast to yeast, the endosomal system plays an essential role in mammalian autophagy (see Section II.D.3). This divergence between species has been highlighted by the discovery that SKD1 is necessary for autophagosome maturation in mouse cells (Nara *et al.*, 2002). SKD1 is the mouse homolog of yeast Vps4 and, as its counterpart, it is also essential to maintain endosome morphology and endosomal transport (Yoshimori *et al.*, 2000). As mentioned, Vps4 is not required for autophagy in yeast (Section II.D.3) (Reggiori *et al.*, 2004b; Shirahama *et al.*, 1997).

It is unclear why mammalian autophagosomes need the additional maturation step characterized by their fusion with endosome- and/or *trans*-Golgi network (TGN)-derived transport vesicles and MVB. In yeast, double-membrane vesicles fuse with a much larger vacuole one after the other. Therefore, their cargoes do not influence the hydrolytic capacity of this compartment by altering, for example, the pH because the volume of their contents is just a fraction of that of the entire vacuole. Lysosomes, in contrast, are much smaller than vacuoles and their size is comparable to that of autophagosomes.

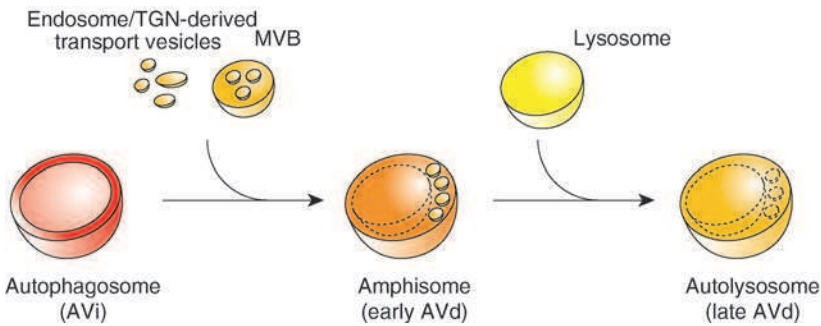

Figure 2 Autophagosome maturation in mammalian cells. Once sealed, the autophagosome (or AVi) fuses with endosome- and/or TGN-derived transport vesicles and the MVB becoming an amphisome (or early AVd). This event leads to the acquisition of hydrolytic enzymes that initiate the consumption of the autophagosome cargo. The amphisome then fuses with a lysosome generating a new organelle termed autolysosome (or late AVd) where the degradation of the content of the initial autophagosome is completed.

Consequently, if these two structures would immediately fuse together, an important dilution of the lysosome content could occur impairing its internal enzymatic activity. Addition of extra hydrolytic enzymes prior to autolysosome formation could help to compensate for this dilution phenomenon.

E. Pathogens

Autophagy provides a cellular defense against invading pathogens but unfortunately, some of them have developed systems to avoid the sequestration and elimination by double-membrane vesicles (Deretic, 2005; Kirkegaard *et al.*, 2004; Levine, 2005). In addition, there are virus and bacteria that exploit this transport route to enter and replicate inside the host cell (Kirkegaard *et al.*, 2004). The study of this latter class of pathogens has furnished some indications about the possible origin of autophagosome membranes even if it should be kept in mind that these invading microorganisms are also altering other cellular pathways, and therefore autophagy could progress in part differently in the infected cells.

1. Virus

Upon infection, positive-strand viruses disassemble and release their genomic RNA into the cytoplasm of the host cell. The genomic RNA is subsequently translated to produce the replicase proteins that induce the formation of the RNA replication complexes. These complexes are assembled and anchored on membrane surfaces and this is an essential requisite for their virulence. Some of the positive-strand viruses, such as the poliovirus, the mouse hepatitis virus (MHV), the equine arterivirus (EAV), and the severe acute respiratory syndrome (SARS) coronavirus, seen to use autophagosomes as a membrane platform (Kirkegaard *et al.*, 2004).

Factors of the poliovirus RNA-replication complex localize to double-membrane vesicles that are derived from the ER by the action of viral proteins 2BC and 3A by a mechanism that excludes resident host proteins (Schlegel *et al.*, 1996; Suhy *et al.*, 2000). Importantly, these structures contain LC3/Atg8 and are highlighted with the fluorophore monodansylcadaverine, a dye that specifically stains autophagosomes (Jackson *et al.*, 2005). The idea that poliovirus subverts components of the cellular autophagy machinery to promote its replication is also supported by the fact that inhibition of this pathway by 3-methyladenine or by RNA interference against mRNAs that encode two different Atg proteins (LC3/Atg8 and Atg12) decrease the poliovirus yield (Jackson *et al.*, 2005).

Coronaviruses (MHV and SARS) and arteriviruses (EAV) are the two families within the order Nidovirales. Cells infected by these viruses accumulate double-membrane vesicles and the viral RNA-replication complexes are associated with them (Goldsmith *et al.*, 2004; Gosert *et al.*, 2002; Pedersen *et al.*, 1999; Shi *et al.*, 1999; van der Meer *et al.*, 1998). In the case of the MHV and SARS coronaviruses, it has also been shown that these structures are decorated with LC3/Atg8, revealing that they are autophagosomes (Prentice *et al.*, 2004a,b). For the MHV in addition, it has been demonstrated that the autophagy machinery is required to generate these compartments and in its absence the virus replication is severely blocked (Prentice *et al.*, 2004a). Importantly, studies about the origin of these double-membrane vesicles generated in cells infected by nidoviruses indicate that they are derived from the ER (Pedersen *et al.*, 1999; Prentice *et al.*, 2004a; Shi *et al.*, 1999; van der Meer *et al.*, 1998).

2. Bacteria

After endosomal uptake of *Porphyromonas gingivalis* and *Brucella abortus*, by the host cell, the endosomes that contain these bacteria immediately fuse with structures resembling autophagosomes (Dorn *et al.*, 2001; Pizarro-Cerda *et al.*, 1998a,b; Progulske-Fox *et al.*, 1999). This event prevents their delivery to the lysosome where they would be eliminated. In addition containing endosomal factors, the double membranes surrounding these two pathogens are decorated with ER protein markers and their formation is blocked by autophagy inhibitors such as 3-methyladenine and wortmannin (Rich *et al.*, 2003).

Legionella pneumophila is a Gram-negative bacterium that can replicate within human macrophages. After being taken up by phagosomes, this pathogen becomes enwrapped within a double-membrane compartment that contains the ER resident chaperone BiP through an unknown mechanism, and starts to replicate (Coers *et al.*, 2000; Joshi *et al.*, 2001; Sturgill-Koszycki and Swanson, 2000; Swanson and Isberg, 1995). It has been shown that this compartment also progressively becomes decorated with typical autophagosome markers such as Atg7 and Atg8 (Amer and Swanson, 2005). However, it remains unclear if these structures are autophagosomes or similar conformations derived from the ER that at successive stage acquire autophagosomal membranes or subvert the autophagy machinery to complete their biogenesis (Kagan and Roy, 2002; Tilney *et al.*, 2001). In *Dictyostelium discoideum*, a natural host for *L. pneumophila*, deletion of *ATG* genes leads to a defect in autophagy without affecting the formation of the double-membrane compartment and therefore the replication of this invading microorganism is unaffected (Otto *et al.*, 2004). But this could just reflect host-specific differences.

Listeria monocytogenes is another Gram-negative bacterium that after entering into host cells destroys the phagosome membrane using hemolysin to gain access to the cytoplasm where it starts to multiply. However, when infected cells are treated with chloramphenicol, an inhibitor of bacterial protein synthesis, or lack the *actA* gene, the bacteria become trapped into double-membrane compartments shortly after phagosome lysis (Rich *et al.*, 2003). These structures are autophagosomes because *L. monocytogenes* sequestration is enhanced by autophagic induction through serum withdrawal and blocked by autophagy inhibitors such as 3-methyladenine and wortmannin (Rich *et al.*, 2003). The formation of these autophagosomes seems to be mediated by the assembly of small vesicles and cisternae with variable morphology, which contain the ER protein marker protein disulfide isomerase (PDI). Importantly, PDI-positive vesicular structures are accumulated around the cytoplasmic bacteria during the early stages of autophagosome biogenesis but not at later stages when these structures begin to acquire endosomal makers (Rich *et al.*, 2003).

IV. Perspectives

Our knowledge about the physiological roles of autophagy has enormously increased and we have realized how important this pathway is for cell survival in several extreme situations. Despite the identification and partial characterization of the Atg proteins, however, the molecular mechanism of this catabolic transport route remains largely unknown. A major challenge in studying this process arises from the fact that the origin and the transport mode of the lipids employed to compose these structures is unknown. Investigations on this topic seem to indicate that the ER and possibly the Golgi complex are involved in supplying the nascent autophagosomes with membranes. Endosomal compartments, in contrast, play a relevant role only in mammalian cells and at a later stage during autophagosome maturation.

The large majority of the morphological characterization of autophagosome formation was done 10–15 years ago, when specific autophagy markers were unavailable. Atg proteins provide now the researchers with the long-awaited markers that could be used to at least dissect this transport route at an ultrastructural level, thus solving some of the mysteries that surround the double-membrane vesicle origin and biogenesis. Analysis of pathogens and their gene products has helped in the past to unveil and analyze numerous cellular pathways. The discovery of the existence of viruses and bacteria subverting autophagy will probably have a similar impact. The study of these microorganisms will help us to understand how lipid bilayers are derived from the membrane source(s) but will also potentially lead to the isolation of agents that will allow investigators to manipulate this process.

Acknowledgments

The author thanks Daniel Klionsky, Judith Klumperman, Catherine Rabouille, and Ger Strous for critically reading the chapter. The author also wishes to thank Marc van Peski and René Scriwanek for Figs. 1 and 2.

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2

Chromatin Assembly with H3 Histones: Full Throttle Down Multiple Pathways

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The typical eukaryotic genome packages roughly 6 feet of DNA into a nucleus about 5 μm in diameter, yet this compaction blocks access to the DNA. At the first level of compaction, DNA is wrapped around octamers of core histone proteins to form arrays of nucleosomes. Nucleosomes are sufficient to block access to DNA, and cells must therefore manipulate nucleosomes in the course of activating the genome. Dramatic progress has been made in understanding the mechanisms by which nucleosomes are manipulated. In addition to the major core histones, most eukaryotic genomes also encode additional variant histones, which have some structural similarity. These are targeted to specific loci by coupling specialized nucleosome assembly pathways to DNA replication, transcription, or to developmental processes. We review evidence that nucleosome assembly pathways are interlinked with histone-modification systems, and may thereby perpetuate epigenetic chromatin states.

Current Topics in Developmental Biology, Vol. 74

0070-2153/06 $35.00
DOI: 10.1016/S0070-2153(06)74002-9

I. Introduction

Bulk DNA is packaged into nucleosomes containing the major H2A, H2B, H3, and H4 core histones. The structure of the nucleosome has been detailed with exquisite resolution (Luger *et al.*, 1997). This confirmed earlier studies that described the octamer as being composed of three subunits: a central tetramer of two H3:H4 dimers, flanked by a dimer of H2A:H2B on either side. These three subparticles form a ramp around which ~146 bp of DNA is wrapped. The subunit nature of the nucleosome is thought to be key to its properties during chromatin assembly. Specifically, nucleosomes are assembled in steps, where first the H3:H4 tetramer associates with DNA and then two dimers of H2A:H2B are added (Eickbush and Moudrianakis, 1978). Conversely, disassembly of a nucleosome must reverse these steps.

Studies of nucleosome dynamics have distinguished between assembly and positioning once associated with DNA. In many cases, changes in nucleosome positioning are critical for gene regulation (Wolffe, 1994). However, shifting a nucleosome means that DNA–histone contacts must be broken, and these contacts also hold the nucleosome together. Thus, the same processes that shift nucleosomes can lead to nucleosome disassembly. Therefore, while the structure of the nucleosome appears robust, *in vivo* it may often be much less stable. Cells appear to deal with this instability by regulating nucleosome assembly and disassembly. While the biochemistry of nucleosome assembly has been extensively reviewed (Nakatani *et al.*, 2004; Verreault, 2000), it has become clear that cells have a number of distinct activities that assemble nucleosomes during DNA replication, during transcription, or during particular developmental stages. We focus here on work that starts to elucidate how DNA replication and transcription deploy chromatin assembly pathways.

II. DNA Replication and Nucleosome Assembly

A. Bulk Chromatin is Packaged by Replication-Coupled Nucleosome Assembly

The requirement for nucleosome assembly during DNA synthesis has long been recognized and is the best-studied histone assembly system. Histones from parental nucleosomes are retained on newly synthesized daughter chromatids. These old histones appear to be transiently released and then recaptured by the daughter chromatid. The remaining gaps in the nucleosome array are filled by *de novo* nucleosome assembly (Jackson and Chalkley, 1985). Coupling of nucleosome assembly with DNA synthesis is critical for maintaining genomic stability and preventing DNA damage

(Ye *et al.*, 2003), and histone production during S phase responds to cell cycle cues to ensure supplies of free histones for new DNA (Zhao, 2004). Many of the proteins responsible for replication-coupled (RC) nucleosome assembly are conserved across eukaryotes, and human chromatin assembly factor (CAF) is the archetypal complex. It is a heterotrimer that binds histones H3 and H4 and targets them to DNA replication forks via interaction with the processivity factor proliferating cell nuclear antigen (PCNA) (Krawitz *et al.*, 2002; Marheineke and Krude, 1998; Martini *et al.*, 1998; Moggs *et al.*, 2000; Shibahara and Stillman, 1999; Smith and Stillman, 1989). Replication-coupling assembly factor (RCAF) is a second biochemically identified complex, which contains the ASF1 protein. ASF1 also interacts with H3 and H4 and cooperates with CAF1 in nucleosome assembly during DNA replication and repair (Mello *et al.*, 2002; Tyler *et al.*, 1999). The nucleosome particle is completed with the addition of two H2A/H2B dimers, which is thought to be transferred from the chaperone Nucleosome Assembly Protein-1 (Ito *et al.*, 1996). Finally, the addition of linker histones and an array of posttranslational histone modifications completes chromatin maturation.

B. Histone Modifications and Variants Distinguish Chromatin States: Road Signs Along Chromatin

All chromatin is packaged during replication, but there are important differences between chromatin regions. Distinct patterns for covalent modifications of histones are found throughout the genome. Modifications, such as hyperacetylation of histone tails and methylation at H3 lysine 4 and lysine 79 (H3K4Me and H3K79Me, respectively), correlate with gene activity, while hypoacetylation and methylation at H3 lysine 9 (H3K9Me) are associated with silencing. In many cases, these modifications serve as binding platforms for chromatin proteins. Additionally, functionally distinct histone variants are found at certain loci where they may set up specialized chromatin structures. Many modifications are catalyzed by transcription-associated enzymes and are probably a consequence of active transcription. However, the histone code hypothesis proposes that some histone modifications are the basis for heritable epigenetic traits (Jenuwein and Allis, 2001). In this scheme, some modifications would be the result of transcriptional states and also act to perpetuate them.

Histones arrive to the nucleus with modifications, and these must be reset as chromatin matures. A number of acetylations at lysine residues in newly synthesized histones have been described, and at least two of these have roles in controlling electrostatic interactions between histones or with DNA. In yeast, new H3 arrives in the nucleus acetylated at lysine 56 (H3K56Ac) (Masumoto *et al.*, 2005; Ozdemir *et al.*, 2005), which weaken

histone–DNA interactions. This acetylation is removed from most of the genome except for some active genes, where it may destabilize the nucleosome and promote transcription (Xu *et al.*, 2005). Similarly, new H4 is acetylated at lysine 91 (H4K91Ac) (Ye *et al.*, 2005). This acetylation is retained in active regions and decreases stability of the histone octamer. Thus, this modification may also promote transcription by structurally altering the nucleosome.

The functions of other predeposition modifications are less clear. Lysines 5 and 12 of H4, for example, are acetylated from *Drosophila* to humans (Chicoine *et al.*, 1986; Sobel *et al.*, 1995). One might therefore expect that these modifications facilitate histone deposition. However, mutations at lysines 5 and 12 of histone H4 do not reduce nucleosome assembly in yeast although an additional mutation at lysine 8 does (Ma *et al.*, 1998). The histone H4 and H3 N-terminal tails are also redundant for assembly (Morgan *et al.*, 1991). These redundancies make the functions of predeposition acetylation unclear. Some of these complexities appear to be due to the fact that multiple nucleosome assembly pathways package chromatin, as we discuss later.

C. Can RC Assembly Duplicate Chromatin States?

Considering that nucleosomes are disrupted and then reassembled during S phase, it is apparent that RC assembly must affect the transmission of chromatin epigenetic states to daughter cells. Several experiments have sought to determine the fate of nucleosomes as they are segregated onto daughter strands. The general conclusion is that nucleosome inheritance is a conservative process, where old H3:H4 tetramers are transferred to daughter DNA strands intact, the gaps being filled in by newly synthesized histone octamers (Henikoff *et al.*, 2004) H2A:H2B dimers, on the other hand, are randomly distributed onto new and old H3:H4 tetramers behind the replication fork (Gruss *et al.*, 1993; Jackson, 1987, 1990). Because of these inheritance patterns, it is difficult to see how histone modifications can be accurately retained in both daughter cells in specific chromatin segments. However, an alternative possibility is appealing—if each daughter strand inherits half a tetramer and then completed by the deposition of a new H3:H4 dimer, some histone modifications at every nucleosome could be retained and then copied to the new dimer. This semiconservative mode of nucleosome inheritance was dismissed by classic biochemical experiments, but has been recently resurrected by biochemical characterization of CAF deposition complexes. These contain H3:H4 dimers and not tetramers (Tagami *et al.*, 2004). In spite of this, it is clear that nucleosome assembly in bulk chromatin is conservative, and at best only a small fraction of chromatin

may inherit histones in a semiconservative manner during replication. As parental histones randomly distribute between daughter chromatids during replication, all specific histone modifications should be diluted as the replication fork passes, if unmarked new nucleosomes are deposited through the RC pathway. Consistent with this, gene silencing in yeast causes a gradual loss of active state H3K4Me and H3K79Me over a few cell divisions (Katan-Khaykovich and Struhl, 2005). Thus, most patterns of modifications in the genome cannot survive replication, and other mechanisms must perpetuate them.

One type of model proposes that replication fork complexes differ at certain sites in the genome (McNairn and Gilbert, 2003). In general, active euchromatic regions replicate early in the S-phase period, and heterochromatic silenced regions are replicated from late-firing origins. Distinctive components of late replication forks have been detected, including DNA methyltransferases and chromatin remodeler complexes. Distinct components might have the ability to modulate nucleosome assembly, thereby contributing to the repression of late-replicating regions. In support of this, expression of an ectopic reporter depends on whether it replicates in early or late S phase, implying the late replication is inherently repressive (Zhang *et al.*, 2002). Thus, one way to maintain chromatin states may be to alter replication timing of the locus.

A separate mechanism linking specific repressive H3 modifications and the RC pathway has been demonstrated (Sarraf and Stancheva, 2004). In this case, the MBD1 protein binds methylated DNA and recruits H3 histones premethylated at lysine 9 to the replication fork. This mechanism only deposits methylated H3 at DNA sequences that have high levels of DNA methylation, and both DNA methylation and histone methylation are required for gene repression. In this way, the RC assembly apparatus propagates epigenetic information to new chromatids. It will be interesting to see if other modifications are copied by similar mechanisms.

III. Replication-Independent Nucleosome Assembly

Although the RC pathway accounts for the bulk of nucleosome assembly during S phase of the cell cycle, a fraction of assembly occurs outside S phase (Ahmad and Henikoff, 2001, 2002). This has been termed replication-independent (RI) nucleosome assembly and specifically uses histone variants instead of canonical core histones. There are multiple RI assembly systems: one of the best-characterized examples uses the highly conserved H3.3 variant histone instead of the major H3 version. The consequences of RI assembly are particularly dramatic in long-lived neuronal cells, which no

longer undergo replication, and thus do not accumulate new H3 (Pina and Suau, 1987). However, assembly using H3.3 continues, and this eventually becomes the dominant subtype in neuronal chromatin. The basis of using H3 for RC assembly and H3.3 for RI assembly is due to three of the four amino acid residue differences between these two histones (Ahmad and Henikoff, 2002). While these residues presumably bind different assembly proteins before deposition, they are likely to have little effect on nucleosome structure.

A. Active Genes Undergo Transcription-Coupled Nucleosome Assembly

Experiments in *Drosophila* have shown that one RI system using H3.3 is a transcription-coupled (TC) nucleosome assembly pathway (Ahmad and Henikoff, 2002; Schwartz and Ahmad, 2005). Green fluorescent protein (GFP)-tagged H3.3 is distributed throughout the polytene chromosomes of *Drosophila*, implying that H3.3 deposition occurs at all transcribed genes (Schwartz and Ahmad, 2005). These experiments demonstrated that TC assembly occurs throughout the transcription units of induced developmental genes. However, other studies have detected H3.3 primarily in promoters of active genes (Chow *et al.*, 2005). These authors suggested that H3.3 deposition occurs mainly through the action of chromatin remodeling activities associated with transcription initiation, rather than elongation, although deposition was seen throughout transcription units in a few genes. Their results highlight the wide variation in chromatin constitution that occurs even among active genes.

A comprehensive and high-resolution chromatin immunoprecipitation study in *Drosophila* cells has clarified the genomic distribution of histone H3.3 (Mito *et al.*, 2005). Across roughly 2000 genes examined at nucleosome level resolution, H3.3 is generally concentrated in upstream regions and in the first few kilobases of transcription units. H3.3 enrichment tapered off along the body of the longer genes but was still detectable even beyond the annotated 3′ end. Importantly, the amount of H3.3 positively correlated with transcription level. The large number of genes surveyed in this study more accurately represents the distribution of H3.3 and allows one to speculate on the processes that led to deposition. Based on these studies, it appears that both promoter remodeling and RNA polymerase II (Pol II) elongation contribute to H3.3 deposition. We argue for two mechanistically distinct processes: in promoters, transcription factor binding may efficiently disassemble nucleosomes, and throughout transcription units Pol II elongation pushes histones off the template (Fig. 1). Nucleosomes in both regions appear to be restored by H3.3 RI assembly.

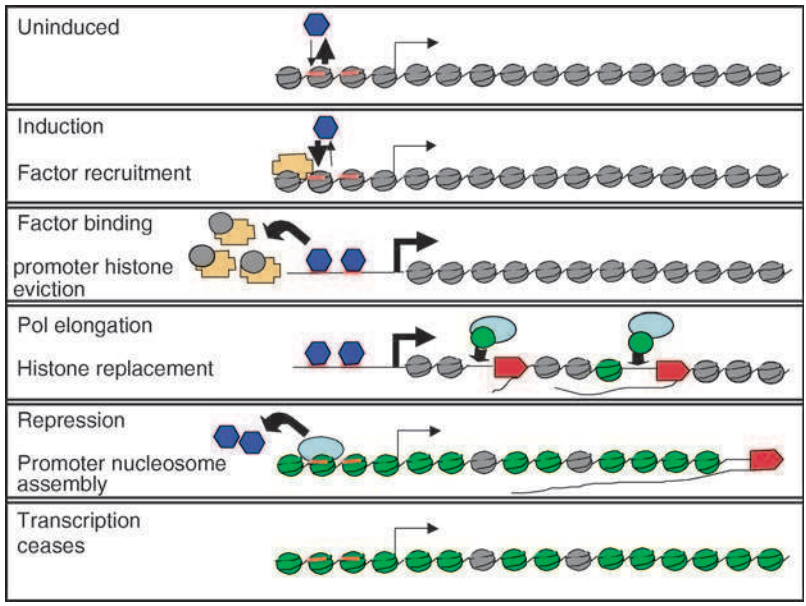

Figure 1 TC nucleosome assembly in promoters and in transcription units. In promoters, the probability of transcription factor binding depends on the availability of activator protein (blue hexagon) and the accessibility of the DNA binding site (red). Random fluctuations in nucleosome positioning may determine how often sites are exposed, but can be increased using chromatin remodelers or histone chaperones to destabilize nucleosomes. Histones will preferentially associate with DNA (free activator ⇀↽ DNA complex ⇀↽ free histones), but with chaperones present the equilibrium shifts, allowing activator binding and evicting histones (activator ⇀↽ DNA complex ⇀↽ histones ⇀↽ chaperones). RNA polymerase (red) elongation in active genes drives nucleosome replacement in transcription units, occasionally displacing histones at a rate that is proportional to transcription frequency. New H3.3 histones (green) are delivered to the gene by assembly factors (light blue), reassembling nucleosomes in the wake of the polymerase. Histone remodelers or chaperones that accompany the polymerase may have a role in disassembly and reassembly. Upon repression, activators leave DNA, and restoration of promoter nucleosomes occurs by RI assembly using histone H3.3.

B. Promoter Remodeling: Turning the Ignition

A long held notion is that nucleosomes can occlude the binding of transcriptional activators. However, even nucleosomes positioned over factor binding sites do not completely block binding. To understand how factors can bind to DNA packaged in nucleosomes, the dynamics of site exposure must be factored in (Polach and Widom, 1995). According to this model, DNA wraps and unwraps from a nucleosome in equilibrium. Thus, binding sites will usually be inaccessible but will be briefly exposed as DNA unwraps,

allowing factor binding. Because histones and factors are in competition for dynamically exposed promoter DNA, the presence of activators will displace histones. In this model, the enrichment of H3.3 in promoter regions results from rounds of activator binding alternating with reassembly of nucleosomes.

Although factor binding itself may be sufficient to displace nucleosomes, the probability may be low because initial site exposure is rare. One role for chromatin remodeling enzymes may be to increase nucleosome dynamics, shifting the equilibrium toward increased exposure. This increases the probability of factor binding and also promotes nucleosome disassembly. This model predicts that three major determinants of histone replacement in promoters are: (1) the frequency of site exposure, (2) the availability of factors, and (3) the activity of chromatin remodelers. These variables provide the combinatorial complexity required to selectively activate genes. One or more of these variables can be eliminated in cases where the speed of activation is crucial. The promoters of *Drosophila* heat shock genes contain a 200 bp segment that is constitutively nucleosome-free, owing to the combined action of the DNA-binding GAGA factor and NURF nucleosome remodeler enzyme (Costlow and Lis, 1984; Tsukiyama *et al.*, 1994). Because of this arrangement, the rate-limiting step in heat shock gene activation is the binding of activator, heat shock factor, which occurs within 30 sec of heat shock (Boehm *et al.*, 2003). Genome-scale profiling of nucleosome positions in yeast revealed that many gene promoters are nucleosome-free (Yuan *et al.*, 2005).

There are many other situations where positioned nucleosomes in promoters are used instead of nucleosome-free regions. For example, the yeast PHO5 promoter contains four positioned nucleosomes that prevent activators from binding their cognate sites under repressive conditions. Under inducing conditions, the Pho4p activator binds its site and this leads to hyperacetylation and then disassembly of the nucleosomes (Boeger *et al.*, 2003; Reinke and Horz, 2003). The CAF ASF1 is required for efficient disruption of promoter nucleosomes (Adkins *et al.*, 2004). In this situation, disrupting nucleosomes is a regulatory step in gene activation and serves as a model for how the displacement and reassembly of histones within gene promoters may exert antagonistic effects on gene expression.

C. Nucleosome Assembly in Transcription Units: Shifting Gears

Transcription regulation in eukaryotes requires the orchestrated recruitment of dozens of general and gene-specific factors, yet the underlying need for a TC nucleosome assembly machine can be inferred from simple *in vitro* experiments. In a purified transcription assay, Pol II cannot elongate

through chromatin templates unless nucleosomes are artificially disrupted with high salt concentrations (Izban and Luse, 1991, 1992; Kireeva *et al.*, 2002). This is in contrast to bacterial SP6, T7 RNA polymerase, and yeast RNA polymerase III, which in similar assays can traverse nucleosomal templates without the need for increased ionic conditions (Clark and Felsenfeld, 1992; Kirov *et al.*, 1992; O'Neill *et al.*, 1993; Studitsky *et al.*, 1994, 1995, 1997). Therefore, in the absence of auxiliary factors, the nucleosome acts as a barrier to transcription by Pol II and may be regarded as a form of gene regulation where the default state for most genes is "off." Cells have evolved a number of ways to overcome this impediment.

Early studies on actively transcribed chromatin suggested that nucleosomes within the body of the gene are perturbed during Pol II elongation since active genes display hypersensitivity to DNase I (Elgin, 1988). It has been difficult to determine, however, whether this hypersensitivity was due to histones being lost from DNA or merely becoming unfolded while remaining in contact with DNA. The heat shock genes have proven to be a useful model for addressing this question. *In vitro* nuclease digestion assays as well as dimethyl sulfate mediated protein–DNA crosslinking studies indicated that induced *HSP70* genes lacked histones (Levinger and Varshavsky, 1982; Levy and Noll, 1981; Wu *et al.*, 1979). Similar results were obtained by *in vivo* "protein-imaging" (Karpov *et al.*, 1984). However, later experiments using formaldehyde to crosslink proteins to the same gene revealed that H4 is mostly retained during activation although there appears to be a modest decrease compared to the inactive gene (Solomon *et al.*, 1988). Experiments that followed a pulse of GFP-tagged histone H3.3 showed that newly made histones are detectable at *HSP70* genes after a recovery from heat shock, arguing that at least some core histones were completely removed from DNA during transcription (Schwartz and Ahmad, 2005). A similar temporary eviction of histones was observed by Wirbelauer *et al.* (2005) on *Drosophila HSP70*, the yeast heat shock genes (Zhao *et al.*, 2005), and the activated yeast *GAL10* gene (Schwabish and Struhl, 2004).

How does one reconcile these results? It appears that active genes are in equilibrium between the two opposing processes of nucleosome disassembly and reassembly (Fig. 2). Potentially, each round of transcription has some chance of displacing histones. As the frequency of transcription increases, the probability of losing H3:H4 histones increases. For the vast majority of genes, nucleosome reassembly in the wake of an elongating polymerase is very efficient. However, the heat shock genes represent an extreme case of transcriptional activation, where nucleosome disassembly dominates. At fully induced *HSP70* genes, the rate of disassembly would outpace reassembly, and they would be stripped of histones at the height of activation. Lis and coworkers (O'Brien and Lis, 1991) have estimated that the activated *Drosophila HSP70* gene is fully occupied by elongating Pol II molecules. It is

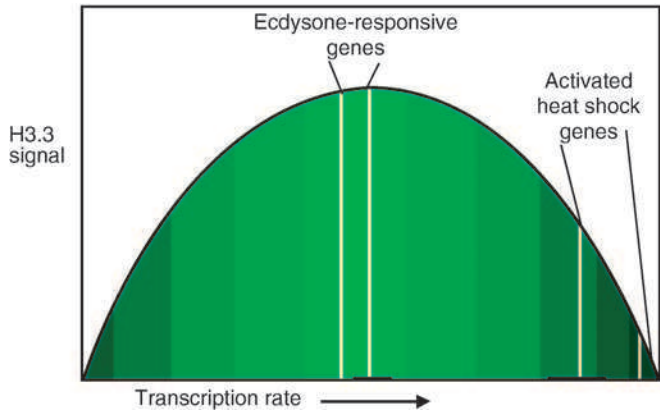

Figure 2 H3.3 deposition as a function of transcription rate. Chromatin of genes with low transcription rates rarely loses nucleosomes as RNA polymerase passes. At genes where the frequency of transcription is higher, nucleosomes are lost more frequently and H3.3 becomes progressively more enriched in chromatin. At very high transcription rates the rate of nucleosome loss exceeds the rate of nucleosome reassembly and the gene becomes depleted for all histones. Most genes are transcribed at low rates, the ecdysone-response genes are transcribed at moderate rates, and the *HSP70* genes are transcribed at very high rates.

unlikely that nucleosomes could be retained although it is not clear if this is due to the high density of polymerase, progression of the polymerase, or the activity of accompanying chromatin remodelers (Zhao *et al.*, 2005). However, after the heat shock transcription and disassembly cease, allowing nucleosome reassembly, newly made histone H3.3 can be detected at these loci. This sequence of chromatin states can explain the early *in vitro* experiments that could not detect histones during induction (Levinger and Varshavsky, 1982; Levy and Noll, 1981; Wu *et al.*, 1979).

It is important to point out that at most genes disassembly probably does not outpace reassembly. Rates of nucleosome reassembly have been measured during transcriptional repression of the *GAL10* gene in yeast, and it takes less than 1 min to completely restore nucleosomes to the body of the gene (Schwabish and Struhl, 2004). Our own analysis of polytene chromosomes from flies containing GFP-tagged H3.3 gave us an opportunity to visualize nucleosome assembly in a spectrum of genes with different rates of transcription. The moderately transcribed developmental genes *Eip74* and *Eip75* appear typical of most genes, and these loci were consistently and uniformly labeled with GFP when transcribed. We interpreted this as indicating that each round of transcription-mediated nucleosome disassembly is rapidly followed by deposition of H3.3. In contrast, while some new

histone could be seen at active *HSP70* genes, labeling varied in intensity and uniformity between cells (Schwartz and Ahmad, 2005 and Fig. 3). Perhaps in some cells nucleosome reassembly matches the rate of disassembly, enriching H3.3 at these genes, while in other cells slightly faster disassembly outpaces

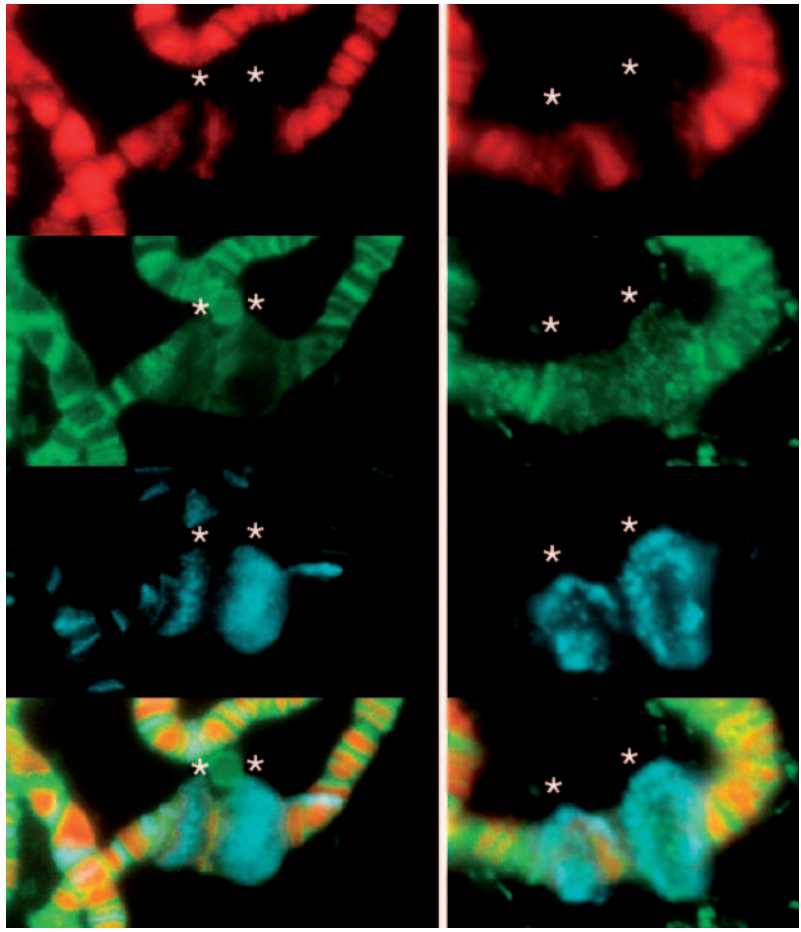

Figure 3 Variable intensity of H3.3-GFP in heat shock puffs. Two examples of HSP70 heat shock puffs in *Drosophila* polytene chromosomes during a 20 min heat shock treatment. Chromosomes were fixed and immunostained with antibodies against GFP (green) and the active form of RNA Pol II (blue). DNA is in red. The two loci containing *HSP70* genes are puffed during active transcription. In most chromosome spreads, the induced loci are coated with new H3.3-GFP. In other spreads, induced loci become depleted for histones. Depletion may result if nucleosomes are disassembled faster than they can be reassembled.

reassembly and leads to depletion of all histones from the locus. Additionally, the staining of some heat shock genes appeared qualitatively different from more moderately transcribed genes. This may be due in part to histones that were dislodged from chromatin but retained in the vicinity of the gene. In this regard, crosslinking and ChIP studies of histone dynamics *in vivo* may be ambiguous because displaced histones might be crosslinked to DNA with formaldehyde, which nonspecifically creates protein–protein and protein–DNA crosslinks (Solomon *et al.*, 1988).

D. Factors that Promote or Inhibit TC Assembly: One Foot on the Brake, One Foot on the Gas

Progress has been made in purifying predeposition complexes containing histone variants, including H3.3 (Tagami *et al.*, 2004). H3.3 predeposition complexes share many subunits with the H3 CAF complex, including ASF1 and the small CAF-1subunit, p48. One notable distinction is the histone regulator A (HIRA) protein, which is only found with H3.3. HIRA interacts with histones and had been previously identified in *Xenopus* egg extracts as a factor required for RI assembly (Ray-Gallet *et al.*, 2002). However, it is not clear that the HIRA-containing complex is responsible for TC histone deposition. In yeast, individual mutations in the HIR1/2 homologs have effects on gene silencing and more severe effects when combined with CAF mutations (Kaufman *et al.*, 1998; Sharp *et al.*, 2002). Perhaps HIR-dependent TC assembly suffices to package the transcriptionally active yeast genome in CAF-deficient cells, but the loss of both assembly pathways is lethal. However, HIR1/2 are also involved in transcriptional regulation of the histone genes, which may be the cause of synthetic interactions with CAF. Similarly, a role for HIRA in TC assembly is unclear in higher eukaryotes. There is as yet no evidence linking the HIRA-containing complex to transcription, and HIRA is known to play a role in another developmentally specific RI assembly process (see later). It is important to note that Tagami *et al.* also isolated H3.3-containing complexes that do not contain HIRA. This suggests that there may be multiple distinct RI pathways that use H3.3 in higher eukaryotes, and it is possible that another H3.3-specific complex performs TC nucleosome assembly.

Of known histone chaperones, both Facilitates Chromatin Transcription (FACT) and SPT6 may be key players in TC assembly. FACT permits Pol II transcription through chromatin by destabilizing H2A:H2B dimers in front of elongating Pol II and redepositing them after polymerase passage (Belotserkovskaya and Reinberg, 2004). This shuffling of dimers in active chromatin allows for free exchange between nucleosomes, and an enhanced mobility of H2A in active chromatin has been observed (Jackson, 1990).

SPT6 has been shown to interact directly with histone H3 in yeast (Bortvin and Winston, 1996). Furthermore, it localizes to transcribed loci in polytene chromosomes and is rapidly recruited to activated heat shock genes in *Drosophila* (Andrulis *et al.*, 2000; Kaplan *et al.*, 2000). FACT and SPT6 may therefore act to facilitate Pol II elongation by promoting nucleosome disassembly.

Three studies (Carrozza *et al.*, 2005; Keogh *et al.*, 2005; Rao *et al.*, 2005) raise the possibility that there are factors that also suppress TC disassembly and reassembly. The SET2 histone methyltransferase accompanies elongating RNA Pol II during transcription and methylates histone H3 at lysine 36 (H3K36Me) (Strahl *et al.*, 2002). This modification is not uniform throughout active genes but is enriched downstream of the promoter. H3K36Me is only found in active genes, but does not correlate with transcription rates, suggesting that a single passage of polymerase is sufficient to methylate the template (Rao *et al.*, 2005). Biochemical and genetic studies show that H3K36Me serves as a binding platform for the Eaf3 chromodomain, which in turn recruits the Rpd3S histone deacetylase complex. A deacetylase would accompany transcribing Pol II because histone deacetylation normally favors chromatin compaction and represses transcription. Mutant phenotypes for these components, however, suggest that they control nucleosome disassembly during transcription. Mutants in SET2, Eaf3, or at the H3K36 residue all reduce expression and cause improper transcription from cryptic promoters within genes (Carrozza *et al.*; Keogh *et al.*, both in press). This is reminiscent of *spt6* mutants, where transcription causes an aberrant depletion of histones within the bodies of genes and permits initiation from cryptic promoters (Kaplan *et al.*, 2003). Thus, the purpose of recruiting Rpd3S to genes may be to inhibit nucleosome disassembly by deacetylating chromatin. This would allow active genes to retain their original histones, and allow proper regulation of transcription *in vivo*. This is a clear example of the antagonism between polymerase-mediated disruption of nucleosomes and factors that modulate chromatin assembly.

This model is consistent with data showing that H3.3 deposition in *Drosophila* is higher in 5′ ends of genes than in 3′ ends, and is the inverse of H3K36Me patterns (Mito *et al.*, 2005; Wirbelauer *et al.*, 2005). The emerging theme is TC nucleosome disassembly and reassembly can be regulated, and we speculate that many components of the transcriptional machinery act as drivers or brakes to adjust the structure of the chromatin template.

E. The Intersection of TC Assembly and Chromatin States

H3 and H3.3 histones are deposited by RC and TC assembly pathways, respectively. These histones also differ in the abundance of histone modifications (McKittrick *et al.*, 2004). Bulk H3 is enriched for repressive

modifications, such as H3K9Me, and is generally hypoacetylated. The bulk of active modifications, such as H3K4Me and H3K79Me, are found on H3.3. At least part of these distinctions is due to the different localizations of the two histones in the genome (Schwartz and Ahmad, 2005). However, the different assembly pathways must also affect the stability and inheritance of histone modifications. Since new nucleosome assembly occurs throughout coding regions with transcription, any modifications on these histones will be removed as nucleosomes disassemble. This mechanism has been observed to act as a switch to activate genes in repressed chromatin, where repressive H3K9Me modifications are removed and replaced with H3.3 (Ahmad and Henikoff, 2002; Janicki *et al.*, 2004; Johnson *et al.*, 2005; Stopka *et al.*, 2005).

While histone replacement can act to switch genes from repressed into active chromatin, one can imagine that TC assembly specifically in active genes might also be involved in maintaining histone modifications. For example, if new histones are hypoacetylated in S phase but hyperacetylated in gap phases of the cell cycle, bulk chromatin will tend to be repressed because it is assembled only by RC systems in S phase. In contrast, since TC assembly occurs throughout the cell cycle, active chromatin would be enriched for acetylation. This model is analogous to the early/late replication-timing model for perpetuating states, except that it is the timing of nucleosome assembly that determines chromatin states.

The TC nucleosome assembly pathway is responsible for depositing new H3.3:H4 histones (Schwartz and Ahmad, 2005). However, it is unclear if TC assembly replaces dimers or tetramers since the predeposition complexes contain H3.3:H4 dimers (Tagami *et al.*, 2004). Therefore, if the H3:H4 tetramer is disassembled during transcription, some reassembled nucleosomes could contain a mixture of old and new H3:H4 dimers. In fact, this is observed if active chromatin is separated from bulk chromatin (Kumar and Leffak, 1986). Theoretically, this provides a mechanism for faithfully propagating active histone modifications onto new histones in active chromatin as suggested by Tagami *et al.* (2004) because here old histone dimers could serve as a template for copying identical marks on the new histones within the same nucleosome.

F. RI Nucleosome Assembly During Fertilization

A number of developmental transitions appear to involve global changes in chromatin composition. It has become clear that some of these changes are driven by RI nucleosome assembly activities. Two dramatic examples occur during spermatogenesis to package and then unpackage sperm chromatin. Spermatogenesis begins with the generation of spermatogonia from stem

cells, which undergo several rounds of mitotic divisions to form 16 primary spermatocytes. Primary spermatocytes are transcriptionally active and increase in cell volume many times before starting meiotic divisions. In postmeiotic cells, the chromatin becomes condensed and transcription ceases. The germ cells then progress through a series of sperm head elongation stages to become a mature sperm. Postmeiotic gametes undergo dramatic stepwise repackaging of their chromatin, where the major histones are replaced by protamines. These are small basic proteins that are thought to tightly pack the DNA into the sperm head. Once a sperm fertilizes an egg, protamines must be removed and replaced by maternally supplied histones to form the male pronucleus. The deposition and removal of protamines from the sperm genome are both RI processes.

Strikingly, the H3.3 histone variant plays a role in the deposition and removal of protamines. One of the more detailed studies of H3.3 deposition during spermatogenesis was performed in *Drosophila* (Akhmanova *et al.*, 1997). Using antibodies that discriminate between H3 and H3.3, they found that the nuclei of premeiotic germ cells consisted of H3-containing nucleosomes and H3.3 was not detectable until the transcriptionally active primary spermatocyte stage. Here, H3.3 was confined to the decondensed Y chromosome, while the autosomes maintained H3 as the dominant subtype. In postmeiotic cells, more H3.3 was deposited into chromatin and the abundance of this histone was maintained throughout spermatid elongation stages. A striking change occurred in spermatid cysts, when the H3.3 pattern became dispersed and uneven, suggesting that histones were being replaced by protamines at this stage. The authors were careful to acknowledge the possibility that this result could also be explained by the inability of the antibodies to penetrate the highly condensed chromatin. However, the existence of sperm protamines in *Drosophila* was demonstrated (Jayaramaiah Raja and Renkawitz-Pohl, 2005). Thus, it appears that gametic chromatin undergoes a global replacement of histones with H3.3, which is subsequently replaced with protamines. A similar progression occurs in mammals although a set of transition proteins (TP1 and TP2) follows H3.3 replacement and precedes protamine deposition. Deposition of H3.3-containing nucleosomes in this process represents yet another specialized and highly conserved nucleosome assembly pathway. These intermediates may be necessary because core histones and protamines tightly associate with DNA, and a direct transition is difficult.

A second deployment of H3.3 occurs during fertilization when protamines are stripped from sperm DNA and replaced with maternally stored histones. Cytologically, this process can be visualized as the global decondensation of the tightly packed sperm genome in the egg, forming the large male pronucleus. Decondensation is an RI process but is not accompanied by transcription (Wright, 1999). The maternal effect *sesame* allele in *Drosophila*

has been identified as a missense mutation in the *HIRA* gene (Loppin *et al.*, 2005). The *sesame* mutation blocks decondensation (Loppin *et al.*, 2000, 2001). Further studies showed that normal decondensation is accompanied by maternal histone H3.3 deposition. Thus, one function of the biochemically identified HIRA-H3.3 complexes is to remove protamines and replace them with H3.3. This is RI assembly but is not TC. Loppin *et al.*, 2005 have pointed that this is sufficient to generate global differences in histone modifications between maternal and paternal contributions to zygotes because the rounds of replacement in sperm nuclei eliminates all previous histone modifications. This may play a role in inheritance patterns of gametically imprinted genes. In any case, it is clear that there are multiple RI systems that use the H3.3 histone, coupled to different processes.

G. Targeting Centromeres with RI Nucleosome Assembly

An additional assembly system in eukaryotes specifically uses a different H3 variant from H3.3. Centromeres in all eukaryotes are packaged with a specialized H3 histone variant, collectively referred to as CenH3 histones (Malik and Henikoff, 2003). CenH3 histones are extremely diverged between species but retain sequence and structural features of the H3 family. Differences between H3 and CenH3 histones are thought to be important for signaling the position of the centromere, thereby directing nucleation of the kinetochore and spindle attachment at that site (Sullivan *et al.*, 2001). CenH3 histones in yeast, *Drosophila*, and mammals are deposited by RI assembly systems (Ahmad and Henikoff, 2001; Collins *et al.*, 2004; Shelby *et al.*, 1997). The reason why RI pathways are used may be that this is an effective way to target the variant to specific sites in the genome since RC assembly packages all chromatin. Although mutations that affect centromeric assembly have been identified, these systems remain poorly understood. In budding yeast, both *CAF-1* and *HIR1/2* mutants delocalize the yeast CenH3 histone Cse4p (Kaufman *et al.*, 1998; Sharp *et al.*, 2002). These factors are also involved in the deposition of histone H3. In fission yeast, the Mis6-Sim4 complex, including histone chaperones and GATA transcription factors, localizes to centromeres and is required for loading the SpCNPA histone (Takahashi *et al.*, 2005). However, the budding yeast and mammalian Mis6 homologs are not required for CenH3 targeting (Measday *et al.*, 2002; Nishihashi *et al.*, 2002). Finally, simple overexpression of CenH3 histone is sufficient to mislocalize the histone in mammals, *Drosophila*, and in budding yeast (Ahmad and Henikoff, 2001; Collins *et al.*, 2004; Sullivan *et al.*, 2001). These results led to the suggestion that CenH3 may not have specific assembly factors but use general factors in limited ways.

Targeting the centromere using general factors may rely on sequestering CenH3 histones in the nucleus. If predeposition CenH3 histones were localized near centromeres, they would only be available for deposition at those sites. CenH3 variants from a variety of species tend to localize in heterochromatin near centromeres in human and *Drosophila* cells (Henikoff *et al.*, 2000). Expression of the budding yeast Cse4 protein can even functionally substitute for CENPA in human cell lines (Wieland *et al.*, 2004). It remains unresolved how CenH3 histones are normally limited to centromeres, but the general factor model suggests that many centromeric defects in specific chromatin assembly mutants could be due to the indirect consequences of affecting other histones. For example, overexpression of H3 also reduces CenH3 function, suggesting that these two histones are in competition (Glowczewski *et al.*, 2000). These observations underscore the point that different nucleosome assembly pathways in a cell share histone substrates and must affect each other.

IV. Conclusions

It has become clear that there are many pathways for nucleosome assembly in cells. These systems differ in the histone types they use, the complexes that assemble nucleosomes, and the regions in the genome that they package. However, the phenotypes of mutants defective for specific nucleosome assembly factors suggest that remaining pathways could assemble enough nucleosomes for the whole genome. It was initially surprising that budding yeast *CAF* mutants are viable, since this appears to be responsible for most RC nucleosome assembly (Enomoto and Berman, 1998; Kaufman *et al.*, 1997). This tolerance may be because the small yeast genome is predominantly transcriptionally active and can assemble nucleosomes by TC pathways. *CAF* and *HIR1/2* double mutants do have greatly reduced growth rates (Kaufman *et al.*, 1998), suggesting that there is redundancy between the RC and TC assembly pathways for maintaining a normal density of nucleosomes in chromatin. In this model, elimination of the RC assembly pathway is not lethal because new DNA is packaged with TC assembly machinery. Functional redundancy for nucleosome assembly in higher eukaryotes must also exist because *CAF* mutants in *Arabidopsis* are viable (Kaya *et al.*, 2001).

While each pathway may be sufficient for nucleosome assembly, they are not equivalent. We have already discussed how RC assembly tends to dilute histone modifications over cell generations, while TC assembly drives more rapid dynamics of histone modifications in active genes. The multiplicity of assembly systems has additional implications for histone-modification states throughout the genome. Maintaining active state modifications requires ongoing transcription (Kouskouti and Talianidis, 2005). This requirement

can be understood by the fact that transcriptional machinery includes histone-modifying activities that improve binding of transcription complexes (Gerber and Shilatifard, 2003). These kinds of enzymes are thought to act at any time on stable chromatin. The use of a separate nucleosome assembly pathway at active chromatin provides two other mechanisms to maintain patterns of histone modifications. First, some modifications may be catalyzed onto predeposition histones, which are then delivered to active chromatin. Predeposition histones are acetylated at many residues, and these residues overlap with acetylation sites found in active chromatin (Chang *et al.*, 1997). It will be interesting to determine if other active state modifications are preset on free histones. Second, each specific assembly machinery may include histone-modifying activities that act only as nucleosomes are being assembled. Although no examples for active chromatin are known, one example where RC nucleosome assembly is interlinked with histone-modification states has been shown, where the SETDB1 H3 lysine 9 methyltransferase is recruited to replication forks (Sarraf and Stancheva, 2004).

Is it possible that nucleosome assembly pathways and histone modifications are generally interlinked? In both budding yeast and in *Arabidopsis*, elimination of the CAF assembly system is accompanied by defects in gene silencing (Kaya *et al.*, 2001; Krawitz *et al.*, 2002). Thus, compensation by alternative nucleosome assembly pathways alters chromatin states, presumably by affecting histone modifications. It is striking that silenced genes become derepressed in *CAF* mutants, as if using alternative pathways are producing chromatin that is more active than normal. Furthermore, the elimination of the histone H4K91 and H3K56 predeposition acetylations in yeast have very similar silencing defects as *CAF* mutants (Xu *et al.*, 2005; Ye *et al.*, 2005). These observations argue that histone-modification systems are interlinked with assembly pathways. Human cell lines appear exceptional, in that CAF knockdowns are lethal. However, it is not clear if lethality is due to a failure to assemble a normal density of nucleosomes or if human cells lacking CAF develop epigenetic defects that are lethal. Chromatin from CAF knockdown cells is more sensitive to micrococcal nuclease (Nabatiyan and Krude, 2004). This is consistent with failures in nucleosome assembly or with chromatin being generally more active and accessible. Since the normal RC assembly system must rapidly package newly synthesized DNA during S phase, lethality may also result if alternate systems are less efficient. CAF deficiencies in human cells do show extended S-phase periods (Ye *et al.*, 2003). This may obscure epigenetic defects in human cells lacking CAF.

There are likely to be additional roles for specialized nucleosome assembly pathways. We have focused on changes to the nucleosome involving the H3:H4 subunit of the nucleosome, but disassembly of the nucleosome also provides the opportunity to exchange H2A:H2B subunits. FACT is an example of a specialized chaperone that manipulates dimers to potentiate

transcription (Belotserkovskaya *et al.*, 2003). There are also numerous variants for H2A in most eukaryotes (Malik and Henikoff, 2003), and the conserved H2A.Z variant has roles in transcriptional regulation and chromosome segregation (Dryhurst *et al.*, 2004). Other biological processes are likely to require histone variants and specialized nucleosome assembly. For example, the consequences of double strand DNA breaks for chromatin has only been partially elucidated. DNA breaks recruit PCNA and CAF-1 proteins, and is therefore similar to S-phase RC assembly (Moggs *et al.*, 2000). However, H3 is only available in S phase, so DNA breaks induced in gap phases of the cell cycle cannot use this histone for new assembly. It stands to reason that H3.3 would be used to assemble new nucleosomes at breaks, and this may distinguish repaired chromatin from undamaged templates. It is clear that specialized nucleosome assembly using H2A variants play a large role in DNA repair. In a variety of species, the C-terminus of H2A.X becomes phosphorylated around a DNA break, which acts as a signal to recruit repair proteins and cohesins (Foster and Downs, 2005). The *Drosophila* H2A.X analog (H2Av) is similarly phosphorylated around DNA breaks and then acetylated by the Tip60 chromatin-remodeling complex (Kusch *et al.*, 2004). This complex then disassembles the modified histone from chromatin and replaces it with unmodified H2Av. Exchange may be important to attenuate DNA damage responses once the break has been repaired.

The multiple mechanisms that ensure the specific targeting of histone variants to discrete regions of chromatin attest to the importance of specialized histones in orchestrating many cellular processes. For processes, such as transcription, much is known and some fundamental principles regarding the influence of histone modifications on chromatin structure and gene expression are coming into focus. Still, we have only just opened the hood on nucleosome assembly machines, and many more discoveries will be made in coming years.

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Protein–Protein Interactions of the Developing Enamel Matrix

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Current Topics in Developmental Biology, Vol. 74

0070-2153/06 $35.00
DOI: 10.1016/S0070-2153(06)74003-0

Extracellular matrix proteins control the formation of the inorganic component of hard tissues including bone, dentin, and enamel. The structural proteins expressed primarily in the enamel matrix are amelogenin, ameloblastin, enamelin, and amelotin. Other proteins, like biglycan, are also present in the enamel matrix as well as in other mineralizing and nonmineralizing tissues of mammals. In addition, the presence of sulfated enamel proteins, and "tuft" proteins has been examined and discussed in relation to enamel formation. The structural proteins of the enamel matrix must have specific protein–protein interactions to produce a matrix capable of directing the highly ordered structure of the enamel crystallites. Protein–protein interactions are also likely to occur between the secreted enamel proteins and the plasma membrane of the enamel producing cells, the ameloblasts. Such protein–protein interactions are hypothesized to influence the secretion of enamel proteins, establish short-term order of the forming matrix, and to mediate feedback signals to the transcriptional machinery of these cells. Membrane-bound proteins identified in ameloblasts, and which interact with the structural enamel proteins, include Cd63 (cluster of differentiation 63 antigen), annexin A2 (Anxa2), and lysosomal-associated glycoprotein 1 (Lamp1). These and related data help explain the molecular and cellular mechanisms responsible for the removal of the organic enamel matrix during the events of enamel mineralization, and how the enamel matrix influences its own fate through signaling initiated at the cell surface. The knowledge gained from enamel developmental studies may lead to better dental and nondental materials, or materials inspired by Nature. These data will be critical to scientists, engineers, and dentists in their pursuits to regenerate an entire tooth. For tooth regeneration to become a reality, the protein–protein interactions involving the key dental proteins must be identified and understood. The scope of this review is to discuss the current understanding of protein–protein interactions of the developing enamel matrix, and relate this knowledge to enamel biomineralization.

I. Introduction

The extracellular assembly of the enamel organic matrix, and the subsequent process of enamel biomineralization, occurs in the extracellular space bounded by ameloblast cells and odontoblast cells or dentin. As is true for all extracellular biological matrices, much of the enamel organic matrix is assembled without direct contiguous cellular intervention. Enamel matrix assembly follows the example of basement membrane assembly. Reminiscent of enamel, the basement membrane is a structure formed through the contributions of multiple protein members and is a structure,

which assembles solely by virtue of information contained within the protein constituents themselves (Borradori and Sonnenberg, 1999; Ekblom *et al.*, 1998). Some basement membrane proteins contain multiple domains, with each domain contributing a unique interaction with another protein that leads to protein assemblies. The physiologic function of this basement membrane is dependent upon the assembled grouping of the membrane proteins (Borradori and Sonnenberg, 1999; Miner, 1998; Yamada and Kleinman, 1992). Unlike the basement membrane, enamel does not remodel nor does it remain in close contact with the cells that synthesize the enamel proteins. The cells that produce enamel (ameloblasts) move away from the forming matrix with simultaneous mineral deposition (Simmer and Fincham, 1995; Smith, 1998). Once enamel has matured, the ameloblasts remain latent until tooth eruption at which time ameloblasts are lost from the enamel surface to the oral cavity.

There are many unresolved issues within the complex of enamel proteins, including the relationship between an assembly of enamel proteins and their subsequent interactions on the mineral crystallite. Also unknown at present are the mechanisms by which the ameloblast remains in registry over a prescribed field of enamel organic matrix. Protein–protein interactions may guide each ameloblast movements during amelogenesis. As an example of this, data suggests that the adherens junctions in the ameloblasts are involved in their cell-to-cell movements by either limiting or allowing such activity (Nishikawa *et al.*, 1990). Proteomic studies that are aimed at identifying proteins that interact directly with the known enamel matrix proteins are currently being done (Paine *et al.*, 1998a; Wang *et al.*, 2005, in press). Methodologies used in such proteomic studies include the yeast two-hybrid (Y2H) system (Fields and Song, 1989; Paine *et al.*, 2002), surface plasmon resonance (Paine *et al.*, 2002), and other supportive techniques (Moradian-Oldak *et al.*, 2000; Paine *et al.*, 2002) that decipher protein-to-protein interactions. Recent data has demonstrated that enamel matrix proteins interact not only with secreted proteins of the enamel matrix but also a number of integral membrane proteins shown to be present on ameloblasts (Wang *et al.*, 2005, in press).

II. The Unique Mechanical Properties of Dental Enamel

Dental enamel is a composite bioceramic composed largely of a carbonated form of hydroxyapatite (HAP), dahlite, and small amounts of protein and water (Boyde, 1979; Lowenstam and Weiner, 1989). Enamel rarely undergoes catastrophic mechanical failure despite a lifetime of repeated masticatory, parafunctional, and occasional impact loading in a wet environment of varying pH. The durability of enamel is somewhat surprising given that it is

largely composed of weak and brittle HAP crystallites. The discussion of the mechanical properties of enamel presented here is focused primarily upon the human condition because the majority of this published mechanical data has been collected from human teeth.

Enamel structure reflects its two main mechanical functions, wear resistance and fracture resistance; but wear resistant engineered ceramics are usually brittle and easily fractured. We propose that enamel structure is designed to achieve a balance between these two opposing properties. The dominant rods orientation presents the component crystallites to the outer tooth surface in an approximately perpendicular orientation in order to reduce wear or to control wear faceting; however, interconnections and complex cleavage planes limit crack propagation and fracture but allow limited deformation (Boyde, 1997).

Human enamel is much tougher, approximately three times tougher, than crystalline HAP (White *et al.*, 2001). Enamel is somewhat more flexible, approximately 1.4 times more flexible, than crystalline HAP (Clark, 1966; Craig *et al.*, 1961; Habelitz *et al.*, 2001; White *et al.*, 2001; Xu *et al.*, 1998a; Yoon and Newham, 1969). Bulk enamel (Paine *et al.*, 2005) is substantially softer, approximately 0.6 times softer, than HAP. Despite the dominance of rod orientations, bulk enamel is only moderately anisotropic (White *et al.*, 2001). The amount of anisotropy probably reflects a balance between protecting enamel from the most common functional stresses as well as protecting it from less commonly directed functional or accidental stresses. Varying degrees of moderate anisotropy may help to direct stresses from the geometrically complex enamel occlusal surfaces to the resilient underlying dentin (Spears *et al.*, 1993; Xu *et al.*, 1998a). These data demonstrate the remarkable biological processing to form tough, flexible, relatively plastic, and functionally graded enamel from much weaker, stiffer, and harder HAP crystallites. The key to achieving these surprising mechanical properties lies in the complex levels of structural organization that, in turn, are a result of a highly coordinated, matrix-mediated mineralization process that requires key organic components.

Individual crystallites in human enamel are approximately 70×30 nm in cross section (Arends, 1978; Kerebel *et al.*, 1979). These are bundled together to form "rods" approximately 3–4.5 μm in diameter and to form sheets of "interrod." Crystallite orientations are highly organized within rods, and in rod to interrod connections, and within interrod. In bulk enamel, rods are largely surrounded by interrod to produce the characteristic "honeycomb" appearance. Interrod takes the form of an undulating sheet of HAP crystallites approximately 0.5 μm thick. Most of the crystallites within each rod run in a direction approximately parallel to the course of the rods. However, the interrod crystallites are at an angle of approximately 60° to the long axes of the rods, and transitional angulations are found in the connections between rod and interrod (White *et al.*, 2001).

Face-on or surface views show that the incisal surface and the lateral sides of rods are typically rounded, clearly defined, and surrounded by a sheet of interrod. However, in human enamel the apical surfaces of rods tend to be continuous with the interrod phase. Thus, rod and interrod form a single partly interrupted continuum. The incisal and lateral surfaces of the rods produce the outline of the characteristic "fish scale" appearance, but the apical surface or base of the "scale" is less well defined and becomes continuous with interrod.

Rods generally follow highly organized radial paths from the dentino-enamel junction (DEJ) in an outward and incisal direction to reach the tooth surface. Human enamel contains "Hunter-Schreger" bands (Hanaizumi *et al.*, 1998; Osborn, 1965). Each band or cohort is composed of approximately 10 rods at a large angle of decussation with adjacent cohorts. Within single bands or cohorts, adjacent layers of rods have a small angle of decussation. Therefore, human enamel has two separate levels of decussation.

The paths followed by the rods, and cohorts of rods, are determined by the paths of secretory ameloblasts as they migrate away from the DEJ to the outer tooth surface leaving organized proteinaceous matrices in their wakes (Boyde, 1987; Nanci and Warshawsky, 1984; Rinses, 1998; Smith, 1998; Warshawsky *et al.*, 1981; White *et al.*, 2001) (Fig. 1). The mineralization of crystallites and their organization within rod, interrod, the resultant continuum, and its defined interruptions are the direct reflections of the proteinaceous matrices that self-assemble following secretion by the ameloblasts (Boyde, 1987; Helmcke, 1967; Rinses, 1998; White *et al.*, 2001).

The complex matrix not only creates connections between rods, and between rod and interrod but also creates and maintains discontinuities. The importance of such discontinuities should not be underestimated. Protein remnants help to define the rounded discontinuity seen around the top of the "fish scale" pattern on rods viewed in cross section as well as the linear demarcations between rods viewed in long section. These proteinaceous remnants have very important mechanical functions. They define complex convoluted three-dimensional cleavage planes to deflect cracks, diffuse damage, and prevent catastrophic fracture (Boyde, 1997; White *et al.*, 2001). Wet proteinaceous remnants may also facilitate limited differential movement between adjacent rods, or stress reduction during dynamic loading, again preventing catastrophic failure (Haines, 1968; White *et al.*, 2001).

All mammalian enamel has many common components, including the ameloblast cells, which are wholly responsible for the generation of the enamel organic component, the individual proteins that comprise the enamel matrix, and the physicochemical composition of the inorganic component. The size and shape of the enamel crystallites are remarkably similar across mammalian species. However, human enamel differs from that of other commonly studied mammals (Koenigswald and Clemens, 1992). Some differences are due to the

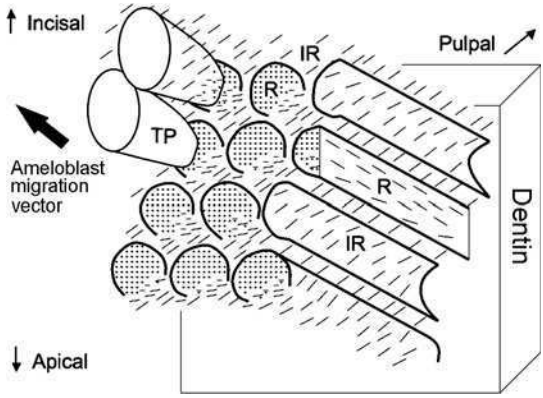

Figure 1 A schematic model of enamel microstructure as described by White *et al.* (2001). The paths of the ameloblasts, specifically of their trailing Tomes' processes, create a staggering between adjacent layers of rod (R), governing the possible spatial and temporal relationships among forming rods, and their possible connections. The orientation of the crystallites within rod and interrod is related to shape and orientation of the secretory sites of the Tomes' processes (TP) as well as to the vectors of movement of the ameloblasts. Rod is primarily contributed by the basal tip of the Tomes' process; whereas, interrod (IR) is contributed by the sides of the process, especially by the longer incisal surfaces. Rod slightly lags rod in formation and is largely formed within a sheath of interrod. The dominant rod and interrod crystallite orientations differ by an angle of approximately 60° in the sagittal plane. Boundaries or potential fracture planes are maintained at the incisal surfaces of the rods (solid black lines) where rod–interrrod continuity is limited, but the apical surfaces of the rods blend into a continuum with interrod.

migratory paths of ameloblasts, for example murine enamel has only a single large angle of decussation between alternating layers of rods. Other differences may be due to the morphology and configuration of the Tomes' processes and of the intracellular organization of the ameloblast, for example differences between rod and interrod organization or in their proportions (Salomon *et al.*, 1991; Warshawsky *et al.*, 1981; White *et al.*, 2001). Additional subtle differences may be related to differences among proteins, within proteins, or in their expression. The following components of this chapter address the proteins of the developing enamel matrix. The spatiotemporal dynamics of matrix formation and maturation govern enamel mineralization, organization, and mechanical function.

III. Proteins of the Enamel Matrix

Early attempts to define the chemical composition of human enamel proteins isolated the insoluble protein components of mature human teeth (decalcified enamel) (Hess *et al.*, 1953; Losee *et al.*, 1950; Stack, 1954). These early

attempts used either various microbiologic methods available at the time (Hess *et al*., 1953; Losee *et al*., 1950) or paper chromatography (Stack, 1954) to crudely define the amino acid composition; both methodologies resulting in similar data. Because of the mature stage of the enamel used in these studies, the bulk of the enamel proteins were not included nor considered. Early attempts to define the chemical composition of enamel proteins from forming enamel, using resin chromatography, isolated the acid-soluble proteins from upper central incisors collected from a 33-week human fetus (Eastoe, 1960, 1964). The amino acid composition was determined, and it was noted that the enamel proteins were unique to enamel and contained no collagen (Eastoe, 1960). Even in this early study, proline was attributed as composing 25% of the total amino acids in the enamel extract, a figure that today we can directly relate to the 28% proline content in human amelogenin. It appears that the acid-soluble component of the forming enamel matrix was collectively referred to as the amelogenin proteins, a name that entered the literature in 1965 (Eastoe, 1965a,b, 1966). Twenty years after the recognition that the enamel amelogenins represented a unique class of protein, the complementary DNA (cDNA) of mouse amelogenin was discovered (Snead *et al*., 1983, 1985), a protein that has high homology across all mammalian species.

Since the discovery of a cDNA sequence for murine amelogenin in 1983 (Snead *et al*., 1983), our understanding of enamel formation has been significantly aided by the subsequent discoveries of additional structural organic components essentially exclusive to the enamel extracellular matrix, including ameloblastin (Cerny *et al*., 1996; Fong *et al*., 1996; Lee *et al*., 1996), enamelin (Hu *et al*., 1997a), and possibly amelotin (Iwasaki *et al*., 2005). In addition, data from an animal model null for the *biglycan* (*Bgn*) gene (Xu *et al*., 1998b; Young *et al*., 2002) indicates that the biglycan protein, while not unique to the enamel matrix environment, plays a role in amelogenesis (Goldberg *et al*., 2002, 2005). Two enamel-specific proteases (kallikrein-4 and matrix metalloproteinase-20) have also been recently characterized and discussed (Bartlett *et al*., 1996; Caterina *et al*., 2002; Hu *et al*., 2000b). The spatiotemporal expression of each of these enamel proteins has been, or continues to be defined, but what remains to be investigated is how each of these enamel matrix components interacts with one another to form a self-assembled matrix competent to initiate and orchestrate the events of mineralization. These events of mineralization ultimately result in mature enamel that is almost completely absent of any history of its protein origins. The important role that each individual protein plays toward the creation of prismatic enamel can be appreciated from the well-ordered hierarchical structure seen in mature enamel (Paine *et al*., 2001), but their individual roles in creating this elegant architecture has yet to be fully illuminated.

Forming enamel is a dynamic composite of a number of individual components, and undoubtedly many more than those discussed. In this review chapter we have limited our focus to the organic extracellular components without discussing the mineral components in any depth. Many other genetic factors clearly impact on amelogenesis. Amelogenin, ameloblastin, enamelin, and amelotin represent those proteins whose expression remains essentially unique to the mineralized tooth structure, primarily to the enamel, but also, albeit at much lower levels, in dentin during odontogenesis (Begue-Kirn *et al.*, 1998; Iwasaki *et al.*, 2005; Lee *et al.*, 2003; Nanci *et al.*, 1998). The expression of biglycan is not unique to enamel, but biglycan does play a significant role in amelogenesis and enamel biomineralization (Goldberg *et al.*, 2002, 2005). It is apparent that the timing during which these gene products are presented to the enamel matrix must be finely controlled and regulated. Understanding the regulatory mechanisms of their gene transcription and identifying the various transcriptional factors that govern mRNA expression are of major significance in the quest to understand amelogenesis. In the laboratory this requires careful dissection of gene promoter regions. This work has just begun for amelogenin (Adeleke-Stainback *et al.*, 1995; Chen *et al.*, 1994; Snead *et al.*, 1996, 1998; Zhou and Snead, 2000), ameloblastin (Dhamija and Krebsbach, 2001; Dhamija *et al.*, 1999), and biglycan (Ungefroren *et al.*, 1998, 2003), but still has to be initiated for enamelin and amelotin. Posttranscriptional modifications to enamel proteins such as alternative splicing (Simmer, 1995) and posttranslational modifications such as phosphorylation (e.g., in amelogenin and enamelin (Fincham *et al.*, 1994a; Fukae *et al.*, 1996; Hu *et al.*, 2000a)) ensure that, even within these five secreted enamel proteins (amelogenin, ameloblastin, enamelin, amelotin, and biglycan), functional diversity from a single gene is possible. Because of the limited tissue expression of these enamel proteins, it appears that their removal from the enamel matrix during enamel maturation has required specific proteases whose spatiotemporal expression must also be exquisitely regulated. This need is met partially or fully with the serine protease kallikrein-4 (KLK4) (Hu *et al.*, 2000b; Nelson *et al.*, 1999; Ryu *et al.*, 2002) and matrix metalloproteinase-20 (MMP20) (Bourd-Boittin *et al.*, 2005; Caterina *et al.*, 2002; Li *et al.*, 1999), both of which are relatively specific to the enamel matrix.

There has been much interest in the evolution of the enamel matrix proteins with the realization that many proteins of mineralized tissues, including enamelin, ameloblastin, and amelotin, map to a relatively small region on the q arm of human chromosome 4 (4q13) (Huq *et al.*, 2005). It has been proposed that the *enamelin*, *ameloblastin*, and *amelogenin* genes arose from a single ancestral gene from which a gene duplication generated the *amelogenin* gene that was then translocated to the X and Y chromosomes, while *enamelin* and *ameloblastin* remained on this original chromosome

(Iwase *et al*., 2003; Kawasaki and Weiss, 2003; Sire *et al*., 2005). The common ancestral gene proposed is *SPARCL1*, located on human chromosome 4q22.1, which is a close relative of *SPARC* (also known as *osteonectin*) (Delgado *et al*., 2001; Kawasaki *et al*., 2004; Sire *et al*., 2005). *Amelotin* may also be a distant relative of *SPARC1*, but the discovery of *amelotin* is so recent (Iwasaki *et al*., 2005), that this type of phylogenic analysis is yet to be done on amelotin.

A. Amelogenin

Amelogenin is the most prevalent protein in the developing enamel extracellular matrix. In humans, an *amelogenin* gene exists on both the X (*AMELX*; locus Xp22.3-p22.1) and Y (*AMELY*; locus Yp11) chromosomes (Lau *et al*., 1989). Both of the human *amelogenin* genes contain 7 exons. In males, while both the X and Y chromosomal-derived amelogenins are expressed, it is the X-chromosome–derived amelogenin that predominates (Salido *et al*., 1992). Recently it has been shown that a more extravagant exon architecture exists in the mouse and rat genomes, with 2 additional exons (exon 8 and 9) being described that are probably infrequently expressed through alternative splicing (Baba *et al*., 2002; Li *et al*., 1998; Papagerakis *et al*., 2005). Amelogenin is absolutely essential for the organic extracellular assembly of the enamel matrix. Humans affected by the inherited enamel defect *amelogenesis imperfecta* (AI) often exhibit alterations in the amelogenin X-chromosome gene locus affecting proper amelogenin expression (Wright *et al*., 2003). *Amelogenin* knockout mice also display an extremely severe AI phenotype (Gibson *et al*., 2001). Enamel phenotypes resulting from gene mutations are broadly characterized as hypoplastic or hypomineralized (Hart *et al*., 2000; Wright *et al*., 2003) by researchers and dental practitioners. Hypoplastic enamel or hypomineralized enamel implies that the defect is uniformly displayed throughout the enamel, yet in patients with AI this is rarely the case and is an often-confounding aspect of enamel developmental biology.

The supramolecular assembly of amelogenin into ''nanospheres'' (Du *et al*., 2005; Fincham and Moradian-Oldak, 1995; Veis, 2005) has been assumed to be critical for the function of this structural protein during enamel formation. Two human pedigrees with an X-linked AI (AIH1) phenotype (Collier *et al*., 1997; Lench and Winter, 1995) have point mutations in the amino-terminal, tyrosine-rich amelogenin peptide (TRAP) segment of amelogenin (circa amino acid residues 1–44). Both of these documented AIH1 point mutations have been experimentally reproduced as recombinant proteins and, by comparing these mutated amelogenins to wild-type amelogenin, altered nanosphere dimensions (Moradian-Oldak

et al., 2000), and altered amelogenin assembly kinetics (Paine *et al.*, 2002) were observed. Amelogenin nanosphere assembly is also observed adjacent to HAP crystallites during *in vivo* enamel formation (Fincham and Moradian-Oldak, 1995; Robinson *et al.*, 1981) suggesting that amelogenin self-assembly is an essential property required to direct the mineral phase. The hydrophilic carboxyl terminal of amelogenin binds HAP as demonstrated *in vitro*, and this suggests that the carboxyl-terminal region facilitates initial orientation of amelogenin along the forming enamel crystallites (Aoba *et al.*, 1989; Iijima *et al.*, 2002; Kirkham *et al.*, 2000; Shaw *et al.*, 2004).

Based upon this information, the Y2H assay has been used to search for amelogenin-to-amelogenin interacting peptide domains (Paine and Snead, 1997). This experimental strategy revealed that the mouse amelogenin self-assembly was dependent upon the amino-terminal residues 1–42 (domain A) and the carboxyl-terminal residues 157–173 (domain B) (Paine and Snead, 1997). Domain A also includes the phosphorylated serine-16 site, which has also been implicated in enamel biomineralization (Fincham *et al.*, 1994a; Torres-Quintana *et al.*, 2000). Amelogenin self-assembly domains are also relevant to the formation of normal enamel *in vivo,* as demonstrated by transgenic animal studies (Paine *et al.*, 2000b).

B. Enamelin

cDNAs to messenger RNAs (mRNAs) for porcine enamelin were first cloned and characterized in 1997 (Hu *et al.*, 1997a). In the year 2001, the human and mouse enamelin cDNAs were also cloned and characterized (Hu and Yamakoshi, 2003; Hu *et al.*, 2001). The human gene for *enamelin* (*ENAM*) maps chromosome 4q21 (Dong *et al.*, 2000; Hu *et al.*, 2000a), as does the *ameloblastin* gene (Krebsbach *et al.*, 1996; Mardh *et al.*, 2001). In mice both genes are syntenic to chromosome 5 (5E1–5E2) (http://www.ncbi.nlm.nih.gov/). Human *enamelin* is a 9 exon-containing gene, and is secreted as a 186 kDa precursor phosphorylated glycoprotein which, once secreted, undergoes a series of proteolytic cleavages (Fukae *et al.*, 1996; Hu *et al.*, 1997a, 2000a). Rajpar *et al.* (2001) analyzed a family with an autosomal dominant, hypoplastic form of AI (AIH2) and found that the *enamelin* gene had a mutation in the splice donor site of intron 7 of *enamelin*. The position of this mutation appears to impact mRNA splicing, and subsequently protein expression. A second family has been described with a mutation in the *enamelin* gene at an exon–intron boundary resulting in AIH2 (Kida *et al.*, 2002; Kim *et al.*, 2005a). Mardh *et al.* (2002) have described a nonsense mutation in the N-terminal region of the *enamelin* gene that effectively generated an *enamelin*-null phenotype also causing AIH2. The most recent reports of mutations to the *enamelin* gene resulting in an autosomal-dominant AI phenotype are from

Kim *et al.* (2005a). All these data imply the importance of enamelin in amelogenesis, although the particular role that this protein plays is yet to be established (Hu and Yamakoshi, 2003). The authors Snead and Lau (1987) made a similar prediction for morbid anatomy of amelogenin as the molecular biology of the amelogenin locus was explored in the 1980s. One suggestion has been that enamelin has a role to play in crystallite elongation; this predicted function is based upon the presence of the fully mature and intact enamelin at the mineralization front of enamel (Hu *et al.*, 1997a; Kim *et al.*, 2005a).

In an elegant study in which the chemical agent *N*-ethyl-*N*-nitrosourea (ENU) was used to generate large-scale and phenotype-driven mouse mutant lines, three unique lines were studied in which mutations to the *enamelin* gene resulted in alterations to the enamel resembling clinical cases of AI (Masuya *et al.*, 2005). The detailed protocol for ENU mutagenesis is available at http://www.gsc.riken.go.jp/Mouse/. The identification of *enamelin* as the cause of these phenotypes was by using linkage analysis which suggested that each of the three mutations occurred in a region of chromosome 5 that contained both *enamelin* and *ameloblastin*. Sequencing of the coding regions and splice sites of these enamel proteins followed. Missense mutations were identified in two lines, while the third line included a T to A substitution at the splicing donor site of intron 4 which resulted in a premature stop codon (Masuya *et al.*, 2005). This methodology is a powerful search-tool that uses a novel approach to study the genotype–phenotype relationship. Thus, both human and animal data suggest that enamelin is an essential component of the enamel matrix, and is necessary for the correct biomineralization of this enamel matrix to mature enamel.

C. Ameloblastin

Ameloblastin was simultaneously characterized by three different groups of investigators, two groups using rat incisors (Cerny *et al.*, 1996; Fong *et al.*, 1996; Krebsbach *et al.*, 1996) and one group using porcine teeth (Hu *et al.*, 1997b). Ameloblastin is a tooth-specific glycoprotein that is expressed in secretory ameloblasts but its expression lessens during enamel maturation (Cerny *et al.*, 1996; Fong *et al.*, 1996; Fukumoto *et al.*, 2004; Krebsbach *et al.*, 1996; Uchida *et al.*, 1997, 1998). The human *ameloblastin* (*AMBN*) gene has been localized to chromosome 4q21 and contains 13 exons (Krebsbach *et al.*, 1996; Mardh *et al.*, 2001). As is true for amelogenin, multiple isoforms of ameloblastin exist in the developing enamel of all species studied and each isoform may serve a unique physiological role (Lee *et al.*, 2003; MacDougall *et al.*, 2000; Simmons *et al.*, 1998). Immunologic identification of ameloblastin during secretory amelogenesis (enamel formation) (Hu *et al.*, 1997b; Nanci *et al.*, 1998) reveals an amelo-

blastin distribution within the enamel extracellular matrix that follows the ameloblast outline, resulting in a "fishnet"-like partitioning (Snead, 1996). Ameloblastin can also be immunolocalized to Tomes' process, the highly specialized plasma membrane component of secretory ameloblast cells. The rat and mouse ameloblastin molecule has a "DGEA" domain (Cerny *et al.*, 1996) that has been identified in collagen type I as a recognition site for $\alpha 2\beta 1$ integrin (McCarthy *et al.*, 2004; Staatz *et al.*, 1991), as well as a thrombospondin cell adhesion domain, "VTXG" (Yamada and Kleinman, 1992). These data prompted the suggestion that ameloblastin might serve as part of the linkage between ameloblasts and the enamel extracellular matrix (Cerny *et al.*, 1996; Snead, 1996), however neither of these peptide domains exists in the human or porcine ameloblastin proteins. An animal model, in which the overexpression of ameloblastin in the enamel organ resulted in a phenotype characteristic of AI, has also recently been published (Paine *et al.*, 2003). More recent data from a murine *ameloblastin*-null animal model adds additional support to the notion that ameloblastin acts as a cell adhesion molecule as it is required for the maintenance of ameloblast differentiation state (Fukumoto *et al.*, 2004). Human linkage data is yet to identify mutations in the *ameloblastin* gene that result in an AI phenotype. Undoubtedly this human genetic data will be forthcoming with the animal data already suggesting that either gain-of-function or loss-of-function for ameloblastin in the enamel matrix can result in disruptions to normal enamel formation. Ameloblastin has also been suggested to serve as a nucleator of crystallization, this because it is expressed at mineralization initiation sites within enamel (Dhamija and Krebsbach, 2001; Nanci *et al.*, 1998).

D. Amelotin

Discovered just recently, murine amelotin is the newest described enamel-specific protein (Iwasaki *et al.*, 2005). Amelotin cDNA fragments were initially isolated by a differential display polymerase chain reaction (PCR) analysis of microdissected dental tissues from 10-day-old mice, where the gene fragment appeared to be uniquely expressed in ameloblasts. Homology searches using this partial sequence in various bioinformatics databases revealed its identity as clone 5430427O21Rik, which had been obtained as a result of large-scale cloning and sequencing efforts of cDNAs expressed in murine 6-day neonate head tissue (Okazaki *et al.*, 2002). The sequence of the murine amelotin appears to be unique and shows significant similarity only with its predicted orthologues from human, rat, cattle, and dog. The encoded 22 kDa protein displays an N-terminal signal sequence typical of secreted proteins, but the processed protein likely undergoes few, if any,

posttranslational modifications. No other conserved motifs are obvious in the protein sequence. The protein is indeed effectively secreted in cultured cells (Iwasaki *et al.*, 2005), but more detailed ultrastructural immunohistochemical studies are required to determine the localization of the amelotin protein *in vivo*. Both *murine* and human *amelotin* genes consist of 9 exons and 8 introns, and are located on chromosome 5 and 4q13.3, respectively, thus localizing in a cluster close to the *ameloblastin* and *enamelin* genes. The expression of the approximately 1.2 kb amelotin mRNA transcript is, at least in mice, essentially restricted to postsecretory stage ameloblasts; a sharp increase in expression levels occurs at the transition from secretory to maturation stage ameloblasts, and this high expression gradually declines toward the zone of reduced ameloblasts. Although Northern analyses do not suggest the existence of alternative splice variants, the relatively small size of some exons calls for a more thorough analysis of this issue. Studies on the amelotin promoter, like functional studies on the recombinant protein, are currently underway (in the laboratory of author Bernhard Ganss) to understand the reasons for its highly restricted expression, and to determine if amelotin is indeed a structural protein that assists in the formation of the enamel ultrastructure, or rather a proteolytic enzyme that is involved in the degradation of the enamel organic matrix. The potential role of *amelotin* as a candidate gene for AI also remains to be confirmed.

E. Biglycan

The human *biglycan* (*BGN*) gene contains 8 exons and is located on chromosome Xq28 (Fisher *et al.*, 1991; Traupe *et al.*, 1992). The protein encoded by this gene is a small cellular or pericellular matrix proteoglycan that is closely related in structure to two other small proteoglycans, decorin and fibromodulin (Hardingham and Fosang, 1992; Young *et al.*, 2002). Decorin contains one attached glycosaminoglycan (GAG) chain, while biglycan is thought to contain two GAG chains (hence the name biglycan). Biglycan is thought to function in connective tissue metabolism by binding to collagen fibrils and transforming growth factor-β1 (TGF-β1) (Redini, 2001; Tasheva *et al.*, 2004b). High levels of TGF-β1 mRNA and protein have been localized in developing cartilage, bone, skin, and teeth, suggesting that they play a role in the growth and differentiation of these tissues (Dickinson *et al.*, 1990; D'Souza *et al.*, 1990; Vaahtokari *et al.*, 1991). In the first molar tooth germs of newborn mice, biglycan labeling is very strong in the stratum intermedium, and still visible to a lesser degree in the central and distal cell body of secretory ameloblasts. This pattern is not detectable in presumably matched transverse sections of incisor teeth.

Biglycan-null (*Bgn*-null) mice have been generated to study the role of biglycan (Bgn) *in vivo* (Xu *et al.*, 1998b). These transgenic animals appear normal at birth, but as these mice age they display a phenotype characterized by reduced growth rate and decreased bone mass. While this type of phenotype is commonly observed in specific collagen-deficient animals, it is rarely observed in skeletal abnormalities in animals lacking noncollagenous proteins. Biglycan is also expressed in teeth, including dentin and enamel (Septier *et al.*, 2001). Goldberg *et al.* (2002, 2005) studied these *Bgn*-null animals with an interest in how dentin is impacted by a *Bgn*-null genotype/phenotype. In addition to changes to the dentin (hypomineralization), significant changes were noted in the enamel of these animals. Biglycan is expressed in ameloblast cells during normal tooth development, and is present in the enamel extracellular matrix (Goldberg *et al.*, 2002). In *Bgn*-null mice the forming enamel (immature enamel) is about sevenfold thicker than normal control animals. Ultrastructural examination of the enamel of these *Bgn*-null animals has shown that the enamel is mostly formed in its outer zone by interrod enamel, with the rods being filled later (Goldberg *et al.*, 2005). Protracted Tomes' processes fill rod spaces, together with increased quantity of stippled material. The differences between these *Bgn*-null and wild-type animals may be explained by two different phenomena. First, ameloblasts move gradually backward in a synchronized movement with enamel formation. In the *Bgn*-null mice, secretory ameloblasts withdraw probably more rapidly, and may be under a greater migratory influence by the cells of the stratum intermedium where very high biglycan labeling is observed. Second, the level of amelogenin synthesis (and secretion) may be enhanced in secretory ameloblasts and also in odontoblasts (Goldberg *et al.*, 2002). From their observations, Goldberg *et al.* (2002, 2005) concluded that biglycan either directly or indirectly acts as a repressor of amelogenin expression during amelogenesis. However, in the *Bgn*-null adult mice, no difference in the enamel structure and its thickness could be detected when compared to the wild-type animals. Therefore, this effect seems to be either transitory or gradually reduced (restricted) by compensatory mechanisms of ameloblasts and the surrounding cells. Other observations from these *Bgn*-null animals include dentin sialophosphoprotein downregulation, and dentin matrix protein-1, integrin-binding sialoprotein [bone sialoprotein (BSP)], and osteopontin upregulation in secretory ameloblasts (Goldberg *et al.*, 2005).

All these data support, first, that biglycan plays a role as a tooth-related extracellular matrix component, with the staining pattern visible not only inside or around ameloblast cells but also in the predentin (Goldberg *et al.*, 2005). Previous data using radiosulfate labeling (Blumen and Merzel, 1976), histochemical investigation with lectins (Jowett *et al.*, 1992, 1994; Nakai *et al.*, 1985), ultrastructural histochemistry (Goldberg and Septier, 1986),

and detection with enzyme–gold complex (Chardin *et al*., 1990) support the presence of sulfated glycoconjugates also in the forming enamel, at least in the outer zone where enamel is just secreted and in the process of lengthening. This labeling is seen during early formation, and disappears when enamel reaches a certain thickness. Second, many matrix molecules are recognized as being multifunctional. It has been reported that biglycan is involved in cell signaling, or at least takes part in the TGF-β1 pathway (Dickinson *et al*., 1990).

In the various tissues that have been studied so far, two different isoforms of biglycan have been identified: these being a nonproteoglycan and a proteoglycan form. Biglycan, thus, can be considered as a ''part-time'' proteoglycan that may sequester TGF-β1 in the extracellular matrix, while the fully processed proteoglycan form may regulate collagen fibrillation (Hocking *et al*., 1996; Neame and Kay, 2000). The composition of the GAGs population (including biglycan) may vary from one tissue to another, and consequently, the affinity to binding with other extracellular matrix molecules (Grzesik *et al*., 2002; Wegrowski *et al*., 2000). We suggest that in tissues that do not contain collagen, such as forming enamel, biglycan may also contribute to regulate the three-dimensional organization of matrix components, through interactions with other matrix molecules (Paine *et al*., 2001; Wang *et al*., 2005).

The mechanisms of interaction between biglycan and TGF-β1 have been elucidated to some extent. TGF-β1 interacts with some of the TGF-β receptors, and certain signaling molecules and transcription factors. Gene products implicated in this cascade include Smad2 and Smad3, Mapk14 and Runx2 (Aberg *et al*., 2004; Bronckers *et al*., 2005; D'Souza *et al*., 1999; Groth *et al*., 2005; Ungefroren *et al*., 2003; Watanabe *et al*., 2002). TGF-β1 influences *biglycan* gene transcription and expression (Heegaard *et al*., 2004), as well as other *small leucine-rich proteoglycan* (*SLRP*) genes, which in turn can influence TGF-β1 activity (Groth *et al*., 2005; Heegaard *et al*., 2004; Tasheva *et al*., 2004a,b; Ungefroren *et al*., 2003, 2005). It has been also recognized that biglycan is a new extracellular component to the Chordin-BMP4 signaling pathway (Moreno *et al*., 2005). Biglycan, through its expression, is also implicated in cell adhesion, growth, and survival. In this context, it has been shown that biglycan decreases the levels of caspase 3 (CASP3) in mesangial cells (Schaefer *et al*., 2003). These activities related to biglycan expression are yet to be explored in secretory ameloblasts.

The variations in the degree of sulfation, and the secondary structure of biglycan, seem to be crucial with respect to the potential functions of biglycan. In this context, nothing is yet known on the specificity of tooth proteoglycans, their secondary structure, and on the nature of the GAGs that are associated with the core protein.

F. Other Proteins of the Enamel Matrix

The enamel tuft protein(s) (including tuftelin) (Deutsch *et al.*, 1987, 2002; Robinson *et al.*, 1975, 1989), tuftelin-interacting protein 11 (TFIP11) (Paine *et al.*, 2000a; Wen *et al.*, 2005), and a glycosylated and sulfated "nonamelogenin" protein of 65 kDa (Nanci *et al.*, 1996b, 1998; Smith and Nanci, 1996; Smith *et al.*, 1995) have also been described as components of the enamel matrix. Tuftelin, while not unique to ameloblast cells or the enamel matrix, was originally identified and classified as an enamel matrix protein (Deutsch *et al.*, 1997). Tuftelin is an acidic protein originally identified from a tooth-derived cDNA library, and shown to be present in the developing and mature extracellular enamel (Deutsch *et al.*, 1991). Much of the history related to the discovery of the *tuftelin* gene (*TUFT1*) and its resulting protein, in various species, has been described in a number of publications including recent reviews (Bashir *et al.*, 1998; Deutsch *et al.*, 2002; MacDougall *et al.*, 1998; Mao *et al.*, 2001). The human *tuftelin* gene localizes to chromosome 1q21. To date, all published tuftelin immunolocalization data suggest that tuftelin is a product of a wide range of cell types, including cells responsible for mineralizing hard tissues (including the enamel extracellular matrix), as well as cells responsible for nonmineralizing tissues (Bashir *et al.*, 1998; Deutsch *et al.*, 2002; Luo *et al.*, 2004; MacDougall *et al.*, 1998; Mao *et al.*, 2001; Paine *et al.*, 2000a; Saarikoski *et al.*, 2002). Under physiologic circumstances the amelogenin secretory pathway in secretory-phase ameloblasts is from the Golgi apparatus to secretory granules, and then to the Tomes' processes (Arana-Chavez and Nanci, 2001). One would expect the transport of tuftelin to the extracellular space to follow this same route. However, *in vivo* localization data show a minimal degree of amelogenin and tuftelin colocalization in the cell cytoplasm (Deutsch *et al.*, 1995; Luo *et al.*, 2004). These data (Deutsch *et al.*, 1995; Luo *et al.*, 2004; MacDougall *et al.*, 1998) suggest that tuftelin accumulates in definite cytoplasmic regions other than the Golgi apparatus and secretory granules, and in various cell lineages that range from cells responsible for both mineralizing and nonmineralizing tissues. None of the various species reported tuftelin cDNAs contain a traditional signal peptide nor do they contain a recognizable endoplasmic reticulum (ER) retention or retrieval signal (Teasdale and Jackson, 1996). Despite the lack of evidence to explain a physiologic transport pathway from the cytoplasm to the extracellular matrix, tuftelin has been repeatedly localized to the enamel matrix (Paine *et al.*, 2000a; Satchell *et al.*, 2002). While the pathway of tuftelin to the extracellular matrix is being debated (Luo *et al.*, 2004), the presence of tuftelin in the enamel matrix continues to suggest that it may contribute to amelogenesis. Similarly, the TFIP11 has been immunolocalized to Tomes' processes and the enamel matrix (Paine *et al.*, 2000a), however TFIP11 appears to be a phosphorylated

RNA splicing factor and is primarily localized—within the cell nucleus (Beausoleil *et al*., 2004; Wen *et al*., 2005). Previous studies have demonstrated that cell membrane phospholipids accumulate in the dentin and enamel extracellular matrices during the process of biomineralization (Dunglas *et al*., 1999; Girija and Stephen, 2003; Goldberg *et al*., 1999). With respect to enamel, one explanation of this phenomenon is that the distal portions of individual Tomes' processes are fragmented during the secretory stages of amelogenesis, and the contents are trapped within the extracellular matrix (Goldberg *et al*., 1998). For a structure such as enamel that has very specific matrix proteases that target the tooth-specific proteins (Bartlett and Simmer, 1999; Den Besten *et al*., 1998), it is conceivable that nonsecreted, ameloblast-associated proteins can become trapped in the immature or mature enamel. This may explain the localization of tuftelin and TFIP11 in the enamel matrix.

Less information is available describing the sulfated enamel matrix proteins. *In vivo* radiolabeling was used to identify the sulfated enamel proteins (Smith and Nanci, 1996; Smith *et al*., 1995), but no specific gene identity has been identified for this particular group of enamel proteins. Being able to assign a specific gene is important because once identified, the spatiotemporal expression patterns for the gene product, along with functional studies, can then be determined and defined.

IV. Proteolytic Enzymes of the Enamel Matrix

Two proteinases are known to be secreted into the enamel matrix. One is MMP20 (also known as enamelysin) (Bartlett *et al*., 1996), and the other is a serine proteinase named kallikrein-4 (KLK4; also known as enamel matrix serine proteinase-1 or serine proteinase 17). Both of these proteinases were originally discovered in porcine enamel organ (Bartlett *et al*., 1996; Simmer *et al*., 1998).

A. Matrix Metalloproteinase-20

Human *matrix metalloproteinase-20* (*MMP20*) gene contains 10 exons and is part of the *MMP* gene cluster that localize to human chromosome 11q22.3 (Llano *et al*., 1997). Members of the MMP family are involved in the breakdown of extracellular matrix in normal physiological processes, such as embryonic development and tissue remodeling (Brinckerhoff and Matrisian, 2002; Vu and Werb, 2000), and are also involved in disease processes, such as arthritis and tumor metastasis (Lynch and Matrisian, 2002). Most MMPs are secreted as inactive proproteins and are activated after delivery to the extracellular environment.

Expression of *MMP20* in normal physiological tissues is limited to the tooth. The ameloblasts of the enamel organ and the odontoblasts of the dental papilla in porcine teeth were demonstrated to express MMP20 (Hu *et al.*, 2002). MMP20 was definitively demonstrated to be secreted into the enamel matrix when a Western blot confirmed that the doublet of approximately 41 and 46 kDa observed on zymograms was a result of MMP20 activity (Fukae *et al.*, 1998). It was also shown that MMP20 activity accounts for virtually all of the known cleavage sites in amelogenin that occur during the early stages of enamel development (Ryu *et al.*, 1999). More recently, it was demonstrated that homozygous deletion of a portion of *Mmp20* encoding the catalytic domain (Caterina *et al.*, 2002) and homozygous mutation of *MMP20* (Kim *et al.*, 2005b) cause malformed dental enamel in mice and humans respectively. The *Mmp20*-null mouse (Caterina *et al.*, 2002) has a severe and profound tooth phenotype. Specifically, the null mouse does not process amelogenin properly, possesses an altered enamel matrix and rod pattern, has hypoplastic enamel which delaminates from the dentin, and has a deteriorating enamel organ morphology as development progresses. Enamel from the family with the homozygous *MMP20* mutation was heavily pigmented, brittle, and was less radiodense indicating that the enamel was soft. The effects of *MMP20* mutation were confined to the teeth so MMP20 is considered a tooth-specific MMP.

B. Kallikrein-4

The human *KLK4* gene is located on chromosome 19q13.41. Although KLK4 was originally discovered in teeth (Simmer *et al.*, 1998), others have since discovered it in the prostate (Nelson *et al.*, 1999). *KLK4* encodes a 254 amino acid protein with a conserved serine protease catalytic triad. Also, as is typical of secreted proteases, KLK4 contains an amino-terminal prepropeptide sequence. Today it is known that KLK4 is expressed in limited tissues including prostate and developing teeth (Hu *et al.*, 2000b; Nelson *et al.*, 1999; Ryu *et al.*, 2002). In teeth, KLK4 is produced by odontoblasts, and also by late-secretory and maturation stage ameloblasts (Hu *et al.*, 2000b; Ryu *et al.*, 2002). Klk4 expression in the enamel matrix correlates with the disappearance of enamel proteins (such as amelogenin) from the enamel matrix (Hu *et al.*, 2000b). Thus, the expression of proteolytic enzymes, including KLK4/Klk4, during enamel maturation appears to be necessary for enamel to achieve its high degree of mineralization (Bartlett and Simmer, 1999; Smith, 1998). To date, homozygous deletion of the *Klk4* gene has not been achieved in mice. However, a *KLK4* mutation was discovered in a family with autosomal recessive hypomaturation AI (Hart *et al.*, 2004). These teeth had a yellow-brown discoloration and were

excessively sensitive to hot and cold. The enamel fractured from the teeth appeared to be of normal thickness but had a decreased mineral content. The affected family members were female, so it is not known if KLK4 plays a significant role in the prostate. Even so, only the teeth were apparently affected by the homozygous *KLK4* mutation (Hart *et al*., 2004).

V. Protein–Protein Interactions Within the Enamel Matrix

A number of enamel matrix protein–protein interactions have been described in the literature, and these will be mentioned and discussed in reference to their discovery and in chronological order. The bulk of the literature in this area of science relates to amelogenin assemblies commonly referred to as amelogenin "nanospheres" (Du *et al*., 2005; Fincham and Moradian-Oldak, 1995; Robinson *et al*., 1981; Veis, 2005). Amelogenin nanospheres have been visualized *in vivo* using transmission electron microscopy (Fincham *et al*., 1995), demonstrated by *in vivo* chemical cross-linking studies (Brookes *et al*., 2000), and (re)produced *in vitro* from recombinant amelogenins (Moradian-Oldak *et al*., 2000).

In 2001 and 2003 there were single reports of amelogenin–cytokeratin K14 (Ravindranath *et al*., 2001) and amelogenin–cytokeratin K5 (Ravindranath *et al*., 2003) interactions *in vivo*, however, these interactions appear unlikely physiologically because secreted proteins are at all times physically isolated from the cell cytoskeleton (Lewin, 2000), and thus unable to directly interact (Luo *et al*., 2004). There is a single report from an *in vitro* study of an amelogenin–enamelin interaction (Yamakoshi *et al*., 2003), and there is also a single report demonstrating amelogenin–ameloblastin interactions *in vitro* (Ravindranath *et al*., 2004). Another study using the Y2H assay system has demonstrated a physiological interaction of the enamel matrix proteins amelogenin, ameloblastin, and enamelin with biglycan (Wang *et al*., 2005). Biglycan is a small secreted proteoglycan that is present in enamel (Goldberg *et al*., 2002). Biglycan appears to influence amelogenesis by retarding *amelogenin* gene transcription (Goldberg *et al*., 2002, 2005).

More protein–protein interactions involving proteins of the enamel matrix have been discovered recently in a comprehensive screening of a gene expression library using the Y2H assay (Wang *et al*., 2005). From this study a small number of known secreted proteins were identified. Two such proteins were calnexin and α-2-HS-glycoprotein (AHSG or Fetuin-A). Calnexin contains a C-terminal endoplasmic reticulum (ER) retention motif "RKPRRE" and is implicated to play a role in a number of cell activities including ER membrane related protein folding, calcium ion binding, calcium ion storage, and protein secretion via secretory vesicles (Michalak *et al*.,

2002; Okazaki *et al.*, 2000; Rajagopalan and Brenner, 1994; Schrag *et al.*, 2001). Using software for the Signal P3.0 server (http://www.cbs.dtu.dk/services/SignalP/), the N-terminal 20 amino acids of calnexin appear to code a signal peptide (with a 100% probability figure for both human and mouse proteins). This signal peptide domain for calnexin has not previously been recognized or discussed in the literature, but it is an intriguing finding and suggests yet another role for this multifunctional protein. AHSG is a glycoprotein present in the serum, and is synthesized by hepatocytes (Denecke *et al.*, 2003; Jahnen-Dechent *et al.*, 1997; Kalabay *et al.*, 2002). The AHSG molecule consists of two polypeptide chains, which are both cleaved from a proprotein encoded from a single mRNA (Magnuson *et al.*, 1998; Szweras *et al.*, 2002). AHSG is involved in several functions, such as endocytosis, brain development, and the formation of bone tissue (Denecke *et al.*, 2003; Jahnen-Dechent *et al.*, 1997; Kalabay *et al.*, 2002). It is also quite possible that AHSG, thus, has a regulatory role in the mineralization process of amelogenesis. We hypothesize that such a unique mineralized tissue-like enamel is likely to have multiple regulatory protein controls that contribute to the inhibition, initiation, and growth of individual crystallites.

In addition to the known secreted proteins (identified by this Y2H screening) interacting directly with enamel matrix proteins (Wang *et al.*, 2005), two amelogenin-interacting proteins were identified that are designated unknown expressed sequence tags (ESTs). These two gene products have the National Center for Biotechnology Information (NCBI) gene accession numbers NM_026325 and NM_026808. Messenger RNAs (mRNAs) of both these unknown ESTs are expressed in multiple tissues including both mineralized and nonmineralized tissues (Wang *et al.*, 2005). The translated products of both genes have a conceptual signal peptide, and NM_026325 has a cell adhesion arginyl-glycyl-aspartic acid (RGD) motif (D'Souza *et al.*, 1991; Ganss *et al.*, 1999; Takagi, 2004) at its C-terminal region (Wang *et al.*, 2005). No other functional peptide domain could be identified in these two unknown ESTs (Wang *et al.*, 2005).

A. Amelogenin–Amelogenin Interactions

Katz *et al.* (1965), using proteins isolated from embryonic bovine enamel, reported for the first time, the notion that the protein components of the enamel matrix appear as polymerizing multi- or single-components. Later, in a sedimentation equilibrium study of bovine enamel proteins, unusually high molecular weight complexes ranging from 1 to 4 million Dalton were detected (Katz *et al.*, 1969). Chaotropic agents such as guanidine hydrochloride could not break these enamel protein "aggregates" which consisted mainly of amelogenins (Mechanic *et al.*, 1967). Using a freeze-fracture

technique Robinson *et al.* (1981) investigated rat incisor enamel and observed that the forming region of enamel consisted largely of spherical units that are approximately 30–50 nm in diameter. Again using freeze-fracture techniques and electron microscopy to observe secretory-stage and mature enamel, other investigators had described globular particles between enamel crystallites that they believed were composed of amelogenin proteins (Bai and Warshawsky, 1985).

The availability of a recombinant form of amelogenin following its cDNA cloning allowed investigators to systematically study the "aggregative" behavior of this hydrophobic protein (Simmer *et al.*, 1994; Snead *et al.*, 1985). During the last decade understanding the assembly properties of amelogenins has significantly been advanced. The self-assembly properties of a recombinant mouse amelogenin (rM179) have been studied employing dynamic light scattering, size exclusion chromatography, atomic force, and transmission electron microscopy (Fincham *et al.*, 1995, 1994b; Moradian-Oldak *et al.*, 1994, 2004). Full-length amelogenin molecules undergo a self-assembly process to generate spherical structures that were named "nanospheres" (Fincham *et al.*, 1995), and these structures are 15–20 nm in hydrodynamic radii (Moradian-Oldak *et al.*, 1994, 2000). It was proposed that the nanospheres were formed through intermolecular hydrophobic interactions when the hydrophilic segment is exposed on the surface of the nanospheres. The formation of nanospheres by a recombinant human amelogenin in a native state (vitreous ice system) was independently confirmed by cryotransmission electron microscopy (Leytin *et al.*, 1998). Formation of nanospheres by a native porcine amelogenin, isolated from scrapings of fourth mandibular molars, has also been observed (Moradian-Oldak *et al.*, 2002).

The availability of a highly sensitive DynaPro MS/X-TC light scattering instrument, with a size detection range of 1 nm–1 μm, has allowed a systematic analysis of the size distribution of amelogenin nanospheres and their intermediate subunits in different solutions (Du *et al.*, 2005). It was then found that typical nanospheres of hydrodynamic radii (R_H) of 13–27 nm were the most stable assembling form of the recombinant porcine amelogenin (rP172) in either buffer or water. Smaller particles with R_H less than 10 nm have been detected in short measurement intervals indicating the presence of smaller subunits prior to nanosphere formation (Fincham *et al.*, 1998). Amelogenin monomers, and discrete oligomers such as dimers, trimers, and hexamers are detected when light scattering measurements were performed in 60% aqueous acetonitrile and at low protein concentration (~20 mg/ml). These small oligomers have been defined as the basic subunits of typical $R_H = 13$–27 nm nanospheres (Du *et al.*, 2005).

In order to investigate nanosphere formation *in vivo*, Brookes *et al.* (2000, in press) have used rat enamel organs treated with a bifunctional chemical

cross-linking agent which unite lysine groups in close molecular proximity. From these studies it was shown that intracellular amelogenin monomers are in close contact, and can form oligomers including dimers, tetramers, pentamers, and hexamers. These authors then suggested that these subunits are preassembled intracellularly prior to their secretion. These *in vivo* findings are in a good agreement with previous *in vitro* studies on amelogenin nanosphere subunits analyzed by dynamic light scattering (Du *et al.*, 2005).

Direct experimental evidence that amelogenin molecules interact was obtained in a series of *in vitro* studies, employing the Y2H system (Paine and Snead, 1997). It was found that amelogenin–amelogenin interactions occur through the amino-terminal 42 residues called A-domain (Paine and Snead, 1997). In addition, it was reported that the 17-residue domains in the carboxyl-terminal region; called B-domain, are involved in amelogenin–amelogenin interactions (Paine and Snead, 1997). Alteration of these self-assembly domains in engineered amelogenin proteins resulted in interruption of nanospheres formation (Moradian-Oldak *et al.*, 2000). While deletion of the N-terminal A-domain caused disassembly, deletion of B-domain resulted in the aggregation of the nanospheres (Moradian-Oldak *et al.*, 2000). Transgenic animals in which either the A-domain or B-domain of amelogenin was removed from the full-length murine amelogenin cDNA showed enamel formation with defective structural organization (Paine *et al.*, 2000b). These animal studies support the view that amelogenin self-assembly into nanospheres is a key factor in controlling normal enamel biomineralization *in vivo* (Paine *et al.*, 2001).

Studies were also designed to explore the reversible temperature-sensitive aggregation properties of amelogenins, originally demonstrated by Nikiforuk and Simmons (1965). The structure of concentrated solutions of amelogenins that have been referred to as a ''gel-like'' matrix (100–300 mg/ml), at two different temperatures, has been studied using a number of different methodologies (Moradian-Oldak and Goldberg, in press). Preparations that rendered amelogenin translucent at 4°C and opaque at room temperature were fixed using Karnovsky fixation technique, and examined by scanning and transmission electron microscopy (SEM and TEM) as well as atomic force microscopy (AFM) (Wen *et al.*, 1999b). The gel-like matrices were composed of spherical structures ranging in diameter size from 20 to 200 nm. The clear to opaque transition with the temperature raise appeared to be the result of the formation of numerous globular ''voids'' in the gel, presumably as the result of increased hydrophobic interactions between the spherical subunits creating more empty space. This change in size distribution of the spherical structures with temperature supports the theory that an increase in temperature promotes hydrophobic forces between the nanospheres, resulting in the formation of larger subunits.

The classic "hanging drop" technique for protein crystallization has been recently used in an attempt to crystallize a rP172 (Du *et al*., 2005). Using this technique, the full-length rP172 generated elongated fibrous structures that have been referred to as "microribbons" which are hundreds of microns in length, tens of microns in width, and a few microns in thickness (Du *et al*., 2005). Transmission electron microscope examination of amelogenin solution drops prior to microribbon formation revealed nanosphere chains in intermediary stages of formation (Du *et al*., 2005; Moradian-Oldak and Goldberg, in press). Although the microribbons did not have the defined X-ray diffraction pattern as was originally reported (this was later shown to be an X-ray diffraction pattern from contaminating cellulose in the samples; see an Erratum published in Du *et al*., 2005), subsequent and additional experiments have been performed on the amelogenin microribbons confirming their existence (Moradian-Oldak and Goldberg, in press). For this data set, Raman microscopy and Fourier transform infrared spectroscopy (FTIR) have been used to establish the amelogenin composition of microribbons, and the birefringence of these amelogenin ribbons indicates some level of ordered alignment (Moradian-Oldak *et al*., in press).

B. Amelogenin–Enamelin Interactions

To the authors' knowledge, there has only been a single report suggesting that amelogenin and enamelin interact with each other (Yamakoshi *et al*., 2003). In this study, the *in vivo* derived 32 kDa porcine enamelin peptide (a fragment located close to the N-terminal region), with an exposed *N*-acetylglucosamine (GlcNAc), was able to interact directly with the N-terminal region (circa amino acids 1–65) of porcine amelogenin. Larger amelogenin fragments, and 32 kDa enamelin without the exposed GlcNAc, showed no such ability to interact. This, and other data may be suggestive that amelogenin can interact with the sugar chains of glycoproteins, although not necessarily with enamelin (Yamakoshi *et al*., 2003). For example, this type of amelogenin interaction may also be apparent with ameloblastin, another enamel glycoprotein (Ravindranath *et al*., 1999).

C. Amelogenin–Ameloblastin Interactions

As with the scant data describing amelogenin–enamelin interactions, there is a similar lack of data supporting any interaction between amelogenin and ameloblastin, with just a single study demonstrating this possibility (Ravindranath *et al*., 2004). In this single study, using Western blots and ELISA, the recombinant full-length mouse amelogenin, and its amelogenin

trityrosyl motif peptide (ATMP motif: amino acids PYPSYGYEPMGGW) were able to bind to a nonglycosylated form mouse recombinant ameloblastin (Ravindranath *et al.*, 2004). Previously, the ATMP region of amelogenin had been identified as a potential binding region for GlcNAc and for GlcNAc-mimicking peptides (GMps) (Ravindranath *et al.*, 1999, 2000). While these data are seemingly contradictory, it does suggest (as with enamelin) that a number of enamel matrix protein–protein interactions are possible, and that a full, or partial state of glycosylation of individual enamel proteins may be a requirement.

Finally, and also contradictory, in another study using the Y2H assay to critically examine enamel matrix protein–protein interactions, there was no evidence to support an amelogenin–ameloblastin interaction (Paine *et al.*, 1998b). Because the yeast cell is eukaryotic, and thus capable of posttranslational modifications including glycosylation, the assumption is that the ameloblastin protein studied in the Y2H assay was in the glycosylated form.

D. Role of Biglycan in Enamel Matrix Assembly

Data recently published using the Y2H assay demonstrated that biglycan interacts directly with amelogenin, ameloblastin, and enamelin (Wang *et al.*, 2005). *In vivo* evidence has shown that biglycan directly or indirectly represses *amelogenin* gene transcription (Goldberg *et al.*, 2002, 2005). Biglycan is expressed in ameloblast cells and is present in the developing enamel matrix (Goldberg *et al.*, 2002). In a cell model system, biglycan is also robustly expressed in ameloblast-like LS8 cells (Wang *et al.*, 2005). Not only has biglycan been shown to interact with amelogenin using the Y2H assay (Wang *et al.*, 2005), but there is also evidence from *biglycan*-null animals (Xu *et al.*, 1998b) that amelogenin expression is downregulated by biglycan (Goldberg *et al.*, 2002, 2005), suggesting the existence of quite an elaborate feedback mechanism from the enamel extracellular matrix to ameloblast cells. Such a feedback mechanism would be triggered by enamel matrix protein–protein interactions, and in this case the interaction between amelogenin and biglycan.

As an initial experiment to confirm amelogenin–biglycan interaction, *in vitro* coimmunoprecipitation was performed. The Y2H vectors used previously to demonstrate amelogenin–biglycan interactions included the full-length mouse amelogenin tagged with the c-Myc epitope at the N-terminal, and the full-length human biglycan tagged with the hemagglutinin (HA) epitope at the N-terminal (Wang *et al.*, 2005). Both Y2H vectors include the T7 promoter prior to the epitope, but following either the GAL4 DNA binding domain or the GAL4 transcriptional activating domains. This plasmid arrangement allows for transcription (using T7 RNA polymerase) and

translation reactions *in vitro*, with the resulting proteins being composed only of the N-terminal epitope tag and the protein of interest. Using the TNT® Quick Coupled (reticulocyte lysate) Transcription/translation System (Promega, Maddison, WI), ^{35}S-methionine–labeled mouse amelogenin and human biglycan were synthesized. We will refer to these two isotope-labeled proteins as ^{35}S-Myc-Amel and ^{35}S-HA-BGN. Using the BD Matchmaker Co-IP Kit (BD Biosciences Clontech, Palo Alto, CA) and following standard methodology (Sambrook and Russell, 2001), we demonstrate the coimmunoprecipitation of ^{35}S-Myc-Amel and ^{35}S-HA-BGN *in vitro* (Fig. 2). This data is complementary and supportive of the previous Y2H data (Wang *et al.*, 2005), which demonstrated an amelogenin–biglycan interaction within the yeast cytoplasm.

Amelogenin is composed primarily by three domains: the N-terminal TRAP region of approximately 44 amino acids; internal region; and the short carboxylated hydrophilic C-terminal of approximately 13 amino acids (Fincham *et al.*, 1995, 2000; Shaw *et al.*, 2004). There is a single phosphate at

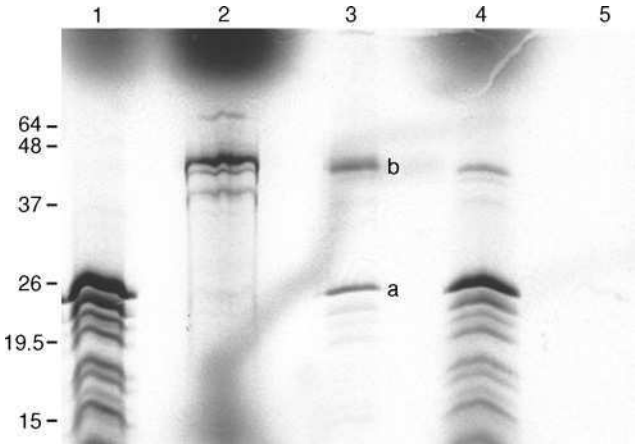

Figure 2 Amelogenin and biglycan coimmunoprecipitation. The c-Myc–tagged mouse amelogenin (Myc-Amel) and the HA-tagged human biglycan (HA-BGN) were *in vitro* translated using ^{35}S-methionine. Myc-tagged amelogenin (^{35}S-Myc-Amel) and HA-tagged biglycan (^{35}S-HA-BGN) were incubated together, and coimmunoprecipitated using an HA monoclonal antibody and analyzed by SDS-PAGE and autoradiography. Lane 1: ^{35}S-Myc-Amel. Lane 2: ^{35}S-HA-BGN. Lane 3: ^{35}S-Myc-Amel plus ^{35}S-HA-BGN plus HA monoclonal antibody coimmunopreciptiation. Lane 4: Supernatant from coimmunoprecipitation reaction. Lane 5: ^{35}S-Myc-Amel plus HA monoclonal antibody coimmunoprecipitation (negative control). Biglycan-specific band with a calculated molecular weight of 41.6 kDa is identified by "b," and amelogenin-specific band with a calculated molecular weight of 22.6 kDa is identified by "a" in lane 3. No cross-reaction was detectable (Lane 5). Protein size marker in kDa included on left.

serine 16 in the TRAP N-terminal region, which may be implicated in the mineralization process (Fincham *et al.*, 1994a). The N-terminal region of amelogenin has lectin-like properties being able to bind to *N*-acetyl-D-glucosamine (Ravindranath *et al.*, 1999, 2000). These recent data remind the authors of earlier data that looked at the lectin distribution in the forming tooth, and the identification of glucosyl, *N*-acetyl-D-glucosaminyl and D-galactosyl residues in the forming enamel (Blottner and Lindner, 1987). The sugar moieties interacting with amelogenin are components of heparan sulfate (HS) and keratan sulfate (KS) GAGs, rather than the chondroitin sulfate/dermatan sulfate (CS/DS) GAGs (Blottner and Lindner, 1987; McKee *et al.*, 1996). Yamakoshi *et al.* (2002) have shown that the deficiency of *N*-galactosamine 6-sulfatase induces enamel structural defects in a mucopolysaccharidosis type IVA (Morquio A syndrome, MPS IVA). Other characteristic features of MPS IVA are corneal clouding, aortic valve disease, and the urinary excretion of keratosulfate (Morquio, 1929). KS and chondroitin 6-sulfate (C6S), shown to be expressed by secretory ameloblasts, accumulate in lysosomes in this MPS IVA disease. This provides a clear-cut demonstration that the two GAGs are present in the cells responsible for amelogenesis, and is indirect evidence of the crucial role of these two GAGs in enamel formation. All these data may have future implications in enamel and tooth formation (Paine *et al.*, 2000b).

VI. Amelogenin–Mineral Interactions

In order to assess the functional significance of amelogenin in controlling certain aspects of crystal formation, different investigators have adapted various *in vitro* experimental strategies to investigate interactions of amelogenin with calcium phosphate crystals. These *in vitro* experiments include; quantitative determination of protein adsorption affinity onto synthetic or commercially available crystals (Aoba *et al.*, 1989; Bouropoulos and Moradian-Oldak, 2003), evaluation of inhibitory effect of the protein on the kinetics of crystal growth in solution (Aoba *et al.*, 1989; Doi *et al.*, 1984; Moradian-Oldak *et al.*, 1998), evaluation of the morphological changes of crystals grown in amelogenin containing "gel-like" matrices (Iijima *et al.*, 2002; Moradian-Oldak, 2001; Wen *et al.*, 1999a), effect on the induction time of crystal formation in the presence of amelogenin (Bouropoulos and Moradian-Oldak, 2003), and the effect of amelogenin on the organization of crystals grown on bioactive surfaces such as titanium and bioglass (Wen *et al.*, 1999a, 2000), fluoroapatite glass ceramics (Habelitz *et al.*, 2004), and in solution (Beniash *et al.*, 2005). More recently, amelogenin microribbons have also been used to grow organized and oriented crystals (Du *et al.*, 2005; Moradian-Oldak *et al.*, in press).

Aoba *et al.* (1987) originally reported the adsorption affinity of porcine amelogenin and its proteolytic products onto synthetic HAP crystals. The adsorption affinity of extracted, full-length 25-kDa amelogenin was found to be stronger than the 20-kDa amelogenin proteolytic product (Aoba *et al.*, 1987). These same authors later reported that the effect of amelogenin is associated with the whole molecular structure and not partial molecular sequence (Aoba *et al.*, 1989). Earlier, in seeded crystal growth experiments, it was found that amelogenin can inhibit the growth of apatite in solution, but its effect is weaker than achieved by the acidic class of enamel proteins then referred to as the enamelins (Doi *et al.*, 1984). Using a series of purified recombinant and native amelogenins and some of its various isoforms it was shown that the binding affinity of proteins on the HAP is decreased after cleaving the hydrophilic C-terminal segment (Moradian-Oldak *et al.*, 2002). Direct evidence for the proximity of the charged C-terminal region of amelogenin protein on the HAP surface was based on recent solid-state NMR studies (Shaw *et al.*, 2004).

The interactions of amelogenin nanospheres with apatite have been investigated since the early description and characterization of amelogenin nanospheres (Bouropoulos and Moradian-Oldak, 2003; Fincham *et al.*, 1994b; Moradian-Oldak *et al.*, 1994). The adsorption isotherm of assembled amelogenin nanospheres onto HAP can be described with a good correlation coefficient ($R^2 = 0.99$) indicating that amelogenin nanospheres can bind to the surface of apatite crystals at defined adsorption sites (Bouropoulos and Moradian-Oldak, 2003). Another interesting effect of amelogenin on growing HAP crystals is that the assembled full-length amelogenin causes aggregation of apatite crystals more effectively than the C-terminally cleaved amelogenin (Moradian-Oldak *et al.*, 1998). This aggregation (or bundling) effect was interpreted as the ''bridging'' of apatite crystals by the preassembled amelogenin nanospheres (Moradian-Oldak *et al.*, 1998). The formation of apatite crystals, organized parallel to their *c*-axial direction and in the presence of preassembled amelogenin in solution has been recently demonstrated (Beniash *et al.*, 2005). In a mineralization solution and in the presence of amelogenin, organized bundles of elongated apatite crystals can form on bioactive glass and on titanium (Wen *et al.*, 1999a). Amelogenin can also control the rate and direction of crystal growth on fluoroapatite glass ceramic (Habelitz *et al.*, 2004).

In vitro studies have shown that amelogenins also have potential to selectively affect crystal morphology. It has recently been shown that amelogenins affected the morphology of octacalcium phosphate crystals in a dose-dependent manner changing crystal morphology from plate-like to a more elongated ribbon-like shape (Iijima and Moradian-Oldak, 2004). While the presence of the hydrophilic C-terminal of amelogenin decreased the affinity of amelogenin to bind to apatite, and inhibited the kinetics of its

crystal growth (Moradian-Oldak *et al.*, 1998), the effect on octacalcium phosphate and apatite crystal shape is reported to be independent of the presence of the hydrophilic C-terminal segment (Beniash *et al.*, 2005; Iijima *et al.*, 2002). These *in vitro* studies collectively demonstrate that amelogenin nanospheres interact with synthetic calcium HAP and octacalcium phosphate crystal faces in a selective manner, and therefore give some control over the crystallite morphology and habit. Specific binding of amelogenin nanospheres on side faces of *in vivo* derived enamel crystals has also been demonstrated (Kirkham *et al.*, 2000; Wallwork *et al.*, 2001, 2002).

The above *in vitro* experimental studies used strategies to examine the effect of amelogenin proteins on crystal growth in solution, in ''gel-like'' matrices, and on bioactive surfaces. Amelogenin microribbons have also been used as a substrate for the growth of crystals (Du *et al.*, 2005). Amelogenin microribbons that have been soaked in a metastable calcium phosphate solution allow for the formation of ordered and oriented crystals that align along the microribbon's long axis (Du *et al.*, 2005). The *c*-axial orientation of the crystals was confirmed by TEM, and also from its corresponding electron diffraction patterns (Du *et al.*, 2005). From these studies it has been proposed that the linear alignment of amelogenin nanospheres *in vitro* (amelogenin microribbons) creates a framework that facilitates the oriented growth of apatite crystals regardless of the presence of acidic proteins such as phosvitin. The oriented nucleation of such crystals could be promoted through the interactions of the structured amelogenin framework. In the enamel extracellular matrix this control could be achieved by the coassembly of amelogenin and the nonamelogenin proteins such as the acidic enamelin (Bouropoulos and Moradian-Oldak, 2004; Hu and Yamakoshi, 2003) or alternatively, the ordered hydrophobic/hydrophilic partitioning of amelogenin molecules could create an oriented array of acidic peptides on a hydrophobic substrate.

VII. Interactions of Enamel Matrix Proteins with the Cell Surface

There is little published information regarding ameloblast-specific endocytotic pathways despite the fact that this theme has been specifically addressed over the years. For endocytosis to occur one would have to be able to visualize, or accept that, enamel matrix proteins directly interact with proteins intimately associated with Tomes' processes. Immunohistochemistry data suggests that high concentrations of ameloblastin and enamelin are present proximal to Tomes' processes compared to the bulk enamel matrix (Fukumoto *et al.*, 2004; Hu *et al.*, 1997a,b; Nanci *et al.*, 1998; Uchida *et al.*, 1997). The intensity of an immunolocalization signal to secreted amelogenin is relatively low proximal to Tomes' processes, with a noticeable increase

toward a distance greater than 1.25 μm (away from Tomes' processes) (Nanci *et al.*, 1998). In the case of ameloblastin, gene knockout studies have suggested that ameloblastin acts as a cell-adhesion molecule, and without ameloblastin ameloblast cells are unable to fully differentiate, while losing their ability to synthesize and secrete enamel matrix proteins (Fukumoto *et al.*, 2004). This data implies that at least some of the secreted ameloblastin is in intimate contact with the plasma membrane of ameloblast cells. One question that baffles enamel investigators is how the enamel matrix is lost to its environment to allow for the almost complete mineralization of the enamel volume. Is the activity of the enamel proteinases so efficient and complete that only single amino acids or very short peptides remain that can diffuse away from the local (enamel) environment during the events biomineralization, or alternatively, are these protein reminants pinocytosed by the surrounding cells? A reasonable alternative explanation is that ameloblast cells remove (endocytose) the enamel protein matrix as efficiently as they secrete it. Probably more likely is that, to varying degrees, all of these mechanisms (diffusion, pinocytosis, and endocytosis) operate to remove the organic components of the enamel matrix during biomineralization. This alternative explanation of endocytosis of the enamel organic matrix is a reasonable hypothesis; and is currently being pursued by a number of investigators (Tompkins *et al.*, 2006; Wang *et al.*, 2005).

A fairly exhaustive screening of a cDNA expression library was recently completed to identify enamel matrix proteins that interact directly with amelogenin, ameloblastin, or enamelin (Wang *et al.*, 2005). From this screening a small number of identified proteins were targeted as of particular interest because they were identified multiple times. These were integral membrane proteins, proteins that had a role in calcium transport and hard-tissue mineralization, or collagens. Among this group was the cluster of differentiation-63 protein (Cd63; also known as melanoma 1 antigen, or lysosomal-associated membrane protein-3 or Lamp-3), annexin A2 (Anxa2), α-2-HS-glycoprotein (Ahsg), and types I, II, and V collagen. The possible significance of each of these proteins to enamel formation has been discussed (Wang *et al.*, 2005). In addition, lysosomal-associated protein-1 (Lamp1) has been proposed as a membrane-bound amelogenin receptor (Tompkins *et al.*, 2006). Both Cd63 and Lamp1 are also present in the membranes of the endosome/late endosome and lysosome (Cook *et al.*, 2004; Duffield *et al.*, 2003). Anxa2 has been shown to play a key role in the organization and dynamics of the endosome membrane (Gruenberg and Stenmark, 2004). These data support the existence of an indirect signaling pathway, mediated through membrane-bound receptors, which allow for the transfer of information from the enamel matrix to affect ameloblast-specific gene transcription (Tompkins *et al.*, 2006). These data support an anchoring mechanism for orientating the enamel matrix relative to the secretory surface

of ameloblast cells, referred to as Tomes' processes. Additionally, these data support a common theme in amelogenesis; the existence of a specific receptor-mediated mechanism for the removal of the large masses of degraded extracellular enamel proteins during enamel biomineralization. This theme has been addressed previously (Nanci *et al.*, 1996a, 1998; Reith and Cotty, 1967; Smith, 1979, 1998), but no specific mechanism/pathway of ameloblast-related endocytosis has been described. In the following section is a brief description of three membrane-bound proteins whose role in amelogenesis we would like to speculate on: these proteins are Cd63, Anxa2, and Lamp1.

A. Lysosomal-Associated Membrane Protein 1

The human *lysosomal-associated membrane protein 1* (*LAMP1*) gene is located on chromosome 13q34. At steady state, LAMP1 is a transmembrane protein highly expressed in late endosomes and lysosomes and is often used as a marker for these two organelles (Cook *et al.*, 2004). Thus, most of the literature on LAMP1 relates to its involvement in endocytosis, pinocytosis, or phagocytosis (Cook *et al.*, 2004). The movement of LAMP1 from the rough ER and Golgi to the lysosome membrane has been documented (Cook *et al.*, 2004), and this pathway is independent of trafficking of LAMP1 through the plasma membrane. In many cell types, LAMP1 immunoreactivity is also observed at the plasma membrane, and it can also be observed in early endocytic compartments (Kannan *et al.*, 1996). This is true for peripheral blood lymphocytes (Holcombe *et al.*, 1993) and platelets (Silverstein and Febbraio, 1992). LAMP1 expression levels in the plasma membrane have also been related to the metastatic potential of different colon cancer cell lines (Saitoh *et al.*, 1992). The presence of LAMP1 on the plasma membrane is suggestive of LAMP1 acting as a cell surface intermediary that can be shuttled to the lysosome through endocytosis. The specific molecular requirements for such activity are yet to be identified or discussed in the literature. To date, no *Lamp1*-null animals have been reported on. Recently, Lamp1 has been shown to interact with an alternatively spliced isoform of amelogenein (Tompkins *et al.*, 2006), leading to speculation that specific enamel proteins could trigger a cell-signaling mechanism that is responsible for cell differentiation events, including those leading up to periodontal ligament regeneration (Gestrelius *et al.*, 2000; Veis, 2003; Veis *et al.*, 2000; Viswanathan *et al.*, 2003).

For our studies, a monoclonal antibody to rat Lamp1 (Developmental Studies Hybridoma Bank, University of Iowa; catalogue # 1D4B) was used. Using this antibody we are able to demonstrate Lamp1 in presecretory, secretory, and postsecretory ameloblasts (Fig. 3). This is supportive evidence that LAMP1/Lamp1 plays a role in amelogenesis.

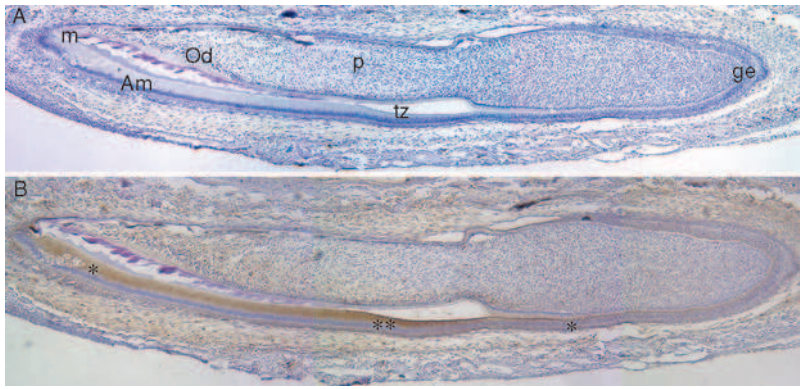

Figure 3 Immunolocalization of Lamp1 to ameloblast cells in a 4-day-old mouse mandibular incisor. Panel A is a no primary antibody control section. Abbreviations: mature end of incisor (m); transition zone ameloblasts (tz); growing end of incisor (ge); and dental pulp (p). Presecretory ameloblasts are located at the growing end, secretory ameloblasts are within and beyond the transition zone up to the postsecretory ameloblasts. Postsecretory ameloblasts are located at the mature end of the incisor teeth. Both sections counterstained with hematoxylin prior to photographing. No antibody staining is seen in the negative control section seen in panel A. Both images taken at 10× magnification. Panel B is using a monoclonal antibody to rat Lamp1 was purchased from the Developmental Studies Hybridoma Bank (catalogue # 1D4B; University of Iowa) and used for this study at a 1:50 dilution. Lamp1 is clearly and evenly expressed in the cytoplasm of ameloblasts at all stages of amelogenesis (brown chromophore staining; identified by a single and double asterisk), and most highly expressed in the secretory ameloblasts around the transition zone of enamel formation (identified by two asterisks).

B. CD63 Antigen

The human *CD63 antigen* (CD63; also known as melanoma 1 antigen or the lysosomal-associated membrane protein-3 or LAMP-3) gene is located on chromosome 12q12. CD63 is a member of the transmembrane-4 glycoprotein superfamily, which is also known as the tetraspanin family. Most of these family members are cell-surface proteins that are characterized by the presence of four hydrophobic (transmembrane) domains (Stipp *et al.*, 2003; Yunta and Lazo, 2003). These proteins mediate signal transduction events that play a role in the regulation of cell development, activation, growth, and motility (Mantegazza *et al.*, 2004; Yunta and Lazo, 2003). In particular, as a cell surface glycoprotein, CD63 and other tetraspanins are known to complex with integrins (Berditchevski, 2001; Yunta and Lazo, 2003). Recent studies relating the tetraspanins–integrin protein interactions suggest that as a class of proteins, the tetraspanins act as organizers of membrane microdomains and signaling complexes (Yunta and Lazo, 2003). Of particular

note is that CD63 resides not only in the cytoplasmic membranes of most cell types but also in late endosomes, lysosomes, and secretory vesicles, and CD63 traffics between these different compartments (Duffield *et al.*, 2003). This has led to the suggestion that CD63 may play a role in the recycling of membrane components, and also the uptake of degraded proteins from the extracellular matrix (Duffield *et al.*, 2003). Both these activities are likely apparent in ameloblasts with their rapid movement and the need to remove matrix proteins as they are replaced with mineral. To date, no *Cd63*-null animals have been reported.

A rabbit antipeptide polyclonal mouse Cd63 antibody was generated against a unique Cd63 peptide region (amino acids 177–190; N-terminal CGNDFKESTIHTQG) by Zymed Laboratories Inc. (South San Francisco, CA, USA). This polyclonal antibody has been used to examine Cd63 distribution in developing mice mandibular incisor teeth. Initial data shows that Cd63 is immunolocalized to the Tomes' processes of ameloblast cells, and is also distributed throughout the cytoplasm of ameloblasts and all surrounding cell types (Fig. 4). While the Y2H data implies that Cd63 has the ability to interact directly with secreted enamel proteins, this immunolocalization data also suggest that Cd63 has a functional role in amelogenesis, and this role is likely to relate directly to protein–protein interactions with individual enamel matrix proteins, or their protein fragments, at Tomes' processes.

C. Annexin A2

The human *annexin A2* (*ANXA2*) gene is located on chromosome 15q21-q22. ANXA2 is a 339 amino acid Ca^{2+} and phospholipid-binding protein (Gerke and Moss, 2002). Members of the annexin protein family play a role in the regulation of cellular growth and in signal transduction pathways. This protein functions as an autocrine factor that stimulates osteoclast formation and bone resorption (Gerke and Moss, 2002; Menaa *et al.*, 1999; Takahashi *et al.*, 1994). *Anxa2*-null mice display deposition of fibrin in the microvasculature and incomplete clearance of injury-induced arterial thrombi (Ling *et al.*, 2004). These *Anxa2*-null mice demonstrated normal lysis of fibrin-containing plasma clots, but tissue plasminogen activator-dependent plasmin generation at the endothelial cell surface was markedly reduced (Ling *et al.*, 2004). *Anxa2*-null mice also displayed markedly diminished neovascularization of fibroblast growth factor (FGF)-stimulated cornea and of oxygen-primed neonatal retina. No dental anomalies were reported in these *Anxa2*-null animals, however it is clear from this publication that the dentition of these animals was not investigated (Ling *et al.*, 2004).

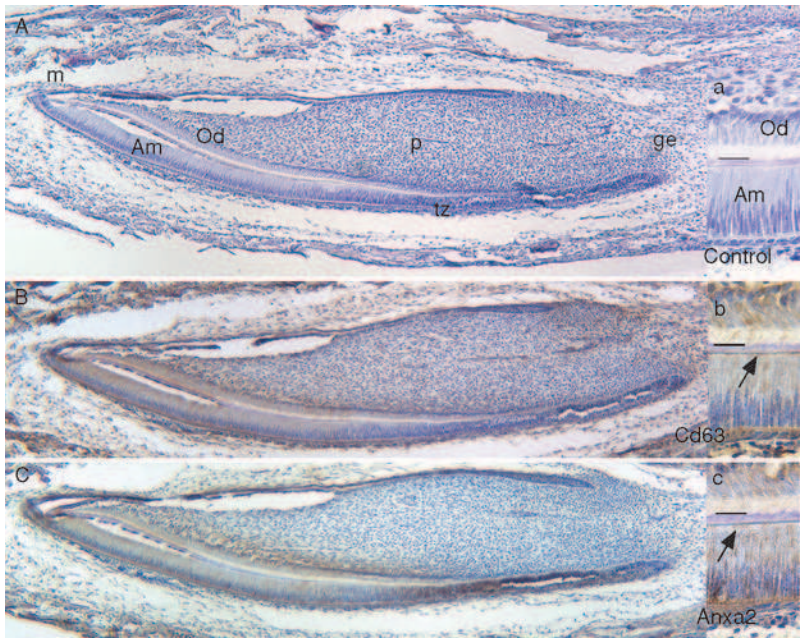

Figure 4 Immunolocalization of Cd63, and Anxa2 to ameloblast cells in a 3-day-old mouse mandibular incisor. Panel A is a no primary antibody control section. Panels A, B, and C are from a single 3-day-old mouse mandibular incisor. Abbreviations: mature end of incisor (m); transition zone ameloblasts (tz); growing end of incisor (ge); and dental pulp (p). Presecretory ameloblasts are located at the growing end, secretory ameloblasts are within and beyond the transition zone up to the postsecretory ameloblasts. Postsecretory ameloblasts are located at the mature end of the incisor teeth. The inset in each panel (a, b, and c) identifies secretory ameloblasts (Am), odontoblasts (Od), Tomes' processes (arrows) and dentino-enamel junction (straight line). All sections counterstained with hematoxylin prior to photographing. No antibody staining is seen in the negative control section seen in panel A. Panels A, B, and C are taken at 4× magnification while inset panels (a, b, and c) are 40× magnification. Panel B: A rabbit antipeptide polyclonal mouse Cd63 antibody was generated against a unique Cd63 peptide region (amino acids 177–190; N-terminal CGNDFKESTIHTQG) by Zymed Laboratories Inc. (South San Francisco, CA, USA) and used at a dilution of 1:30. The dominant location of immunostaining (brown chromophore) within ameloblasts is cytoplasmic (unevenly distributed within individual ameloblasts), with some staining also apparent at Tomes' processes (arrow). Panel C: A rabbit antipeptide (amino acids 1–50) polyclonal antibody to human ANXA2 was purchased from Santa Cruz Biotechnology (catalogue #sc-9061; Santa Cruz, CA, USA) and used at a dilution of 1:100. Immunostaining (brown chromophore) within the ameloblasts is cytoplasmic (unevenly distributed within individual ameloblasts), with some staining apparent at Tomes' processes (arrow).

ANXA2/Anxa2 also appears to be a necessary component of the machinery controlling endosomal membrane dynamics and multivesicular endosome biogenesis (Gruenberg and Stenmark, 2004; Mayran *et al.*, 2003). There are a few reports describing Anxa2 as a membrane-bound receptor, the best characterized ligand being tissue-type plasminogen activator (tPA), where tPA interacts with the amino-terminal amino acids 7–12 (Roda *et al.*, 2003). Anxa2 is reported to be a receptor for the ligand β_2-glycoprotein I, a phospholipid-binding protein from plasma known as an autoantigen in the antiphospholipid antibody syndrome (Gerke and Moss, 2002). ANXA2 can also act as a receptor for procathepsin B as demonstrated in human breast cancer cells (Mai *et al.*, 2000). Few reports describe annexin expression in the developing tooth. One report describes the distribution of annexins I–VI within secretory ameloblasts (Goldberg *et al.*, 1990). In this study annexin II/Anxa2 was present in the cytosol of secretory ameloblasts near Tomes' processes, and Anxa2 was also evident in the secretory vesicles of these same cells (Goldberg *et al.*, 1990). The role of annexins in tooth development thus appears related to exocytosis and endocytosis. Further, annexins are implied as having a role in the regulation of cell calcium (Goldberg *et al.*, 1990). Another report describes Anax2 mRNA in tooth germs as being stage-specific to amelogenesis (Fukumoto *et al.*, 2005). This report found strong expression of Anax2 mRNA in presecretory ameloblasts, weak expression in secretory ameloblasts, and strong expression in the early and late maturation stages of ameloblast cells (Fukumoto *et al.*, 2005).

For our studies, a rabbit antipeptide (amino acids 1–50) polyclonal antibody to human ANXA2 was purchased from Santa Cruz Biotechnology (catalog #sc-9061; Santa Cruz, CA, USA). Using this antibody we are able to demonstrate Anxa2 in presecretory, secretory, and postsecretory mouse ameloblast cells (Fig. 4), which is supportive evidence that Anxa2 plays a role in amelogenesis. In addition, the protein expression profile of Anxa2 throughout the various stages of amelogenesis is directly reflective of the mRNA profile previously described (Fukumoto *et al.*, 2005); that is higher levels of Anxa2 mRNA and protein are apparent in presecretory and postsecretory ameloblasts when compared to the secretory ameloblasts (Fig. 4).

VIII. Enamel Matrix Protein Isoforms as Signaling Molecules

Amelogenin is the most abundant protein of the enamel organic matrix and is a structural protein indispensable for enamel formation via self-assembly (Aldred *et al.*, 1992; Diekwisch *et al.*, 1993; Gibson *et al.*, 2001; Lagerstrom-Fermer *et al.*, 1995; Langerstrom *et al.*, 1991; Lyngstadaas *et al.*, 1995; Paine

and Snead, 1997; Paine *et al*., 2000b). The structural functions for amelogenin are becoming better known and have been previously reviewed (Paine *et al*., 2001). Recent evidence suggests that amelogenin can also function as a signaling molecule (Giannobile and Somerman, 2003; Veis, 2003), and this topic is discussed later.

Some three decades ago, leading investigators in the field of odontogenesis focused their attention on reciprocal epithelial–mesenchymal signal exchanges during tooth formation. These investigators postulated the exchange of biochemical signals, RNA, and other effector molecules believed to be responsible for instructing epithelia-fate or mesenchyme-fate during tooth development (Kollar and Baird, 1970a,b; Kollar and Lumsden, 1979; Slavkin and Bringas, 1976; Thesleff *et al*., 1977). Moreover, they postulated that signaling occurring in the crown of the tooth might also occur during root formation (Thomas and Kollar, 1989). Enamel matrix proteins secreted during root formation by Hertwig's root sheath cells, the apical extension at the cervical loop of the inner enamel epithelium, were shown to be involved in the formation of acellular cementum during nascent tooth development (Slavkin, 1976; Slavkin and Boyde, 1975; Slavkin *et al*., 1989a,b). Amelogenin is detected in the acellular cementum (Hammarstrom, 1997a; Slavkin *et al*., 1989a). Exposure of mesenchymal cells from the dental follicle to enamel matrix *in vivo* induces the formation of an acellular cementum-like layer at the surface for the matrix (Hammarstrom, 1997a,b).

Based on the insight from developmental biology discussed earlier, investigators extended their hypothesis to state that epithelial–mesenchymal interactions that occurred during root formation might be replayed in adult tissues to regenerate or repair lost root tissues and supporting tissue. Toward this end, enamel matrix proteins were tested on monkeys in a buccal dehiscence model. Application of homogenized enamel matrix or an acidic extract of the matrix containing the hydrophobic, low molecular weight proteins, amelogenins, results in an almost complete regeneration of acellular cementum, firmly attached to the dentin and with collagenous fibers extending over to newly formed alveolar bone (Hammarstrom, 1997a). Such studies lead to the development of a porcine enamel matrix derivative, known as EMDOGAIN® (EMD), which has been shown to promote periodontal regeneration (Gestrelius *et al*., 1997a,b; Hammarstrom, 1997a,b; Heijl, 1997; Heijl *et al*., 1997; Tokiyasu *et al*., 2000; Zetterstrom *et al*., 1997). Interestingly, differential effects of EMD on mesenchymal and epithelial cells are observed. Cell attachment rate, growth rate, and metabolism of human mesenchymal periodontal ligament cells are all significantly increased when EMD is present in culture; however, the proliferation and growth of epithelial cells are inhibited by EMD (Lyngstadaas *et al*., 2001). Treatment of oral epithelial cells (SCC25) with EMD results in p21WAF1/cip1-mediated G1 arrest without discernible increase of the number of apoptotic cells,

suggesting that EMD acts as a cytostatic agent rather than a cytotoxic agent on epithelial cells (Kawase *et al.*, 2000). The inhibitory role on epithelium was viewed as a therapeutic advantage, since ingrowth (lowering) of the epithelial attachment would favor periodontal pocket formation rather than the repair of surrounding tissues that would improve anchorage. On the other hand, the regulatory effects of EMD on mesenchymal osteoblast proliferation and differentiation are cell type and maturation stage specific (Schwartz *et al.*, 2000). The ability of EMD to induce new bone formation in nude mouse calf muscle or to enhance bone induction of a demineralized freeze-dried bone allograft was examined. The conclusion drawn is that EMD is not osteoinductive; however, it is osteopromotive above a threshold concentration (Boyan *et al.*, 2000). In contrast, human fetal enamel proteins have been shown to have bone inductive activity when implanted into mouse thigh muscle (Wang, 1993). The discrepancy could be due to species difference or contamination of bone morphogenetic proteins (BMPs) in the human fetal enamel proteins. In fact, significant level of TGF-β1 is detected in EMD preparations and EMD rapidly stimulates translocation of smad2 into the nucleus of both oral epithelial and fibroblastic cells (Kawase *et al.*, 2001). In a study using an anti-TGF-β neutralizing antibody, TGF-β1 functions as a principal bioactive factor in EMD to inhibit epithelial cell proliferation through a smad2-mediated p21WAF1/cip1-dependent mechanism, whereas other mitogenic factors act in combination with TGF-β to fully stimulate fibroblastic proliferation (Kawase *et al.*, 2002).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analyses of proteins contained within the EMD indicate that EMD is very heterogeneous. The amelogenin components consisting of degradation products and splicing variants are concentrated; whereas albumin, enamelin, and ameloblastin/amelin are absent in EMD. Zymography showed that both metalloendoprotease and serine protease activity are present in EMD (Maycock *et al.*, 2002). The complexity of EMD components makes it difficult to relate the biological activity of EMD to any single component.

Low molecular weight amelogenin-related polypeptides extracted from mineralized dentin have been shown to induce chondrogenesis in cultures of embryonic muscle-derived fibroblasts (EMF) *in vitro* and lead to the formation of mineralized matrix in *in vivo* implants (Nebgen *et al.*, 1999). Furthermore, two splicing products of rat amelogenin ([A+4] and [A-4]) have shown signaling capacity, inducing chondrogenic and osteogenic differentiation both *in vitro* and *in vivo* (Veis *et al.*, 2000). Both products at ng/ml level enhance *in vitro* sulfate incorporation into proteoglycan and induce the expression of type II collagen, a chondrocyte marker. However, the inductive activity on osteogenic differentiation is restricted to [A-4], evidenced

by the upregulation of Cbfa1 expression in [A-4]-treated rat EMF but not in [A+4]-treated cells. *In vivo* implant assays further demonstrate that both [A+4] and [A-4], each to a different extent, are active in inducing cellular ingrowth into the implants, followed by extracellular matrix production, vascularization, and mineralization. The [A-4] fraction induces a more copious and diffuse mineral deposition than [A+4], which produces more focal and more highly vascularized mineralized areas. The [A+4] and [A-4] proteins are orthologs of murine amelogenin isoform M73 and M59 respectively, with sequences identical between the two species. The isoform M59 is best known as leucine rich amelogenin polypeptide (LRAP). LRAP has been shown to have a direct effect on cementoblast activity and the involvement of MAPK pathway is suggested by inhibitor assays (Boabaid *et al*., 2004). Decreased expression of BSP at both the mRNA and protein level in cementoblasts and surrounding osteoblasts is observed in *amelogenin*-null mice. In immortalized murine cementoblasts (OCCM-30), BSP expression is enhanced by low concentration (0.1 μg/ml) whereas BSP is downregulated by high concentration (10 μg/ml) of recombinant full-length murine amelogenin protein (Viswanathan *et al*., 2003). In a recent study using BSP reporter constructs, recombinant full-length rat amelogenin protein (1 μg/ml) stimulates BSP expression in rat osteoblast-like ROS 17/2.8 cells and rat stromal bone marrow (BM) cells. The activation is mediated by FGF2 response element and TGF-β1 activation element in the BSP promoter (Shimizu *et al*., 2005). The difference in amelogenin concentration required to activate BSP in these two studies could result from differences in species (mouse vs rat), cell lines, and/or purity of the recombinant proteins.

Given that two splicing products of rat amelogenin ([A+4] and [A-4]) were identified by a screen of a rat pulp-odontoblast cDNA library (Veis *et al*., 2000), Veis and colleagues tested the hypothesis that the two amelogenin molecules have signaling effects on ameloblast and odontoblast development. When lower first molars from postnatal days 1 and 2 CD1 mice were cultured *in vitro*, exogenous [A-4] induced the expression of cementum attachment protein (CAP) in the mesenchymal cells of the dental follicle, whereas [A+4] induced the expression of dentin matrix protein 2 (DMP2) in the odontoblasts (Tompkins and Veis, 2002). In a related study, E15/E16 tooth germs were used to better examine the development of the enamel organ. Tooth germs cultured with [A+4] had well-polarized odontoblasts with robust dentin production and concomitant ameloblast polarization. On the other hand, cultures with [A-4] gave rise to disorganized preameloblast layer and nonpolarized ameloblasts along the dentin surface, and odontoblast polarization and dentin production were reduced compared with [A+4]-treated samples (Tompkins *et al*., 2005). The conclusion drawn

was that [A-4] had an inhibitory effect on ameloblast development, whereas [A+4] strongly stimulated odontoblast development. The observation also led to the hypothesis that [A-4] produced in the developing odontoblasts delays preameloblast maturation until the dentin layer is adequately formed. These seminal observations are clouded by some questions regarding experimental design. For example, in the tooth germ culture setting, it remains unclear whether the supposed target tissue (i.e., ameloblasts) is accessible to exogenous [A-4]. Tight junctions seal the enamel organ and the peptides would have to find an access route to influence the ameloblast if a direct effect of the peptide is anticipated. Alternatively, another population of cells in the enamel organ could have been the target and these cells then generated the signal that induced the changes observed in the ameloblast population. The expression profiles of [A-4] and [A+4] during tooth development also need to be determined to ascertain if the amount of endogenous material could be overcome by the exogenously added peptides or if there are any binding proteins for the [A+4] or [A-4] signaling molecules, as shown for other signals such as FGFs (Eswarakumar *et al.*, 2005) (with GAGs) or for BMPs (which are antagonized by noggin) (Chen *et al.*, 2004a,b; Yu *et al.*, 2002).

To determine the signaling effect of LRAP on human periodontal ligament stem cells (hPDLSC) (Seo *et al.*, 2004), we cultured hPDLSC in media supplemented with 100 μM ascorbic acid and 10 μM β-glycerophosphate for 4 weeks *in vitro* to induce mineralization. Alizarin red-positive nodules formed in the presence of 10 nM dexamethasone, indicating calcium accumulation *in vitro* (Fig. 5); however, no nodule formation was detected in the absence of dexamethasone (Fig. 4). When bacterially produced purified amelogenin protein LRAP was added to the culture media in place of dexamethasone, efficient nodule formation was evident in the three LRAP concentrations tested (1 μg/ml, 100 ng/ml, and 10 ng/ml; Fig. 4 panels C, D, and E). The fact that LRAP induced osteogenic differentiation of human periodontal ligament stem cells at the concentration of 10 ng/ml suggested that LRAP functioned as a signaling molecule.

Accumulating evidence indicates that amelogenin has signaling effects on various cell types, in addition to its structural role during enamel formation. To understand the underlying mechanism, it is essential to delineate signaling transduction pathways that mediate amelogenin's signaling effects, including receptor identification and target gene analysis. The effect of amelogenin on stem cell differentiation, such as the osteogenic effect of LRAP on hPDLSC differentiation, is a promising area that needs to be pursued. Knowledge gained in this field will be applicable to stem cell-based therapy, for example, tooth or bone regeneration.

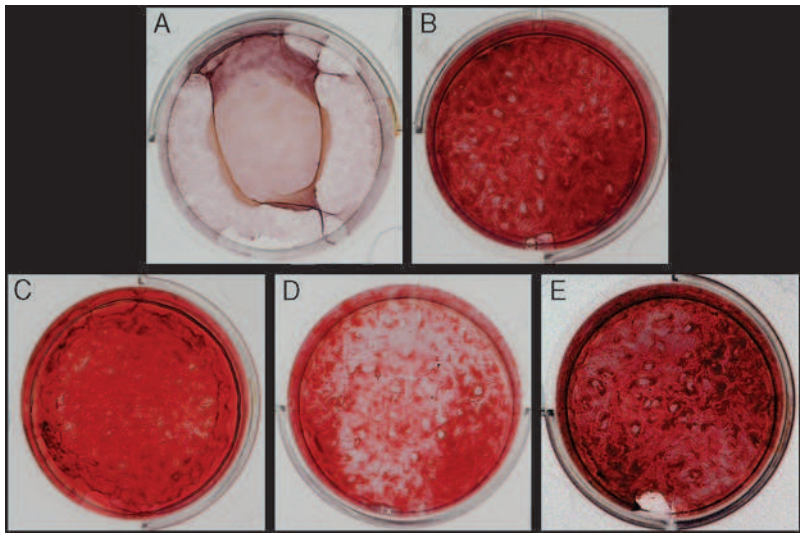

Figure 5 Mineralization of human periodontal ligament stem cells induced by amelogenin isoform LRAP. Human periodontal ligament stem cells were cultured in media supplemented with ascorbic acid and β-glycerophosphate for 4 weeks, and stained with alizarin red. Dexamethasone or bacterially produced purified LRAP was added in panels B–E. Panel A, control; Panel B, 100 nM dexamethasone; Panel C, 1 μg/ml LRAP; Panel D, 100 ng/ml LRAP; and Panel E, 10 ng/ml LRAP.

IX. Conclusions

Secreted enamel proteins assemble in an ordered manner to create a matrix competent to direct HAP crystallite habit and orientation into the resulting enamel prismatic structure. With recent data derived from the Y2H assay, a number of likely scenarios have evolved to explain these ordered, enamel matrix protein assembly properties. For example, both amelogenin and enamelin interact with a number of collagens and this may help explain the DEJ as a transition zone of mechanical properties rather than an abrupt interface between two dissimilar biological materials (Imbeni *et al.*, 2005; White *et al.*, 2000, 2005). A molecular mechanism to explain the DEJ as a transition zone has yet to be identified. Second, amelogenin, ameloblastin, and enamelin all interact with biglycan (itself a proteoglycan), leading to the suggestion that biglycan may allow for the union of these three unique enamel matrix proteins. Biglycan, through its sugar side chains, could act as a bridge to unite these three enamel matrix proteins. Third, enamel matrix proteins have been shown to interact with a number of membrane-bound proteins, including Cd63, Anxa2, and Lamp1. Cd63, Anxa2, and Lamp1 are

all products of ameloblast cells (Fukumoto *et al.*, 2005; Tompkins *et al.*, 2006; Wang *et al.*, 2005). Cd63 and Lamp1 also coat endosomes, lysosomes, and secretory vesicles (Duffield *et al.*, 2003; Goldberg *et al.*, 1990; Mayran *et al.*, 2003). Lamp1 has been described recently as an integral membrane protein that directly interacts with amelogenein (Tompkins *et al.*, 2006). This leads to the suggestion that Cd63, Anxa2, and Lamp1 may play a prominent role in the regulation and organization of secreted enamel proteins, and also the uptake of enamel matrix proteolytic byproducts. These events would necessarily occur at the Tomes' processes of secretory ameloblast cells.

X. Future Directions

To study enamel formation, scientists recently have focused their attention on identifying proteins of interest using protein-based technologies to govern their experimental strategy; a so-called proteomic approach to discovery. Now, with the sequencing of the mouse and human genomes, experimental approaches to study enamel formation are focused on molecular biology studies, and structure–function relationships of individual genes and their proteins to biomineralization. Despite the power of new technologies, we still do not have a complete catalogue of all the "enamel" genes, their products, and their function during enamel formation. However, as more investigators focus their attention to the unique biologic problems of enamel, new and valuable insights have, and continue to be, gained. Not least among these strategies is the use of gain-of-function and loss-of-function genetic manipulation of amelogenesis in mice. Mouse genetics, and our abilities to scan human haplotypes for defects in genes that regulate enamel biomineralization, offer the potential for rapid advancement in this field.

To view these studies from a different perspective, understanding protein–protein interactions as they relate to enamel matrix assembly and enamel biomineralization will be essential in our quest to regenerate a mammalian tooth. A similar level of understanding will also be required for the proteins involved with the formation of dental pulp, dentin, and the periodontal ligament (alveolar bone, cementum, Sharpey's fibers, and so on). The regeneration of the mammalian tooth is arguably the ultimate goal of current research into a greater understanding of the various stages and aspects of odontogenesis. It is now possible to imagine a future where a biologically inspired enamel substitute is available for clinical use, and tooth regeneration and implantation is a reality for patients with a compromised dentition. Current studies being done will help lay the foundation for both these very ambitious and challenging endeavors.

Acknowledgments

The corresponding author (MLP) would like to thank Dr Gerald Schatten for extending the invitation to my colleagues and myself to submit this chapter to "Current Topics in Developmental Biology," and also Cindy Minor from Elsevier/Academic Press for her assistance. All authors contributed equally to this chapter and all authors have proofread and accepted this chapter in its final form. The authors wish to thank HongJun Wang, Pablo Bringas Jr., and Drs Wen Luo and Curtis Okamoto for their technical expertise and insightful discussions over recent years; their expertise has contributed much to the data referred to, or presented, in this chapter.

Support for this research was provided from grants DE006988 and DE013045 (MLS), DE014084 (JDB), DE013404 and DE014867 (MLP), DE013414, DE015332 and DE015644 (JMO), and DE014189 (SNW) from the National Institute of Dental and Craniofacial Research, National Institutes of Health.

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Stem and Progenitor Cells in the Formation of the Pulmonary Vasculature

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The pulmonary vasculature is formed by two distinct mechanisms: vasculogenesis and angiogenesis. During vasculogenesis vessels form by *de novo* synthesis from cells residing within the distal mesenchyme, while in angiogenesis new vessels sprout from preexisting structures. Both processes require the activity of vascular stem/progenitor cells to differentiate and form the components of the vessel wall. In general, blood vessels are composed of two cell types, endothelial and vascular supporting cells. Isolation of these cells from the lung demonstrates remarkable heterogeneity. In part, this heterogeneity may relate to the various stem and progenitor cells involved in the formation of the pulmonary circulation. Reports indicate that multiple stem/progenitor cells, which have unique phenotypes and possess variable differentiation capacity, exist in the lung. Moreover, these cells are derived from separate tissues and contribute only to selected regions of the pulmonary circulation. In this chapter, we will summarize what is known about pulmonary vascular stem/progenitor cells, discuss their role in the development of the arterial and venous systems, and expound upon the factors limiting their study.

Current Topics in Developmental Biology, Vol. 74

0070-2153/06 $35.00
DOI: 10.1016/S0070-2153(06)74004-2

I. Introduction

The developing lung consists of an array of branching epithelial tubes enveloped by a network of mesenchymal cells. Residing within the network of mesenchymal cells are the stem and progenitor cells that serve as precursors to the supporting structures of the lung and the pulmonary vasculature. In part, the identification and isolation of these stem and progenitor cells has been limited due to the absence of cell-specific markers and the inherent complexities associated with isolating live cells from solid organs. Despite these limitations, reports have identified and even isolated putative vascular stem and progenitors from the developing lung. In this chapter, we will provide an introduction to lung vascular development and review what is known about the involvement of stem and progenitor cells in this process.

II. Mechanisms of Pulmonary Vascular Development: Vasculogenesis and Angiogenesis

The formation of vascular structures occurs by two principle mechanisms: angiogenesis and vasculogenesis (Fig. 1). During vasculogenesis, locally derived vascular progenitors proliferate to form a loose complex of cells that serve as a nidus for the developing blood vessel. Following formation of a cellular network, proliferating cells assemble into tube-like structures, and further development proceeds by angiogenesis.

Angiogenesis is the process by which new blood vessels sprout from preexisting ones. It is a complex process driven by growth factors, which stimulate proliferation and sprouting of endothelial cells, regulate lumen formation, and direct the recruitment of perivascular components (pericytes and smooth muscle cells) to the vessel wall. Angiogenesis is required for the extension of preexisting blood vessels and for establishing links between neighboring structures.

Although there remains some debate as to the exact mechanisms by which the pulmonary vasculature forms a widely held belief is that both angiogenesis and vasculogenesis play essential roles. Demello *et al.* (1997) used a combination of light microscopy, transmission electron microscopy, and vascular casts to examine the developing pulmonary arterial system. They demonstrated that proximal and distal blood vessels develop simultaneously but independently. This work along with that of others suggests that proximal hilar vessels form by central sprouting (angiogenesis), and distal vessels form by *de novo* synthesis through progenitors residing within the splanchnopleural mesenchyme (vasculogenesis). Reports suggest that similar mechanisms are likely involved in the formation of the proximal and distal

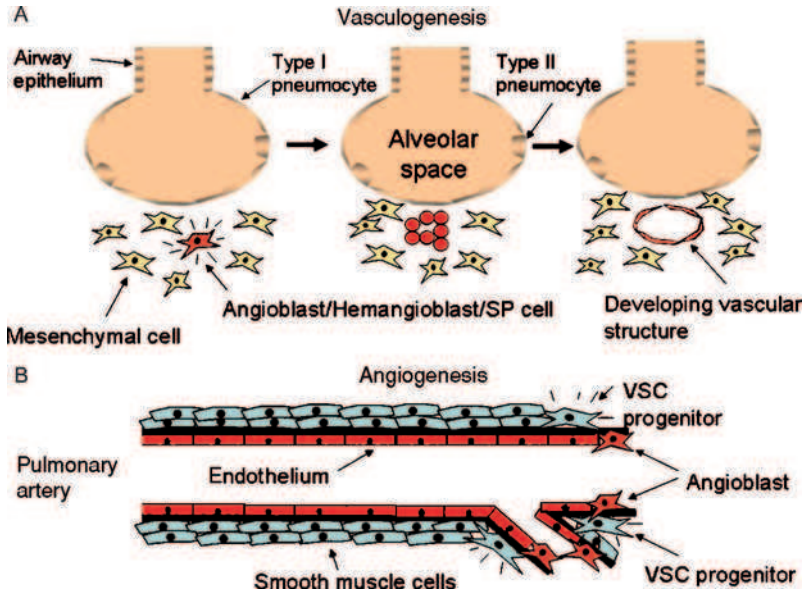

Figure 1 Mechanisms involved in the formation of the pulmonary vasculature. (A) Intrapulmonary blood vessels form by *de novo* synthesis from stem/progenitors residing within the distal mesenchyme. This process is referred to as vasculogenesis. (B) Proximal pulmonary vascular structures form by angiogenesis; a process in which new vessels form from preexisting ones. VSC, vascular supporting cell.

venous circulation (Anderson-Berry *et al.*, 2005; Hall *et al.*, 2002; Webb *et al.*, 2001). At this point, it is speculated that distinct stem/progenitor cells are involved in each of these processes.

III. Time Course of Embryonic Pulmonary Vascular Development

To identify and isolate lung vascular stem/progenitor cells requires an understanding of the time course of pulmonary vascular development. During embryogenesis, a distinct time point has been identified that is associated with the initiation of the pulmonary circulation (Schachtner *et al.*, 2000). Notably, the induction of the pulmonary circulation correlates with the onset of epithelial branching morphogenesis, which is believed to serve as a template for pulmonary vascular development. Consistent with this, intrapulmonary endothelial and smooth muscle precursors are thought to reside within the mesenchyme, surrounding the developing airways. Over time,

these precursor cells coalesce to form muscularized tube-like structures and acquire features of mature vessels. While the onset of pulmonary vascular development is restricted to specific embryonic time points, it should be noted that vascular development persists throughout embryogenesis and extends into the postnatal period (Burri and Tarek, 1990; Jones and Reid, 2004). This ongoing proliferation suggests that vascular stem and/or progenitor cells might exist in the late fetal and even the adult lung.

IV. Single or Multiple Vascular Progenitors

It has become increasingly apparent that significant heterogeneity exists among lung vascular cells. Differences between proximal and distal endothelial cells have been widely characterized. Endothelial cells isolated from the pulmonary artery and from the lung microvasculature have different growth properties, distinct signal transduction mechanisms, and express unique surface markers (Gebb and Stevens, 2004; Kelly *et al*., 1998; Parker and Yoshikawa, 2002). Moreover, endothelial cells derived from arteries, capillaries, and veins can be reliably differentiated on the basis of unique patterns of gene expression (Chi *et al*., 2003). Remarkably, even within the same vessel segment endothelial cells respond differently to noxious stimuli, suggesting that heterogeneity is not limited to anatomically distinct populations. Similarly, vascular smooth muscle cells (VSMCs) isolated from the same site in the medial layer of the bovine pulmonary artery exhibit structural and phenotypic heterogeneity (Frid *et al*., 1994). Whether differences in mature endothelial and smooth muscle cell phenotypes result from distinct environmental cues or reflect different developmental origins is not known. Support for the latter relates to findings demonstrating that phenotypically distinct vascular progenitors exist, they arise from separate embryonic tissues, and they localize to discrete anatomic niches (Akeson *et al*., 2000; Ali *et al*., 2003; Bergwerff *et al*., 1998; Demello *et al*., 1997; Hall *et al*., 2002; Jones *et al*., 1994).

V. The Principal Players: Vascular Stem Cells and Progenitors

In the scientific literature, the terms stem and progenitor cells are often used interchangeably, and as a result cells are often mistakenly labeled. In this chapter, we will only refer to cells as stem cells when strict criteria have been met. Stem cells are defined based on their unlimited self-renewal capacity and their broad differentiation potential. Importantly, not all stem cells possess the same differentiation capacity. Unlike stem cells, progenitor

cells have a limited differentiation and self-renewal capacity. Despite these limitations, progenitors play a key role in tissue development and maintenance of its integrity. In the remaining sections of this chapter, we will discuss the role of stem and progenitor cells in pulmonary vascular development. The hierarchical classification and the anatomic localization of these cells are depicted in Figs. 2 and 3, respectively.

VI. Vascular Progenitor Cells (Endothelial and Smooth Muscle Precursors)

Blood vessels are composed of a thin layer of endothelium surrounded by a layer of supporting cells. Numerous studies have shown that critical interactions occur between endothelial cells and supporting cells. For example, endothelial cells secrete a variety of factors that stimulate and inhibit smooth muscle growth. The interdependence between these cell types is further demonstrated by murine knockout models. Animals deficient in the smooth muscle ligand angiopoietin 1 (Ang1) or the endothelial receptor for Ang1, Tie2, develop severe vascular defects (Sato *et al*., 1995; Suri *et al*., 1996). Given these anatomic and molecular associations between endothelial and VSMCs it is not surprising that reports suggest they might be derived from a similar precursor.

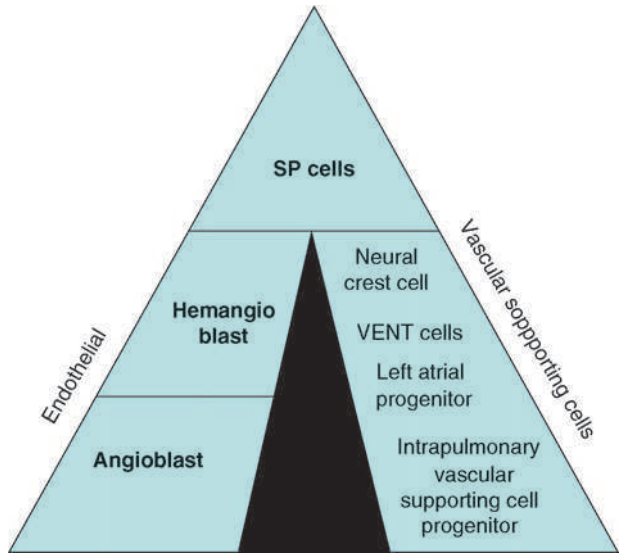

Figure 2 Proposed hierarchy of lung vascular stem/progenitor cells.

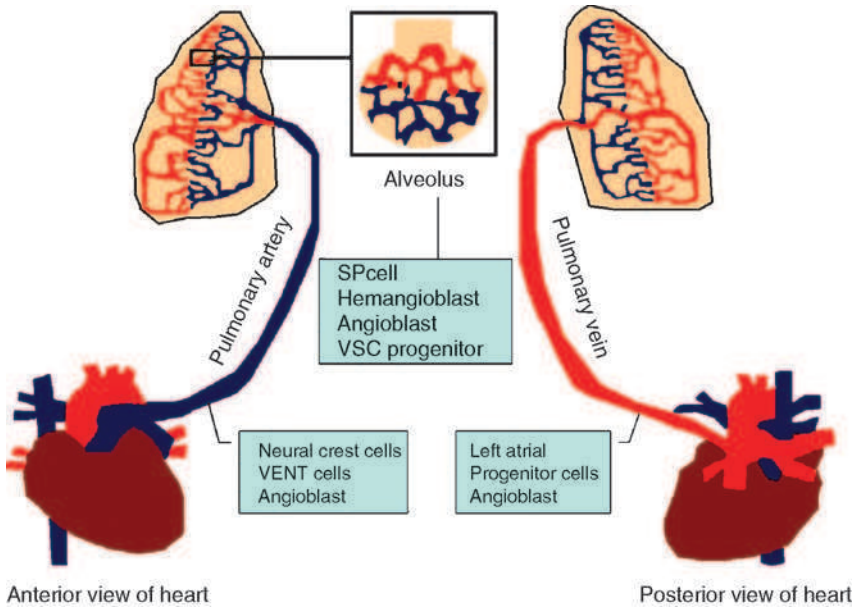

Figure 3 Anatomic location of putative pulmonary vascular stem/progenitors.

In one such report, Yamashita *et al.* (2000) isolated and grew clonally derived embryonic stem (ES) cells in specific conditions, including a collagen I matrix, and selected growth factors. During culture, ES cells differentiated into complex tube-like structures that resembled blood vessels histologically with an inner endothelial lining and an outer layer of smooth muscle. Importantly, these findings were reproduced *in vivo*. After injection into a chick blastocyst, ES cells incorporated into numerous vascular structures. While cells with the capacity to form endothelial cells and VSMCs have not been definitively identified in the lung, these findings provide support for a common endothelial and smooth muscle cell precursor.

Although less convincing, recent work suggests that a common vascular progenitor may reside within or traffic through the developing lung. In this study, Summer *et al.* (2005) used a novel approach to isolate stem/progenitor cells from the embryonic lung. After enzyme digestion, these investigators isolated lung cells based on their absence of staining to Hoechst dye. This strategy was selected based on discoveries in other tissues demonstrating that Hoechst-effluxing cells are enriched for stem/progenitor cell activity (Goodell *et al.*, 1996). In the embryonic lung, Hoechst-effluxing cells, also termed side population (SP) cells, are heterogeneous based on the expression

of the pan-hematopoietic marker CD45. Long-term competitive blood reconstitution studies demonstrated that hematopoietic stem cell (HSC) activity was restricted to the CD45+ subset. On the other hand, limited gene expression profiling suggested that the CD45− fraction might be a mesenchymal precursor. Consistent with this, *in vitro* assays demonstrated that these cells could differentiate to smooth muscle cells, endothelial cells, and form complex tube-like structures during culture. Unlike the ES cells described previously, these putative vascular progenitors did not express fetal liver kinase (flk1). To date, the capacity of CD45− lung SP cells to form vascular structures *in vivo* has yet to be tested; however, the ability to isolate purified populations by high-speed cell sorting should facilitate such studies.

VII. Hemangioblasts

The existence of a common precursor of hematopoietic and endothelial cells, termed a hemangioblast, has long been hypothesized (Murray, 1932; Sabin, 1920; Wagner, 1980). Indirect evidence for such a cell comes from the fact that endothelial and hematopoietic cells express common antigens (Flk-1, CD34) and develop in close proximity. The existence of a hemangioblast is further supported by the fact that mice deficient in Flk-1 develop defects in both hematopoiesis and vasculogenesis (Shalaby *et al*., 1997). *In vitro* studies utilizing ES cells have provided additional evidence for the presence of a hemangioblast. In these studies, murine ES cells expressing the marker Flk-1 were found to differentiate into blood and endothelial cell types. Similarly, cells derived from embryoid bodies also demonstrate the capacity to form hematopoietic and endothelial cells during culture (Choi *et al*., 1998). Hemangioblasts have been identified in humans and mice at later gestational time points (Guo *et al*., 2003). In mice, these cells coexpress Flk-1 and brachyury and localize to the mesoderm along the posterior primitive streak. Clonal assays have confirmed that single cells can differentiate to hematopoietic and vascular cell types. The fraction of hemangioblasts varies with gestational age. Cells are first detected at the mid-streak stage, peak at late-streak to early neural plate stage, and sharply decline thereafter. Since these studies are limited to early developmental time points (embryonic day 7.5) it is unclear whether a hemangioblast exists at induction of the pulmonary circulation. (Huber *et al*., 2004).

Maina (2004) provides the first report of a possible pulmonary hemangioblast; however, this work is limited to the avian lung. Using scanning and transmission electron microscopy at various stages of development the presence of homogenous appearing, tightly packed mesenchymal cells were identified. Within days of their appearance, these cells were noted to differentiate along two separate pathways: hematopoietic and angiogenic. Based on

this finding, Maina purports to have identified a hemangioblast that resides within the avian lung mesenchyme. While not as convincing, similar observations also have been made during mammalian lung development. In both humans and mice, blood-lakes can be identified in the lung at sites of ongoing vasculogenesis. These lakes are thought to exist prior to the formation of an intact pulmonary circulation, suggesting that blood cells are derived locally in the mesenchyme. While these findings might suggest that hematopoietic development occurs in the lung, this by no means demonstrates that endothelial and hematopoietic cells are derived from a similar precursor.

VIII. Angioblasts

Endothelial cell progenitors, or angioblasts, are precursors to mature endothelial cells. There is no clear consensus as to what defines an angioblast, and evidence for their existence is mostly indirect. Localization of tissue angioblasts is limited to immunohistochemistry data demonstrating the presence of cells with an "undifferentiated" endothelial phenotype at sites of ongoing blood vessel formation. Markers characteristic of an angioblast include the expression of "early" endothelial markers (Tie2, Tal1, flk1) and the lack of expression of "late" endothelial markers (Drake and Fleming, 2000). Based on these criteria, angioblasts have been identified in various species. These cells are initially localized to sites around the developing heart, and aortic primordia, and subsequently throughout the embryo at sites of ongoing vasculogenesis. After the formation of an intact vascular system, cells with an angioblast phenotype are no longer detectable in tissue sections (Drake and Fleming, 2000).

In the developing lung, cells with an angioblast phenotype have been identified in various species including murine, human, and avian. Immunohistochemistry data indicate these cells reside in the intrapulmonary mesenchyme surrounding the developing airway (Gebb and Shannon, 2000; Schachtner *et al*., 2000). Pardanaud *et al*. (1989) performed quail/chick interspecies lung bud transplantation and found that the pulmonary endothelium was entirely of the donor organ species, demonstrating the presence of resident lung angioblasts; however, they did not isolate or characterize these cells. Subsequently, Akeson *et al*. (2000) have developed a method for isolating angioblasts from the murine lung. In these studies, embryonic mesenchymal cells were isolated, immortalized, and screened for cells possessing an angioblast phenotype. Consistent with the multiple progenitor hypothesis, two distinct angioblast phenotypes were identified. Comparison of each population by flow cytometry found variable expression of endothelial specific genes [*Tie1*, *Tie2*, *Flk1*, *CD34*, and angiotensin converting

enzyme (ACE)]. Importantly, both populations were negative for the late endothelial marker CD31 and were capable of generating vascular tube-like networks during culture (Akeson *et al.*, 2000). The limitations of using clonally derived, virally transduced cells should be noted. However, this work may suggest a strategy for isolating endothelial cell progenitors from the lung. Future studies to determine whether clonally derived angioblast precursors are specific to a particular vascular bed and/or developmental time point need to be performed.

IX. Circulating Endothelial Progenitor Cells

In the adult, a role for circulating endothelial progenitor cells (EPCs) in the repair of tissues has been suggested. These cells are thought to originate in the bone marrow (BM) and are recruited to tissues after injury. Alterations in EPC number and function have been implicated in the pathogenesis of various diseases including coronary artery disease, systemic hypertension, and pulmonary hypertension (Del Papa *et al.*, 2004; Imanishi *et al.*, 2005; Schmidt-Lucke *et al.*, 2005; Vasa *et al.*, 2001). Data has suggested a role for circulating EPCs in pulmonary vascular repair. Ishizawa *et al.* (2004) demonstrated mobilization of EPCs from the BM in response to hepatocyte growth factor (HGF). Following elastase-induced lung injury, mice treated with HGF were found to have significantly increased bone marrow-derived cells in their lungs compared with non-HGF treated controls. These cells were morphologically consistent with endothelial cells, and expressed the endothelial cell marker CD34, suggesting that BM derived, circulating EPCs may participate in pulmonary vascular repair. However, without confocal microscopy, the BM-derived green fluorescent protein (GFP)+ cells found in the lung in this experiment cannot be definitely identified as endothelial cells based on the methods utilized.

EPCs have not yet been implicated in the development of the pulmonary circulation; however, the contribution of circulating progenitor cells to developing blood vessels seems quite logical.

X. Progenitors of the Vascular Supporting Cells

Vascular supporting cells (VSCs) are a heterogeneous population of cells that help to maintain vessel integrity and regulate its tone. In medium to larger vessels, supporting cells are composed of contractile and noncontractile smooth muscle, while in capillaries nonsmooth muscle cells, called pericytes, are present. Little is known about the precursors involved in VSC development; however, findings suggest that distinct progenitors exist

for the proximal and distal supporting cells, and the arterial and venous supporting cells.

Supporting cells of the proximal pulmonary artery are derived, at least in part, from neural crest cells. Neural crest cells are multipotent stem cells that are important in the development of various tissues. These cells can be identified based on the fact that they originate at the border of the neural plate and have a characteristic gene expression profile (Huang and Saint-Jeannet, 2004). Consistent with their key role in development, neural crest cells have been identified in virtually all species and have the capacity to differentiate into multiple cell types including neurons, melanocytes, endocrine cells, and relevant to our discussion, a diverse number of mesenchymal supporting cells (Le Douarin *et al.*, 2004).

A population of neural crest cells, which originate between the midotic placode and the third somite, termed the cardiac neural crest, plays a significant role in cardiovascular development (Kirby *et al.*, 1985). Miyagawa-Tomita *et al.* (1991) demonstrated that the cardiac neural crest also makes contributions to the proximal pulmonary arteries. This contribution was further characterized via both interspecies grafting (quail/chick) and a genetic labeling technique to track the migration of chick neural crest cells (Bergwerff *et al.*, 1998; Waldo and Kirby, 1993). Using each of these methods, neural crest cells were only detected in the most proximal aspect of the pulmonary artery where they localized to the mesenchyme and the outer part of the vessel wall during early development. As VSMCs have been reported to be derived from the neural crest and that neural crest cells have been identified in the mesenchyme and vessel wall suggests that neural crest cells may give rise to VSCs of the proximal pulmonary artery (Le Lievre and Le Douarin, 1975). However, at later developmental time points, these cells were no longer present. Together these findings suggest that additional source(s) of VSC progenitors participate in pulmonary arterial development.

One such candidate source of VSC progenitors that arises near the neural plate has been identified. These cells, termed ventrally emigrating cells from the neural tube (VENT) cells, originate in the ventral part of the hindbrain and emigrate to their target tissues. Ali *et al.* (2003) demonstrated using a retroviral tag that VENT cells directly contribute to the mesenchymal components of the ventricles, the atria, and the great vessels of the heart. Immunohistochemistry data confirmed their differentiation to proximal pulmonary artery smooth muscle. Moreover, ablation of VENT cells prior to their departure from the neural tube results in severe developmental defects, including pulmonary artery stenosis, demonstrating the critical role that VENT cells play during vascular development (Ali *et al.*, 2003). Like neural crest cells, much of the work on VENT cells has been limited to avian species, and it is not clear if these findings can be extrapolated to other species.

VSCs of the proximal pulmonary veins are thought to arise from an entirely different source, the atrial myocardium. This finding has led some investigators to refer to these cells as the ''pulmonary myocardium'' (Favaro, 1910). Consistent with their origin, mural cells along the pulmonary vein express various atrial myocardial markers including alpha-myosin heavy chain, myosin light chain 1A, atrial natriuretic factor, and cardiac troponin I (cTNI) (Jones *et al.*, 1994; Lyons *et al.*, 1990). Using confocal microscopy, Millino identified three distinct layers of the adult mouse pulmonary vein: an inner CD31+ endothelial layer, a middle smooth muscle actin (SMA)/smooth muscle myosin layer, and an outer pulmonary myocardium that expresses cTNI. Developmentally, these cTNI+ cells first appear in the proximal pulmonary vein around embryonic day 12.5. Studies in late gestation indicate that these cells extend only to the third bifurcation, and do not exist along the pulmonary artery, in keeping with the hypothesis that the VSCs of the pulmonary artery and pulmonary vein arise from different progenitor populations (Millino *et al.*, 2000).

Intrapulmonary VSCs, unlike those of the extrapulmonary circulation, have been suggested to arise from three possible sources, including migrating bronchial smooth muscle cells, transdifferentiating endothelial cells, and relevant to this chapter, progenitor cells located within the mesenchyme surrounding the developing lung (Hall *et al.*, 2000). Examination of serial reconstructions of human fetal lung has revealed initially undifferentiated (α-SMA negative) mesenchymal cells surrounding the intrapulmonary artery. At later time points these mesenchymal cells are found to express α-SMA and to be actively replicating, suggesting a VSC progenitor cell phenotype (Hall *et al.*, 2000).

These cells have not been further characterized or isolated due to the absence of cell-specific markers. However, a report suggests that a member of the annexin family of proteins, annexin A5, is a candidate VSC progenitor cell marker. During murine development, annexin A5 expressing cells are confined to areas of ongoing vasculogenesis, and dual immunofluorescent staining has confirmed that these cells are separate from but adjacent to the developing endothelium. Isolation and gene expression profiling of freshly isolated cells demonstrate expression of early smooth muscle and pericyte-specific genes. Finally, these cells have been shown *in vitro* to differentiate to multiple mature mesenchymal cells, including adipocytes, chondrogenic, and osteogenic cell types (Brachvogel *et al.*, 2005). Thus, based on their location, early gene expression profile, and broad mesenchymal differentiation capacity, annexin A5 expressing cells are attractive candidates as possible VSC progenitors. Further work is warranted to determine if annexin A5 expressing cells can form vascular smooth muscle and if they can be found within the lung mesenchyme.

XI. Conclusions

Stem cells are essential in the formation and regeneration of many tissues. Studies of stem cells have significantly advanced the understanding of organ development, differentiation pathways, and gene expression patterns during development. Significant progress has been made in identifying progenitor cells that give rise to elements of vessel walls, including endothelial cell and VSC progenitors. These studies have provided insight into the molecular signals and interactions that are critical for vascular development.

Much of our understanding of which stem and progenitor cells give rise to the pulmonary vasculature are based on findings from interspecies grafting studies and immunolocalization data. Together this work has provided clues to the origin and mechanisms of pulmonary vascular development. Despite these advances, there are significant limitations with these approaches. Interspecies grafting techniques are performed primarily in avian species, and the morphologic and functional differences between avian and mammalian lung vascular systems make generalization difficult. Second, immunohistochemistry studies rely on the expression of early but not late endothelial cell markers to localize vascular stem and/or progenitor cells. This strategy is largely descriptive and fails to test whether cells possess functional stem or progenitor cell properties.

At present, significant obstacles have limited the study and our understanding of lung vascular stem and progenitors. Principal among these is the absence of cell-specific markers and the complexity associated with isolating live cells from solid organs. In addition, the late development of the pulmonary vascular system in relation to other vascular beds further complicates matters. Mice genetically engineered to be deficient in key endothelial specific molecules frequently die prior to the formation of the pulmonary circulation, thus, limiting the utility of these models. In turn, progress in this area of research will likely require: (1) the identification of new lung vascular stem and progenitor cell markers, (2) the improvement in cell isolation techniques, and (3) the development of conditional knockouts.

In spite of the above limitations, considerable progress has been made in identifying at least some of the stem and progenitor cells that give rise to the cellular components of the pulmonary vasculature. There appear to be differences in the origin of the proximal and distal vasculature, supporting the notion that lung vessels form by angiogenesis proximally and vasculogenesis distally. For example, the neural crest cell and pulmonary myocardium have been demonstrated to contribute to the VSCs of the proximal pulmonary vessels. By contrast, the work of Pardanaud suggests that intrapulmonary vessels arise from local vascular progenitor cells residing within the distal mesenchyme rather than from invading nonlung cell types. Thus far,

numerous potential progenitors of the intrapulmonary vasculature have been identified, including a common endothelial and VSC progenitor (possibly a subset of the SP cells of the lung), a possible hemangioblast in the avian lung, and Flk-1 (+) angioblasts. Less progress has been made in identifying progenitors of distal lung vascular smooth muscle, but annexin A5 appears to be a promising candidate marker of such cells.

The pulmonary vasculature is an extremely complex structure. Remarkable heterogeneity exists even among endothelial and smooth muscle cells derived from the same blood vessel. Further elucidating the derivation and plasticity of these cells and the extent that this heterogeneity is accounted for by differences in progenitor cell sources will contribute significantly to the understanding of pulmonary vascular formation, function, and its repair after injury.

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Mechanisms of Disordered Granulopoiesis in Congenital Neutropenia

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Current Topics in Developmental Biology, Vol. 74

0070-2153/06 $35.00
DOI: 10.1016/S0070-2153(06)74005-4

Neutrophils are critical components of the innate immune response, and persistent neutropenia is associated with a marked susceptibility to infection. There are a number of inherited clinical syndromes in which neutropenia is a prominent feature. A study of these rare disorders has provided insight into the mechanisms regulating normal neutrophil homeostasis. Tremendous progress has been made at defining the genetic basis of these disorders. Herein, progress in understanding the genetic basis and molecular mechanisms of these disorders is discussed. We have focused our discussion on inherited disorders in which neutropenia is the sole or major hematopoietic defect.

I. Neutrophil Homeostasis

Neutrophil homeostasis in the blood is regulated at three levels: (1) neutrophil production in the bone marrow (granulopoiesis), (2) neutrophil release from the bone marrow to blood, and (3) neutrophil clearance from the blood. Under normal conditions, neutrophils are produced exclusively in the bone marrow, where it is estimated that 10^{12} neutrophils are generated on a daily basis (Boxer and Dale, 2002). Mature neutrophils are selectively released from the bone marrow to the blood in a regulated fashion. Once in the circulation, neutrophils are cleared rapidly with a half-life of only 6–8 hours (Lord *et al.*, 1989).

A. Granulopoiesis

Granulocytic differentiation of hematopoietic stem cells is regulated by the coordinated expression of a number of key myeloid transcription factors, including PU.1, CCAAT enhancer-binding protein α (C/EBPα), C/EBPε, and GFI-1. The contribution of these and other transcription factors to the regulation of granulopoiesis has been reviewed previously (Rosmarin *et al.*, 2005).

Granulocyte colony-stimulating factor (G-CSF) is the principal cytokine regulating granulopoiesis. It is widely used in the clinical setting to treat or prevent neutropenia. G-CSF stimulates the proliferation of granulocytic precursors, reduces the average transit time through the granulocytic

compartment, and stimulates neutrophil release from the bone marrow (Anderlini *et al.*, 1996). The biological effects of G-CSF are mediated through the G-CSF receptor (G-CSFR), a member of the hematopoietic (class I) cytokine receptor family. The role of G-CSF in basal granulopoiesis has been confirmed by the severe, but not absolute, neutropenia present in G-CSF deficient and G-CSFR deficient (G-CSFR$^{-/-}$) mice (Lieschke *et al.*, 1994; Liu *et al.*, 1996). Granulocyte-macrophage colony-stimulating factor (GM-CSF) has also been used to stimulate both the production and release of neutrophils. However, mice lacking GM-CSF have normal granulopoiesis, suggesting a redundant role for this cytokine in granulopoiesis (Stanley *et al.*, 1994).

B. Neutrophil Release

Neutrophils, under normal conditions, are produced solely in the bone marrow and are released into the blood in a regulated fashion to maintain homeostatic levels of circulating neutrophils. The bone marrow provides a large reservoir of mature neutrophils that can be readily mobilized in response to infection. In mice, only 1–2% of the total number of mature neutrophils is found in the blood with the great majority remaining in the bone marrow (Semerad *et al.*, 2002). A broad range of substances has been shown to induce neutrophil release from the bone marrow, including chemokines, cytokines, microbial products, and various other inflammatory mediators (e.g., C5a) (Opdenakker *et al.*, 1998). Evidence suggests that the chemokine stromal-derived-factor-1 (SDF-1, CXCL12) plays a key role in regulating neutrophil trafficking in the bone marrow; this issue will be discussed in detail in the section on warts, hypogammaglobulinemia, infections, and myelokathexis (WHIM) syndrome.

II. Congenital Neutropenia (Overview)

Congenital neutropenia is defined as an inappropriately low number of circulating neutrophils (usually less than 1000 per microliter of peripheral blood) present at or shortly after birth. Depending upon the severity and chronicity of neutropenia, these patients are prone to develop bacterial infections. The infections, which are typically caused by endogenous flora and involve mucous membranes, include gingivitis, stomatitis, perirectal abscesses, and cellulitis. Pneumonia and septicemia also occur, although less frequently. Fungal infections are rare and generally result from prolonged antibiotic therapy and the subsequent disruption of normal flora. Patients with isolated neutropenia do not have an increase susceptibility to viral or

parasitic infections, although many causes of congenital neutropenia are associated with derangements in cellular and humoral immunity as well.

Table I contains a representative list of congenital neutropenia syndromes, their mode of inheritance, and the genetic lesion(s) responsible, where known. Severe congenital neutropenia (including Kostmann syndrome), cyclic neutropenia, and WHIM syndrome are syndromes in which neutropenia is the sole or major clinical feature; they are discussed in detail in this chapter. There are a number of systemic disorders in which neutropenia is a frequent and often important clinical feature (Table I). These syndromes are briefly discussed in this chapter with the exception of dsykeratosis congenita and fanconi anemia, for which excellent reviews have been published (Mason *et al.*, 2005; Tischkowitz and Hodgson, 2003; Wang and D'Andrea, 2004). There are a number of primary immunodeficiency syndromes that are associated with neutropenia. In these conditions, the loss of cellular and/or humoral immunity leads to an inappropriate mobilization of the innate immune system against infection. Consequently, the defect in neutrophil production seen in these syndromes is not generally attributed to an intrinsic myeloid defect. Likewise, autoimmune neutropenia results from accelerated neutrophil destruction rather than disordered granulopoiesis. Since granulopoiesis is thought to be normal in these later two groups of congenital neutropenia, they will not be discussed further in this chapter.

III. Severe Congenital Neutropenia/Cyclic Neutropenia

A. Clinical Presentation

Severe congenital neutropenia (SCN) is a heterogeneous group of disorders first described in an extended consanguineous family by the Swedish physician Rolf Kostmann in the 1950s (Kostmann, 1956). The disease has thus also been known as Kostmann's syndrome, although this eponym has since been used to define a subset of patients with autosomal recessively inherited SCN. SCN is characterized by severe neutropenia present at birth with absolute neutrophil counts generally below 200 cells per microliter. The bone marrow invariably shows an arrest in myeloid maturation with an accumulation of promyelocytes or myelocytes. Other hematological parameters are generally normal, although a peripheral monocytosis is often observed.

Historically, affected patients had a poor prognosis and often succumbed in the first or second decade of life with recurrent severe bacterial infections. The use of G-CSF has changed the natural history of this disease; the results of a randomized phase III trial comparing G-CSF with no treatment in patients with SCN demonstrated that the majority (>90%) of patients had a significant increase in circulating levels of neutrophils (Dale *et al.*, 1993).

Table I Causes of Congenital Neutropenia

	Inheritance[a]	Genetics
Isolated disorders of the myeloid lineage		
Severe congenital neutropenia	AD, XL	*ELA2* (sporadic); *GFI1*, *CSF3R* (AD); *WAS* (XL)
Kostmann syndrome[b]	AR	
Cyclic neutropenia	AD	*ELA2*
WHIM syndrome[c]	AD, AR	*CXCR4* (AD)
Systematic disorders affecting bone marrow function		
Shwachman–Diamond syndrome	AR	*SBDS*
Barth syndrome	XL	*TAZ*
Pearson's syndrome	mitochondrial	variable deletions
Glycogen storage disease Type Ib	AR	*G6PT1*
Chediak–Higashi syndrome	AR	*LYST*
Griscelli syndrome (type II)[d]	AR	*RAB27A*
Cartilage-hair hypoplasia	AR	*RMRP*
Dyskeratosis congenita	XL; AD	*DKC1* (XL); *TERC* (AD)
Fanconi anemia	AR; X-linked	*FANCA*, C, *BRCA2* (D1), D2, E, F, *XRCC9* (G), I, J, *PHF9* (L) (AR); *FAAP95* (FANCB) (XL)
Primary immunodeficiencies associated with neutropenia		
Hyper IgM syndrome	XL; AR	*HIGM1*, *IKBKG* (XL); *AICDA* (AR)
Agammaglobulinemia[e]	XL; AD	*BTK* (XL); *LRRC8* (AD)
Severe combined immunodeficiency	XL; AR	*IL2RG* (XL); *ADA*, *RAG1 & -2*, *DCLRE1C* (AR)
Reticular dysgenesis[f]	AR	
Autoimmune neutropenias		
Alloimmune neonatal neutropenia		
Primary autoimmune neutropenia of infancy		

[a]AD, autosomal dominant; AR, autosomal recessive; XL, X-linked.

[b]Kostmann syndrome typically refers to the autosomal recessive form of SCN first described by Kostmann.

[c]WHIM syndrome is also associated with significant B cell dysfunction.

[d]Griscelli syndrome has been classified as GS1, GS2, and GS3; depending on the gene mutated.

[e]Mutations in the BTK gene may directly impair neutrophil production and function.

[f]Reticular dysgenesis is associated with a myeloid-intrinsic defect in neutrophil production.

Furthermore, the incidence and severity of bacterial infections was significantly reduced. These data have led to the widespread use of G-CSF in patients with SCN.

One of the hallmarks of SCN is a pronounced predisposition to myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML) with a crude rate of malignant transformation that exceeds 10%. Disturbingly, a study noted that the cumulative incidence of MDS/AML among patients on G-CSF for 6 years was 9% (Rosenberg, 2003). This figure rose to 23 and 33% for patients on G-CSF therapy for 10 or 12 years, respectively. Furthermore, the risk of MDS/AML also correlates with G-CSF dose, as patients receiving G-CSF doses above the median had a 2.7-fold higher incidence of malignant transformation. Whether higher doses of G-CSF directly contribute to leukemogenesis or instead simply define a more severe SCN phenotype remains to be answered. There have also been three reported cases of acute lymphoblastic leukemia (ALL) secondary to SCN (Cassinat *et al.*, 2004; Tschan *et al.*, 2001; Yetgin *et al.*, 2005).

Cyclic neutropenia (CN) is a related disorder of granulopoiesis, characterized by 21-day oscillations in the number of circulating neutrophils (Haurie *et al.*, 1998). In CN patients, neutrophil counts often fluctuate between normal or near normal levels to less than 200 per microliter. Reticulocytes, platelets, and monocyte numbers in the blood also oscillate (with monocytes out of phase with respect to oscillations in the neutrophil counts), and the amplitude of these oscillations is generally smaller (Wright *et al.*, 1994). The studies have found evidence of cycling in patients classified as having SCN (Haurie *et al.*, 1999), suggesting that SCN and CN are related and may fall along a continuum of disorders of granulopoiesis.

B. Genetics

Cyclic neutropenia is inherited in an autosomal dominant fashion (Briars *et al.*, 1996), whereas SCN demonstrates multiple modes of inheritance, including autosomal recessive, autosomal dominant, X-linked, and sporadic patterns. Accordingly, genetic studies have identified a single gene (*ELA2*) associated with CN but multiple gene mutations in SCN, including *ELA2*, *GFI1*, *CSF3R*, and *WAS*. The genetic cause of autosomal recessively inherited SCN (Kostmann syndrome) remains unknown.

1. *ELA2* Mutations

Genomewide genetic linkage analysis and positional cloning localized the gene responsible for cyclic neutropenia to a 400-kb region on chromosome 19p13.3 (Horwitz *et al.*, 1999). Using a candidate gene approach, mutations

were identified in the *ELA2* gene in 13 independent pedigrees. The *ELA2* gene encodes neutrophil elastase (NE), a serine protease found in the primary granules of neutrophils. Reports suggest that all cases of CN are associated with *ELA2* mutations. Subsequent studies have found that between 35 and 88% of patients with SCN (all with sporadic or autosomal dominant SCN) also have mutations of the *ELA2* gene, strengthening the hypothesis that CN and SCN are related diseases lying along a continuum of disorders of granulopoiesis (Ancliff *et al.*, 2001; Bellanne-Chantelot *et al.*, 2004; Dale *et al.*, 2000; Germeshausen *et al.*, 2001). To date, 47 different heterozygous mutations have been identified in patients with CN or SCN (Bellanne-Chantelot *et al.*, 2004). Most of the mutations (~80%) are missense mutations, although nonsense mutations (~10%) and splicing defects leading to in-frame deletions and insertions (~10%) have also been observed (Fig. 1). While most of the mutations identified segregate with one phenotype (i.e., CN or SCN), a number of mutations, notably S97L, P110L, and ΔV161–F170, have been associated with both phenotypes, suggesting the presence of other genetic disease modifiers. Bellanne-Chantelot *et al.* also noted that SCN patients with *ELA2* mutations have more severe neutropenia, increased rates of infectious complications, and a greater risk of leukemic transformation than SCN patients without *ELA2* mutations.

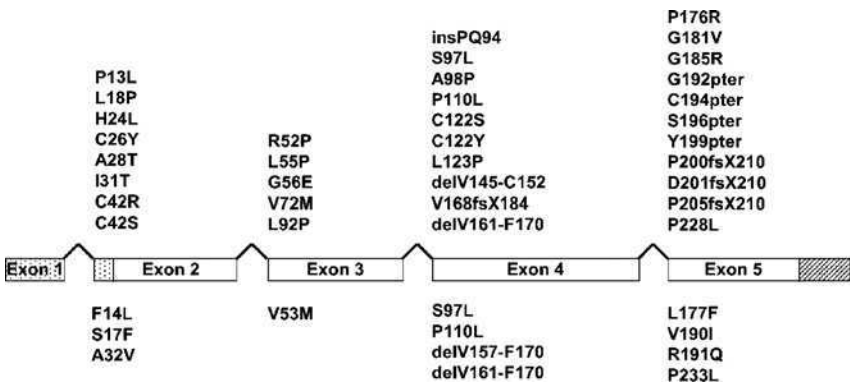

Figure 1 *ELA2* mutations in severe congenital neutropenia and cyclic neutropenia. Mutations listed above the gene are associated with SCN; those listed below are associated with CN. A subset of mutations has been associated with both phenotypes; these mutations are listed twice. Mutations are listed above or below the exon that they affect. In addition to those listed, a promoter mutation and a mutation of the start codon to valine have also been identified; the significance of these mutations is unclear.

The description of a case of paternal mosaicism in a family with SCN provides further evidence implicating *ELA2* gene mutations in the pathogenesis of SCN (Ancliff *et al.*, 2002). The father of a patient with SCN and an *ELA2* mutation was found to have the same mutation but was not neutropenic. While approximately 50% of his T lymphocytes were heterozygous for the *ELA2* mutation, it was nearly absent in his peripheral blood neutrophils. These results suggest that this individual is a mosaic who acquired a mutation in one of his *ELA2* alleles at the two-cell stage of development. Since NE expression is normally restricted to myeloid cells, and since no toxic paracrine effects on wild type neutrophils were seen, these observations suggest that expression of mutant NE inhibits granulopoiesis in a cell intrinsic manner.

2. *CSF3R* (G-CSF Receptor) Mutations

There are three case reports of patients with SCN who have germline mutations in the extracellular domain of their G-CSF receptor in the absence of *ELA2* mutations (Dror *et al.*, 2000; Druhan *et al.*, 2004; Sinha *et al.*, 2003). As noted in an earlier section, G-CSF is the principal cytokine-regulating granulopoiesis, and mice lacking G-CSF or the G-CSF receptor display severe chronic neutropenia. The germline G-CSFR mutations found in patients with SCN are thought to act in a dominant-negative fashion by inhibiting receptor trafficking to the cell surface and heterodimerization in response to G-CSF. There are two distinct features of SCN associated with these G-CSFR mutations. First, these patients display unusually severe neutropenia that is refractory to suprapharmacologic doses of G-CSF. Second, the bone marrow of these patients displays hypocellularity throughout the myeloid lineage rather than the accumulation of promyelocytes characteristic of most cases of SCN.

3. *GFI1* Mutations

There is a report describing heterozygous germline mutations of *GFI1* in two families with persistent neutropenia and lymphopenia (Person *et al.*, 2003). *GFI1* encodes for a zinc-finger domain transcriptional repressor. Mice lacking *GFI1* display severe neutropenia, monocytosis, and lymphopenia (Hock *et al.*, 2003; Karsunky *et al.*, 2002). The mutations of *GFI1* in patients are in the zinc-finger domains and are thought to generate a dominant-negative *GFI1* mutant protein. The phenotype of SCN associated with GFI1 mutations is distinct from that observed in classic SCN, as perturbations in lymphocyte production and function are not typically seen in SCN associated with *ELA2* mutations.

4. Wiscott–Aldrich Syndrome Protein (WAS)

An X-linked form of SCN has been identified that is linked to germline mutations in the *WAS* gene (Ancliff, 2003; Devriendt *et al.*, 2001). Unlike classic Wiscott–Aldrich syndrome, which results from loss-of-function mutations, WAS-associated X-linked neutropenia appears to result from gain-of-function mutations of *WAS* that disrupt an autoinhibitory domain of the WAS protein.

C. Molecular Pathogenesis

1. Cell Biology

Prior to the discovery of distinct genetic loci responsible for different subtypes of SCN and CN, studies of the molecular pathogenesis of SCN were focused on cell biology. Several groups characterized the growth and differentiation of hematopoietic progenitors isolated from patients with SCN. Hestdal *et al.* (1993) showed that a 10–100-fold higher concentration of G-CSF (but not IL-3 or GM-CSF) was required for maximal colony formation of progenitors isolated from SCN patients versus healthy controls. The number of mature neutrophils in these colonies was reduced by greater than 50%. Surprisingly, the defect in G-CSF proliferation and granulocytic differentiation could be rescued by inclusion of kit ligand in the culture media. Likewise, Konishi *et al.* (1999) showed that G-CSF-induced proliferation and granulocytic differentiation of $CD34^+$/c-kit^+ progenitors isolated from four patients with SCN was impaired. There are two reports showing that the number and cytokine responsiveness of $CD34^+$/G-$CSFR^+$ progenitors but not $CD34^+$/G-$CSFR^-$ progenitors is reduced in patients with SCN (Kawaguchi *et al.*, 2003; Nakamura *et al.*, 2000). Collectively, these data suggest a cell intrinsic defect in G-CSF-induced granulocytic differentiation of myeloid progenitors in patients with SCN.

There is an evidence that myeloid progenitors and granulocytic precursors from patients with SCN have an increased susceptibility to apoptosis (Aprikyan *et al.*, 2003; Carlsson *et al.*, 2004; Mackey *et al.*, 2003). In fact, one study estimated the rate of apoptosis in postmitotic granulocytic precursors to be 13-fold higher than in normal cells (Mackey *et al.*, 2003). The increased susceptibility to apoptosis could be partially rescued by treatment with G-CSF *in vitro*, providing a potential mechanism for the therapeutic efficacy of G-CSF in patients with SCN (Carlsson *et al.*, 2004). Increased rates of apoptosis have also been observed in myeloid cells from patients with cyclic neutropenia (Aprikyan *et al.*, 2001). These data support the hypothesis that an increased susceptibility to apoptosis may contribute to the defect in granulopoiesis in SCN.

Neutrophils from patients with SCN display impaired chemotaxis and superoxide ion generation (Elsner *et al.*, 1993). The clinical relevance of these functional neutrophil defects is unclear since most infectious complications resolve once neutrophil counts are greater than 1000 per microliter. Several biochemical studies have also been performed on patient-derived neutrophils. These studies have found differential expression of GTPases, guanine diphosphate-dissociation inhibitors, and phosphatases in patient neutrophils (Kasper *et al.*, 2000; Tidow *et al.*, 1999). However, it remains unclear whether these biochemical alterations directly contribute to the defect in neutrophil function or simply reflect an underlying perturbation in granulopoiesis.

Finally, there is evidence that expression of primary granule proteins is globally reduced in myeloid precursors from patients with SCN (Kawaguchi *et al.*, 2003; Sera *et al.*, 2005). Specifically, mRNA expression of NE, proteinase-3, and myeloperoxidase was reduced. This effect does not appear to be directly related to *ELA2* mutations, since it is observed in *ELA2*-normal SCN samples. Moreover, primary granule protein expression is normal in myeloid precursors from patients with CN, all of whom have *ELA2* mutations. Whether decreased primary granule protein expression contributes to the pathogenesis of SCN is unclear.

2. *ELA2* Mutations

As reviewed earlier, there is convincing genetic evidence that mutations of *ELA2* are causative for most cases of sporadic or autosomal dominantly inherited SCN. *ELA2* encodes for NE, a serine protease found in the primary (azurophilic) granules of neutrophils and monocytes, where it is stored in an active state (Borregaard and Cowland, 1997). Upon stimulation, neutrophils and monocytes may release their granule contents, including NE, into the extracellular space. Although the physiological substrates have not been fully defined, there are many recognized and potential substrates for NE, including coagulation proteins, growth factors, and extracellular matrix components (Bieth, 1998).

Previous studies have suggested that G-CSF and the G-CSFR may be substrates for NE, thus providing a potential direct link between NE and the regulation of granulopoiesis (El Ouriaghli *et al.*, 2003; Hunter *et al.*, 2003). Incubation of G-CSF or the extracellular portion of the G-CSF receptor with recombinant NE resulted in rapid proteolytic cleavage of these two proteins, and cells pretreated with NE demonstrate decreased surface G-CSF receptor expression. Moreover, the addition of NE to cultures dramatically reduced hematopoietic progenitor cell growth in response to G-CSF but not other cytokines. Collectively, these data suggested that NE may regulate G-CSF signaling by direct cleavage of G-CSF and the G-CSF

receptor. However, the physiological significance of this finding is unclear because it is based exclusively on *in vitro* data, where concentrations of NE in excess of 1 μg/mL were required for many of the observed effects. Mice lacking NE display normal granulopoiesis. Furthermore, patients with Papillon–Lefevre syndrome who lack dipeptidyl peptidase I (DPP1), an enzyme required for production of proteolytically active NE, have normal neutrophil counts (de Haar *et al.*, 2004; Pham *et al.*, 2004; Toomes *et al.*, 1999). Together, these data suggest that, in both humans and mice, loss of functional NE is not sufficient to perturb granulopoiesis. Thus, it is unlikely that the *ELA2* mutations observed in SCN contribute to disease pathogenesis through a dominant-negative inhibition of NE activity or through haploinsufficiency.

To directly assess the effect of *ELA2* mutations on NE function, Li and Horwitz (2001) performed an extensive *in vitro* biochemical characterization of 15 SCN-related NE mutants. Surprisingly, no consistent effect of these mutations on NE proteolytic activity, substrate specificity, or serpin inhibition was identified. An expression of mutant NE was not sufficient to induce apoptosis in the rat basophil leukemia (RBL-1) cell line used in this study. In contrast, Massullo *et al.* (2005) reported the expression of the G185R NE mutant in HL60 cells, a human promyelocytic cell line, induced apoptosis when the cells were induced to undergo granulocytic differentiation. Whether differences in the level of NE expression, type of NE mutant studied, or cell line used in each of these studies account for the difference in results remains to be determined.

To study the effect of mutant NE expression in a more physiological context, Grenda *et al.* (2002) generated transgenic mice carrying a targeted mutation of their *ELA2* gene ("V72M") reproducing a mutation found in two unrelated patients with SCN. Mice heterozygous and homozygous for the V72M allele had normal numbers of circulating neutrophils, and no accumulation of myeloid precursors in the bone marrow was observed. Rates of apoptosis following cytokine deprivation were similar in wild type and mutant neutrophils, as were the frequency and cytokine responsiveness of myeloid progenitors. The stress granulopoiesis response, as measured by neutrophil recovery after cyclophosphamide-induced myelosuppresion, was normal. Finally, a tumor watch failed to detect the development of leukemia in mice heterozygous for the mutant allele. These findings showed that expression of the murine V72M NE mutant in primary hematopoietic cells is not sufficient to impair granulopoiesis.

Dogs with canine cyclic hematopoiesis, also known as gray collie syndrome, display cyclic changes in blood neutrophil number that resembles those seen in patients with CN. Genetic linkage studies in these dogs identified homozygous mutations in the $\beta 1$ subunit of the AP3 complex. Of note, AP3 is a heterotetrameric adaptor complex associated with the transport of transmembrane proteins to lysosome-related organelles; mutations in the

AP3b3A subunit of the AP3 complex have also been identified as the cause of Hermansky-Pudlak syndrome type II in humans (Jackson, 1998; Starcevic *et al.*, 2002). NE protein expression in neutrophils from dogs with cyclic hematopoiesis was markedly reduced, suggesting that AP3 is required for the normal trafficking of NE to primary granules. To determine whether mistrafficking of NE is a common mechanism by which AP3 mutations and NE mutations result in neutropenia, the authors characterized the subcellular localization of a number of NE mutants. They showed that many but not all of the tested SCN mutations resulted in aberrant trafficking of NE to the membrane while mutations associated with CN resulted in excessive granule localization. Based on these data, the authors propose a model in which mutations associated with SCN disrupt the ability of NE to bind to AP3, leading to increased membrane localization. However, inherent in this model is the assumption that NE, at least transiently, can adopt a transmembrane configuration to interact with AP3, an assumption that conflicts with the published crystal structure of NE. Thus, the role of differential trafficking of mutant NE proteins in the pathogenesis of SCN and CN remains controversial.

3. Leukemogenesis

As noted earlier, one of the hallmarks of SCN is a pronounced predisposition to myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML), with a crude rate of transformation of 23% for patients on G-CSF therapy for 10 years (Rosenberg, 2003). An analysis of patients from the French SCN registry found a statistically significant association between *ELA2* mutations and leukemia, noting that all four cases of AML occurred in patients with *ELA2* mutations (Bellanne-Chantelot *et al.*, 2004). However, the presence of an *ELA2* mutation is not an absolute requirement to develop leukemia, since a study reported that a patient with SCN without an *ELA2* mutation developed acute lymphoblastic leukemia (Yetgin *et al.*, 2005).

Acquired mutations of the G-CSFR are present in a subset of patients with SCN (Dong *et al.*, 1994, 1995, 1997; Sandoval *et al.*, 1995; Tidow *et al.*, 1997). In the largest published series, G-CSFR mutations were detected in 34 out of 97 patients with SCN (Germeshausen *et al.*, 2001b). These mutations are distinct from the previously discussed germline mutations, affecting the extracellular domain of the G-CSFR that are thought to inhibit G-CSF signaling in a dominant-negative fashion. Instead, these are acquired mutations that introduce a premature stop codon, resulting in the truncation of the distal cytoplasmic portion of the G-CSFR. A role for G-CSFR truncation mutations in the pathogenesis of SCN has been postulated. However, these mutations have no apparent effect on the severity of neutropenia or response to G-CSF (Tidow *et al.*, 1997). Moreover, transgenic mice expressing a representative G-CSFR mutation have normal or near normal basal granulopoiesis (Hermans *et al.*,

1998; McLemore *et al.*, 1998). Thus, G-CSFR truncation mutations are not responsible for the block in myeloid maturation in SCN.

The G-CSFR truncation mutations are strongly associated with the development of AML/MDS (Bernard *et al.*, 1998; Dong *et al.*, 1995, 1997; Tschan *et al.*, 2001; Zeidler and Welte, 2002). In the largest series, 14 out of 35 patients with G-CSFR mutations developed MDS or AML, whereas only 2 out of 63 patients without G-CSFR mutations developed leukemia (Germeshausen, 2001b). A review of the literature shows that 17 out of 21 SCN patients progressing to MDS/AML harbored G-CSF receptor mutations, making such mutations the most common abnormality in leukemia secondary to SCN (Ancliff *et al.*, 2003; Cassinat *et al.*, 2004; Chen *et al.*, 1996; Germeshausen, 2001b). The premature truncation mutations are observed almost exclusively in the context of SCN with only one case of such a mutation in a patient who did not have SCN prior to developing AML (Carapeti *et al.*, 1997). Other common genetic abnormalities found in patients with SCN who developed AML/MDS include partial or complete loss of one copy of chromosome 7 or 5 and activating *ras* mutations (Ancliff, 2003; Freedman *et al.*, 2000; Kalra *et al.*, 1995).

The strong association of G-CSFR truncation mutations with AML/MDS suggests that these mutations may be leukemogenic. In support of this hypothesis, transgenic mice expressing the mutant G-CSFR display a hyperproliferative response to G-CSF (Hermans *et al.*, 1998; McLemore *et al.*, 1998). In fact, a preliminary study showed that expression of the mutant receptor conferred a striking clonal advantage at the hematopoietic stem level (Grenda *et al.*, 2004). Together, these observations suggest that patients with SCN who acquire G-CSFR mutations are at high risk for developing AML/MDS and should be monitored closely. Moreover, it would seem prudent to consider allogeneic bone marrow transplantation in such patients where a suitable donor can be found.

IV. WHIM Syndrome

A. Clinical Presentation

WHIM syndrome is a rare inherited disorder characterized by neutropenia, hypogammaglobulinemia, and extensive human papillomavirus (HPV) infection. Affected individuals typically present with recurrent bacterial infections from birth with absolute neutrophils count of less than 1000 per microliter. Despite the peripheral neutropenia, the bone marrow of affected patients is generally hypercellular with increased numbers of mature neutrophils (a condition termed myelokathexis). First described in 1964 by Zuelzer and colleagues, myelokathexis can present with isolated neutropenia

or in association with other hematopoietic abnormalities, as in WHIM syndrome (Bohinjec, 1981; Krill *et al.*, 1964; O'Regan *et al.*, 1977; Zuelzer, 1964). Bone marrow neutrophils in patients with myelokathexis frequently contain hypersegmented nuclei with condensed chromatin and vacuolated cytoplasm. Treatment with G-CSF or GM-CSF is effective in correcting the neutropenia. The neutropenia often corrects within hours after cytokine administration. The rapid kinetics of neutrophil recovery following cytokine treatment has led to speculation that the peripheral neutropenia observed in WHIM syndrome is secondary to impaired neutrophil release from the bone marrow rather than impaired neutrophil production.

Patients with WHIM syndrome commonly have lymphopenia or T-cell dysfunction, yet immunity to most viral pathogens is normal. The major exception is human papillomavirus, which is the cause of warts in patients with WHIM syndrome. While some patients have few if any warts, the majority of patients suffer from extensive verrucosis. They typically appear in the first or second decades of life and can involve any mucocutaneous surface. There are two case reports of the development of Epstein–Barr virus-associated lymphoprolifierative disease in patients with WHIM syndrome, suggesting that immunity against Epstein–Barr virus may also be defective. Hypogammaglobulinemia is variable ranging from normal to modestly decreased serum IgG, IgM, and IgA (Gorlin *et al.*, 2000).

B. Genetics

Although most cases of WHIM syndrome are inherited in an autosomal dominant fashion, sporadic and autosomal recessive inheritance patterns have also been documented. Genetic linkage analysis of several pedigrees affected with WHIM syndrome mapped the locus of this autosomal dominant form of myelokathexis to chromosome 2q21. Analysis of candidate genes revealed heterozygous germline mutations in the gene encoding the CXCR4 chemokine receptor (Hernandez *et al.*, 2003). To date, 32 patients with WHIM syndrome have been reported. Of those patients in whom the *CXCR4* gene has been sequenced, 20 out of 22 displayed heterozygous, germline mutations (Balabanian *et al.*, 2005; Diaz, 2005). All of the mutations reported to date result in the truncation of the carboxy-terminal (cytoplasmic) tail of the CXCR4 protein.

C. Molecular Pathogenesis

The normal to increased number of mature neutrophils in the bone marrow indicates that neutrophil production and maturation are normal and suggests that neutrophil release from the bone marrow may be defective. This

hypothesis is strengthened by observations that patients with myelokathexis can rapidly mobilize large numbers of neutrophils during periods of acute infection or during challenge with lipopolysaccharide, subcutaneous epinephrine, or cortisone (Diaz, 2005; Krill *et al.*, 1964; Zuelzer, 1964). Alternatively, it is possible that neutrophil survival may be decreased. Consistent with the later possibility, two studies reported accelerated apoptosis in patients with myelokathexis (Aprikyan *et al.*, 2000; Taniuchi *et al.*, 1999). The increased apoptosis observed was found to correlate with impaired expression of bcl-x while expression of bcl-2 and Fas remained normal.

As noted in an earlier section, germline heterozygous mutations of the *CXCR4* gene are associated with the majority of cases of WHIM syndrome. CXCR4 is a G-protein coupled–heptahelical receptor that also functions as a coreceptor for T-tropic strains of HIV-1 (Bleul *et al.*, 1996a; Oberlin *et al.*, 1996). It is broadly expressed on hematopoietic cells, including neutrophils. The ligand for CXCR4, stromal derived factor SDF-1 or CXCL12, is a CXC chemokine that was cloned from a bone marrow stromal cell line (Aiuti *et al.*, 1997). SDF-1 is a chemoattractant for many leukocytes, including neutrophils, monocytes, subpopulations of B and T lymphocytes, and hematopoietic progenitor cells (Aiuti *et al.*, 1997; Bleul *et al.*, 1996b; Chan *et al.*, 2001; Nagase *et al.*, 2002). It has also been shown to regulate cell adhesion, survival, and proliferation (Lataillade *et al.*, 2000, 2002; Peled *et al.*, 2000). All of the biological actions of SDF-1 are mediated through its interaction with CXCR4.

There is emerging evidence suggesting that SDF-1/CXCR4 signaling is a key regulator of neutrophil trafficking from the bone marrow. First, SDF-1 is constitutively produced by stromal cells in the bone marrow (Aiuti *et al.*, 1997). Second, while mice deficient in *SDF-1* and *CXCR4* are embryonic lethal, studies utilizing fetal liver cells from *CXCR4* deficient mice have established a critical role for these genes in the retention of myeloid cells in the bone marrow (Ma *et al.*, 1998; Nagasawa *et al.*, 1996). Specifically, in wild type mice reconstituted with CXCR4 deficient bone marrow cells by bone marrow transplantation, there is premature release of myeloid cells into the blood (Ma *et al.*, 1999). Finally, treatment with AMD3100, a selective antagonist of CXCR4, leads to the rapid mobilization of neutrophils into the blood that begins within 1 hour and peaks by 3–6 hours (Liles *et al.*, 2003). These data support a model in which the constitutively high concentration of SDF-1 in the bone marrow provides a key retention signal for neutrophils in the bone marrow.

Given the likely importance of SDF-1/CXCR4 signaling in the regulation of neutrophil trafficking in the bone marrow, several studies have examined its importance in regulating neutrophil release from the bone marrow. As noted earlier, disruption of CXCR4 signaling by the specific antagonist AMD3100 results in rapid neutrophil release. G-CSF, the prototypical

mobilizing cytokine, also appears to mobilize neutrophils through disruption of SDF-1/CXCR4 signaling. G-CSF treatment significantly reduces SDF-1 protein expression in the bone marrow and results in the secretion of proteases in the bone marrow microenvironment that result in CXCR4 cleavage on leukocytes (Levesque *et al.*, 2003; Petit *et al.*, 2002; Semerad *et al.*, 2002). Suratt *et al.* (2004) showed that stimulation of neutrophils with the CXCR2 ligand KC leads to heterologous desensitization to SDF-1, suggesting that neutrophil mobilization by inflammatory CXC chemokines may also be mediated, in part, by disruption of SDF-1/CXCR4 signaling. To summarize, these data suggest that the modulation of SDF-1/CXCR4 signaling is a key step in regulating neutrophil release from the bone marrow. Of note, Martin *et al.*(2003) reported that SDF-1/CXCR4 signaling may also contribute to the return of senescent neutrophils to the bone marrow. They showed that freshly isolated blood neutrophils express a low level of CXCR4. As neutrophils age, CXCR4 surface expression and SDF-1 responsiveness increases. Thus, aged neutrophils expressing high levels of CXCR4 may preferentially home to the bone marrow, where they are destroyed.

In WHIM syndrome, all of the mutations reported to date are predicted to result in the production of a truncated protein lacking the carboxy-terminal 10–19 amino acids. Previous studies of an engineered CXCR4 receptor lacking the carboxyl terminal 34 amino acids found increased G-protein activation and inositol phosphate generation, and a more sustained calcium elevation (Haribabu *et al.*, 1997). Similarly, EBV-immortalized lymphoblastoid cell lines generated from patients with WHIM syndrome displayed both higher initial peak calcium levels, as well as longer sustained calcium release. A follow-up study of three additional patients with *CXCR4* mutations failed to detect any alterations in calcium flux following SDF-1 stimulation. However, both neutrophils and lymphocytes displayed an increased chemotactic response to SDF-1 (Gulino *et al.*, 2004). These observations suggest a model in which enhanced signaling by the *CXCR4* mutants in WHIM syndrome leads to abnormal neutrophil retention in the bone marrow. Consistent with this model, Vroon *et al.* (2004) showed that G-protein coupled–receptor kinase-6 (GRK6) deficiency, which causes enhanced CXCR4 signaling, also results in impaired G-CSF induced neutrophil release. An exaggerated chemotactic response to SDF-1 was also observed in the granulocytes and lymphocytes from two WHIM patients with normal *CXCR4* alleles (Balabanian *et al.*, 2005). Furthermore, wild type CXCR4, when ectopically expressed in fibroblasts derived from patients with WHIM syndrome, failed to internalize appropriately in response to SDF-1. Together these data suggest that mutations of genes that regulate CXCR4 signaling pathway may be responsible for WHIM syndrome in patients with normal *CXCR4* alleles.

The defect in B lymphocyte function (i.e., hypogammaglobulinemia) in WHIM syndrome may also be directly related to abnormal leukocyte retention in the bone marrow. While the number of B lymphocytes in the bone marrow is normal in patients with WHIM syndrome (suggesting that B-lymphopoiesis is normal), the number of $CD27^+$ memory B cells is significantly reduced, suggesting a model in which B cell precursors may be inappropriately sequestered in the bone marrow preventing their homing to lymph nodes and subsequent maturation to memory B cells. This model may explain the observation that G-CSF treatment has been reported to result in increased levels of serum immunoglobulins in patients with WHIM syndrome. Specifically, the disruption of SDF-1/CXCR4 signaling in the bone marrow by G-CSF treatment might improve B lymphocyte mobilization from the bone marrow and a normalization of B cell trafficking to lymph nodes.

In summary, evidence suggests that most cases of WHIM syndrome are associated with gain-of-function mutations of the *CXCR4* gene. These mutations appear to result in elevated CXCR4 signaling, leading to the abnormal retention of neutrophils and B lymphocytes in the bone marrow.

V. Shwachman–Diamond Syndrome

A. Clinical Features

Shwachman–Diamond syndrome (SDS) is a rare multisystem disorder characterized by exocrine pancreatic insufficiency, bone marrow dysfunction, and skeletal abnormalities, first described in the 1960s (Bodian *et al.*, 1964; Shwachman *et al.*, 1964). Maldigestion caused by pancreatic insufficiency is present in nearly all patients in early life. In the largest published series of patients with SDS (88 patients), steatorrhea was present in 86 and 91% displayed a low serum trypsinogen level (Ginzberg *et al.*, 1999). Fatty infiltration of the pancreas is typical in patients with SDS and has been used as a diagnostic tool (Lacaille *et al.*, 1996). The pancreatic insufficiency improves with increasing age in most patients (Cipolli *et al.*, 1999). Most patients with SDS have bony abnormalities with rib cage abnormalities and metaphyseal dysostosis the most common features (Ginzberg *et al.*, 1999; Makitie *et al.*, 2004). Growth retardation is also common in SDS patients, but it is not thought to be secondary to malnutrition. Bone marrow dysfunction is present in nearly all patients with SDS. In the largest series, 86 of 88 patients with SDS displayed chronic or intermittent neutropenia (Ginzberg *et al.*, 1999). Moreover, defects in neutrophil function, most notably impaired chemotaxis and motility, have been reported (Stepanovic *et al.*, 2004). Anemia and thrombocytopenia, though less common, are present in more

than a third of patients (Ginzberg *et al.*, 1999). Defects in B- and/or T-lymphocyte function have been described, including impaired specific immunoglobulin response and altered CD4 or CD8 T-cell subsets (Dror *et al.*, 2001). Patients with SDS have a marked propensity to develop myelodysplasia or acute myeloid leukemia. A series of 21 patients with SDS showed that the overall risk of leukemic transformation was 24% (Smith *et al.*, 1996).

Treatment of SDS includes pancreatic enzyme replacement and fat-soluble vitamins for pancreatic insufficiency. Neutropenia is usually treated with G-CSF. Stem cell transplantation is generally reserved for patients with bone marrow failure or who have transformed to AML/MDS. A study reported 5-year event free survival of 60% inpatients with SDS following stem cell transplantation (Donadieu *et al.*, 2005).

B. Genetics

SDS is inherited in an autosomal recessive fashion (Ginzberg *et al.*, 1999). Previous genetic studies localized the gene responsible for SDS to an approximately 10-Mb span in the centromeric region of chromosome 7 (Goobie *et al.*, 2001). Boocock *et al.* (2003) reported that compound heterozygous mutations of the Shwachman–Bodian-Diamond syndrome (SBDS) gene located within this region on chromosome 7 were present in the majority of patients with SDS. Most of these mutations resulted from gene conversion with a neighboring pseudogene (*SBDSP*). Subsequent studies have confirmed that SBDS mutations are present in approximately 90% of clinically diagnosed cases of SDS (Nakashima *et al.*, 2004; Nicolis *et al.*, 2005; Woloszynek *et al.*, 2004). The majority of mutations occurs within exon 2 and is predicted to result in the production of a truncated protein. The remaining mutations are missense and nonsense mutations scattered throughout the protein. The most common genotype, present in approximately 50% of patients, is compound heterozygosity with 183–184TA→CT and 258+2T→C mutations. In the subset of patients who do not have SBDS mutations, SBDS protein expression in leukocytes is normal, indicating that other genetic mutations may be responsible for these cases of SDS (Woloszynek *et al.*, 2004).

C. Molecular Pathogenesis

To explore the pathogenesis of bone marrow failure, Dror and Freedman (1999) characterized the proliferation and differentiation of bone marrow cells isolated from patients with SDS and showed significant defects in both

hematopoietic progenitor cells and bone marrow stromal cells. They showed that SDS hematopoietic progenitors display increased apoptosis that was linked to increased Fas expression (Dror and Freedman, 2001). In contrast, Kuijpers *et al.* (2005) reported no increase in susceptibility to apoptosis in SDS neutrophils. Whether increased apoptosis of myeloid cells *in vivo* contributes to neutropenia in patients with SDS remains an open question.

The mechanism for the increased susceptibility to AML and MDS in SDS is unclear. Remarkably, 5 of 19 patients with SDS had cytogenetic abnormalities in the absence of MDS or AML, suggesting genomic instability within hematopoietic progenitors (Kuijpers *et al.*, 2005). Although additional study is needed, a potential mechanism for this increased genomic instability is provided by the report that telomeres are short in the leukocytes of patients with SDS (Thornley *et al.*, 2002).

As described earlier, the majority of patients with SDS have compound heterozygous mutations of their SBDS gene. The SBDS gene encodes for a protein of 250 amino acids that is highly conserved from archaea through vertebrates. Though the function of the SBDS protein is unknown, the following indirect evidences support the hypothesis that it may be involved in RNA metabolism. First, the putative yeast ortholog (YLR022c) is coordinately regulated with other yeast genes involved in RNA-processing (Wu *et al.*, 2002). Second, archaeal orthologs are located within a highly conserved operon that includes RNA-processing genes (Koonin *et al.*, 2001). Third, the crystal structure of the *Archaeoglobus fulgidus* SBDS ortholog shows structural homology with known RNA-binding proteins (Shammas *et al.*, 2005). Finally, proteomic analysis of the yeast SBDS ortholog (YLR022c) revealed an association with proteins involved in ribosomal RNA biosynthesis (Savchenko *et al.*, 2005). Consistent with a role in ribosomal RNA processing, Austin *et al.* (2005) showed that the human SBDS protein prominently localized to the nucleolus of cells in a cell cycle dependent fashion.

The most common mutations observed in SDS are predicted to generate truncated protein. The 183–184TA→CT mutation generates an in-frame stop codon (K62X) and the 258+2T→C mutations results in an 8-bp deletion causing a frameshift and premature truncation (84Cfs3) (Boocock *et al.*, 2003). Accordingly, no full length SBDS protein is detected in leukocytes isolated from patients with the 183–184TA→CT plus 258+2T→C mutations (Woloszynek *et al.*, 2004). Homozygosity for the 183–184TA→CT allele has not been observed despite its relatively high frequency in families with SDS, suggesting that complete loss of SBDS function may be lethal. Consistent with this hypothesis, yeast lacking the SBDS ortholog YLR022c is nonviable (Giaever *et al.*, 2002). Moreover, complementation studies of human SBDS

mutants in yeast lacking YLR022c showed that wild-type human SBDS, but not the K62X SBDS protein, rescued cell viability (Savchenko *et al.*, 2005).

In summary, evidence suggests that most but not all cases of clinically diagnosed SDS have compound heterozygous mutations of the SBDS gene. These mutations lead to reduced SBDS protein function, which is hypothesized to result in impaired RNA metabolism.

VI. Barth Syndrome

A. Clinical Presentation

In 1983, Barth *et al.* (1983) described a rare inherited condition characterized by cardiac and skeletal myopathy, growth retardation, and neutropenia. The phenotype was later expanded to include 3-methylglutaconic aciduria, a feature that aids in the diagnosis of Barth syndrome. Most patients display at least some degree of neutropenia ranging from mild decreases in neutrophil number to their near complete absence. Serial sampling is occasionally necessary to document neutropenia, since neutrophil numbers can fluctuate significantly in a noncyclic manner. Similar to severe congenital neutropenia, bone marrow evaluation shows an arrest of granulocytic differentiation at the promyelocyte stage. The neutropenia is treated with chronic G-CSF therapy and, where indicated, aggressive antibiotic prophylaxis (Barth *et al.*, 2004).

Cardiac dysfunction usually presents in the first year of life as a dilated cardiomyopathy. Although it can spontaneously improve in some patients, the cardiac dysfunction can progress to severe heart failure with ventricular tachycardias, requiring cardiac transplantation. Skeletal myopathy typically presents as proximal weakness, although most patients are able to walk without assistance by 2 years of age (Barth *et al.*, 2004).

B. Genetics

Barth syndrome is inherited in an X-linked recessive fashion. Genetic linkage studies localized the locus for this disease to the distal region of chromosome Xq28 (Bione *et al.*, 1996; Bolhuis *et al.*, 1991). Using a candidate gene approach, Bione and colleagues sequenced genes in this region that were expressed both in muscle and in leucocytes and identified point mutations in the *G4.5* gene. This gene, which has subsequently been identified as the *TAZ* gene, generates a number of alternatively spliced transcripts (Gonzalez, 2005); the translation products of these transcripts are known as "taffazins." A wide spectrum of mutations of the *TAZ* gene in Barth syndrome has been

identified, including deletions, insertions, splice-site, missense, and nonsense mutations. In fact, study showed that 73 different mutations in the *TAZ* gene were identified in 91 unrelated patients with Barth syndrome. At least some of these mutations (e.g., stop codons in exon 1 or 2) are predicted to generate loss-of-function alleles. No specific genotype–phenotype correlations have been identified (Johnston *et al.*, 1997), with the possible exception of a single large family with a severe form of Barth syndrome in which all affected members died within the first months of life; these patients had a frameshift mutation of exon 8 (Gedeon *et al.*, 1995).

C. Molecular Pathogenesis

The *TAZ* gene belongs to a family of putative acyltransferases (Neuwald, 1997). Studies of fibroblasts obtained from patients with Barth syndrome revealed an isolated defect in the transfer of linoleic acid to phosphatidylglycerol and cardiolipin (Vreken *et al.*, 2000). Cardiolipin is a phospholipid found exclusively in the inner mitochondrial membrane. It interacts both hydrophobically and electrostatically with cytochrome c in the mitochondria, thus preventing the release of cytochrome c into the cytoplasm where it may exert its proapoptotic effects (McMillin and Dowhan, 2002). Decreases in cardiolipin are associated with apoptosis in a variety of primary cells and cell lines. To test the hypothesis that increased levels of linoleic acid might rescue the defect in cardiolipin acylation observed in Barth syndrome, patient-derived fibroblasts were cultured in the presence of linoleic acid (Valianpour *et al.*, 2003). Cardiolipin levels were restored; leading the authors to conclude that linoleic acid supplementation could be of benefit in patients with Barth syndrome.

The pathogenesis of neutropenia in Barth syndrome is unclear. Orstavik and colleagues performed X-inactivation studies in six families with Barth syndrome. They reported that the majority of carriers of *TAZ* mutations demonstrated extreme skewing of their blood leukocytes toward cells without the *TAZ* mutation (Orstavik *et al.*, 1998). This observation suggests that *TAZ* mutations affect granulopoiesis in a cell-intrinsic fashion.

As noted earlier, one hypothesis is that *TAZ* mutations induce cell apoptosis through inhibition of cardiolipin biosynthesis. However, increased apoptosis does not appear to be responsible for the neutropenia in Barth syndrome (Kuijpers *et al.*, 2004). While increased cell surface binding of Annexin V was observed, no increase in phosphatidylserine on the cell surface of neutrophils was observed. Moreover, the rate of apoptosis in Barth neutrophils was similar to that of control neutrophils both at baseline and after overnight culture either in the presence or absence of G-CSF. Finally, the authors noted that neutrophil turnover *in vivo*, as measured by

plasma levels of FcγRIII, was comparable to that observed in control patients, suggesting that neutrophils in the periphery do not have a shortened half-life. While the cardiolipin levels found in Barth neutrophils are significantly lower than those seen in controls, ATP production by neutrophil mitochondria and the expression of cytochrome c in neutrophils is quite low (Maianski *et al.*, 2004; Murphy *et al.*, 2003). Thus, the proapoptotic effects of cardiolipin deficiency may not be nearly as pronounced in neutrophils compared with other cell types. These observations do not rule out elevated rates of apoptosis in a population of granulocytic precursors that might account for the impaired generation of mature neutrophils.

In summary, the majority of patients with Barth syndrome have loss-of-function mutations of the *TAZ* gene. This mutation disrupts cardiolipin biosynthesis, resulting in reduced mitochondrial cardiolipin expression and possibly apoptosis, particularly in cardiac tissues. The molecular pathogenesis of neutropenia is unclear.

VII. Pearson's Syndrome

A. Clinical Presentation

In 1979, Howard Pearson described four young patients with a complex set of clinical and laboratory features that included cytoplasmic vacuolization of hematopoietic cells in the bone marrow, sideroblastic anemia, and exocrine pancreatic insufficiency (Pearson *et al.*, 1979). Severe intermittent neutropenia (ANC $<$1000 cells per microliter) was present in all patients. Additionally, three of four cases had hepatic dysfunction. Subsequent studies documented persistent metabolic acidosis and elevated serum lactate levels (Rotig *et al.*, 1990). The treatment of Pearson's syndrome is largely supportive, including the administration of hematopoietic growth factors, where appropriate.

B. Genetics

Pursuing the hypothesis that metabolic derangements might reflect a defect of mitochondrial energy production, Rotig *et al.* (1989) analyzed lymphocyte-derived mitochondrial (mt) DNA from a patient with Pearson's syndrome. They showed that a majority of the patient's mtDNA harbored a 4977 bp deletion. A 13 bp repeat located immediately upstream and just before the end of the deleted mtDNA sequence was identified and hypothesized to promote intramolecular recombination. Mita *et al.* (1990) showed that such flanking repeats promote recombination in a majority of mtDNA deletions. Further

analysis of patients with Pearson's syndrome as well as Kearns–Sayre syndrome (KSS) and chronic progressive external ophthalmoplegia (CPEO) have identified several distinct types of mtDNA deletions, although the 4977 bp deletion described by Rotig *et al.* (1990, 1991) is the most common.

C. Molecular Pathogenesis

Most human cells contain hundreds to thousands of copies of mtDNA. In most normal cells, wild type DNA coexists with deleted or mutated mtDNA, a condition termed heteroplasmy. While the proportion of mutant mtDNA does appear to increase with age and has been linked to some of the effects of aging on cellular function, the percentage of mutated or deleted mtDNA typically remains less than 1%. In contrast, in Pearson's syndrome the percentage of deleted mtDNA is much higher. To directly assess the effects of mtDNA deletions on cellular bioenergetics, Porteous *et al.* (1998) constructed a series of cybrids by fusing an osteosarcoma cell line lacking mtDNA with enucleated fibroblasts from a patient bearing the common 4977 bp deletion. The deleted mtDNA content in these various cybrids ranged 0–86%. They found that reductions in mitochondrial membrane potential and ATP production correlated with the percentage of deleted mtDNA, although they noted that there appeared to be a threshold of deleted mtDNA ($\approx$50%) below which no obvious perturbations in cellular energy production were observed.

One of the more perplexing aspects of Pearson's syndrome (and of related disorders arising from mtDNA deletions) is the heterogeneity of clinical findings and deletions (Kerr, 1997; Lacbawan *et al.*, 2000). Identical deletions can give rise to a primarily hematologic syndrome in one patient while affecting primarily neuromuscular tissue in another. Conversely, distinct mutations can result in a very similar clinical phenotype. The same deletion can give rise to differing clinical presentations in successive family generations, that is, a mother and her offspring may present with different clinical syndromes despite identical mtDNA deletions. The heterogeneity of affected tissue types is thought to result from the random distribution of mitochondria during cell division in early embryogenesis (Jenuth *et al.*, 1996). Because of this random distribution, the degree of heteroplasmy in different cell types can vary within an individual and between generations. The pathogenesis of neutropenia in Pearson's syndrome is unknown, although the process of granulocytic differentiation is highly energy-dependent and thus may be quite susceptible to perturbations in oxidative phosphorylation and ATP production.

Thus, evidence suggests that Pearson's syndrome is caused by deletions of mtDNA that lead to mitochondrial dysfunction. The heterogeneity of clinical features is perhaps secondary to differences in the percentage of deleted mtDNA per cell in different tissues. The pathogenesis of neutropenia in this syndrome is unknown.

VIII. Glycogen Storage Disease Type Ib

A. Clinical Features

Glycogen storage disease type I (GSDI) is an inborn disorder of metabolism with an incidence in the population of approximately 1 in 100,000. Two major forms of this disease have been identified. Type Ia is due to mutations in the *glucose-6-phosphatase* gene and is associated with an impairment in the dephosphorylation of glucose-6-phosphate to glucose in the endoplasmic reticulum. Type Ib is caused by mutations of the gene encoding for microsomal glucose-6-phosphate translocase and is associated with defective glucose transport from the cytosol into the endoplasmic reticulum (ER). Patients with both types Ia and Ib display severe fasting hypoglycemia, hepatomegaly, and laboratory abnormalities, including elevated lactate, uric acid, and triglyceride levels. However, patients with GSD type Ib (who make up $\approx$20% of total GSD type I cases) often display severe neutropenia as well. In reports, as many as two-thirds of patients presented the clinical signs of neutropenia by age 1 (Visser *et al.*, 2000, 2002a). The neutropenia in these patients is often accompanied by oral ulcers, periodontal disease, and a Crohn's disease—like inflammatory bowel disease (Visser *et al.*, 2000). The use of G-CSF has dramatically improved the quality of life in these patients, and it remains the mainstay of treatment (Visser *et al.*, 2002a,b).

B. Genetics

Both type Ia and type Ib GSDs are inherited in an autosomal recessive manner. Loss-of-function mutations in the glucose-6-phosphate enzyme were found to be responsible for type Ia (Lei *et al.*, 1995). However, the genetic lesion responsible for type Ib remained unknown until mutations in the gene encoding a glucose-6-phosphate translocase (G6PTl) on chromosome 11 were identified (Veiga-da-Cunha *et al.*, 1998). A review noted that 70 mutations have been identified in the *G6PT1* gene; however, no correlation could be established between the genotype of patients and the degree of neutropenia, number of infections, or systemic complications (Melis *et al.*, 2005).

C. Molecular Pathogenesis

As noted in an earlier section, in GSD type Ib, the enzyme complex responsible for the translocation of glucose-6-phosphate into the ER is defective. Our understanding of the pathogenesis of neutropenia in this disease has been facilitated by the development of a murine model of GSD type Ib. Mice lacking the murine homolog of the *G6PT1* gene display most of the features of patients with GSD type Ib, including hypoglycemia, hyperlipidemia, and growth retardation (Chen *et al.*, 2003). In addition, these mice display a transient neutropenia that resolves by 6 weeks of age. The bone marrow in these mice is hypocellular but with normal to increased numbers of myeloid progenitors, suggesting a defect in terminal granulocytic differentiation. Consistent with this conclusion, a recent study reported that neutrophils from patients with GSD type Ib display elevated rates of apoptosis (Kuijpers *et al.*, 2003). Similar to affected humans, mice lacking *G6PT1* display persistent defects in neutrophil chemotaxis and oxidative burst.

In summary, GSD type Ib is associated with severe neutropenia and is due to loss-of-function mutations of the *glucose-6-phosphate* transporter gene. The resulting disruption in glucose metabolism disrupts terminal granulocytic differentiation, possibly through increased neutrophil apoptosis.

IX. Chediak–Higashi Syndrome

A. Clinical Presentation

Chediak–Higashi syndrome (CHS) is a rare inherited syndrome characterized by partial albinism, a mild bleeding diathesis, severe immunodeficiency with lack of natural killer cell activity, and progressive neurological defects (Beguez-Cesar, 1943; Chediak, 1952; Higashi, 1954; Steinbrinck, 1948). The pathognomonic feature of CHS is the presence of giant inclusion bodies in virtually all granulated cells, particularly neutrophils. Neutropenia is common and the residual neutrophils display functional defects. Approximately 85% of patients will progress to an "accelerated phase" characterized by a nonclonal lymphohistiocytic infiltration of multiple organs, leading to multiorgan system failure. The development of the accelerated phase has been attributed to viral infections, including Epstein–Barr infection, although no definitive link has been established (Rubin *et al.*, 1985). A subset of patients survives to adulthood free of the numerous infections and bleeding episodes characteristically associated with CHS. Other than allogeneic bone marrow transplantation, treatment is largely supportive.

B. Genetics

CHS is inherited in an autosomal recessive fashion. The genetic basis of CHS was largely elucidated through the study of *beige* mice, a murine model of CHS (Lutzner *et al.*, 1967). The murine *beige* locus was mapped to murine chromosome 13 by YAC complementation and positional cloning; the locus was found to correspond to the syntenic region of human chromosome 1q42–44 to which the locus for CHS had been mapped (Perou *et al.*, 1996). A candidate gene approach was used to identify mutations of the *Lyst* gene as the likely cause of the *beige* phenotype in mice. The *Lyst* gene, named for lysosome trafficking regulator, is a very large gene containing 55 exons, and producing an mRNA of approximately 11.5 kb; the translation product is a protein of 3801 amino acids with a molecular weight of 430 kDa. Subsequent studies identified loss-of-function mutations in the human ortholog, termed as *CHS1* or *LYST*, in most patients with CHS. At least 26 different mutations of the *LYST* gene have been identified in patients with CHS, including frameshift, nonsense, and missense mutations (Karim *et al.*, 1997, 2002). Karim *et al.* (2002) reported that a genotype–phenotype correlation exists in CHS. Whereas in patients with severe childhood CHS, only functionally null *LYST* alleles were observed, and patients with milder forms of CHS had hypomorphic *LYST* alleles.

C. Molecular Pathogenesis

In CHS, the formation and/or maturation of secretory lysosomes is impaired. Secretory lysosomes are modified lysosomes that undergo regulated secretion. They are present in many different cell types including melanocytes, platelets, and cells of the immune system. Neutrophils contain three major classes of granules (Borregaard and Cowland, 1997). Primary, or azurophilic granules are produced during the promyelocyte stage of differentiation and contain cathepsins, defensins, serine proteases including neutrophil elastase and proteinase 3, lysozyme, and myeloperoxidase (hence the term myeloperoxidase positive granules). These granules are acidified and are closely related to lysosomes, expressing the lysosomal membrane markers CD63, CD68, and a vacuolar-type H^+ATPase. Secondary, or specific granules contain lactoferrin and enzymes involved in the degradation of extracellular matrix proteins. Tertiary, or gelatinase granules contain mainly lysozyme and gelatinase or matrix metalloprotease-9. Of the neutrophil granules, only primary granules are classified as secretory lysosomes (Blott and Griffiths, 2002; Stinchcombe *et al.*, 2004). Accordingly, Kjeldsen *et al.* (1998) demonstrated by immunogold electron microscopy that the giant granules observed in CHS neutrophils are derived primarily from azurophil

granules and appear to contain little, if any, secondary or tertiary granule content. These granules mobilize poorly in response to ionomycin; since they contain a number of important antimicrobial peptides, their failure to fuse with endosomal compartments is consistent with the functional impairment seen in CHS neutrophils.

Although expression of the LYST protein normalizes lysosome size in beige cells, its function remains poorly understood. Sequence analysis has revealed the presence of three domains including an ARM/HEAT domain thought to be involved in vesicle trafficking, several WD-40 repeat domains thought to mediate protein–protein interactions, and a novel BEACH (for *BE*ige *A*nd *CH*ediak-Higashi) domain. Although its function is unknown, the BEACH domain defines a large family of proteins involved in vesicle trafficking, membrane dynamics, and receptor signaling. Several studies have attempted to identify the precise mechanism by which loss of LYST protein function impacts subcellular trafficking in CHS. These studies show the large lysosome-like organelles, seen in a perinuclear distribution, have a nearly full complement of lysosomal enzymes and are able to degrade endocytosed material, albeit at a substantially reduced rate. Thus, it is neither the fusion of lysosomes with endosomal organelles nor their ability to function once such fusion has taken place that is impaired. Rather, it appears that the coordinated trafficking of lysosomal proteins and endosomes to a common location in the cell is affected in CHS. Moreover, a study reported that the ability of CHS cells to repair plasma-membranes lesions may also be impaired (Huynh *et al.*, 2004).

In summary, evidence shows that most if not all cases of CHS are due to loss-of-function mutations of the *LYST* gene. The loss of LYST protein disrupts the normal regulation of secretory lysosomes, leading to hypopigmentation and dysregulated immune cell function. The pathogenesis of neutropenia is unclear.

X. Griscelli Syndrome

A. Clinical Features

Griscelli syndrome (GS) was first described as a syndrome of partial albinism, frequent infections, and thrombocytopenia (Griscelli *et al.*, 1978). The major clinical features of GS are partial or complete albinism, including white or silvery hair, variable cellular immune deficiency, progressive neurological dysfunction, and the development of a lymphohistiocytic or hemophagocytic syndrome similar to that observed in CHS. Although GS has a clinical presentation similar to that of CHS, the distinctive granules seen in leukocytes from CHS patients are not seen in GS. Neutropenia and

neutrophil dysfunction are common in patients with GS. The episodes of hemophagocytic syndrome are thought to be secondary to uncontrolled immune cell activation, resulting in the infiltration of organs (particularly the liver and central nervous system) with lymphocytes and/or macrophages, ultimately leading to progressive organ dysfunction and often death.

B. Genetics

GS is inherited in an autosomal recessive fashion. Early genetic studies mapped the locus for "Griscelli syndrome" to chromosome 15q21–22. Analysis of candidate genes identified mutations in the *MYO5A* gene, and GS was initially defined by the presence of mutations in this gene (Pastural *et al.*, 1997). However, studies of additional patients presenting with Griscelli syndrome-like features but lacking *MYO5A* mutations led to further genetic analysis of affected pedigrees. These additional studies determined the presence of a second gene responsible for GS located within 7 cM of *MYO5A* (Pastural *et al.*, 2000). Menasche *et al.* (2000) subsequently identified mutations in the *RAB27A* gene with a form of GS associated with a hemophagocytic syndrome. *MLPH*, a third gene responsible for GS has been mapped to 15q21. The mutations in all three genes are thought to be loss-of-function mutations. Consequently, three distinct types of Griscelli syndrome are now genetically defined: (1) type I GS (also called Elejalde syndrome) is caused by mutations in the *MYO5A* gene, (2) type II GS is caused by *RAB27A* mutations, and (3) type III GS is associated with mutations in the *MLPH* gene encoding melanophilin (Menasche *et al.*, 2003b). Only patients with type II GS develop the hemophagocytic syndrome. Spontaneously occurring mouse models for each type of GS have been identified. The *ashen*, *leaden*, and *dilute* mouse strains correspond to mutations of *RAB27A, MLPH* and *MYO5A* genes, respectively.

C. Molecular Pathogenesis

MYO5A encodes for myosin 5A (MYO5A), an organelle motor protein, *MLPH* encodes for melanophilin, and *RAB27A* encodes for a member of the Rab family of GTPases. All three proteins are believed to mediate secretory vesicle movement along actin networks. MLPH, MYO5A, and RAB27A form a protein complex that is necessary for melanosome trafficking, explaining the common defect in pigmentation observed in all three types of GS. In contrast, expression of MLPH and MYO5A appears to be dispensable in the trafficking of secretory lysosomes in cytotoxic T cells. Loss

of *RAB27A* but not *MYO5A* and *MLPH* results in impaired cytotoxic T-cell function and likely accounts for the unique association of type II GS with the hemophagocytic syndrome. A central role for *RAB27*A in this process has been confirmed by the experiments showing that retroviral-mediated expression of *RAB27A* in GS patient-derived cytotoxic T cells rescues the phenotype of defective secretory lysosome mobilization (Bizario *et al.*, 2004).

RAB27A is expressed in a wide variety of cell types that undergo regulated granule secretion. The specificity of Rab proteins for particular membranes are thought to derive from membrane-specific guanine nucleotide exchange factors (GEFs), which catalyze the transition of the Rab protein from inactive (GDP-bound) to active (GTP-bound). Upon GTP binding, Rab family members alter their conformation to expose a lipid-modified tail that then inserts into the lipid bilayer of various membranes. The proper recruitment of vesicles to different locations in the cell is mediated both by Rabs and by Rab effectors, an extremely diverse family of proteins that bind Rab proteins. Thirteen distinct RAB27A binding proteins (or effectors) have been identified to date (Fukuda, 2005). Loss-of-function mutations in the *UNC13D* gene encoding Munc13–4, a known *RAB27A* effector, also result in a form of familial hemophagocytic syndrome, suggesting that these two proteins (along with others) are required for the generation of secretory lysosomes and/or their regulated fusion with the plasma membrane. Biochemical characterization of *RAB27A* mutants has found unexpected complexity in the molecular defects caused by mutations in the *RAB27A* gene (Bahadoran *et al.*, 2003; Menasche *et al.*, 2003a). These defects include impaired GTP and Rab effector binding and decreased protein stability.

In summary, three distinct types of GS can be identified based on genetic mutations of the *MYO5A, RAB27A*, or *MLPH* genes. Loss-of-function mutations of each of these genes is associated with impaired secretory vesicle trafficking. Similar to Chediak–Higashi syndrome, the resulting defect in secretory lysosomes results in hypopigmentation and, in the case of type II GS, defects in cytotoxic T-cell function. The pathogenesis of neutropenia is unclear.

XI. Cartilage-Hair Hypoplasia

A. Clinical Presentation

Cartilage-hair hypoplasia (CHH), also referred to as McKusick-type metaphyseal chondrodysplasia, is characterized by short-limbed short stature, hypoplastic hair, and variable hematologic and immune abnormalities. A subset of cases also present with a Hirschprung-like aganglionic megacolon

(Makitie and Kaitila, 1993). The major clinical feature of CHH is short stature with adult heights of 110–135 cm. Most patients also present with impaired cellular immunity manifested as lymphopenia, decreased delayed hypersensitivity, or decreased serum immunoglobulins. Neutropenia was observed in 21 out of 79 patients (Makitie and Kaitila, 1993). A mild macrocytic anemia is also frequently observed, but severe anemia is rare. The incidence of cancer, and in particular non-Hodgkins lymphoma and skin cancer, is significantly higher in patients with CHH than in the general population (Makitie *et al.*, 1999). The successful use of G-CSF to treat CHH-associated neutropenia has been documented in a single patient with recurrent severe infections (Ammann *et al.*, 2004).

B. Genetics

CHH is inherited in an autosomal recessive inheritance pattern. The carrier frequency is as high as 1:19 in the Old Order Amish population (Sulisalo *et al.*, 1994) and 1:76 in the Finnish population (Makitie, 1992). Genetic linkage analyses localized the gene for CHH to a 1.7-Mb region of chromosome 9p13 (Sulisalo *et al.*, 1993, 1995; Vakkilainen *et al.*, 1999). Ridanpaa *et al.* (2001) identified the putative CHH gene as *RMRP*, a nontranslated gene encoding the RNA subunit of the *R*Nase *M*itochondrial *R*NA *P*rocessing (RMRP) complex. Subsequent analysis determined that the 70A→G *RMRP* mutation is the most common worldwide, accounting for 92% of mutations found in the Finnish population and nearly half of mutations worldwide. Furthermore, this mutation segregates with the same haplotype in both Finnish and Amish population, suggesting the introduction of the mutation to Finland nearly 4000 years ago (Ridanpaa *et al.*, 2002, 2003). A study of Japanese patients with CHH revealed a different spectrum of mutations of *RMRP* (Nakashima *et al.*, 2003).

C. Molecular Pathogenesis

The mechanism of neutropenia in CHH has been ascribed to impaired bone marrow production of neutrophils. Two reports describe the presence of a maturation arrest in the bone marrow similar to that seen in SCN (Ammann *et al.*, 2004; Lux *et al.*, 1970). Moreover, the number of CFU-GM in the bone marrow is decreased compared with controls, although the decrease did not correlate with the degree of neutropenia observed (Juvonen *et al.*, 1995). Consistent with a model of impaired production was the observation

that neither epinephrine nor bacterial polysaccharide increased the numbers of circulating neutrophils (Lux *et al.*, 1970).

The *RMRP* gene encodes the RNA component of the RNase MRP (mitochondrial RNA processing) complex. *RMRP* is a nontranslated, intronless gene that is essential for MRP complex activity. The MRP complex is a large protein–RNA complex believed to play a role in the processing of RNA species involved in the replication of mitochondrial DNA and in the processing of the 5.8S ribosomal RNA (Clayton, 2001; van Eenennaam *et al.*, 2000). Two major classes of mutations have been identified in the *RMRP* gene. "Null" mutations are characterized by insertions and duplications between the TATA box and the transcriptional start site and result in silencing of the allele. No patients have been identified who are homozygous for null mutations, suggesting that homozygosity for null alleles is embryonic lethal. This hypothesis is consistent with the observation that yeast completely lacking function of the *RMRP* homolog is not viable (Ridanpaa *et al.*, 2001). The second class of *RMRP* mutations, including the common 70A→G mutation, appears to produce hypomorphic alleles. They result in a modest decrease in RNA expression but do not appear to impair association of the RNA subunit with the protein subunits found in the MRP complex. A report suggested that the 70A→G mutation may also disrupt normal 5.8S ribosomal RNA processing (Hermanns *et al.*, 2005).

To summarize, most cases of CHH result from partial loss-of-function mutations of *RMRP,* resulting in impaired ribosomal RNA processing and/or mitochondrial dysfunction. The molecular mechanism responsible for neutropenia in patients with CHH remains unknown.

XII. Conclusions

The last 10 years have witnessed tremendous progress in our understanding and treatment of many congenital neutropenic syndromes. Perhaps most importantly, the genetic bases for many of these syndromes have been identified. Genetic testing is becoming an important diagnostic tool in the evaluation of patients with congenital neutropenia. Consequently, syndromes previously classified together on the basis of clinical features can now be subclassified on the basis of different genetic etiologies. In many cases, the identification of specific gene mutations has led to significant advances in our understanding of the molecular pathogenesis of these disorders. The challenge in the future will be to translate this information into novel targeted therapies.

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6

Social Dominance and Serotonin Receptor Genes in Crayfish

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Gene expression affects social behavior only through changes in the excitabilities of neural circuits that govern the release of the relevant motor programs. In turn, social behavior affects gene expression only through patterns of sensory stimulation that produce significant activation of relevant portions of the nervous system. In crayfish, social interactions between pairs of animals lead to changes in behavior that mark the formation of a dominance hierarchy. Those changes in behavior result from changes in the excitability of specific neural circuits. In the new subordinate, circuits for offensive behavior become less excitable and those for defensive behavior become more excitable. Serotonin, which is implicated in mechanisms for social dominance in many animals, modulates circuits for escape and avoidance responses in crayfish. The modulatory effects of serotonin on the escape circuits have been found to change with social dominance, becoming excitatory in dominant crayfish and inhibitory in subordinates. These changes in serotonin's effects on escape affect the synaptic response to sensory input of a single cell, the lateral giant (LG) command neuron for escape. Moreover, these changes occur over a 2-week period and for the subordinate are reversible at any time following a reversal of the animal's status. The results have suggested that a persistent change in social status leads to a gradual change in the expression of serotonin receptors to a pattern that is more appropriate for the new status. To test that hypothesis, the expression patterns of crayfish serotonin receptors must be compared in dominant and subordinate animals. Two of potentially five

Current Topics in Developmental Biology, Vol. 74
0070-2153/06 $35.00

DOI: 10.1016/S0070-2153(06)74006-6

serotonin receptors in crayfish have been cloned, sequenced, and pharmacologically characterized. Measurements of receptor expression in the whole CNS of dominant and subordinate crayfish have produced inconclusive results, probably because each receptor is widespread in the nervous system and is likely to experience opposite expression changes in different areas of the CNS. Both receptors have recently been found in identified neurons that mediate escape responses, and so the next step will be to measure their expression in these identified cells in dominant and subordinate animals.

I. Introduction

The struggle for survival and reproductive success that Darwin described is often most intense among members of the same species, all of whom are competing for the same resources within the same niche. This competition for food, shelter, and mating opportunities can become violent, but social animals have developed a variety of behavioral mechanisms to minimize violence. Among the most important of these is the social dominance hierarchy that is usually established through agonistic interactions. Once established, the hierarchy enables the participating animals to divide resources relatively peacefully, if unevenly (Wilson, 1975). This spares them from fighting and the chance of injury that for most animals in the wild leads directly to death.

The neural and neuroendocrine mechanisms that underlie social dominance are not well understood for any animal, in part because they include dynamic interactions within and between systems at all levels, from gene expression to social interactions (Fig. 1). These interactions form loops, such that cause and effect become indistinct. For example, a new social interaction (''World,'' Fig. 1) will trigger sensory excitation, central activity, new motor patterns, and a behavioral response on a short timescale. The neural responses will also feed back to modify neural circuit function and structure through rapid mechanisms of plasticity and modify patterns of gene expression through slower mechanisms. New patterns of gene expression can promote structural changes through neurogenesis, synaptogenesis, apoptosis, or synapse elimination and functional changes through redistribution of receptors and ion channels, or through changes in second messenger cascades. These then alter circuit function and activity and so affect the animal's subsequent social behavior and experience.

Despite this complexity, some of the neurochemicals that appear to play significant roles in dominance behavior have been identified. Among the most prominent of these across phyla is serotonin (5-hydroxytryptamine or 5-HT) (Edwards and Kravitz, 1997; Miczek *et al.*, 2002). In vertebrates, the

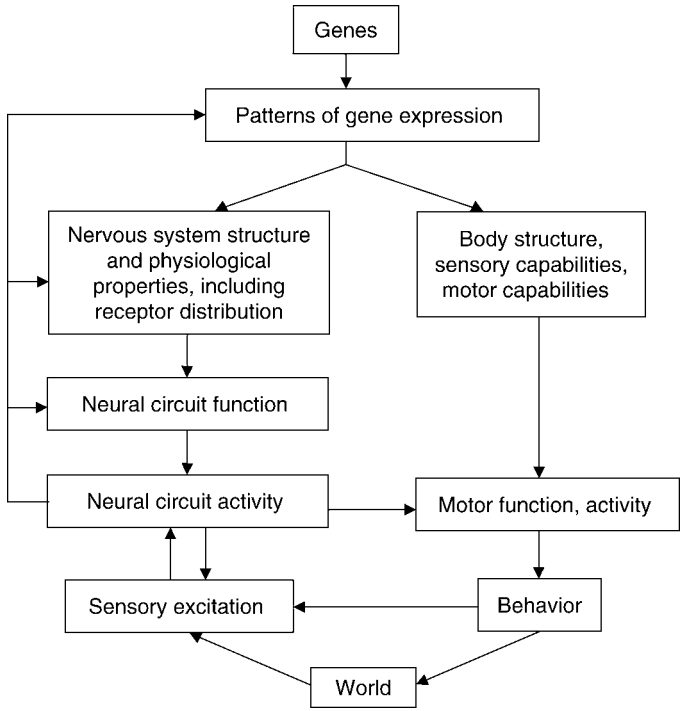

Figure 1 Patterns of influence that govern the relationship between social dominance behavior and serotonin receptors. Social experience ("world") leads to patterns of sensory excitation that prompt neural responses ("neural circuit activity") leading to immediate motor and behavioral responses, and to immediate and longer-term changes in neural circuit function. Immediate changes can occur through neuromodulation, whereas longer-term changes can occur through changes in physiological properties or neural structures mediated by phosphorylation or changes in gene expression.

correlation between dominance and serotonin is largely negative (Manuck *et al.*, 2006; Miczek and Fish, 2006). In fish (Winberg *et al.*, 1997) and monkeys (Fairbanks *et al.*, 2004), levels of 5-HT or its metabolite, 5-HIAA, are lower in dominant animals than in subordinates. In crustaceans, an opposite trend was observed in shore crabs (Sneddon *et al.*, 2000). In those animals, resting 5-HT levels were found to be higher in the blood of winners than in losers, and 5-HT levels increased more following a fight in winners than in losers. In crayfish, however, no differences in CNS levels of 5-HT occurred between new dominant, subordinate, and control animals 24 hours after status differences were established (Panksepp *et al.*, 2003).

The actions of serotonin are mediated through specific receptors that now number about 13 different types in vertebrates and 5 in arthropods (Manuck

et al., 2006). Agonists of vertebrate 5-HT_{1A}, 5-HT_{1B}, and 5-HT_{2A} receptors have been shown to reduce aggression in mammals (Miczek *et al.*, 2002) and in some instances to promote social dominance status. At the same time, they also affect other patterns of motor behavior that, given the widespread distribution of both 5-HT projections and 5-HT receptors within nervous systems, is not surprising (Miczek and Fish, 2006; Peeke *et al.*, 2000).

Only two of the estimated five or more 5-HT receptors have been identified in crayfish, and their role in formation and maintenance of dominance hierarchies has only begun to be studied. However, much is known about the social dominance behavior of crayfish, and some of the neural circuits that mediate aspects of that behavior have been described, together with their modulation by 5-HT. This unique knowledge base provides an opportunity that is missing in other animals to study the interaction between social status, neural circuits that mediate discrete aspects of social behavior, and the serotonergic modulation of those circuits and behavior.

Here we will describe what is known of dominance hierarchies in crayfish, the neural circuits that mediate relevant behavior patterns, and the roles of serotonin in modulating those circuits and behaviors. Finally, we will describe the two 5-HT receptors that have recently been identified in crayfish, and the roles they may play in modulating neural circuits that control behavior patterns relevant to social dominance behavior.

II. Dominance Hierarchies in Crustaceans

Dominance hierarchies are commonly observed in captured populations of decapod crustaceans, particularly in bottom- or shore-dwelling or land-dwelling animals, including many species of lobsters, crabs, crayfish, hermit crabs, and freshwater shrimp. In captivity, where resources of space, shelter, and food are limited, dominance hierarchies are readily established and maintained, at least until a dominant animal molts, when it becomes highly vulnerable. In the wild, territoriality is observed among animals with fixed shelters, including crayfish (Bergman and Moore, 2003), fiddler crabs (Zeil and Layne, 2004), and hermit crabs (Briffa and Elwood, 2004), all of which defend their shelters from conspecific intruders.

Establishment and maintenance of a dominance hierarchy requires a mechanism to determine dominance order and a mechanism to recognize more and less dominant neighbors. Dominance order is decided very quickly between crayfish of different size, but animals of similar size need to exchange more information. A series of escalating exchanges between dominance rivals begins with an elevated posture, waving or spreading the large claws, and displaying the bright undersides of claws. Should these demonstrations prove unable to cause a rival to withdraw, then escalation

continues with antennal whipping, pushing and shoving with the claws, urination directed at the opponent, grappling and wrestling in an attempt to turn the opponent over, offensive tail flips, and strikes and tears with the claws (Breithaupt and Eger, 2002; Bruski and Dunham, 1987; Herberholz *et al*., 2001; Huber and Kravitz, 1995).

The dominance decision is marked by the sudden switch of one animal from offensive to defensive behaviors—a cessation of approaches, strikes and offensive tail flips, and the sudden onset of a retreat or series of backward tail flips. The other animal usually maintains its aggressive attitude toward the defeated opponent, pursuing it as it attempts to break off the contest and escape further punishment (Herberholz *et al*., 2001). Retreat carries the penalty of becoming subordinate because it usually reinforces the dominant and aggressive tendencies of the winner (Goessmann *et al*., 2001).

If the animals remain paired, the dominance hierarchy matures and different patterns of behavior are displayed by the dominant and subordinate animals. Aggression is infrequent between animals in mature hierarchies, so that slow approaches of the dominant elicit retreats from the subordinate before contact occurs (Issa *et al*., 1999). When the subordinate is unable to see the dominant, an unexpected lateral touch causes the subordinate either to lower its posture suddenly and move backward, or to move forward quickly away from the touch. The same unexpected lateral touch of a dominant will cause it to turn quickly toward the source of the touch and raise its claws (Song *et al*., 2000). Subordinate animals will refrain from digging a burrow near a social dominant, whereas the dominant will dig avidly near a subordinate (Herberholz *et al*., 2003). Dominants are seen to attack a crayfish that has been seized by a dragonfly nymph predator, whereas subordinates avoid such situations (Herberholz *et al*., unpublished observations). These examples suggest that the states of the nervous systems in dominant and subordinate animals come to differ significantly as the hierarchy matures.

III. Social Status and the Function of Neural Circuits

The changes in behavior that mark dominance hierarchy formation are produced by changes in the activity and excitability of the neural circuits that mediate them (Fig. 2). It has been possible to observe these changes in two sets of circuits, those that mediate tail flip behaviors and those that mediate responses to an unexpected touch.

Crayfish display four types of tail flip behaviors, each of which is excited by a unique set of stimuli and performs a distinct function. Offensive tail flips (OTs) occur during a fight and are used to thrust an animal above its opponent and then drag the opponent (Herberholz *et al*., 2001). Medial

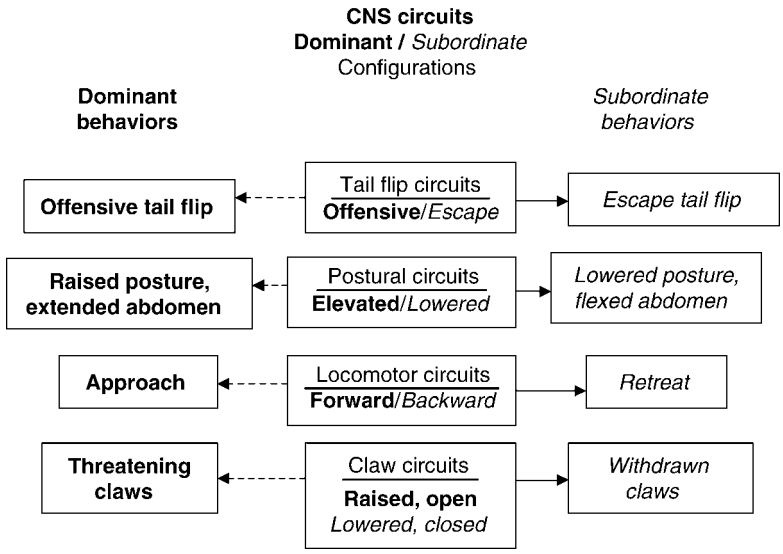

Figure 2 Different behavior patterns that emerge from common neural circuits in animals of opposite social status. The central column identifies several discrete sets of neural circuits that control aspects of behavior used by dominant and subordinate animals. In dominant animals circuits or patterns of activity that mediate the behaviors in the left-hand column (bold) are excited, and those that mediate behaviors in the right-hand column (italics) are inhibited. When the animal is subordinate, the opposite occurs.

giant (MG), LG, and nongiant (NG) tail flips are more powerful abdominal flexions that enable the animal to escape from the triggering stimulus. Herberholz *et al.* (2001) identified activation of each of the four different tail flip neural circuits during a dominance contest by observing tail flip behavior and recording the associated electrical field potentials produced in the aquarium water by the active neurons and muscles. They found that during the height of the contest only the OTs were used by both animals, along with other offensive behaviors. The dominance decision was marked by a sudden flurry of NG and MG tail flips by one animal, which carried it directly away (NG) or backward (MG) from the other (Herberholz *et al.*, 2004). The LG tail flips, which are activated by an attack from the rear, occurred only rarely (once during contests between eight pairs). The LG circuit is strongly inhibited in the subordinate and only weakly inhibited in the dominant during fighting (Krasne *et al.*, 1997).

In many animals an unexpected sensory stimulus will evoke a startle response (Eaton, 1984; Nagayama *et al.*, 1986; Zeil *et al.*, 1985). An unexpected touch to the side of the first abdominal segment usually caused a dominant crayfish to turn toward the source of the touch, and a subordinate

crayfish either to drop its posture and move quickly backward, or retain its posture and move quickly forward (Song *et al*., 2006). Video recordings of these behavioral responses and simultaneous electrical recordings of activity in the leg depressor nerves of freely behaving animals showed how these movements were produced (Issa *et al*., 2004, 2005). In the dominant animal, depressor (Dep) motor neurons in the fifth walking leg ipsilateral to the touch were excited as the leg was planted for the animal's turn. Simultaneously, Dep motor neurons in the contralateral leg were inhibited as the leg was lifted for the turn. In the subordinate crayfish, Dep motor neurons in both ipsi- and contralateral legs were inhibited as the animal dropped its posture before backing away (Issa *et al*., 2004, 2005).

Interestingly, the status-dependent differences in motor neuron responses survived isolation of the thoracic portion of the ventral nerve cord from the rest of the animal (Issa *et al*., 2005). The thoracic ventral nerve cord and leg motor nerves were removed from animals that had earlier been videotaped and recorded. After pinning the nerves out in a saline-filled petri dish, electrical shocks applied to the sensory nerve mediating the response to touch evoked Dep motor neuron responses that reflected the same status-dependent differences seen earlier in the intact animals. This demonstrated that the status-dependent differences in the Dep motor neuron responses to afferent input did not result from a tonic signal of social status descending from the brain to the thoracic walking leg circuitry. Rather, it indicated that the thoracic leg circuitry was reprogrammed by the change in the animal's social status and that the reprogramming, which presumably derived from higher centers, survived separation from those centers.

Earlier work had established that the same ipsilateral nerve stimulus or touch would produce the same social status-dependent pattern of responses in the pair of large serotonergic neurons in both the first abdominal (A1) and fifth thoracic (T5) ganglia (Drummond *et al*., 2002). In dominant animals, the unilateral stimulus excited the ipsilateral serotonergic neurons in those two ganglia, and inhibited or had no effect on the contralateral neurons. In subordinate animals, the unilateral stimulus produced bilateral excitation in half of the 40 animals tested and bilateral inhibition in the other half.

The A1 5-HT neurons project to and appear to contact the ipsilateral Dep motor neurons in T5 at the nerve root exit, and they have no contralateral projections (Issa *et al*., 2005). Electrical stimulation of the A1 5-HT neurons enhances the resistance reflex responses of the Dep motor neurons in some communally housed animals and inhibits the responses in others. Although the social status of each of these animals was unknown, the result suggests that the A1 5-HT cells help produce the differing Dep motor neuron responses to unilateral stimuli that are characteristic of dominant and subordinate animals.

IV. Effects of Applied 5-HT on Crustacean Behavior

Although the effects of serotonin on aggression and social dominance in crustaceans have been studied over the last 20 years, a clear picture has yet to emerge. In a seminal study, Livingstone *et al.* (1980) showed that acutely injected 5-HT would quickly cause both lobsters and crayfish to adopt a posture in which the claws were held forward with depressed tips, the thorax was elevated, and the abdomen was flexed (Livingstone *et al.*, 1980; Tierney and Mangiamele, 2001). The elevated thorax of the 5-HT posture was reminiscent of that of dominant animals, whereas similarly injected octopamine induced an extended, prone posture, reminiscent of subordinate status (Kravitz, 1988). Similar postural differences were been seen in other crustaceans, including squat lobster (Antonsen and Paul, 1997) and prawn (Sosa and Baro, 2002).

The relationship between serotonin and the aggressive behavior of crustaceans was strengthened by a series of studies in which both increases (Huber and Delago, 1998; Panksepp and Huber, 2002; Panksepp *et al.*, 2003) and decreases (Doernberg *et al.*, 2001; Kravitz and Huber, 2003) in serotonin levels were seen to induce increases in aggressive behavior. Acute injections of 5-HT caused changes in the aggressive motivation of subordinate crayfish after the initial postural effects had disappeared (Huber *et al.*, 1997). Serotonin had no effect on their willingness to initiate a fight with a dominant partner, but made them resistant to give up a fight, thereby causing fights to be prolonged. Injected 5-HT also induced squat lobsters to display aggressive behavior that is not normally seen in their natural crowded conditions (Antonsen and Paul, 1997).

More recent studies have made the relationship between 5-HT and aggression in crustaceans less certain. Acute 5-HT injections in lobsters disturbed their ability to locomote and reduced their ability to compete effectively for a shelter (Peeke *et al.*, 2000). Tierney found that the "5-HT posture" described by Livingstone *et al.* (1980) differed from the animal's posture during fights, when the chelae are raised, the thorax is elevated (as in the 5-HT posture), and the abdomen and legs are extended to provide a wide base of support (Tierney and Mangiamele, 2001). Tierney also tested a range of 5-HT concentrations and a set of 5-HT agonists to discover if individual elements of the aggressive posture might be released by a specific 5-HT concentration or a particular agonist. She found that although a broad range of 5-HT concentrations could evoke an elevated posture, these concentrations also reduced the animal's walking behavior by about 80% (Tierney and Mangiamele, 2001). Of the several agonists injected, 5-carboxamidotryptamine maleate (5-CT), which activates 5-HT_1, 5-HT_5,

and 5-HT_7 receptors in vertebrates, was the only one that evoked the elevated posture and did not affect walking. In interactions between an injected animal and a control, 5-CT was also the only agonist that promoted aggressive behavior.

Exposure to chronically injected 5-HT also affects the aggressive behavior of crayfish. Animals that had received a rapid release of 5-HT displayed slower increases in aggressive behavior than control animals, whereas animals that had received a 10-fold slower release escalated aggressive behavior more quickly than controls (Panksepp and Huber, 2002).

The varied effects of injected 5-HT on the behavior of lobsters and crayfish are likely to result from its simultaneous action on a variety of circuits and systems that are not normally activated together in this fashion (Beltz and Kravitz, 2002). Acutely and chronically injected 5-HT enters immediately into the bloodstream that carries it directly to targets in the vascular system, central nervous system, and skeletal muscle (Spitzer *et al.*, 2005a). 5-HT is known to affect heart rate (Florey and Rathmayer, 1978; Listerman *et al.*, 2000), foregut contractions (Flamm and Harris-Warrick, 1986a,b; Tierney *et al.*, 1999), hindgut peristalsis (Musolf and Edwards, 2004), blood sugar levels (Lee *et al.*, 2000; Santos *et al.*, 2001), and sensory and motor processing throughout the CNS and PNS (Beltz, 1999). Many of these targets also receive direct contact from 5-HT–containing neurons that are found throughout the nervous system, whereas others only receive blood-borne 5-HT (Fig. 3) (Beltz, 1999). In addition, some neurons can take up extracellular 5-HT and may repackage it for release (Musolf and Edwards, 2000). This enables injected 5-HT to have a delayed and prolonged effect on neurons and effectors that are innervated by serotonergic neurons. The uptake and delayed release of injected 5-HT might have abnormal effects because the amount of 5-HT released onto targets will likely be supernormal.

5-HT's modulatory effects can be sensitive to the pattern of exposure (Teshiba *et al.*, 2001), suggesting that 5-HT sources are normally activated selectively to produce a pattern of exposure that modulates a subset of systems in a coherent and adaptive manner. The selective targeting of modulation is challenging because the different targets are often closely apposed. For example, in the small terminal abdominal ganglion of crayfish, 5-HT is known to modulate the neural circuitry for escape and for abdominal posture, two targets that may have different modulatory requirements. Appropriate targeting and avoidance of unwanted 5-HT cross talk appear to be achieved by matching hormonal, paracrine, or synaptic delivery mechanisms of release to the proximity and affinity of receptors, and to the efficacy and proximity of rapid reuptake mechanisms (Bunin and Wightman, 1999).

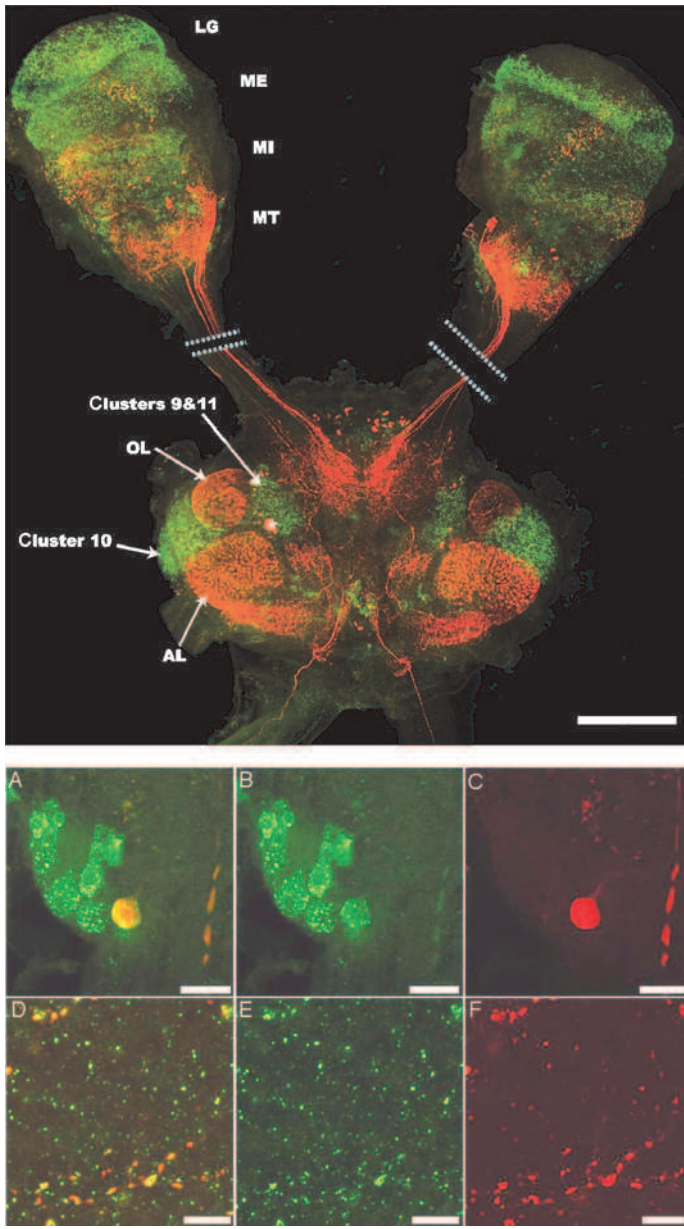

Figure 3 5-HTir (red) and 5-$HT_{1\alpha}$ir (green) in the crayfish nervous system. Top: The crayfish brain and optic lobes. Labeling shows patterns of overlap (e.g., in the olfactory (OL) and accessory (AL) lobes of the brain and the medullae of the optic lobes) and patterns where the receptor distribution and 5-HT projections each occur alone (e.g., protocerebrum: 5-HT alone;

V. Social Dependence of Serotonergic Modulation of Neural Circuit Function

Several behavior patterns that change with the animal's social status are mediated by neural circuits subject to serotonergic modulation. For example, LG escape is strongly inhibited in subordinates during fighting, and only weakly inhibited or facilitated in dominants (Krasne *et al.*, 1987). 5-HT has long been known to inhibit LG's response to inputs from primary afferents and interneurons (Glanzman and Krasne, 1997). Similarly, postural responses to an unexpected touch differ behaviorally (Song *et al.*, 2006) in dominant and subordinate animals; dominants turn and elevate to confront the stimulus while subordinates drop in posture and move away. These differences are reflected in status-related differences in the responses of Dep motor neurons (Issa *et al.*, 2005) and serotonergic interneurons (Drummond *et al.*, 2002) to the same stimulus. The input resistance and resistance reflex responses of the Dep motor neurons are modulated both by applied 5-HT and by stimulation of local 5-HT neurons (Issa *et al.*, 2005). These examples prompted the suggestion that 5-HT modulation could help mediate status-related differences in circuit excitability.

Studies have shown that 5-HT can have either facilitatory or excitatory effects on LG, depending on both the pattern of exposure and the social status of the animal (Teshiba *et al.*, 2001; Yeh *et al.*, 1996). When applied slowly to the exposed ventral nerve cord of crayfish that had been raised in social isolation, 50 μM 5-HT gradually facilitated LG's response to sensory stimulation (Fig. 4). The facilitation persisted for many hours after the 5-HT was washed away. When the same exposure and stimulus were applied to social dominant and subordinate animals from well-established pairs, 5-HT had very different effects—it facilitated the LG's response in the dominants and inhibited it in the subordinates. Moreover, the modulatory effects were removed by the saline wash (Yeh *et al.*, 1996, 1997).

Experiments with 5-HT receptor agonists suggested that the different effects of 5-HT on LG excitability resulted from a difference in the balance of 5-HT receptors in dominant and subordinate animals. The 5-HT agonist α-methyl-5-HT mimicked the facilitatory effects of 5-HT in dominant

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clusters 9 and 10 of olfactory interneuron cell bodies: 5-$HT_{1\alpha}$ir largely alone). Scale bar: 500 μm. Bottom: Labeling in thoracic and abdominal ganglia. (A–C) Several somata on the ventral ganglionic surface display punctate 5-$HT_{1\alpha}$ir labeling in each ganglion (A), (B); at least one colabels for 5-HTir (A), (C). (A) 5-$HT_{1\alpha}$ir and 5-HTir colabeling; (B) 5-$HT_{1\alpha}$ir labeling alone; (C) 5-HTir labeling alone. (D–F) Neuropilar labeling shows processes that are labeled separately (white arrow and arrowhead) and that are colabeled (yellow arrows). (D) Both 5-$HT_{1\alpha}$ir and 5-HTir; (E) 5-$HT_{1\alpha}$ir alone; (F) 5-HTir alone. Scale bar 50 μm for (A–C), 10 μm for (D–E). From Figs. 2 and 7, Spitzer *et al.* (2005a).

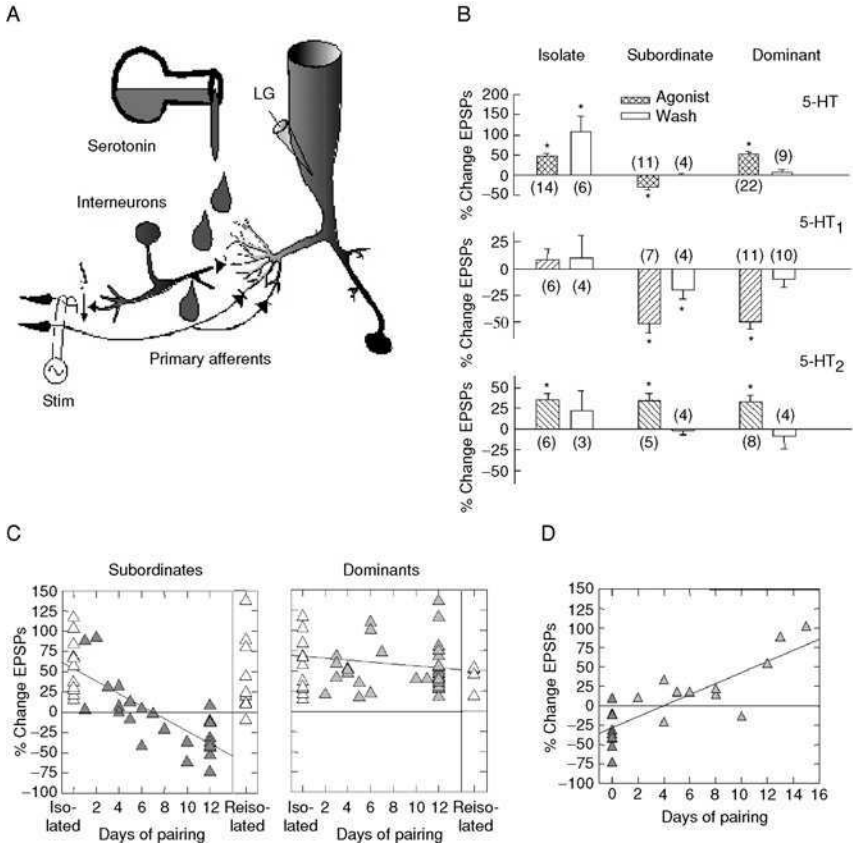

Figure 4 Dominance-dependent effects of 5-HT on the EPSP in the LG neuron. (A) Experimental setup: Recording EPSPs from the initial segment of LG evoked by electrical shock of a sensory nerve before, during and after exposure to 50 μM 5-HT applied to the bath. (B) Average (and S.D.) percent change in the EPSP produced by 5-HT (top row), mCPP (5-HT_1; middle row), and α-Me-5-HT (5-HT_2; bottom row) (filled bars), and after 1-hour saline wash (empty bars). Number of preparations indicated in parentheses above or below each bar. (C) Change in effect of 5-HT following dominance decision on new subordinates (left) and dominants (right). Each symbol represents effect of 5-HT (triangles) on the LG response of one animal. Animals were tested after 1-month isolation (open triangles at left of each panel), after isolation and pairing for different periods (filled triangles), and after isolation, pairing for 12 days and reisolation for 8 days (open triangles at right of each panel). (D) Effect of 5-HT on responses of new dominants (light gray triangles) following pairing of subordinates (dark gray triangles) for up to 15 days. (Adapted from Yeh *et al.*, 1997.) Copyright 1997 by the Society for Neuroscience.

animals, and had similar effects in subordinates. Conversely, another 5-HT agonist, (m-chlorophenyl)-piperazine (m-CPP), mimicked the inhibitory effects of 5-HT in subordinates, and had similar effects in dominants

(Fig. 4) (Yeh *et al.*, 1996, 1997). These results suggested that 5-HT activated both inhibitory and facilitatory mechanisms in LGs of dominant and subordinate crayfish, but to different effects—a net facilitatory effect in dominants and a net inhibitory effect in subordinates.

Perhaps most remarkably, the change in modulatory effect in new subordinates occurred gradually, increasing daily as the animals remained together (Fig. 4C). 5-HT was initially facilitatory in the new subordinates, as it was for social isolates. As they remained subordinate to their dominant partner, the facilitatory effect decreased and, after 1 week, reversed and became inhibitory. During this time, 5-HT retained its facilitatory effect in the dominant members of the pair, but lost its persistence following saline wash. Eight days reisolation restored the facilitatory effect of 5-HT to both animals.

The inhibitory effect of mCPP developed over the same time-course in new subordinates as that of 5-HT. mCPP had no effect on LG in social isolates and no effect in newly paired subordinates or dominants. As pairing continued, however, mCPP increasingly inhibited LG in both dominants and subordinates. Reisolation restored the null effect of mCPP on LG's response.

When pairs of subordinates were placed together, one became dominant, and the effect of 5-HT on LG in those animals gradually changed from inhibitory to facilitatory with time (Fig. 4D). The reverse did not happen—5-HT remained facilitatory for LG's response in former dominant animals that were now subordinate.

VI. 5-HT Receptors and Dominance

Across species, a set of 5-HT receptor types coupled to various second messenger systems exert different neuromodulatory effects. In view of this, the different modulatory effects of 5-HT on the responses of LG in socially dominant and subordinate crayfish might result from different expression patterns of two or more G-protein–coupled 5-HT receptors in the two classes of animals.

In vertebrates, serotonin receptors are a large family of proteins making up seven receptor classes, each with numerous subtypes. With the exception of 5-HT_3-type receptors, which are ligand-gated ion channels, these belong to the seven-transmembrane G-protein–coupled receptors. The signal transduction mechanisms and pharmacology of vertebrate serotonin receptors have been well described.

Several different classes of serotonin receptors have also been cloned from different invertebrates, including flies, marine snails, and nematodes (Saudou and Hen, 1994; Tierney, 2001). Expression studies have shown that

the protein sequences and signal transduction linkages of serotonin receptor classes are conserved between vertebrates and invertebrates so that the sequences of 5-HT_1 receptors from mouse and fly are more similar to each other than to that of a 5-HT_2 receptor from either animal. Also, they both preferentially couple negatively to adenylate cyclase and cause a decrease in cAMP formation upon activation of the receptor with an agonist when expressed in the same cell type. The pharmacology of serotonin receptors, however, is not conserved between vertebrates and invertebrates, so that a specific 5-HT_1 agonist tested in mouse will not necessarily be an agonist of an orthologous invertebrate receptor (Tierney, 2001; Zhang and Harris-Warrick, 1994). In addition, the pharmacological profiles of receptors of the same class can be quite variable between different invertebrates. Because of the paucity of expression data and pharmacological profiling of invertebrate serotonin receptors, it has been difficult to identify the specific roles that different receptor types play in modulating these well-defined circuits.

At present, only two 5-HT receptors have been cloned and sequenced from crustaceans (Clark *et al*., 2004; Spitzer *et al*., 2004, 2005a), although pharmacological evidence suggests that others exist (Tierney, 2001; Tierney *et al*., 2004; Yeh *et al*., 1997; Zhang and Harris-Warrick, 1994).

Sosa *et al*. (2004) cloned the 5-$HT_{1\alpha}$ receptor from spiny lobster, freshwater prawn, and crayfish that has since proved to downregulate cAMP when expressed in HEK293 cells (Spitzer *et al*., 2005b). A detailed map of 5-$HT_{1\alpha crust}$[1] immunoreactivity (ir) and 5-HTir in the crayfish nerve cord showed that 5-$HT_{1\alpha crust}$ is distributed throughout the CNS in somata and in neuropil (Fig. 3) (Spitzer *et al*., 2005a). Colabeling of the receptor and neuronal processes containing 5-HT showed that the receptor is expressed both alone and in close association with 5-HTir neuron profiles (Fig. 3). This labeling pattern suggests that whereas some neurites use the receptor to respond to synaptically released 5-HT, others use it to respond to paracrine- or hormonally released 5-HT. With respect to its possible role in mediating the modulatory effects of 5-HT on LG, the 5-$HT_{1\alpha crust}$ receptor is found closely apposed to the profile of a serotonergic neuron that projects along the ventral aspect of the LG axon and displays varicose endings at the LG initial axon segment (Fig. 5) (Yeh *et al*., 1997).

Immunocytochemistry and quantitative RT-PCR showed that the levels of 5-$HT_{1\alpha Pro}$ expression appear to vary greatly among individuals (Spitzer *et al*., 2005a). No correlation was found between levels of 5-$HT_{1\alpha crust}$

[1] "5-$HT_{1\alpha crust}$" refers to the antibody used for immunocytochemical localization of the receptor in prawn, crayfish, and lobster (Sosa *et al*., 2004). "5-$HT_{1\alpha Pro}$" refers to the receptor cloned from crayfish.

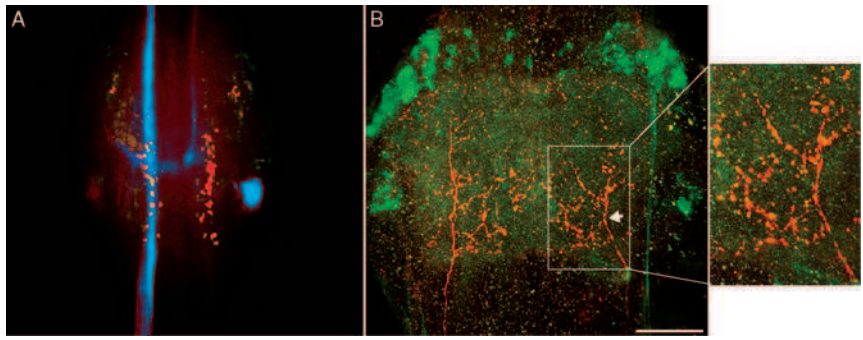

Figure 5 The LG, 5-HTir and 5-$HT_{1\alpha}$ir in the third abdominal ganglion. (A) Dorsal aspect of the ganglion showing varicosities of 5-HT fiber (red) on ventral aspect of LG (blue). Scale bar (in B) is 200 μm. Musolf, B. E., unpublished observation. (B) Dorsal aspect of the ganglion showing the same 5-HTir fiber (red, arrow), its varicosities, and endings of other 5-HT neurons and 5-$HT_{1\alpha}$ir (green). Scale bar is 100 μm. Enlarged version of box is at left, showing the points of overlap (yellow) between 5-HTir fiber and 5-$HT_{1\alpha}$ir. B: From Fig. 9, Spitzer *et al.* (2005a).

immunoreactivity or 5-$HT_{1\alpha Pro}$ mRNA levels and variables, such as the animal's gender, size, molt status, or feeding, nor with external factors such as length of time in the lab or the time of day sacrificed. 5-$HT_{1\alpha Pro}$ mRNA obtained from the entire CNS of nine pairs of dominant and subordinate crayfish showed no differences in mean total content between dominants and subordinates. However, the variance of the total 5-$HT_{1\alpha Pro}$ mRNA content was significantly larger in the dominants than in the subordinates (Fig. 6). The mRNA content of the dominants was within 34% of that of the subordinates for six of the pairs, but was between 83 and 200% greater for the remaining three.

The pattern of 5-$HT_{1\alpha crust}$ir shows that the receptor is distributed across the nervous system in patterns undoubtedly containing many distinct neural circuits that social status could affect. It is not surprising, therefore, that the summed mRNA content should not differ between individuals in many pairs of dominant and subordinate animals. The larger difference expressed in three of the animals suggests that some animals may experience dominant status differently than others. Finally, it is apparent that status-related differences in mRNA content between dominants and subordinates may require a comparison between homologous circuits or single cells from pairs of animals.

Clark *et al.* (2004) cloned a 5-HT_2 receptor (5-$HT_{2\beta Pan}$) from spiny lobster and described its second messenger properties in a heterologous expression system. In 5-$HT_{2\beta Pan}$ the highly conserved DRY motif has evolved to DRF.

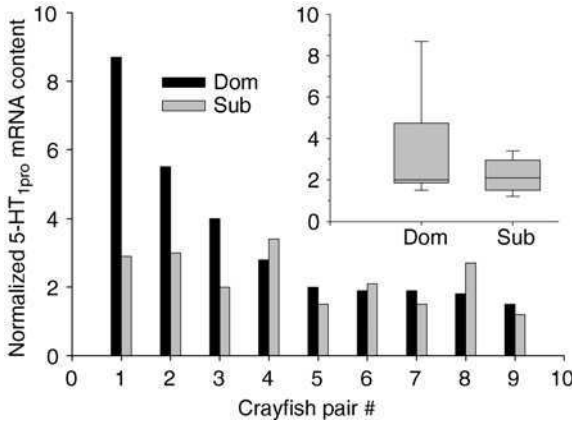

Figure 6 5-$HT_{1\alpha Pro}$ mRNA in CNS from dominant/subordinate pairs of crayfish. Juvenile crayfish were paired for 2 weeks and established consistent dominant/subordinate relationships. 5-$HT_{1\alpha Pro}$ mRNA content was measured from entire ventral nerve cords and brains. There was no significant difference in mean mRNA content between dominant and subordinate animals. However, mRNA content was similar in all subordinates and six of nine dominants. Three dominants had much higher mRNA content than their subordinate partners, causing the variances of the dominant and subordinate mRNA contents to differ significantly ($p < 0.01$, inset). From Fig. 14, Spitzer *et al*. (2005a).

When expressed in cultured HEK293 cells, the DRF motif confers agonist-independent activity to the receptor that is lost upon restoration of the DRY sequence. 5-$HT_{2\beta Pan}$ was found to couple positively to phospholipase C via the traditional G_q pathway, resulting in increased release of DAG and phosphoinositols. These trigger release of Ca^{2+} stores that combine with DAG to activate phosphokinase C.

Preliminary immunocytochemical studies in crayfish show 5-$HT_{2\beta crust}$ immunoreactivity (5-$HT_{2\beta crust}$ir) in the LG neuron, including on the initial axon segment, on the dendrite leading to the cell soma, and in the soma itself. Variations in the expression or immunoreactivity of this receptor with social status have not yet been sought. The complete crayfish ortholog of 5-$HT_{2\beta Pan}$, 5-$HT_{2\beta Pro}$ has recently been sequenced and found to be very similar in sequence, signaling and pharmalogical profile to 5-$HT_{2\beta Pan}$ (Spitzer *et al*., 2005b).

The insect genome projects (*Drosophila*, *Anopheles*, *Apis*) provide a rich resource for crustacean research. By mining these existing databases, genes from other arthorpods can be predicted and cloned by degenerate PCR and hybridization screening. The 5-$HT_{2\beta Pan}$ receptor, for example, was cloned from spiny lobster based on sequence from the *Drosophila* and *Anopheles* genome databases (Clark *et al*., 2004). The crustacean 5-$HT_{1\alpha Pro}$ and

5-$HT_{2\beta Pro}$ receptors have orthologs in *Drosophila*, 5-$HT_{1\alpha Dro}$ and 5-$HT_{2\beta Dro}$. Three additional 5-HTRs have been cloned and characterized from *Drosophila*, 5-$HT_{1\beta Dro}$, 5-$HT_{2\alpha Dro}$, and 5-HT_{7Dro} (Colas *et al.*; 1995; Saudou *et al.*, 1992; Witz *et al.*, 1990) and are likely to have orthologs in crustaceans. In addition, data mining predicts a number of monoamine receptors that are as yet uncharacterized (Clark *et al.*, 2004; Sosa *et al.*, 2004).

VII. The Links Among Social Status, 5-HT, and 5-HT Receptor Expression

Neuromodulators appear to be at the heart of dominance-related behavior in many animals, including crayfish. Although the substances that promote dominance and aggression in crayfish are unknown, in vertebrates they appear to include the peptides arginine vasopressin (AVP) in mammals and arginine vasotocin (AVT) in fish. Released onto targets in the anterior hypothalamus, these substances promote aggressive, dominant-like behavior (Hennessey *et al.*, 1992; Semsar *et al.*, 2004). In crayfish, substances like these may provide a global neuromodulatory signal to promote the excitation of circuits that mediate offensive behavior, such as elevated posture, approaches, attacks, and directed urination, and the inhibition of defensive circuits for escape and retreat.

A sudden change in such a signal may evoke the sudden change in behavior that marks defeat in the new subordinate. Circuits that mediate offensive behavior are inhibited in new subordinate crayfish, while circuits that mediate defensive behavior, including lowered posture, retreats, and MG and NG escapes, are excited or facilitated (Herberholz *et al.*, 2001). In crayfish, these initial changes in circuit excitability are likely to be short-lasting and dependent on the continued neuromodulatory signal. The dominant's persistent attacks on the subordinate after the initial defeat may keep the defensive neuromodulatory signal high in the subordinate, and so help induce development of the longer-lasting plastic changes in circuit excitability described in an earlier section (Arfai and Krasne, 1999). These changes are part of the maturation of the dominance hierarchy that occurs over the first 2 weeks of interaction. In this time the level of aggression declines and the dominance relationship is finally maintained with only infrequent agonistic interactions (Issa *et al.*, 1999).

The maturation of the social hierarchy becomes apparent as circuits in the thorax and abdomen, and presumably throughout the CNS, are altered to favor the release of stimulus responses characteristic of the animal's social status. For example, changes in thoracic and abdominal postural circuits enable the same unilateral touch stimulus to evoke different behavioral

responses in dominant and subordinate animals. Asymmetric responses of local 5-HT neurons and Dep motor neurons help mediate the dominant's turning response toward the stimulus, whereas symmetric reductions in these neurons' basal activities mediate the bilateral drop in posture displayed by subordinates (Issa *et al*., 2005). Similarly, changes in the modulatory effect of 5-HT on LG (Yeh *et al*., 1996, 1997) promote the status-related differences in LG's excitability seen in dominants and subordinates during fighting (Krasne *et al*., 1997).

The persistent differences in circuit function that underlie the status-related differences in behavior are likely to flow from molecular changes in the neurons and synapses that make up the circuit. One set of molecular changes appears to involve the balance of active 5-HT receptors in the LG neurons of dominant and subordinate crayfish. In new subordinates, 5-HT's effect on LG is changed from facilitation to inhibition, presumably by changes in the balance of active receptors to favor those excited by agonists like mCPP, and away from those excited by α-methyl-5-HT. In new dominants, the facilitatory effect of 5-HT is changed from being short-lasting to persistent. This could also occur as a result of changes in the types of active receptors present, or in their efficacy or that of their downstream effectors.

It is an open question at present whether these changes involve either or both of the newly identified receptors, 5-$HT_{2\beta Pro}$ and 5-$HT_{1\alpha Pro}$. Increases in either cAMP (Araki *et al*., 2005; Edwards *et al*., 2002) and Ca^{2+} (Antonsen and Edwards, 2005) facilitate LG's response in the same manner as slowly applied 5-HT does in social isolates and dominants (Yeh *et al*., 1996, 1997). cAMP production could result from activation of a crayfish homolog of the 5-HT_{7Dro} receptor, and internal Ca^{2+} could rise as a downstream product of phospholipase activation via the 5-$HT_{2\beta Pro}$ receptor. Although the crayfish homolog of 5-HT_{7Dro} has not been identified, 5-$HT_{2\beta Pro}$ir is found on the dendrites, soma, and initial segment of LG (unpublished). Activation of this receptor may be responsible for the rise in Ca^{2+} seen at the LG dendrite tips, just postsynaptic to the primary afferents, in response to applied 5-HT (Antonsen and Edwards, 2005). This Ca^{2+} rise is required for the increase in junctional conductance of the primary afferent-to-LG synapses that mediates the 5-HT-induced facilitation of LG. The 5-$HT_{1\alpha Pro}$ receptor reduces cAMP in cell culture; if cAMP were constitutively active in LG, activation of this receptor could reduce that activity and so inhibit LG's response. This receptor is found in proximity to the 5-HTir fiber that is apposed to the ventral aspect of the initial segment of LG (Fig. 5), and so is positioned to help govern LG's firing threshold or its output to motor neurons.

Resolution of these possibilities will have to await experiments that isolate the effects of individual receptors on LG's response, and track the changes in those receptors and their effects through a change in the animal's social

status. Beyond this, we still do not know whether the release of serotonin during a fight can account for the status-dependent change in LG's excitability (Krasne *et al.*, 1997). We also do not know the nature of the signal that alters circuit excitabilities in response to the animal's defeat, or the persistent signal that informs abdominal neurons like LG about the animal's current social status. This latter signal appears to be humoral, and may be 5-HT itself. Finally, 5-HT undoubtedly is not alone in producing the physiological changes that underlie status-dependent difference in behavior. Peptides, like vasopressin and those that modulate digestive motor programs (Harris-Warrick *et al.*, 1992), and other amines, including octopamine and dopamine, may also be involved (Glanzman and Krasne, 1983; Kravitz, 1988).

This discussion makes clear that the feedback loop described in Fig. 1 that includes genes, their molecular products, cells and synapses, circuits, behavior, and the responses of the world, functions for crayfish as it must for all animals. The loop governs not only learning and development but also the responses of animals to both social and environmental challenges that are both unpredictable and of indeterminate duration. Animals must adapt their behavior to survive in the face of such challenges, and this adaptive response occurs at all levels of organization, from molecules to behavior.

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Transplantation of Undifferentiated, Bone Marrow-Derived Stem Cells

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Stem cell research has known an enormous development, and cellular transplantation holds great promise for regenerative medicine. However, some aspects, such as the mechanisms underlying stem cell plasticity (cell fusion vs true transdifferentiation) and the functional improvement after stem cell transplantation, are highly debated. Furthermore, the great variability in methodology used by several groups, sometimes leads to

Current Topics in Developmental Biology, Vol. 74

0070-2153/06 $35.00
DOI: 10.1016/S0070-2153(06)74007-8

confusing, contradicting results. In this chapter, we review a number of studies in this area with an eye on possible technical and other difficulties in interpretation of the obtained results.

I. Introduction

Organ damage or loss can occur from congenital disorders, cancer, trauma, infection, inflammation, iatrogenic injuries, or other conditions and often necessitates reconstruction or replacement. Replacement may take the form of an organ transplant. There is a severe shortage of donor organs that is worsening with the aging of the population. In order to overcome these problems alternative avenues, such as cellular-based therapy, are under intense development. For a long time, it was believed that adult tissue-specific stem cells are committed to supporting only the tissue from which they originated (Armstrong and Svendsen, 2000). Since 1998, many papers began to challenge this fundamental concept of lineage/tissue commitment of adult stem cells by suggesting that the functional potential of stem cells is not restricted to the tissue source from which they are derived. The term stem cell plasticity was introduced and is defined as the capacity of a given (lineage committed) stem cell to differentiate across lineage boundaries to produce cells of other developmentally unrelated tissues, possibly in response to certain changing microenvironmental regenerative signals (Asahara *et al.*, 1999). From then, stem cell research has become an exciting new field in developmental biology, but also for clinicians and the general public, as it might hold the potential for regenerative medicine, especially in replacing cells in tissues that have only minimal intrinsic renewal capacity such as cardiac muscles. However, following the first exciting and promising reports, a second wave of reports were published wherein initial results were challenged, as they could either not be repeated, the degree of plasticity was significantly less obvious or the mechanisms underlying the cell plasticity were debated.

In this chapter, we will mainly focus on the use of bone marrow (BM) stem cells, both mesenchymal stem cells (MSCs) and hematopoietic stem cells (HSCs) in cellular therapy for brain, heart, liver, and pancreas in rodents. Our aim is not to give an extensive overview of all studies published on stem cell therapy but rather to discuss possible mechanisms underlying the apparent stem cell plasticity, as well as point to pitfalls and possible misinterpretations, leading to discrepancies between results.

II. Definition of Stem Cells

By definition, stem cells are defined by three important characteristics that distinguish them from other types of cells. First, they are undifferentiated cells that renew themselves at the single cell level for many cell doublings.

When cell doublings are symmetric, the stem cell pool expands, whereas asymmetric divisions result in maintenance of the stem cell population. In this case, the second daughter cell is lineage committed, giving rise ultimately to one or more differentiated cell type, which constitutes the second characteristic of stem cells. Third, stem cells *functionally and robustly reconstitute a given tissue in vivo* when transplanted in a (damaged) recipient. The latter is easily achieved when HSCs are grafted. However, reconstitution of solid organs, whether the lung, heart, liver, or brain, is more difficult to achieve. Hence, ''robustness'' is a rather relative term (Moore *et al.*, 2003; Quesenberry *et al.*, 2004).

III. Hierarchy in the Potential of Stem Cells

Stem cells can be categorized by their potency (Fig. 1). *Totipotency* refers to the capacity to form both embryonic and extraembryonic cell types, if provided with appropriate maternal support (Verfaillie *et al.*, 2002). The fertilized egg or zygote is totipotent as it is capable of not only forming cells of the mesoderm, endoderm, and ectoderm layer, and germ cells, but also the

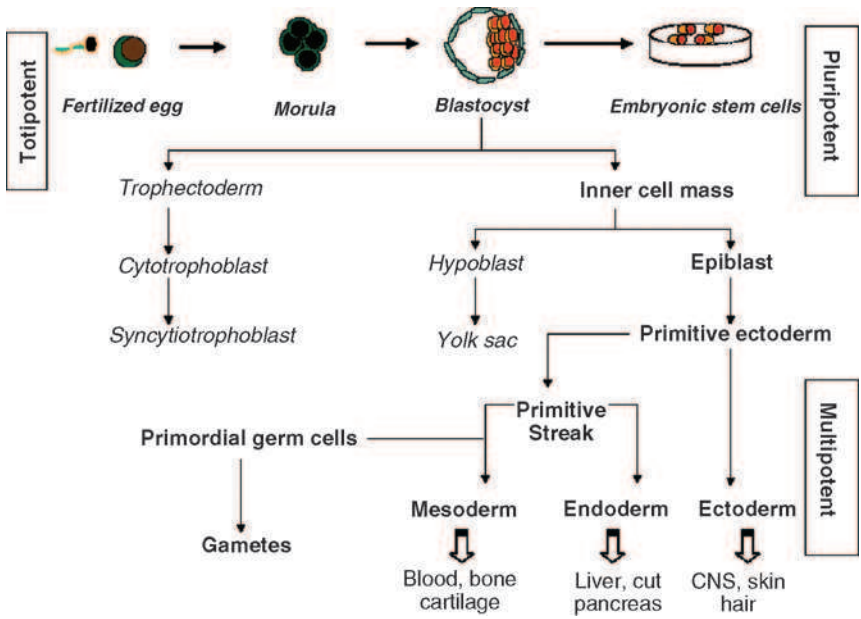

Figure 1 Hierarchy of stem cell potency and overview of early mammalian embryological development. The fertilized egg is the most potent cells, giving rise to both embryonic and extraembryonic tissue. The cells from the inner cell mass (ICM) of the blastocyst are pluripotent as they cannot form trophectoderm. ESCs are derived from this ICM. Multipotent stem cells can differentiate into multiple organ-specific cell types.

supporting trophoblast, required for the survival of the developing embryo (Lakshmipathy and Verfaillie, 2005). By the third to fourth day the embryo develops to a compact ball of 12 or more cells called a morula. After several more divisions, the morula cells begin to specialize and form a hollow sphere of cells called the blastocyst. The outer layer of the blastocyst is the trophectoderm (TE) or trophoblast and the cells inside the blastocyst, the inner cell mass (ICM). The cells of the ICM are *pluripotent* stem cells that can give rise to all cell types of the three embryonic germ layers and the germ cell lineage but not the extraembryonic tissues (Verfaillie *et al.*, 2002). Embryonic stem cells (ESCs) were first derived from the ICM of a mouse blastocyst in 1981 (Evans and Kaufman, 1981; Martin, 1981). Ultimate proof of pluripotency of a cell is its capacity to give rise to all tissues of the embryo following injection into the blastocyst. During development from blastocyst to fetus, pluripotent stem cells first become committed to the somatic or germ lineage, and cells in the somatic compartment become committed to endoderm, ectoderm and endoderm during gastrulation. Stem cells isolated from various adult organs self-renew and differentiate into multiple organ-specific cell types such as neural stem cells (NSCs) are termed "*multipotent* stem cells." Oligopotent cells are able to give rise to a more restricted subset of cell lineages than multipotent stem cells, and unipotent cells, such as endothelial precursor cells, skeletal muscle stem cells or satellite cells, or corneal epithelial stem cells, are able to contribute to only one mature cell type (Lakshmipathy and Verfaillie, 2005; Wagers and Weissman, 2004). However, the validity of this one-way differentiation pathway has been questioned and has been replaced by the belief/hope that some cells might possess a much broader differentiation capacity than previously thought.

IV. Candidates for Cellular Therapy

Under defined conditions, *ESCs* can be kept in their undifferentiated pluripotent state and will proliferate indefinitely, thereby providing a potentially limitless source of cells. In specific culture conditions, ESCs are capable to differentiate into cell types of all three embryological germ layers. This great property of embryonic stem (ES), which makes them so attractive for regenerative medicine applications, is also its biggest enemy for clinical use. A known characteristic of undifferentiated ESCs is the formation of teratomas, tumors composed of elements from all three embryonic germ layers, upon *in vivo* implantation. This hurdle could be overcome by the derivation of highly purified, homogenous ESC-derived differentiated cell types that are free of undifferentiated, pluripotent ESCs. Different protocols to develop large-scale production of highly purified cell preparation have been proposed (Hadjantonakis and Nagy, 2000; Laflamme and Murry, 2005;

Zandstra *et al.*, 2003). Furthermore, the use of human ESCs is encumbered by ethical considerations. Even if ESC transplantation is not a therapeutic option for the near future, research on ESC remains useful as it will give insight in the molecular pathways involved in the embryonic development of multiple tissues.

A second possibility is stem cells derived from tissues following gastrulation, either during development (fetal) or postnatal life (adult). From a biological point of view, *fetal stem cells* might be the optimal cell for cellular therapy. In contrast to ESCs, fetal stem cells are committed to a well-defined lineage, while at the same time more immature than stem cells harvested postnatally with significantly greater proliferative potential (Heng and Cao, 2005a). Unfortunately, the clinical use of fetal stem cells, like ESCs, is encumbered by ethical consideration. Moreover, it is highly unlikely that sufficient fetal stem cells could be procured necessary to repair adult organs. Shortage of whole organ donors also limits the wide use of *adult tissue-specific stem* cells, such as NSCs, hepatoblasts, or satellite cells, unless ways are established to expand them safely, fast, and in sufficient numbers *in vitro* prior to administration. Another option would be to use presumed more pluripotent stem cells residing in BM or perhaps other tissues**.** They are available in a large enough number, could be used in an autologous setting, and reports suggest that they possess previously unknown plasticity as they may be capable of regenerating tissues from all three germ line lineages (Krause *et al.*, 2001). If such plasticity can be substantiated, BM would constitute an ideal alternative to ESCs as a safer and ethically more acceptable source for cell and gene therapy. However, results reported with BM stem cells are often contradictory, controversial, or not reproducible by other laboratories (Wagers *et al.*, 2002).

V. BM-Derived Stem Cells

BM consists of a nonhomogeneous population of cells and harbors three prototypical stem cell populations. HSCs are the first and most extensively studied stem cells (Siminovitch *et al.*, 1963; Spangrude *et al.*, 1988). They are capable of self-renewal and differentiation into progenitor cells, which in turn mature into end-stage blood cells that are continuously released from the BM (Till and McCulloch, 1980). They have been proven to be clinically useful in the treatment of a wide range of hematological diseases. In general, HSCs express CD45, CD34, Thy-1, and do not express many of the surface antigens (lineage markers) that are characteristic of terminally differentiating hematopoietic cells (Bonnet, 2003). Cells with this phenotype are present in murine BM at a frequency of approximately 1 in 10^5 cells (Krause *et al.*, 2001). However, results both in mouse and in human indicate that CD34 expression appears to be reversible and dependent on the activation state of the HSC and the developmental state of the donor (Dao *et al.*, 2003; Sato

et al., 1999). Research is ongoing to identify the ideal combination of cell surface markers to obtain purified HSCs. HSCs can also be purified based on their *in vivo* ability to home to the BM after transplantation (Jang *et al*., 2004; Krause *et al*., 2001) or on their capacity to efflux the dye Hoeschst 33342 on staining, yielding cells with the so-called side-population (SP)-phenotype (Goodell *et al*., 1996).

MSCs (also called marrow stromal cells) reside in the connective tissue-rich stromal compartment of the BM. MSCs were first identified in the pioneering studies of Friedenstein and Petrakova (1966) and are about 10-fold less abundant than HSCs (Pittenger and Martin, 2004). MSCs are important because they secrete growth factors and cytokines, which support the growth and differentiation of HSCs both *in vivo* and *in vitro* (Tocci and Forte, 2003). They are clonogenic and possess the potential to differentiate into adipocytes, chondrocytes, osteocytes, and skeletal and smooth muscle myoblasts (Pittenger *et al*., 1999). Although their phenotypical characteristics are not exactly defined by a set of universal surface markers to select purified cell populations, they can easily be isolated from other cell fractions by their capability to attach to the tissue culture plastic (Hristov *et al*., 2003). However, Prockop's group noted the persistence of CD45+ and CD11b+ pre-B-cell progenitors and granulocytic and monocytic progenitors when MSCs were purified only on the base of adherence (Phinney *et al*., 1999). MSCs are negative for HSC markers CD31, CD34, and CD45 and express on their surface CD44, CD90, CD105, CD106, and CD166 (Mangi *et al*., 2003). Besides mesodermal-related transcripts, MSCs also express endodermal and neuroectodermal-related transcripts (Woodbury *et al*., 2002). Therefore, MSCs could be defined as "multidifferentiated" rather than "undifferentiated" cells. While HSCs are difficult to isolate and expand in culture, MSCs are much easier to isolate from small aspirates of BM and it is possible to reproducibly generate billions of human MSCs (hMSCs) *in vitro* for cellular therapy from a single BM aspirate.

As a third stem cell type, BM contains *endothelial progenitor cells* (EPCs), which can be isolated from BM and circulate in small numbers in the peripheral blood (PB) (Asahara *et al*., 1999). Human EPCs are characterized by the combined expression of CD34, AC133, and Flk-1 (vascular endothelial growth factor (VEGF)-receptor 2) and acquire CD31, vascular endothelial (VE)-cadherin, and vWF expression during differentiation (Hristov *et al*., 2003).

VI. Stem Cell Plasticity: Possible Mechanisms

Plasticity is a general term denoting the capacity of a cell to convert from one type to another (Moore *et al*., 2003). There are five conceptually different mechanisms that may underlie the observation of apparent stem

cell plasticity: (1) presence of multiple tissue-specific stem cells in one tissue, (2) cell fusion, (3) transdifferentiation/transdetermination, (4) de- and redifferentiation, or (5) the presence of an "ESC-like" adult pluripotent cell (Chen *et al*., 2004; Massengale *et al*., 2005).

A. Multipotent Tissue-Specific Stem Cells

The majority of studies describing BM cell plasticity have used nonpurified BM populations as graft or BM enriched for a HSC phenotype. However, even when enriched HSCs or MSCs were used, in most published studies multiple cells were grafted. It is therefore possible that engraftment outside the hematopoietic system is due to coexistence of multiple stem cells within the BM (Verfaillie *et al*., 2002). Ratacjzak *et al.* (2004) found that a subset of CXCR4 positive cells exist in BM and spleen which express early tissue-specific markers of liver (αFP, CK19), muscle (Myf5, MyoD), neurons (GFAP, Nestin), intestinal epithelium, skin epidermis, and endocrine pancreas, as well as pluripotent stem cell markers such as Oct4, Rex1, and Nanog. They hypothesize that these committed tissue-specific stem cells circulate between the BM and the peripheral tissues and if needed could take part in the regeneration of damaged organs. Avital *et al.* (2001) described that β2m(–)/Thy1(+) cells derived from rat and human BM express hepatocyte-specific markers (i.e., albumin, C/EBP) at base line and are *in vitro* capable of differentiating into cells with functional characteristics (metabolization of ammonia into urea) of mature hepatocytes, when cocultured with hepatocytes, especially in the presence of cholestatic serum. As such, the BM could be considered as a "hide out" for already tissue-committed stem/progenitor cells, and it has been suggested that these cells are responsible for at least some of the occurrence of donor-derived chimerism after systemic infusion or local delivery of BM-derived cells (Kucia *et al*., 2005a). However, this does not constitute stem cell plasticity.

B. Cell Fusion

Cell fusion is a well-known phenomenon that had been successfully exploited in hybridoma technology (Burns, 2005). The evidence that cell fusion might be responsible for the apparent plasticity of BM cells came from *in vitro* studies in which BM-derived cells or NSCs were cocultured with ESCs (Terada *et al*., 2002; Ying *et al*., 2002), yielding cells that expressed some markers of the original NSCs or BM cells but acquired functional characteristics of ESCs. Subsequent evaluation demonstrated that this was the result of fusion between ESCs and the more mature stem

cell populations. Subsequent studies have demonstrated that this phenomenon is also operative *in vivo*. Although these fusion events are rare both *in vivo* and *in vitro*, cell fusion may underlie some observations previously attributed to an intrinsic plasticity of tissue stem cells. Fusion of BM-derived cells with recipient cells results in the coexpression of markers/genes of both cell types. Cell fusion, like transdifferentiation, is associated with changes in gene expression or "nuclear reprogramming" (Horwitz, 2003; Toma *et al*., 2002; Wakayama *et al*., 1998). Whereas in the case of transdifferentiation, BMDCs undergo a change in gene expression and function in response to signals in the local environment and differentiate accordingly, in the case of cell fusion, BMDCs undergo at least partial nuclear reprogramming as a result of signals within the cells to which they fuse (Weimann *et al*., 2003).

1. How to Exclude or Prove Cell Fusion?

Fused cells normally express cell surface and cytoplasmic markers derived from both parental cells. However, proving cell fusion can be difficult as it is well known that a fraction of the expressed genes in both the donor cell and the cell to which the donor cell fuses are silenced. Although fusion leads initially to binucleated cells ($2 \times 2n$), subsequent nuclear fusion can occur, resulting in mononuclear $4n$ cells. Additionally, a reductive division can happen with the expulsion of an entire or partial set of chromosomes and the formation of a new (near)diploid cell, which might conceal the fusion history (Wang *et al*., 2003c). To determine whether presumed lineage switch is due to fusion, multiple complementary approaches should be used (Fig. 2). Transplantation of female cells in male recipients and identifying a presumed donor cell with tissue-specific markers indicative of "transdifferentiation" but with the Y-chromosome present proves cell fusion (Alison *et al*., 2004). One caveat is that sections can be thinner than the volume of the nucleus that is investigated; it is highly possible that sex chromosomes distributed in a large nucleus can be missed when thin sections are examined. Moreover, loss of the Y-chromosome is a frequent occurrence in normal cells (Mitchen *et al*., 1997). Xenograft experiments, in which human cells are transplanted into immunodeficient animals, allow relatively straightforward fluorescence *in situ* hybridization (FISH)-based murine versus human chromosomal analysis to evaluate presence of donor and host chromosomes in the same cell. An additional approach to rule in/out cell fusion is experiments in which a reporter gene is activated only when cells fuse such as the Cre/Lox recombination method. This method relies on the ability of the Cre-recombinase enzyme to excise a floxed stop cassette that prevents expression of a reporter gene, leading to the activation of expression of a reporter gene *β-galactosidase* (*β*-Gal), green fluorescent protein (GFP), and alkaline phosphatase (AP) in the fused cells. This was first employed by Alvarez-Dolado

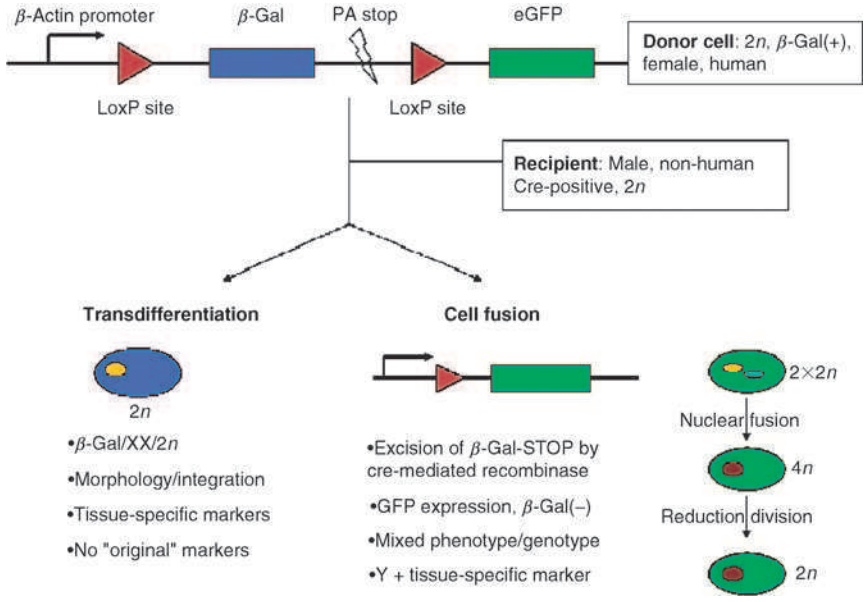

Figure 2 Complementary approaches to exclude cell fusion. (1) Identification of a presumed donor cell, expressing tissue-specific markers indicative of transdifferentiation but with a Y-chromosome is a prove of cell fusion. (2) In a xenotransplant setting, colocalization of a human-specific and non-human–specific marker is indicative for cell fusion. (3) The Z/EG mouse is a Cre reporter strain which normally expresses β-Gal. However, upon exposition to Cre-recombinase, the β-Gal-STOP-DNA part of the construct will be excised. Consequently, the eGFP transgene will be expressed, driven by the β-actin promoter. Cell fusion can be proven if BM cells from a Z/EG mouse, transplanted into mouse strain that ubiquitously expresses Cre-recombinase, turn on the eGFP transgene. (4) Fused cells are initially binucleated ($2 \times 2n$) and can undergo a nuclear fusion to form mononuclear $4n$-cells. Ultimately, a reduction division can occur, resulting in a new diploid ($2n$) cell. (5) Fused cells will express both donor and recipient genes.

who injected Cre-eGFP BM cells into an irradiated LoxP-LacZ transgenic recipient without any selective organ damage (Alvarez-Dolado *et al.*, 2003). They visualized β-Gal positive cells in the heart, cerebellum, and liver, suggesting cell fusion had occurred. The fused cells had the morphological and phenotypical characteristics of cardiomyocytes, purkinje cells, and hepatocytes, respectively, and were multinucleated. The only GFP+ cells, and thus nonfused donor cells, that were detected in these organs were macrophage-like cells. After cell fusion, some of the donor genes, such as *CD45* or *GFP*, may be inactivated/eliminated over time, suggestive for genetic instability/reprogramming in heterokaryons. Some have argued, however, that by metabolic cooperation, a cell can acquire the Cre-recombinase from

a neighboring cell and hence undergo excision of the flox-flanked DNA segment in the absence of cell fusion (Kajstura *et al*., 2005). In addition, detection of fusion using this approach may fail, if one or both of the transgenes are silenced, in case of loss of the genomic DNA containing the transgene through reductive division, or inaccessibility of the LoxP sites to Cre-recombinase (Harris *et al*., 2004). By transplanting male Cre-BM into β-Gal-Flox-Stop-eGFP protein expressing (Z/EG mice) female mice, Krause's group demonstrated that, although at a low frequency, epithelial cells can be derived from BM cells without evidence for cell fusion (Harris *et al*., 2004). They demonstrated in elaborate studies that the Cre-recombinase was expressed as well as the β-Gal-Flox-Stop-eGFP protein, hence avoiding conclusions that might be incorrect as a result of silencing of either one of these genes. With only total body irradiation as tissue injury, 18 out of 36,000 hepatocytes investigated were male derived and none expressed eGFP, CD45. In the lung and skin, 0.2 and 0.1%, respectively, of the epithelial cells were donor derived. Only when animals were exposed to tissue injury (muscle and liver) other than radiation, were there signs of cell fusion.

To which degree cell fusion is responsible for presumed lineage switch is a matter of intense debate in a number of different model systems. In contrast to initial studies by Anversa's group (Orlic *et al*., 2001a), suggesting that BM cells could themselves differentiate into cardiac myoblasts, Jacobsen's group, in agreement with the findings of Alvarez-Dolado, demonstrated high levels of engraftment of hematopoietic cells in the ischemic myocardium and very low levels of cardiomyocytes derived from fusion with BM-derived cells outside the infarct area (Alvarez-Dolado *et al*., 2003; Nygren *et al*., 2004). However, more studies from Anversa continue to suggest that grafted c-kit$^+$ BM cells differentiate into myocytes in the absence of fusion (based on male into female sex-mismatched transplantations). As additional arguments against the notion that the presumed lineage switch would be due to fusion, they argued that no living cardiomyocyte fusion partners are available in ischemic myocardium and that the "new" myocytes are immature, smaller versions of normal cardiomyocytes, which unlike mature, postnatal cardiomyocytes continue to possess proliferative capacity (Kajstura *et al*., 2005). Nevertheless, the generally accepted view is that true transdifferentiation into cardiomyocytes, if it happens, occurs at a very low frequency (Jackson *et al*., 2001).

Controversy also exists in the field of *BM-derived hepatocytes*. The group of Grompe and Lagasse performed a series of experiments to test the utility of liver repopulation by BM-derived stem cells in the fumarylacetoacetate hydrolase (FAH)-deficient mouse model, an animal model of fatal hereditary tyrosinemia type I. FAH is a key component of the tyrosine catabolic pathway. Mutant mice have progressive liver failure and renal

tubular damage unless they are treated with 2-(2-nitro-4-trifluoro-methyl-benzyol)-1,3-cyclohexanedione (NTBC), a compound that prevents the accumulation of toxic metabolites in the tyrosine catabolic pathway by inhibiting an enzyme upstream of FAH. In a first report (Lagasse *et al.*, 2000), they intravenously transplanted 1×10^6 male, LacZ positive, unfractioned BM cells, or >50 highly purified HSCs into lethally irradiated female FAH mutant mice. Three weeks after transplantation, NTBC diet was discontinued, which exerted a strong selection pressure on the engrafted cells. Though the initial engraftment was low, approximately 1 BM cell for every million endogenous hepatocytes at 7 weeks, the strong selection pressures resulted in clonal expansion of the healthy donor-derived cells such that by 7 months after transplantation, donor cells repopulated up to 30–50% of the liver. Biochemically, the liver function of the transplanted, surviving mice (4) was substantially improved. In a subsequent report (Wang *et al.*, 2003c), however, they described that the clusters of BM-derived hepatocytes were mostly, if not exclusively, derived from fusion events between host hepatocytes and BM cells. BM-derived hepatocytes contained markers of both donor and recipient cells. Presumably, the FAH+/+ donor nucleus fused with the FAH−/− host hepatocyte and provided the FAH protein leading to functional improvement of the liver disease. Of note the karyotype of the FAH+ hepatocytes was variable ranging from 80, XXXY, 120, XXXXYY, suggesting fusion between BM cells and diploid hepatocytes, or tetraploid hepatocytes as well as to 40, XY, indicative of direct transdifferentiation or reductive cell division. Some cells were aneuploid, suggesting partial reductionary cell divisions, or random shedding of chromosomes (Alison *et al.*, 2004). The group of Russell came to the same conclusion as they found that the mean level of donor DNA in the regenerating FAH+ noduli was only 26% (Vassilopoulos *et al.*, 2003). Finally, Grompe's group (Willenbring *et al.*, 2004) and the lab of Goodell (Camargo *et al.*, 2004) reported simultaneously that not the transplanted HSCs themselves but a more differentiated myelomonocytic progeny were sufficient for therapeutic cell fusion in their FAH-deficient mouse model. Others, however, concluded that fusion is not the underlying mechanism for the derivation of hepatocytes from HSCs (Harris *et al.*, 2004; Jang *et al.*, 2004). In the study of Jang, highly purified HSCs (retrieved from the BM 48 hours after transplantation of labeled Lin^- BM into a lethally irradiated mouse) were cocultured with injured liver tissue for 48 hours and 3% of the HSCs acquired liver and cholangiocyte-specific markers, after only 8 hours, probably induced by humoral factors released from the injured tissue. In addition, they observed tetraploid cells purely from male origin. Two days after systemic injection of male purified HSCs into irradiated female mice that received CCl_4, 7.6% of the total liver cells were positive for E-Cadherin (epithelial marker), albumin, and

the male chromosome, either XY or XYXY, and 0.01% XXXY. The transplanted cells proliferated, yielding an even higher conversion after 7 days. Transplantation of a less enriched fraction of HSCs showed minimal engraftment and conversion and there was a higher ratio of fused cells. They concluded that the target cell for fusion is a more mature cell type and not the HSC. The experimental settings, described by Harris and Jang, are different from the severe liver injury induced by withdrawal of NTBC in the FAH-deficient mice. The FAH-deficient mouse has inherent chromosomal abnormalities, including aberrant karyokinesis or cytokinesis and multinucleation, not seen after CCl_4 exposure (Jorquera and Tanguay, 2001). It has therefore been speculated that the FAH environment might promote cell fusion more than other types of liver injury. In addition, fusion is observed as a rather late event. While the FAH-deficient mice were analyzed 5 months after transplant, Jang *et al.* sacrificied the animals as soon as 2–7 days following grafting. Nevertheless, the observation of Jang (Jang *et al*., 2004), indicating that hepatic markers are expressed by HSCs after as early as 8 hours following coculture *in vitro* with damaged hepatocytes, and transdifferentiation to hepatocyte-like cells *in vivo* after 2 days, is surprising.

Other studies investigated the generation of cerebellar Purkinje cells from BM. Priller *et al.* (2001) transplanted BM cells, retrovirally transduced with GFP, into lethally irradiated mice. While no GFP-expressing neuron-like cells were found 4 months after BMT, 12–15 months after transplantation, up to 0.1% of fully developed cerebellar neurons were donor derived. These cells were identified as Purkinje cells by their characteristic morphology, the expression of calbindin-D28K—uniquely expressed in mouse Purkinje neurons, the presence of γ-aminobutyric acid (GABA)-synthesizing enzyme, glutamic acid decarboxylase—indicating neurotransmitter synthesis, and by the presence of multiple synaptic contacts, suggesting functional activity. To exclude the possibility of GFP uptake by the recipient Purkinje cells, the experiment was repeated using BM-derived from transgenic mice that ubiquitously express GFP. Also in this setting, they found the same results. Still, transdifferentiation of BM cells to Purkinje cells was a rare event and occured very late after BMT. The authors speculated that, as 30% of Purkinje cells is lost with ageing, the occurrence of BM-derived Purkinje cells late post-BMT may be a physiological response to this ageing phenomenon. Thus, these findings might imply that BM-derived stem cells regenerate neurons that are normally lost to aging, and that cell therapy may be clinically applicable for CNS disorders associated with Purkinje cell loss. In contrast to the study of Priller, Blau's lab undertook an in-depth analysis of the possibility of cell fusion as the underlying mechanism of the newly formed Purkinje cells (Weimann *et al*., 2003). As in the previous study, they transplanted GFP-labeled whole BM cells into irradiated mice.

Several months after BMT, GFP+, calbindin-positive Purkinje neurons (a maximum of 60 neurons after 1.5 years), indistinguishable from normal Purkinje neurons were detected in the cerebellum. The GFP+ Purkinje cells did not express the hematopoietic markers CD45, CD11b, F4/80, and Iba1, suggestive of true transdifferentiation. However, serial 1-μm optical sections through the entire cell body of a GFP+ Purkinje cell revealed the presence of two nuclei: one endogenous and one BM-derived nucleus. Definitive proof that the binucleated cells resulted from fusion was obtained by sex-mismatched transplantation and the detection of a Y-chromosome in one of the two nuclei. Thus, the new Purkinje cells did not arise *de novo* from BM cells but through cell fusion to endogenous Purkinje cells. Of note, with time the BM-derived nucleus assumed the morphology of the Purkinje nucleus to which it fused. The results of the study of Weimann are distinct from the fusion events described in the liver (Wang *et al.*, 2003c). Whereas the fusion of BM cells with mature Purkinje cells occurred spontaneously (under physiological conditions and without ongoing selective pressure) to form stable, nondividing, binucleate, chromosomally balanced heterokaryons, the hepatocyte/BM fusion products arise under high selective pressure conditions yielding unstable heterokaryon, that proliferate extensively, resulting in the repopulation of the diseased liver. It should be noted that the resulting karyotypic instability in the liver was well tolerated presumably because adult hepatocytes are typically poly- and even aneuploid (Bohm and Noltemeyer, 1981).

The group of Hussain used the Cre/Lox method to exclude cell fusion as the underlying mechanism of differentiation of BM cells towards pancreatic β-cells (Ianus *et al.*, 2003). Although they did not perform any functional experiments, they suggested that BM progenitors might play a role in the physiological maintenance of the β-cell pool. However, other groups failed to reproduce these findings (Choi *et al.*, 2003; Hess *et al.*, 2003; Kojima *et al.*, 2004; Lechner *et al.*, 2004; Li *et al.*, 2003; Mathews *et al.*, 2004).

The question remains whether cell fusion is a rare, clinically unimportant phenomenon or could, in some circumstances, represent a mechanism whereby nomadic stem cells initiate differentiation and repair damaged tissues, which could be exploited clinically? The hybrid cells formed by cell fusion in the studies by Grompe are apparently able to modify diseased hepatocytes into functionally competent hepatocytes; hence the therapeutic potential of BM cells for repair of certain liver diseases should not be completely negated. Likewise, BM cells might be exploited to correct muscular dystrophy. Finally, fusion of BM-derived cells might also serve to deliver tumor suppressor genes (Blau, 2002). Further experiments will be needed to determine whether the generation of cells with an aneuploidie may carry an increased risk for tumorogenesis.

C. Transdifferentiation

Given the overwhelming amount of papers describing diverse cell fate transitions, very stringent criteria for the demonstration of a bona fide transdifferentiation event have been proposed (Blau *et al.*, 2001; Wagers and Weissman, 2004; Wagers *et al.*, 2002). First, to claim that a cell has acquired a new identity, proteins indicative of the previous cell fate should be lost; for example, "transdifferentiated" donor HSCs should stain negative for the pan-hematopoietic marker CD45 (Blau, 2002; Massengale *et al.*, 2005). Second, the transdifferentiated donor cell should express one but ideally more tissue-specific markers at the protein level as well as exhibit distinctive morphology and proper integration into the target tissue. Third, and most importantly concomitant demonstration of tissue-specific function (Blau, 2002; Verfaillie *et al.*, 2002) is required. Fourth, transdifferentiated donor cells should be obtained from a single cell transplant to exclude the possibility that multiple distinct tissue-specific stem or progenitor cells present in a potentially heterogeneous test population are responsible for the observed "plasticity" (Wagers and Weissman, 2004) as only this criteria provides proof that one cell can generate both the expected (hematopoietic) and the unexpected (nonhematopoietic progeny) in the same animal. Fifth, in order to assess the intrinsic array of developmental potential of a particular cell type, the cells should be minimally manipulated and preferably analyzed without intervening culture that may affect their gene expression profile and/or chromatin configuration (Goodell, 2003). Sixth, some would argue that the lineage switch should occur without intervening cell division (Shen *et al.*, 2000). Finally, as is true for all scientific observations made, claims of transdifferentiation must be independently repeated by more than one laboratory using more than one experimental model. The best example for transdifferentiation being possible comes from interchanges between hepatic and pancreatic fates. High doses of dexamethasone in combination with oncostatin M induces direct conversion of pancreatic exocrine cells to hepatic-like cells, partially without cell division. The hepatic-like cells progressively acquired multiple liver-specific markers and lost expression of amylase, a marker of the exocrine pancreas. The transcription factor C/EBP β is considered to be the master switch between a pancreatic or hepatic phenotype (Shen *et al.*, 2000). Conversely, transient overexpression of a modified *Xenopus* homolog of Pdx1, a key transcription factor for pancreas development, is sufficient to induce long-term transdifferentiation of differentiating or differentiated liver cells into functional exocrine and endocrine pancreatic cells (Horb *et al.*, 2003; Li *et al.*, 2005).

D. Cells Undergo Dedifferentiation and Redifferentiation

Dedifferentiation describes the process when a cell switches lineage by first reverting to an earlier, more primitive cell type and subsequent redifferentiates along a new tissue lineage pathway (Liu and Rao, 2003; Wagers and Weissman, 2004). An example of this is seen after limb amputation in amphibians, which leads to dedifferentiation of local myocytes, followed by regeneration of cells of different lineages (Nye *et al*., 2003). The same process also occurs during therapeutic or reproductive cloning employing nuclear transfer. The nucleus of a differentiated somatic cell is isolated and injected into the cytoplasm of an enucleated oocyte, leading to epigenetic changes in the DNA of the introduced nucleus, probably by factors present in the oocyte cytoplasm (Wakayama *et al*., 1998). A number of studies have suggested that similar although less dramatic processes may cause dedifferentiation of somatic cells. For instance, when oligodendrocyte progenitors from the optical nerve were maintained in serum-free, low-density culture conditions, they acquired NSC characteristics (Tang *et al*., 2001).

E. True Multi- or Pluripotent Stem Cells Persist in Postnatal Life

A last possibility is that a *rare population of pluripotent, embryonic-like stem cells* resides in the BM, which possibly copurifies in protocols designed to enrich for tissue-specific stem cells such as HSCs or MSCs. Such cells may have persisted beyond the earliest steps of embryogenesis and, depending on the milieu, differentiate to cells different than the organ of origin (Liu and Rao, 2003; Verfaillie *et al*., 2002). Multipotent adult progenitor cells (MAPCs), for instance, are derived from the BM and have almost pluripotent characteristics. *In vitro*, MAPCs express high levels of the embryonic markers Oct4 and Rex1 and can differentiate into cells of mesodermal, ectodermal, and endodermal lineage. *In vivo*, a single MAPC contributes to the three lineages after injection into the blastocyst (Jiang *et al*, 2002). The group of Schiller described the isolation of ''marrow-isolated adult multilineage inducible cells'' (MIAMI) from cadaveric human vertebral bodies (D'Ippolito *et al*., 2004). Undifferentiated MIAMI cells express ESC markers, as well as low levels of linage-specific markers. After induction protocols, they can be further differentiated into osteoblasts, chondrocytes, adipocytes, neuroectoderm, and pancreatic islet-like cells. Unrestricted somatic stem cells (USSCs) are CD45−, adherent cells derived from human cord blood, which possess the *in vitro* capacity to differentiate into cells with a mesodermal and neuroectodermal phenotype. Osteogenesis and chondrogenesis were described after transplantation into femurs of nude rats and

mice, respectively. *In vivo*, USSCs also contributed to the hematopoietic system, hepatic parenchyma (up to 20%), and cardiac tissue in a noninjured preimmune fetal sheep. The high hepatic repopulation seemed not to be based upon fusion events (Kögler *et al.*, 2004). Intramyocardial USSC injection, 4 weeks after infarction, resulted in improved regional perfusion, wall motion, and ejection fraction (Kim *et al.*, 2005). Yet another population, human BM-derived multipotent stem cells (hBMSCs) are clonally expanded cells with unlimited self-renewal capacity (Yoon *et al.*, 2005). These cells do not express markers typical for MSCs such as CD90, CD117, CD105, MHC class I and II, or HSCs. *In vitro*, they show triple lineage differentiation capacity. The investigators also report that transplantation of hBMSCs in an infarcted heart results in the differentiation to cells with markers of cardiomyocytes, endothelial cells, and smooth muscle cells. It should be noted that all these cell populations are derived from *in vitro* culture of blood or marrow, and that it cannot be ruled out that the process of cultivation is responsible for a reprogramming event, endowing the cells with the greater differentiation ability. Finally, although minor differences exist in the cell surface phenotype and expressed gene profile between MAPCs, MIAMI cells, USSCs, and BMSCs, they are likely all related cell populations with more or less primitive features.

VII. Stem Cell Plasticity: Confusion

Sharkis' group was the first to report widespread engraftment and transdifferentiation after transplantation of a single HSC into an irradiated mouse, using BM cells purified through functional isolation involving lineage depletion, elutriation, their ability to home to BM 2 days after transplantation, and separation of small, G_0/G_1 cells (Krause *et al.*, 2001). Male donor-derived epithelial cells were detected 11 months posttransplant in bronchi (2.32%), pneumocytes (12.58%), GI tract (0.22–1.12), and skin (2.1%). No donor-derived hepatocytes, cardiomyocytes, or myoblasts were seen. A study by the lab of Weissman, in contrast, suggested that highly purified HSCs do not possess a robust, intrinsic capacity to transdifferentiate into nonhematopoietic tissue in physiological conditions (Wagers *et al.*, 2002). Four to nine months after transplantation of a single GFP+ KTLS cell, only one GFP+ Purkinje cell and seven GFP+ hepatocytes were found that fulfilled the earlier defined criteria of transdifferentiation. A number of differences between the two studies may explain the seemingly contradictory results, including the age of the donor animal, the method of selection for HSCs, and the method to track donor origin (Y-chromosome vs *GFP* transgene) (Theise *et al.*, 2003). Anversa's lab first reported therapeutic potential of intramyocardial injection of BM-derived cells myocardial

infarction in 2001 (Orlic *et al.*, 2001a). Shortly after coronary ligation, male Lin^- c-kit^+, GFP labeled cells were injected in the nonischemic border surrounding the infarct of female mice. They claimed that newly formed myocardium occupied 68% of the infarcted portion of the ventricle 9 days after transplanting the BM cells. Although, they found colocalization of the GFP+ cells with several cardiomyocyte markers by immunostaining, the engrafted cells did not have the typical morphology of cardiomyocytes and sarcomeres. This therapeutic intervention reduced the infarcted area and improved cardiac hemodynamics. Later, they demonstrated that mobilization of BM cells with stem cell factor (SCF) and granulocyte-colony stimulating factor (G-CSF), from day 5 to day 0 prior to an LAD ligation, might offer a noninvasive alternative therapeutic strategy for the regeneration of the myocardium without mortality of the treated animals (Orlic *et al.*, 2001b). However, a series of subsequent studies have challenged these promising findings. Some investigators could not find any sign of transdifferentiation (Balsam *et al.*, 2004; Murry *et al.*, 2004), whereas others described transdifferentiation but at a much lower frequencies (Jackson *et al.*, 2001), and/or found that rare cell fusion not transdifferentiation is the underlying mechanism (Nygren *et al.*, 2004). Despite lack of engraftment or transdifferentiation, a number of studies found functional impact, suggesting that the BM cells may affect the heart in manners other than cardiomyogenesis (see later).

McKercher's group reported that unfractionated BM cells migrate into the brain and differentiate into cells that express neuron-specific antigens in mice (0.5%) and human (0.025–0.05%), using the Y-chromosome as tracking marker (Mezey *et al.*, 2000). Others injected GFP+ whole BM cells and found engraftment of apparent neural cells but only in the olfactory bulb being an active site of neurogenesis (Brazelton *et al.*, 2000). Several others have questioned these findings. For instance, Castro *et al.* (2002) transplanted SP cells and unfractioned BM cells derived from ROSA26 mice in lethally irradiated mice. After 4 months, rare β-Gal hematopoietic cells were found in the brain and spinal cord, but no donor-derived neural-like cells, even when cortical trauma was added 4 months after transplantation. Somewhat surprising was the fact that only few β-Gal positive microglia were seen (Mezey *et al.*, 2003). That BM cells can contribute to nonhematopoietic tissues but not the brain, was shown by injecting cardiotoxin into the muscle of the transplant recipients, resulting in the presence of β-Gal+ muscle fibers.

In the case of liver repopulation, many variable engraftment levels are described in the CCl_4-induced hepatotoxicity model (Dahlke *et al.*, 2003; Jang *et al.*, 2004; Kanazawa and Verma, 2003; Terai *et al.*, 2004; Wang *et al.*, 2003b). It appears that the type of cell population tested, dose and/or timing of CCl_4 treatment, time posttransplant evaluation, among others may affect

the observed levels of liver engraftment and differentiation. Gilgenkrantz's lab studied the repopulating capacity of unfractionated BM cells from transgenic mice expressing Bcl-2 under the control of a liver-specific pyruvate kinase promoter, rendering hepatocytes resistant to Fas-mediated apoptosis (Mallet *et al*., 2002). They investigated whether BM cells derived from such Bcl-2 transgenic mice could repopulate the liver after irradiation and hepatic injury elicited by repeated injections of the Fas agonist antibody, Jo2. After four injections of Jo2, they estimated that 0.008–0.04% of the total parenchyma were donor-derived hepatocytes. Increasing the number of injections to eight, showed that donor-derived hepatocytes contributed from 0.05% to 0.8% of the total number of cells—a 6- to 20-fold increase over the level observed after four apoptotic challenges. Similarly, the group of Grompe found impressive hepatic repopulation of FAH-deficient mice after transplantation of whole BM and purified KTLS cells but only after prolonged and cycling selective pressure (Lagasse *et al*., 2000; Wang *et al*., 2002b). In comparison, mature hepatocyte transplantation in this model resulted in significant and functional liver repopulation (~50%) in less than 1 month, without the need of preparative irradiation. Thus, hepatocyte replacement by BM cells is intrinsically a slow and rare event, even in severely injured livers. Given the results of Mallet and Grompe, a continued *in vivo* selection strategy will be required to achieve a therapeutic level of liver repopulation with BM-derived hepatocytes. Moreover, the differentiation process, at least in the case of the FAH mice, is not direct transdifferentiation but fusion mediated.

Hence, although the results reported by different groups range from significant levels of transdifferentiation to none, differences in methodology preclude a straight comparison.

In the following sections, we will address some variables that might help explain the contradictory results encountered in different papers.

A. Technical Reasons for Discrepancies

1. Advantages and Disadvantages of Different Cell Tracking Systems

There are several methods to label cells to track them and distinguish the transplanted cells from the host cells (Table I). BM stem cells can be genetically marked, using *GFP or β-Gal* expressed from a universal promoter, such as the ubiquitous β-actin promoter with a cytomegalovirus enhancer, or eF1α, such that all cells and tissues in these mice, except for erythrocytes and hair, are constitutively and irreversibly marked by GFP or β-Gal. Alternatively, the transgene can be placed under the control of a tissue-specific promoter such that only when lineage-specific differentiation

Table I Factors that Play a Role in Possible Stem Cell-Based Therapies, Include the Cell Source to be Used, the Mode of Transplantation, and the Ability to Determine Engraftment

	Possibilities	Pro	Contra
Cell population	Hematopoietic stem cell	Well characterized	Low quantities
		High degree of purification possible	Cannot be expanded
		Can be used without extensive manipulation	Unclear whether transdifferentiates in cells other than blood
	Mesenchymal stem cell	Multipotentiality proven *in vitro*	No good positive selectable markers
		Easy to obtain in high quantity	Culture conditions might alter cell phenotype
		May not be immunogenic	Multidifferentiated rather than undifferentiated
	Unfractioned BM cells	Can be used without extensive manipulation	Impossible to identify which cell is responsible for a given result
Route of administration	Mobilization of endogenous stem cells with G-CSF/SCF	No invasive procedures required	Impossible to identify which cell is responsible for a given result
		No risk of infection transmission	How to specifically mobilize a stem cell/cell population?
	Exogenous administration		
	Intraorganic	Homing problems bypassed	Invasive procedure can have surgical complications
		Can be done with smaller numbers of cells	Cells do not diffuse throughout the organ
		No systemic dissemination	
	Systemic	Easy access	Efficient homing signals required
		Repeated administration possible	Loss of cells in large capillary beds
		Large number of cells can be given	Distribution to nondesired organs
	Local circulation	Limited number of cells required	Patency of artery that feeds the organ needed
			Thrombosis (portal circulation)
			Invasive procedure can have surgical complications

(*Continued*)

Table I *Continued*

	Possibilities	Pro	Contra
Cell tracking systems	Male into female transplantation	Useful in human studies	Confocal microscopy and 3D reconstruction needed to rule in/out fusion
		No additional manipulation of the cells	Loss of morphology
			Fetal microchimerism may yield false positive result
	ROSA26 (*LacZ*) transgenic mice	Widely available	Inhomogeneous expression of β-Gal in some tissues
		No extra manipulation of the cells	Expression is pH dependent
			Silencing of β-Gal expression
			Dilution without fast fixation
			Cell fusion cannot be excluded
	GFP transgenic mice	Widely available	False positivity through autofluorescence
		Naturally inert fluorophore	Silencing of GFP expression
		Immediate visualization by immunofluorescence	Dilution without fast fixation
			Cell fusion cannot be excluded
	Cell type-specific genetic label	Can prove tissue-specific differentiation	Cell fusion cannot be excluded
			Limited animal models available
	BrdU immunoreactivity	Easy to label cells derived from nontransgenic animals	Uptake by surrounding cells
	Membrane binding dyes (PKH26-Dil)	Easy to label cells derived from nontransgenic animals	Dilution with every cell division
			Uptake by surrounding cells

has occurred cells are GFP or β-Gal positive. GFP is a naturally existing, biologically inert fluorophore, derived from the jellyfish *Aequorea aequorea*, that requires no substrates or additional gene products for its fluorescence activity and thus, transplanted GFP+ cells can directly be analyzed by immunofluorescence. However, the images are sometimes difficult to interpret because of autofluorescence, especially after tissue injury. In order to distinguish GFP positivity from background autofluorescence, a dual band ratiometric analysis technique can be used. This technique is based on a comparison of two wavelength bands, one that includes autofluorescence and fluorophore signals and one that includes only autofluorescence (Brazelton and Blau, 2005). Alternatively, classic immunohistochemistry can be performed using a GFP-specific antibody. The *ROSA26* (*LacZ* transgenic) mouse shows ubiquitous but rather weak expression of *Escherichia coli* β-Gal. β-Gal expression is most often detected by the X-Gal histochemical reaction, resulting in a blue coloration or by antibodies specific for β-Gal (Brazelton and Blau, 2005; Tosh and Slack, 2002). The use of ROSA mice as BM donors also requires caution as the ubiquitously but weakly expressed bacterial β-Gal signal can be enhanced by pH changes, which can also upregulate endogenous mammalian β-Gal activity (Blau *et al.*, 2001). Some argue that cells transduced with GFP or β-Gal may lead to rejection. For both GFP and β-Gal, rapid and thorough fixation of tissue is required to avoid diffusion over the entire tissue (Brazelton and Blau, 2005). Finally, silencing of the transgene may preclude identification of the grafted cells *in vivo*. Theise has, for instance, argued that it is uncertain whether all BM cells, derived from a transgenic animal, keep expressing the transgene at the same level, once the cell starts to differentiate into a nonhematopoietic cell type (Theise *et al.*, 2003). In fact, following transplantation of male ROSA26 BM cells into lethally irradiated female mice, >90% engraftment in the spleen was detected by Y-chromosome analysis, whereas <50% of the splenic cells were β-Gal positive. Thus transgene expression, even if driven by a presumed ubiquitously active promoter, may be a nonoptimal means for identifying donor-derived cells.

An alternative approach not susceptible to problems of transgene expression or rejection is to perform *sex-mismatched transplantation.* BM cells derived from a male donor are transplanted into a female recipient, using the Y-chromosome as the identifying marker. The Y-chromosome is typically detected by *in situ* hybridization with fluorescent probes with high sensitivity and specificity. A disadvantage of using the Y-chromosome as tracking method is the fact that the Y-chromosome cannot be detected unless the plane of tissue section being analyzed passes through the correct portion of the cell nucleus (Kotton *et al.*, 2004). For example, in the male liver tissue, only some of the hepatocyte (19%) and cholangiocyte nuclei (29%) stain clearly positive for the Y-chromosome due to this partial sampling of nuclei in the 3-μm thin tissue sections (Theise *et al.*, 2000a,b). This can

underestimate the engraftment frequency but could also conceal cell fusion (Alison *et al*., 2004). Furthermore, the protocol that is required to identify the Y-chromosome, FISH, renders interpretation of the results sometimes difficult. Protease digestion used as part of the FISH, causes loss of typical cell morpohology, an important parameter used to determine transdifferentiation events (Gao *et al*., 2001). Some investigators have multiplied the measured frequency of the Y-chromosome with the FISH efficiency for the Y-chromosome in a given organ (Hocht-Zeisberg *et al*., 2004; Quaini *et al*., 2002; Theise *et al*., 2000b). Some, but not all, studies have suggested that Y-chromosome positive cells can be identified in liver tissues by PCR in about 70% of women who have been pregnant with male children (Alison *et al*., 2000; Tanaka *et al*., 1999) and might suggest donor cell engraftment where this is not the case. It is also commonly known that the Y-chromosome can be lost from male cells without affecting cell function. Hence, lack of Y-chromosome may not necessarily mean lack of engraftment.

Yet another method to identify donor cells is to rely on *cell-type specific genetic* differences between donor and host cells. Several such examples exist, for instance, in the liver literature. The FAH enzyme is exclusively expressed in hepatocytes. Thus, any FAH+ cell in an FAH-deficient mouse is a hepatocyte-like cell and must be donor derived (Lagasse *et al*., 2000; Wang *et al*., 2002b). Other examples of animal models in which cell-type specific gene differences are employed are DPPIV deficient mice/rats (Sigal *et al*., 1995) and the Gunn rats, which lack an enzyme (UGT1A1) necessary for the conjugation of insoluble bilirubin to its soluble form (Guha *et al*., 2002).

BrdUrd immunoreactivity has also been used to follow the fate of transplanted cells. BrdU is a thymidine analog that is incorporated within the DNA of replicating cells. Following pulse labeling, roughly 80% of fresh BM cells are labeled with BrdU. However, with sequential cell divisions, a progressive loss of the intensity of the BrdU occurs. Chopp's group compared the engraftment frequency of BrdU-labeled MSCs and cell tracking based on the Y-chromosome. Using the same animal model of traumatic brain injury, they found much more Y-chromosome–positive cells than BrdU-positive donor. This might have been because the Y-chromosome is a more accurate identification method of the donor cells or BrdU could have a negative effect on cell migration and engraftment (Mahmood *et al*., 2003). There is evidence that BrdU may be reutilized following cell death, and could be incorporated in dividing endogenous cells, or even cells undergoing DNA repair. This could thus yield false positive evidence for cell engraftment.

Transplanted cells can also be labeled with either DiI or PKH26 *membrane-binding dyes*. As for BrdU, the label will progressively be diluted upon cell division. In addition, the label can become incorporated in neighboring

cells following cell death and yield false positive results. Labeling with CFSE that binds irreversibly to intracellular proteins is less prone to false positive results as CFSE will not be transferred to neighboring cells upon cell death because of irreversible modifications following the initial protein-binding event.

In conclusion, every method has its inherent detection limitations. Thus, the presence of BM-derived cells should be confirmed by combining different, independent techniques. Furthermore, appropriate positive and negative controls have to be added to every experiment.

2. Difficulties with Demonstrating Tissue-Specific Differentiation

To prove conversion of BM cells to a nonhematopoietic fate, presence of one or ideally more tissue-specific proteins colocalized with the tracking marker is required but not sufficient. Proof that a cell has truly assumed a different fate also requires that, aside from changes in phenotype, the cell has assumed functional characteristics of the novel tissue (Brazelton *et al.*, 2000).

Immunostaining and fluorescence microscopy are subjective techniques prone to artefacts as antibodies might bind in a nonspecific manner. Incorrect interpretation of the immune-staining techniques may underlie many of the contradictory results reported by different groups (Kotton *et al.*, 2005; No author, Nature, 2004). Correct interpretation requires that the tracking marker and the tissue-specific markers are expressed in the same cell. Superimposition of images may lead to confusion, as host tissue-specific cells can be in close proximity of donor-derived cells but not the same cell (Fausto, 2004). To clearly document that tissue-specific proteins and tracking markers are expressed in the same cell in tissue sections, adequate preparation of tissues and the use of laser scanning confocal or deconvolution microscopic methods, which allow analyses of optical sections less than 1 μm is needed (Brazelton and Blau, 2005). The group of Hasenfuss demonstrated that thorough identification of stem cell-derived tissue-specific cells by excluding the presence of nonparenchymateus markers (i.e., CD45, CD68) and the improvement in imaging technique to minimize artefacts due to overlapping cells, results in a stepwise reduction of cells that could be identified as transdifferentiated cells. Using 3D confocal microscopy, it was shown that some of the presumed donor-derived Y-chromosomes were actually localized outside the presumed transdifferentiated cell (Hocht-Zeisberg *et al.*, 2004). Some organs, such as heart, liver, and lung, have a high intrinsic autofluorescence, which makes immunofluorescent labeling difficult to interpret. This approach requires the establishment of signal thresholds, above which cells are designated as positive for a given marker (Murry *et al.*, 2004). Finally, damaged tissue bind antibodies and probes with much higher background than intact tissue (Wang *et al.*, 2003b).

As briefly discussed earlier, a more objective method to test stem cell plasticity is the use of lineage-specific reporter methodology: cells derived from animals that express transgene reporters under lineage-specific control or by lenti- or retrovirally transducing cells with a marker-gene expressed from a tissue-specific promoter (Kotton *et al.*, 2005; Murry *et al.*, 2004). This method has low background and hence is intrinsically less subjective than immunostaining. A drawback is the possibility of active gene silencing, which may underestimate the plasticity of stem cells.

Transdifferentiation should also be associated with loss of all characteristics of the tissue or cell of origin. Hence, following transplantation of presumed HSCs, proof of transdifferentiation requires acquisition of a phenotype of the destination tissue and loss of CD45 expression. Even then, caution is required as tissue macrophages downregulate CD45 and hence may escape detection as hematopoietic cells. The group of Weissman transplanted GFP-labeled whole BM cells and purified HSCs into irradiated mice and investigated the fate of these cells in noninjured and injured/activated brain (Massengale *et al.*, 2005). In general, the frequency of donor-marker expressing cells coexpressing either CD45 and/or the microglial markers, Iba-1 or Mac-1 ranged from 96 to 100% for BM transplants and from 98.5 to 100% for recipients of a single HSC. These data are consistent with the hypothesis that HSCs give rise to microglia, which are hematopoietically derived, but not neural cells, even though the 1% CD45− cells could represent transdifferentiated cells. After brain injury, using intraperitoneal injections of kainic acid to induce hippocampal injury, findings were similar. The only difference with the noninjured model was a greater number of microglial nodules at the site of CNS lesions in KA-injured recipients. Moore *et al.* (2005) investigated the plasticity of Lin^- BM cells, infused within the intrinsically plastic mammalian olfactory bulb, with or without brain irradiation. Ninety percent of the engrafted cells maintained expression of CD45. They suggested that the CD45− cells could be BM-derived MSCs or the result of incomplete staining with the anti-CD45 antibody. Sawchenko repeated the experiments published by Brazelton by following the fate of BM cells expressing GFP transplanted IV in irradiated mice (Brazelton *et al.*, 2000; Vallières and Sawchenko, 2003). The only BM-derived cells that were discovered throughout the brain were CD45+ and consisted of perivascular microglial cells and leukocytes. None of the GFP+ cells expressed neuronal or astrocytic markers. Even a mechanical insult of the cerebral cortex 5 months after transplantation did not provoke transdifferentiation events nor did the BM cells participate in vascular remodeling after the injury. It is believed that differences in histochemical methods and the use of high-resolution confocal microscope might explain the differences between this study and the study by Brazelton.

GFP-labeled, Lin^- c-kit^+ cells and KTLS, injected in ischemic myocardium, differentiated exclusively into CD45 expressing haematopoietic cell fates but in no instance into cardiac myocytes, smooth muscle cells, or endothelial cells (Balsam *et al.*, 2004). Treatment with c-kit_{Enr} BM cells did provide some long-term benefit (at 6 weeks) in limiting ventricular dilatation and dysfunction after infarction, but it does not limit overall infarct size and nor does it improve overall survival. Again, it was suggested that differences between this study and the studies by Orlic *et al.* may be the result of technically less optimized techniques in the latter study.

B. Scientific Reasons for Discrepancies

1. Does Purity of the Stem Cell Graft Affect the *In Vivo* Behavior?

The purification level of transplanted cells varies greatly between different studies: from unfractionated whole BM to extensively purified HSCs (Table I). Unfractionated BM is a complex mix which contains both CD34+, CD34−, endothelial progenitors, and stromal cells. Negative results with unfractionated BM cells but positive results with stem cell-enriched populations may indicate that environmental cues presumably responsible for a switch in phenotype towards that of the host tissue do not reach the rare stem cells that are "diluted" in this heterogeneous population (Bel *et al.*, 2003). Positive results seen with whole BM studies, however, preclude identification of the cell responsible for the observations. This continues to be true for transplants done with several 100–1000 highly purified stem cell fractions where it remains possible that two rather than a single stem cell population is present. Claims for true transdifferentiation can only be made when reconstitution of a nonhematopoietic tissue is seen after transplantation of a single, clonally expanded cell (Wagers and Weissman, 2004). Obviously, studies that begin with a single stem cell will likely obtain differing results from studies starting off with a population of cells. This raises the question if population studies are more significant as they might represent the more natural and physiological interactions between different cell types (Moore *et al.*, 2003)?

2. Expansion of BM-Derived Cells may Affect Their Phenotype and Behavior

To have sufficient cells for transplantation, *ex vivo* expansion is commonly done. However, once a cell is removed from its original niche, and introduced into a different environment, genetic modification may occur leading to silencing of some and activation of other genes. Such genetic changes may

lead to alterations in cell surface antigen expression that are commonly used to identify/purify a subpopulation of stem cells, may affect the level of transgene expression and therefore make the detection of engraftment more difficult (Theise *et al.*, 2003), and may alter homing determinants on stem cells (Rombouts and Ploemacher, 2003). Hence, minor variations in conditions used to expand and culture cells, such as ESCs, MSCs, or MAPCs, may affect their phenotype and ability to engraft and differentiate *in vivo*. Standardization of culture protocols will thus be needed not only to compare studies between labs but ultimately also to have a reliable clinical cell therapy product.

VIII. Possible Mechanisms Underlying Functional Improvements

Despite the numerous papers describing functional improvement after BM-derived stem cell therapy, the mechanism(s) that underlie the functional effects are unknown.

A. Functional Improvement Mediated by the Direct Contribution of BM-Derived Cells to Tissue Regeneration

One possibility is that transplanted BM cells integrate into the organ of interest, replacing damaged cells and restoring function themselves. As outlined in the earlier sections, only few studies have substantiated that BM stem cells indeed functionally integrate in large numbers in the damaged organ. Robust liver repopulation was seen by purified HSCs in the FAH-deficient mice with biochemical improvement of liver tests and enhanced animal survival (Lagasse *et al.*, 2000). Even though subsequent studies showed that the lineage switch occurred as result of fusion between HSC-derived monocytes and hepatocytes, the acquisition of a hepatocyte fate resulting from the fusigenic event allowed rescue from a fatal liver disease. A highly enriched population of HSCs, transplanted in mice with acute liver failure due to CCl_4 treatment, lead to restored liver function after 2–7 days without evidence of cell fusion (Jang *et al.*, 2004).

To demonstrate that stem cells functionally integrate in the heart and contribute themselves to cardiac function requires that, aside from demonstration that donor-derived cells express cardiac-specific proteins, they contract in synchrony with the host cardiomyocytes. Except for a paper by Anversa (Kajstura *et al.*, 2005), no studies describing the use of BM-derived stem cells in cellular cardiomyoplasty, showed robust evidence of differentiation of BM-derived cells into cells with a real cardiomyocytic phenotype and electromechanical incorporation into the host myocardium.

Similary, although many authors have concluded that transplanted BM cells transdifferentiate into cells with neural characteristics, the proportion of BM cells differentiated in astrocytes or neurons is small (0.02–8%) (Brazelton *et al.*, 2000; Li *et al.*, 2000; Mahmood *et al.*, 2002; Mezey *et al.*, 2000, 2003) and there is no evidence that BM-derived neurons are actively incorporated into the neuronal circuits (Hess *et al.*, 2004b).

Given the modest levels of engraftment of BM cells (<1–2%), whether through cell fusion or transdifferentiation as underlying mechanism, most investigators agree that it is not likely that the observed functional improvement is attributable to the integration of "new" cells (Zhao *et al.*, 2002). Moreover, a number of studies wherein no fate conversion was detected describe functional improvement. This resulted in studies aimed at evaluating other possible mechanisms underlying the effect of BMSCs on organ function.

B. Improvement by Organ Vascularization and Formation of Supporting Cells

Restoration of organ function after an injury not only depends upon the formation of new parenchymal cells but also on revascularization of the injured region and restoration of supporting cells. As suggested by Fausto (2004), candidate cells include endothelial progenitors and macrophages, which enhance blood supply and provide cytokines that may support endogenous stem and progenitor cells to proliferate and/or differentiate.

EPCs, coinjected with the transplanted cell fraction, may aid in revascularization and be responsible for the improved blood supply (Raffi and Lyden, 2003; Shintani *et al.*, 2001). Many studies have shown incorporation of BM cells as endothelial or smooth muscle cells in vascular structures and associated improved neovascularization (Davani *et al.*, 2003; Kathyjo *et al.*, 2001; Tomita *et al.*, 1999). The group of Yamaoka transplanted GFP-labeled BM cells IV in irradiated mice and performed 70% partial hepatectomy 4 weeks after the transplantation (Fujii *et al.*, 2002). As expected, the BM cells did not generate cells within the parenchymal compartment, as hepatocytes themselves can replicate in this model. FACS analysis demonstrated that 11.9% of nonparenchymal cells were GFP+ with 30% Kupffer cells and 70% sinus endothelial cells. The investigators speculated that improved liver regeneration was due to higher local levels of VEGF protein in the grafted animals. Although it is possible that BM-derived EPCs contribute to liver regeneration after partial hepatectomy, by providing nonparenchymal cell types, studies in which EPCs are prevented from migrating into the liver demonstrating delayed liver regeneration have not yet been done (Fausto, 2004). The same group also demonstrated the contribution of

BM cells to the hepatic stellate cell fraction of the liver, after total body irradiation in an animal model of chronic fibrosis, induced by subcutaneous administration of CCl_4 (Baba *et al.*, 2004).

Ogawa and collegues transplanted clonal HSCs into lethally irradiated recipient mice (Hess *et al.*, 2004a). After 3–4 months, some mice underwent middle cerebral artery occlusion (MCAO). The number of HSCs-derived microglial cells and pericytes increased with time after transplantation, and this was enhanced after cerebral ischemia. As pericytes have a phagocytic and antigen presenting function—help keeping the blood–brain barrier intact, regulate endothelial proliferation, are involved in vessel stabilization and initiate central acute phase responses by producing prostaglandins (Thomas, 1999)—higher number of HSC-derived pericytes after cerebral ischemia may suggest that they play a role in angiogenesis or vessel stabilization. Although they detected eGFP+/NeuN+ cells, these may represent microglial cells that phagocytosed damaged neurons.

Whole BM cells can also give rise to the myofibroblast population in multiple organs, especially in areas of damage. Myofibroblasts produce a multitude of chemokines, cytokines, and growth factors; promote the differentiation and proliferation of surrounding parenchymal epithelial cells; and have an important role in healing processes. BM transplantation might be helpful if there is an absence or hypofunction of myofibroblasts, for example, in the platelet-derived growth factor-α knockout mouse. However, overactivation of these cells can result in scaring and in extreme cases, fibrosis (Direkze *et al.*, 2003).

Only one report found substantial conversion of BM to pancreatic β-cells, without evidence of cell fusion, but no functional benefits were demonstrated (Ianus *et al.*, 2003). All other studies demonstrated that cells engrafted in the pancreas were not part of the β-cell compartment. Bathia's group first induced diabetes in immunodeficient mice by streptozotocin (STZ) treatment and subsequently injected whole BM or the c-kit$^+$ fraction from GFP+ mice systemically (Hess *et al.*, 2003). This resulted in substantially reduced hyperglycemia with an increase of insulin production and endogenous β-cell expansion in both groups. Although some Insulin+/GFP+ cells were detected, these cells were PDX1−, suggesting that GFP+ hematopoietic or endothelial cells endocytosed exogenous insulin, as has been described *in vitro* (Rajagopal *et al.*, 2003). Even though 9.2% of the GFP+ cells were PECAM+, no increase in vascular density was observed. Still, the authors proposed that BM progenitors differentiate into endothelial cells in the pancreas and that these indirectly contribute to endogenous β-cell expansion through trophic factors or other supportive roles. Whether the endothelial cells were derived from HSCs or EPCs present in the graft was not addressed. Others also demonstrated BM contribution to pancreatic endothelial cells following partial pancreatectomy but did not report β-cell

expansion or reduced hyperglycemia (Mathews *et al.*, 2004). In contrast to Hess *et al.*, Mathews *et al.* first induced normoglycemia in SZT-treated animals by implanting insulin pellets and subsequently infused BM cells. This might suggest that hyperglycemia is required for the recruitment of cells to the pancreas and the proliferation of the endogenous β-cell compartment. In the setting of exocrine pancreas, proteolytic enzymes can induce malfunction of the islets of Langerhans, leading to diabetes. DeGregori used an animal model ($E2F1^{-/-}E2F2^{-/-}$) with progressively exocrine pancreas insufficiency, reflecting the morphological changes observed during chronic pancreatitis (CP), and pan-hematopoietic defects (Li *et al.*, 2003). Male animals develop diabetes by 19 weeks of age. Transplantation of wild-type BM restored hematopoiesis and prevented or delayed diabetes and, in some cases, normoglycemia was obtained in already diabetic mice. The pancreas showed increased β-cell expansion and islet size, without restored exocrine mass, although there was no direct contribution of the donor cells to the β-cell population. The authors suggested that BM-derived cells and/or their secreted factors protect the islets from the exocrine cell-mediated destruction. In summary, all but one study assessing the ability of BM cells to cure diabetes studies observed the presence of endothelial and hematopoietic cells within the pancreas, which enhanced endogenous β-cell proliferation and survival, not direct β-cell differentiation from BM cells.

C. Paracrine Mechanisms

A number of studies demonstrated that improved end-organ function can be solely attributed to factors secreted by BM cells, which induce angiogenesis, endogenous stem/progenitor proliferation and differentiation, and subsequent functional repair after BM-derived cell therapy. The group of Dzau demonstrated the paracrine cytoprotective effects mediated by biologically active factors secreted by MSCs. In order to augment cell viability and enhance cardiac repair posttransplantation, they first modified rat MSCs (rMSCs) to overexpress *Akt1*, a powerful antiapoptotic prosurvival gene (Mangi *et al.*, 2003). They found that transplantation of 5×10^6 cells, overexpressing Akt, into the ischemic rat heart, inhibited the process of cardiac remodeling, by reducing intramyocardial inflammation, collagen deposition, and cardiomyocyte hypertrophy, regenerated 80–90% of lost myocardial volume, and nearly completely normalized diastolic and systolic cardiac function. Rat-derived MSCs, modified to overexpress the survival gene *Akt1*, were then grown in hypoxic conditions (Gnecchi *et al.*, 2005). After 12 hours, conditioned medium was collected. Concentrated "hypoxic-Akt1" medium was injected at the infarct border zone 30 min after left coronary occlusion with significantly decreased infarct size and apoptotic

index (the percentage of TUNEL positive nuclei) 72 hours later. It has been shown that hMSCs express a broad spectrum of proangiogenic/proarteriogenic genes and proteins (VEGF, bFGF, IL-6, placental growth factor, monocyte chemoattractant protein) *in vitro*, which are upregulated under hypoxic conditions and stimulate endothelial and smooth muscle cells to proliferate and migrate (Kinnaird *et al.*, 2004). When MSC-conditioned medium was injected into the adductor muscle in a mouse model of hindlimb ischemia, a significant improvement in blood flow as well as limb function in the treated mice compared to the mice that received control medium was seen. As the cytokines secreted by the MSCs have additive or synergistic effects on cell proliferation, injection of MSCs or conditioned medium alone may be preferred to the injection of single proteins to augment tissue perfusion. Finally, the studies by Losordo, in which Dil-labeled BMSCs were grafted in infarcted hearts demonstrated aside presumed direct transdifferentiation into cardiomyocytes and vascular cells, also increased endogenous neovascularization and cardiomyogenesis, presumably via paracrine effects exerted by the BMSCs (Yoon *et al.*, 2005). As indicated in the previous section of this chapter, whether the presumed direct transdifferentiation might be overinterpreted as Dil could have been recycled by endogenous vascular and cardiac muscle cells was not addressed. Yet another group reported that following syngeneic rMSC transplantation in an infarcted heart, bFGF, VEGF, and SDF-1α protein levels were significantly increased with a 60% reduction in the levels of the proapoptotic Bax protein. These findings could explain the increased capillary density in the MSC-treated group, with only a low fraction of MSCs integrated in the new-formed blood vessels, associated with a marked reduction in infarct size, and attenuation of ventricular remodeling was seen (Tang *et al.*, 2005a).

The group of Chopp has published a series of papers on the engraftment and the functional effects of hMSCs and rat MSCs in traumatic brain injury or MCAO (Chen *et al.*, 2001; Li *et al.*, 2000, 2001, 2002; Mahmood *et al.*, 2003). Independent of the site of cell injection, species, or tissue injury used, they found a dose-dependent functional benefit of cell treatment within weeks but no beneficial effect on the lesion volume. 4–21% of total injected cells engrafted in the brain parenchyma, but only 1–5% of donor cells expressed neuronal or astrocytic markers, thus suggesting that other mechanisms must be involved in the functional benefit. As hMSCs secrete brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), VEGF, and HGF, which is significantly enhanced when extracts of damaged rat brain were added to the culture medium, *in vivo* production of these growth factors might underly the functional improvements seen (Chen *et al.*, 2002). Following IV transplantation of hMSCs in rats with a MCAO, higher levels of NGF and BDNF were found in the ischemic hemisphere, which was associated with a reduction in cell apoptosis (Li *et al.*, 2002). Others have

suggested that increased levels of insulin-like growth factor in ischemic brains after treatment with hMSCs might contribute to the neurorestorative effects (Zhang *et al*., 2004). hMSCs or rMSCs infused systemically or in the cerebrum-stimulated proliferation and migration of endogenous NSCs that may participate in the brain repair (Chen *et al*., 2001; Li *et al*., 2002; Mahmood *et al*., 2004).Yang's group suggested that recovery after traumatic brain injury after MSC transplantation may be facilitated by attenuated cell death of cholinergic neurons (Chen *et al*., 2005), normally associated with spatial memory deficits seen with in traumatic brain injury and secretion of NGF by the MSCs (Dixon *et al*., 1997).

Treatment of rats, suffering from acute and chronic CCl_4-induced liver injury, with G-CSF mobilized BM, contributed to a faster recovery of the histological damage and offered a strong survival benefit, mainly by promoting increased proliferation of resident hepatocytes. Although the percentage of donor-origin hepatocytes was slightly higher in the CCl_4/G-CSF group compared to CCl_4 group (3.58 vs 1.18%, respectively), this minimal difference cannot explain the improved survival. However, it is possible that BM-derived cells improve the endogenous hepatic repair program via paracrine effects, providing trophic factors that support recipient-origin–mediated restoration of the liver damage (Yannaki *et al*., 2005).

All these findings support the hypothesis that the functional benefit of BM cells is likely in large part due to paracrine signalling by the transplanted cells and not trandifferentiation.

IX. BM-Derived Stem Cell-Based Therapies for Solid Organs: What Needs to be Done?

It should be clear from the previous sections that irrespective of the mechanism underlying the apparent lineage switch that occurs when adult stem cells are grafted in animals, the level of engraftment is low. Hence, it behoves investigators in the field to develop methods that will enhance homing, engraftment, survival, and ultimate differentiation of cells in tissues. This raises a number of questions, such as which route of administration should be used and what are mechanisms underlying homing of stem cells in different tissues, among others. These will be addressed in the following sections.

A. Route of Administration

A critical step for the clinical success of stem cell-based therapy is an efficient method for cell delivery, which supports the optimal survival and growth of the transplanted cells. Two distinct transplantation modalities involving

stem cells can be recognized: transplantation of exogenous cells versus mobilization of endogenous cells (Table I).

1. Stem Cell Mobilization

Initial indications that circulating stem cells might contribute to organ regeneration, came from the detection of Y-chromosome positive cells after transplantation of female solid organs into male recipients. Using archival autopsy and biopsy liver specimens obtained from four male recipients of livers from female donors, it was shown that 4–43% recipient-derived hepatocytes were present. The highest frequency was seen in a case of fibrosing cholestatic recurrent hepatitis C in one of the recipients (Theise *et al.*, 2000b). Using a similar gender mismatch liver transplant, Alison *et al.* (2000) found Y-positive hepatocytes in the female donor liver, with a frequency of 0.5–2%, often growing in clusters. Others demonstrated that levels of chimerism remained steady with time, were not enhanced when damage was present, and cells did not give rise to clusters of hepatocytes, a characteristic expected from stem cells (Wu *et al.*, 2003). The discrepancies between the results reported by Theise and others may be due to the fact that Theise calculated levels of engraftment by multiplying the observed events by 5, to correct for the presumed sampling error in the detection of the Y-chromosome (see above). It should also be noted that yet others such as Lo's lab found that although 0.62% of the total number of hepatocytes were donor-derived 7 months after transplantation, the majority of the host-derived cells were CD68+ macrophages/Kupffer cells (Ng *et al.*, 2003).

Similar variable results have been reported when examining female donor hearts in male recipients. Not only do different reports indicate that different host-derived cells can be identified in the donor heart, including endothelial cells, smooth muscle, cardiomyocytes, and perineural Schwann cells, but there is a great range in the extent to which circulating progenitors repopulate the cardiomyocyte compartment. Most investigators have found cardiomyocyte chimerism to be a very rare phenomenon (0.016–0.2%), with a greater contribution to endothelium and to a lesser extent to smooth muscle (Deb *et al.*, 2003; Laflamme and Murry, 2005; Laflamme *et al.*, 2002), whereas some did not see any evidence that chimerism is present in cardiomyocyte compartment (Glaser *et al.*, 2002). Anversa's group, however, reported that 18% of total cardiomyocytes, 20% of coronary arterioles, and 14% of capillaries were recipient derived (Quaini *et al.*, 2002). To obtain these percentages, the initial percentages were multiplied by 2 given the 50% efficiency of FISH for the Y-chromosome in the heart was taken into account. The highest level of chimerism was found between 4 and 28 days posttransplantation. Y-chromosome–positive inflammatory cells could have mistakenly been considered as cardiomyocytes as most of the patients in

Quaini's study had died of graft failure or other inflammatory causes. Hasenfuss *et al.* investigated autopsy samples from male patients who had received a female donor heart and who developed myocardial infarction after transplantation. Based on the presence of the Y-chromosome, the appropriate morphology on 3D confocal microscopy and the absence of CD45 and CD68, they estimated that 0.02% were male-derived cardiomyocytes, but did not describe a significant increase in the infarcted areas of the heart (Hocht-Zeisberg *et al.*, 2004).

Thus, although stem-cell mobilization to injured organs might be a naturally occurring phenomenon, it is inefficient and occurs most likely at a very low frequency such that the process cannot compensate for the massive tissue loss. However, if correct, it could be further exploited by employing strategies to augment the mobilization of endogenous stem cells, that is, by the administration of cytokines such as G-CSF and SCF (Kronenwett *et al.*, 2000). G-CSF and SCF cause an increase in the release of mainly HSCs from the BM into the circulation (Petit *et al.*, 2002). Such a pharmacology-based stem cell therapy is attractive as it is less invasive, bypasses immunological issues and the risk of transmission of infections. Anversa reported that administration of G-CSF and SCF starting 5 days prior to the induction of MI until 3 days thereafter, resulted in cytokine-mediated translocation of BMC with a significant degree of tissue regeneration 27 days later. Mortality was decreased by 68%, infarct size by 40%, and the ejection fraction progressively increased. They calculated that about 15×10^6 new myocytes were formed (Orlic *et al.*, 2001b). Other groups have reported improved post-infarction ventricular function after cytokine treatment in rats (Sugano *et al.*, 2005) and pigs (Iwanaga *et al.,* 2004). In contrast to these results, Herodin's group did not see decreased infarct size or improved contractile performance after cytokine (G-CSF and SCF) treatment either before or after myocardial infarcts in baboons (Norol *et al.*, 2003). However, treatment did enhance the perfusion of the infarcted area, probably related to the mobilization of endothelial precursors. These findings lead to the hypothesis that some species might exhibit a more limited ability of the cytokines to mobilize cells. In the MAGIC study, G-CSF (with or without intracoronary infusion of CD34+ mononuclear cells) was administered to patients with acute MI who underwent coronary stenting (Kang *et al.*, 2004). Although no serious side effects were reported during the periprocedural period, the trial was stopped prematurely because of an unexpectedly high rate of in-stent restenosis. At 6 months follow-up, however, improved functional capacity and left ventricular ejection fraction, as well as signs of angiogenesis were reported in the group receiving mononuclear cells and G-CSF but not in the G-SCF only arm. They suggested that use of drug-eluting stents to block restenosis could be tested with G-CSF–based therapy. Consistent with the in-stent restenosis seen by Kang *et al.*, another study found that

administration of G-CSF resulted in acute myocardial infarction in 2 out of 12 treated patients with intractable angina (Hill *et al*., 2003). Even if G-CSF and/or SCF have a beneficial effect on cardiac function, the mechanism underlying such an effect may not be transdifferentiation of mobilized cells. G-CSF mobilizes committed granulocytes and monocytes that are major effectors of infarct repair. A marked increase in their availability could modulate infarct repair, independent of regeneration (Laflamme and Murry, 2005). Minatoguchi *et al.* (2004) suggested that G-CSF may act by accelerating absorption of necrotic myocardial tissue due to increased circulating macrophages and by reducing excessive collagen deposition and scar formation via increased expression of MMPs. Harada *et al.* (2005) demonstrated that cardiomyocytes and cardiac fibroblasts express the G-CSF receptor and that G-CSF might have a direct protective effect on cardiomyocytes through the G-CSFR and the Jak-Stat pathway. Upregulation of this pathway may prevent cardiomyocyte and endothelial apoptosis and cardiac dysfunction (Harada *et al*., 2005).

As has been reported for cardiac repair, Shyu *et al.* (2004) described marked recovery in neurological behavior, reduced infarct volume, and improved neural plasticity and vascularization, when G-CSF was administered 1 day after the induction of transient cerebral ischemia. More BrdU positive cells were detected in the brain after G-CSF treatment, compared to PBS-treated animals; however, this does not prove that the proliferating cells were BM derived, and the beneficial effect may be trophic as a result of either HSCs or more mature blood cells that release trophic factors. Yet another hypothesis is that CXCR4-positive tissue-committed stem cells, hypothetically residing in the BM, are released into the PB after G-CSF induced mobilization and attracted to damaged tissue, which express high levels of SDF-1α (Ratajczak *et al*., 2004). However, there exists to date no proof for this hypothesis.

We can conclude that in order to efficiently use mobilization of endogenous stem cells, questions such as which effector cell type is mobilized, which agents in addition to G-CSF/SCF can be used to mobilize the effector cell, and which underlying mechanism (direct differentatiation, angiogenesis, paracrine) is responsible for the beneficial effects, will need to be answered.

2. Exogenous Administration

Stem cells can be delivered systemically, in the local circulation of an organ (i.e., portal vein, coronaries), or directly injected into the organ of interest.

a. Systemic Administration. The systemic delivery of stem cells, as performed during BM transplantation for hematological diseases, is an attractive approach for regenerative medicine for both practical and economical

reasons, as it is the least invasive method and it allows repeated administration of large numbers of cells (Heng *et al.*, 2005c). The downside of this approach, however, is the unwarranted distribution of the transfused cells into multiple organs, other than the ones of interest, and the lower likelihood that the cells will arrive in the damaged tissue as they may be retained in large capillary beds such as lung. Hence, methods to increase migration solely or at least preferentially to the organ of interest should be developed. As will be discussed in more detail later, intravenously infused progenitor cells tend to colonize injured tissue more intensely than normal organs. Barbash *et al.* (2003) compared the distribution and engraftment of IV versus intraventricular injected ^{99m}Tc-labeled MSCs 2 or 14 days after MI in rats. Intravenous infusion resulted in significant retention of the donor cells in the lung with lower retention in liver, kidneys, and bladder. In contrast following intra-LV-cavity infusion significantly more cells could be detected in the infarcted heart. As MSCs are relatively large and express a host of adhesion molecules, it is believed that they are trapped in the lung and the spleen vascular bed, which is bypassed when cells are delivered immediately in the arterial circulation.

b. Administration in the Local Circulation. In contrast to systemic injection, fewer cells might be required when the cells are injected in or near the targeted organ with a greater chance for participation in the ongoing reparative process.

Many studies assessing the effects of BM stem cells in liver regeneration use intrasplenic or intraportal injection. This approach can be associated with (transient) portal hypertension, pulmonary embolism, and portal vein thrombosis. In animal models, intrasplenic injection is technically easier, with lower risk on lethal bleeding complications, compared to intraportal injection. The optimal number of hepatocytes for intrasplenic transplantation has been determined (Guha *et al.*, 2002). Rats transplanted with up to 5 million hepatocytes survived without obvious complications, but transplantation of 10 million hepatocytes resulted in death in most of the animals within 1 week of transplantation. However, the size of transplanted BM-derived cells is smaller than hepatocytes (around 20–25 μm) and may pose lesser problems. One report showed that intracoronary injection of BM-derived MSCs (0.5 million cells per kilogram bodyweight) into a noninjured dog heart caused acute myocardial infarction and subacute myocardial microinfarcts by occluding the coronary circulation. They also found that freshly prepared canine nucleated BM cells are smaller (mean diameter 10–12 μm) than low passage MSCs, which increases the risks of occlusions (Vulliet *et al.*, 2004). Thus, differences in cell size will affect the ability of infusing large doses of different BM cell types in the local circulation.

c. Intraorganic. Perhaps the best mode of delivery would be the direct intratissue transplantation of stem cells. For instance, a series of studies by the Dick group has shown that significantly fewer highly purified human HSCs need to be transplanted directly in the femur compared with infusion in the systemic circulation of NOD-SCID mice to detect human hematopoiesis (Wang *et al.*, 2003a). However, this procedure is more invasive, especially when considering intracerebral grafts, which requires craniotomy and can cause bleeding. Chopp's group compared intracarotid and intracerebral administration of MSCs in a rat model of MCAO and concluded that intracarotid delivery is superior to intracerebral transplantation; safer, a larger cell dose can be administered and better cell survival was seen (21 vs 8.6%, respectively) (Li *et al.*, 2001). Intracerebral injected cells tend to localize in the injected region with a limited penetration distance of 2–3 mm. This limited dispersion of the graft may be disadvantageous in the setting of widespread damage or disease. MSCs injected into the carotid artery appeared to distribute over a wider area of the ischemic core and penumbra (Li *et al.*, 2000, 2001). Comparison of intravenous and intracerebral MSC injection in a rat model of traumatic brain injury demonstrated that both approaches enhanced progenitor cell proliferation and imparted functional improvement (Mahmood *et al.*, 2004). Administration of cells into the brain ventricles limits the number of cells that can be injected but avoids systemic dissemination and ensures adequate numbers of MSCs at the place of injury. However, the cells distribute less well throughout the lesion compared with systemic administration. An intraventricular injection route is clinically relevant because ventriculostomies are often carried out to monitor intracranial pressure in patients with severe traumatic brain injury (Chen *et al.*, 2005). Intrahepatic injection of HSCs has not been shown to enhance engraftment without hepatic preconditioning (Wang *et al.*, 2002b).

B. Enhancing Homing

Upon systemic transplantation, or even within the local circulation of an organ, BM cells must be capable of migrating into the damaged tissues. There is mounting evidence that this homing process is enhanced when tissue damage is present. Transplanted cells preferentially migrate towards injured organs, and the altered microenvironment might subsequently provide the clues necessary for further tissue-specific differentiation and proliferation (Bittira *et al.*, 2003; Martin *et al.*, 2002; Saito *et al.*, 2002). The need for tissue injury in most cases reflects the importance of signals, which are released by the damaged tissue that preferentially attract stem cells to the damaged organ (Blau *et al.*, 2001). Identification of the molecular signals

governing stem cell migration *in vivo* will therefore be of major importance for improving stem cell-based regenerative therapy.

Many cytokines and cell adhesion molecules (i.e., ICAM-1) are upregulated upon tissue injury and might favor migration and homing of transplanted BM cells. MCP-1 and MIP are chemoattractant cytokines, whose expression is increased following brain injury, that are known to induce recruitment of mononuclear cells into injured areas (Kim, 1996; Lu *et al*., 2004). In interface cultures, recombinant MCP-1, MIP-1, and IL8, as well as ischemic brain tissue induced increased migration of hMSCs in a chemotaxic assay, suggesting a facilitating role of these cytokines for the migration of BM-derived cells, together with inflammatory cells, to a damaged site (Wang *et al.*, 2002a). The most intensively investigated regulator of cell trafficking is the chemoattractant cytokine, stromal-derived factor-1 (SDF-1), also named CXCL-12, which binds exclusively to the CXCR4 receptor, a G-protein–coupled seven-span transmembrane receptors. Ratajczak considers CXCR4 as a universal marker of stem cells as it is found on ESCs and most adult stem cells (Kucia *et al*., 2005c).

The best-studied system is the hematopoietic system. During embryogenesis, SDF-1α is produced by osteoblasts, marrow fibroblasts, and endothelial cells. In response to SDF-1α, HSCs that express the CXCR4 receptor leave the fetal liver and home to the BM, where they establish adult hematopoiesis (Kucia *et al*., 2005b). The SDF-1α–CXCR4 axis is also crucial in directing homing/engrafment of HSCs into BM in the transplant setting. *In vitro*, SDF-1α causes migration of CD34+ cells in a transwell migration assay, and on a per cell basis, cells capable of migrating towards SDF-1α engrafted significantly better in NOD-SCID mice. The level of engraftment of human CD34+ cells into NOD-SCID mice was reduced after treatment of the cells with an anti-CXCR4 antibody or after coinjection with antibodies to SDF-1α (Peled *et al*., 1999).

SDF-1α also expressed by epithelial cells in many adult organs, and CXCR4 is expressed by many organ-specific stem cells, murine ESCs, and presumed tissue-committed stem cells present in BM (Kucia *et al*., 2005a,b; Peled *et al*., 1999). Expression of SDF-1α and CXCR4 are positively regulated by transcription factors related to stress/hypoxia and tissue damage, such as hypoxia-inducible factor (HIF)-1 and NF-kB (Kucia *et al*., 2005c), and secretion of SDF-1α increases during different types of tissue damage (Bel *et al*., 2003; Kollet *et al*., 2003; Lin *et al*., 2003). Peterson demonstrated that upregulation of SDF-1α in hepatocytes depends on the severity of the liver injury (Hatch *et al*., 2002). Moderate liver injury is repaired by proliferation of resident hepatocytes, and not by "stem cells," and under these conditions, SDF-1α expression is not upregulated and oval cells are not activated in the liver. By contrast, massive liver injury depends on oval cells for repair, and this injury is associated with upregulation of SDF-1α.

Lapidot studied possible mechanisms that mediate the migration into and retention of CD34+ cells in the liver (Kollet *et al.*, 2003). SDF-1α expression was significantly increased following total body irradiation, especially in bile duct epithelial cells. Injection of human SDF-1α into the hepatic parenchyma of nonirradiated NOD/SCID mice lead to increased homing of enriched human CD34+ cells. Administration of neutralizing CXCR4 antibody almost completely abrogated this homing, indicating the importance of the SDF-1/CXCR4 interaction for stem cell homing. Together with SDF-1α, proteolytic enzymes, such as MMP-2 and MMP-9, and cytokines produced by the stellate cells in the injured liver, such as HGF and SCF, navigate circulating stem cells to the damaged liver. HGF and MMPs do so by inducing CXCR4 upregulation on CD34+ cells. Giordano confirmed the crucial role of SDF-1/CXCR4 interactions in stem cell homing to the heart but found that it acts only in the presence of tissue injury, indicating that the concomitant expression of additional factors, such as VCAM, ICAM, and MMPs, is necessary (Abbott *et al.*, 2004).

Other molecules that have been implicated in adult stem cell homing are nonproteinaceous molecules, such as heparan sulfate (Netelenbos *et al.*, 2002) and hyaluronan (Avigdor *et al.*, 2004), which are carbohydrates of the glycosaminoglycan (GAG) family. GAG oligosaccharides are breakdown products of the extracellular matrix after tissue damage. Avigdor *et al.* (2004) found that upon homing of hematopoietic progenitor cells (HPCs) towards the BM, CD44, a cell adhesion molecule present on the surface of HPCs, interacts with HA expressed on BM sinusoids as one of the crucial events in extravasation of human HPCs to the BM. In addition, they suggest that SDF-1α facilitates this migration by modulation of cell adhesion via increasing the avidity of membranal CD44 to HA in the BM sinusoidal endothelium (Avigdor *et al.*, 2004).

C. Following Homing *In Vivo*

The further development of stem cell-based therapy of solid organs will require a real time assessment of stem cell distribution and eventual engraftment *in vivo* using noninvasive imaging techniques. However, imaging small number of cells in a living subject, over a protracted time period is a difficult task. Frangioni and Hajjar (2004) reviewed the advantages and disadvantages of various imaging modalities. In animal models, noninvasive imaging can be done by transducing the graft with luciferase expressing vectors, which allows whole body imaging (WBI) using the Xenogen luciferase-mediated bioluminescent imaging (BLI). Due to the fact that the cells need to be genetically modified, this approach is not clinically relevant. Alternative approaches employ magnetic resonance imaging (MRI), which

is clinically relevant. For example, rMSCs were magenetically labeled with superparamagnetic iron oxide (SPIO) in combination with a dendrimer transfection agent to enhance its uptake by stem cells (Bos *et al.*, 2004). The magnetic probe concentrates into endosomes and following biodegradation and metabolization incorporates into the normal iron pool. Cells could be visualized up to 7 days after injection in healthy rat kidneys and up to 12 days after injection in a rat liver with a centrolobular necrosis model. *In vitro*, SPIO labeled MSCs and HSCs retained their differentiation potential and phenotypical characteristics (Arbab *et al.*, 2005). The problem with supramagnetic particles include dilution of the agent with every cell division, the potential of transfer of contrast to nonstem-cells, such as macrophages, after stem cell death and difficulties in quantification (Frangioni and Hajjar, 2004).

D. Therapeutic Window for Administration of BM-Derived Stem Cells

Because injury and the concomitant release of chemoattractant cytokines are key components for successful cellular therapy, the temporal course of the expression of these cytokines was investigated to find the optimal timing of cell transplantation. Injection of BM cells should possibly be performed at the early stage of injury, that is, at a time where there is still living tissue that may harbor the appropriate signals and appropriate nourishing vasculature for inducing tissue-specific differentiation of the grafted cells. Sun's group evaluated the spatial and temporal expression of ICAM-1, MCP-1, the activity of MMP-1 and the deposition of collagen in the infarcted area for 21 days after permanent LAD ligation in the rat (Lu *et al.*, 2004). These "homing" factors reached a peak expression at 7 days after MI. With time after MI, collagen deposition increased and by 3 weeks after MI, the infarcted myocardium was replaced by scar tissue leaving no room for new cell growth. They concluded that the optimal time to rebuild myocardium resides within 2 weeks post-MI. Ma *et al.* (2005) investigated the correlation between the levels of SDF-1α and engraftment of MSCs. Levels of SDF-1α were highest 1 day after myocardial infarction, and MSCs were recruited most to the injured heart on day 1 after the infarct. On the other hand, grafted MSCs themselves seem to be the source of SDF-1α when injected into ischemic myocardium (Tang *et al.*, 2005a). In a study by Barbash, however, no differences were seen in the number of LacZ or Brdu labeled rMSCs in the border of infarcted heart, after either early (2 days) or delayed (10–14 days) post-MI infusion (Barbash *et al.*, 2003). In agreement with previous studies, no donor cells were seen in the remote intact myocardium. As reported by Ma *et al.* for cardiamyoplasty, Iihoshi *et al.* (2004) found that the earlier intravenous

administration of autologous BM-derived mononuclear cells was performed after transient MCAO, the greater the beneficial outcome, both on the degree of residual infarct size and functionality. In contrast, Olson's group reported a greater cell survival and improved functionality when MSCs were transplantated 1 week after spinal cord injury compared to immediate delivery. They argue that MSCs infused immediately after injury encounter a hostile environment because of ischemia, necrosis, and toxic compounds (Hofstetter *et al.*, 2002).

Manipulation of these homing factors might aid in recruitment of effector cells to sites of injury and may extend the therapeutic time window. Askari *et al.* (2003) investigated the role of local SDF-1α expression at a time remote of the acute tissue injury. They used a combined strategy of SDF-1α gene transfer and stem cell mobilization to enhance ventricular function 8 weeks after LAD ligation. Stem cell mobilization alone did not lead to significant engraftment of circulating cells in the ischemic cardiomyopathy 8 weeks following the MI. G-CSF administration with transplantation of cardiac fibroblasts stably transfected to express SDF-1α into the peri-infarct zone, induced homing of CD117+ and CD34+ cells and endothelial cells, resulting in greater vascular density and improved ventricular function compared to animals transplanted with control fibroblasts. Tang *et al.* (2005b) performed a similar experiment but overexpressed SDF-1α levels in the ischemic heart by administration of a plasmid containing SDF-1α and found that the transplanted Lin^- c-kit^+ cells preferentially migrated to the sites, expressing SDFα. In addition, increased endogenous stem cell mobilization was seen in the treated group. Likewise, prestimulation of effector cells to more highly express surface homing receptors could be advantageous. Peled *et al.* (1999) demonstrated that prolonged pretreatment with SCF and IL-6 upregulates CXCR-4 expression on mobilized PB CD34+ cells, which in turn potentiated SDF-1α–induced migration *in vitro* and enhanced engraftment following transplantation. This strategy of directed stem cell engraftment and controlled release of chemoattractants could be applicable to other organ systems (Peled *et al.*, 1999). Apart from the transplantation of engineered cells, controlled release of cytokines or chemokines could also be achieved by implantation of scaffolds at the site of tissue damage. The gradient of the released molecules within the PB circulation could then direct the chemotactic migration and homing of not only endogenous adult stem cells from ectopic sites but also stem/progenitor cells that are transfused. Such scaffolds could also contain specific cytokines, growth factors, and extracellular matrix components that favor lineage-specific differentiation of the effector cells (Heng *et al.*, 2005b).

X. Conclusions

There is no question that stem cell plasticity is a real phenomenon. However, most probably it is a rare and slow event with an uncertain physiological role and clinical applicability. The efficiency of stem cell therapy will mainly depend on the isolation of the most potent stem cells, strategies to enhance targeted homing, and the identification of the specific microenvironment, which favors stem cell conversion. The ideal route and time of administration and the optimal degree/nature of tissue injury will vary among different organs. Long-term follow-up will have to determine whether cell fusion could, after all, be exploited therapeutically. Probably the most immediate goal in this field is that simple cell selection and/or culture protocols and animal models are developed, which can easily be reproduced by different laboratories, to further clarify the many confusing results we discussed.

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The Development and Evolution of Division of Labor and Foraging Specialization in a Social Insect (*Apis mellifera* L.)

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How does complex social behavior evolve? What are the developmental building blocks of division of labor and specialization, the hallmarks of insect societies? Studies have revealed the developmental origins in the evolution of division of labor and specialization in foraging worker honeybees, the hallmarks of complex insect societies. Selective breeding for a single social trait, the amount of surplus pollen stored in the nest (pollen hoarding) revealed a phenotypic architecture of correlated traits at multiple levels of biological organization in facultatively sterile female worker honeybees. Verification of this phenotypic architecture in ''wild-type'' bees provided strong support for a ''pollen foraging syndrome'' that involves increased sensomotor responses, motor activity, associative learning, reproductive status, and rates of behavioral development, as well as foraging behavior. This set of traits guided further research into reproductive regulatory systems that were co-opted by natural selection during the evolution of social behavior. Division

Current Topics in Developmental Biology, Vol. 74

0070-2153/06 $35.00
DOI: 10.1016/S0070-2153(06)74008-X

of labor, characterized by changes in the tasks performed by bees, as they age, is controlled by hormones linked to ovary development. Foraging specialization on nectar and pollen results also from different reproductive states of bees where nectar foragers engage in prereproductive behavior, foraging for nectar for self-maintenance, while pollen foragers perform foraging tasks associated with reproduction and maternal care, collecting protein.

I. Introduction

Advanced societies of insects display marked patterns of behavior where reproduction is restricted to elite individuals (queens) who are often anatomically differentiated from nonreproductive individuals (the workers) (Wheeler, 1928). Workers are often further differentiated into anatomically and/or behaviorally differentiated individuals that specialize on the performance of specific behavioral tasks for at least some part of their adult lives. In the honeybee, this differentiation is behavioral without any obvious anatomical differences and expressed by changes in behavior associated with age and change of location in the nest, an age-related polyethism (Seeley, 1982). Typically, bees perform tasks in the center of the brood nest soon after emergence including cleaning brood cells and feeding larvae. After about 1 week they make a transition to performing tasks outside the brood nest area such as comb construction and food processing. When they are in about their third week of life, they make a final transition to foraging outside the nest after which they are seldom observed performing tasks within the nest other than those directly related to foraging, such as unloading pollen and nectar, and performing recruitment dances.

When a worker honeybee makes the transition to foraging, she usually collects pollen (a source of protein) and nectar (a carbohydrate source), though a minority of workers collect water and propolis, a resinous substance collected from plants and used in nest construction. Most food foragers collect both pollen and nectar on a single foraging trip, however, many collect only a single substance (Hunt *et al.*, 1995; Page *et al.*, 2000). The total load collected by a forager is constrained. A maximum nectar load is about 60 mg, while a maximum load of pollen is about 30 mg. So, each 1 mg of pollen "costs" about 2 mg of nectar. Nectar is carried inside the crop, the first chamber of the alimentary canal (Snodgrass, 1956), while pollen is carried on the hind legs and may impose aerodynamic drag, perhaps explaining the differences in maximum load sizes.

Returning nectar foragers pass their nectar loads to younger bees in the nest through trophallaxis. The younger bees then distribute the nectar to other bees, or deposit it in open cells in the comb where it is eventually

processed by other bees into honey. Returning pollen foragers deposit their loads directly into empty cells or cells containing pollen close to the area of the nest where young larvae are raised (Dreller and Tarpy, 2000). Stored pollen is consumed by young bees (Crailsheim *et al.*, 1992). The pollen proteins are converted into glandular secretions that are fed directly to larvae (Crailsheim, 1990). Stored pollen inhibits pollen foraging in colonies (Dreller *et al.*, 1999) while pheromones produced by larvae stimulate pollen foraging (Pankiw *et al.*, 1998). Colonies regulate the amount of stored pollen (Fewell and Winston, 1992), probably through a combination of the inhibiting effects of pollen and stimulating effects of brood. At ''equilibrium'' pollen intake into the colony should equal pollen consumption, and meet the protein demands of developing larvae.

The brood nest is organized spatially with the brood (eggs, larvae, and pupae) located centrally (Winston, 1987). Pollen is stored close to the brood, and honey is stored at the periphery of the nest (Fig. 1). The amount of pollen stored in the comb represents a complex colony-level trait that is a consequence of the interactions of thousands of individual colony members. Younger workers consume pollen and feed the protein to larvae, older workers respond to the foraging stimuli, forage, and recruit other foragers to their resources. The stored pollen phenotype can be selected by artificial selection and is assumed to be under natural selection (Page and Fondrk, 1995).

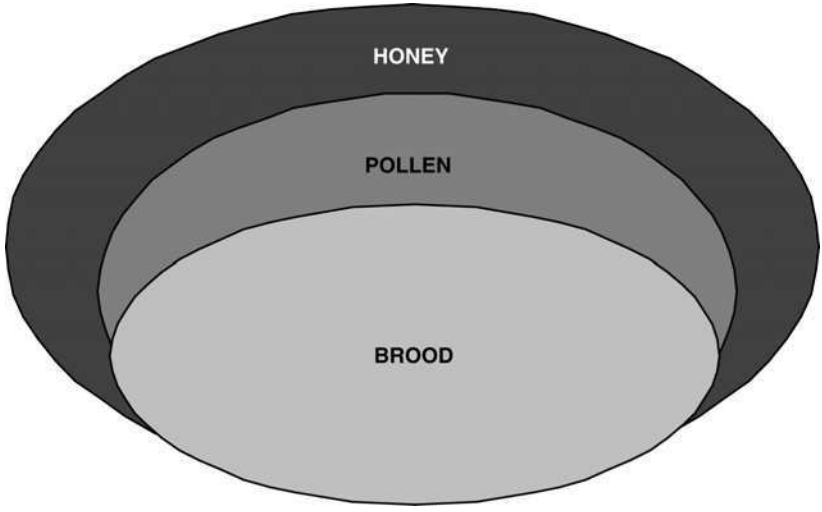

Figure 1 A diagram of a comb drawn from near the center of a honeybee nest showing the spatial orientation of honey, pollen, and brood.

II. Effects of Selection on Pollen Hoarding

A. Colony Level Selection

Hellmich *et al.* (1985) conducted two-way selection for the amount of pollen stored in the comb (pollen hoarding) and demonstrated a strong selective response. Subsequent studies showed that when fostered in the same colony, workers from the high-pollen hoarding strain were more likely to forage for pollen than were bees from the low strain (Calderone and Page, 1988, 1992). Bees from the high strain also foraged about 1 day earlier in life (Calderone and Page, 1988). Page and Fondrk (1995) repeated the selection from a different commercial population and also demonstrated a strong response to selection. After just three generations, colonies of the high strain contained about six times more pollen. Like Hellmich *et al.* (1985), they selected for a single trait, pollen hoarding, however, they also looked at other individual behavioral and physiological traits that might have changed as a consequence of selection on the colony-level phenotype. This enabled them to look for mechanisms at different levels of biological organization that causally underlie the differences in the colony-level phenotype (Page and Erber, 2002).

B. Foraging Behavior of High- and Low-Strain Bees

High-strain bees initiate foraging earlier in life than low-strain bees. Pankiw and Page (2001) demonstrated an average difference of about 10 days in a study of 12 host colonies. High-strain bees are more likely to specialize on pollen, while low-strain bees are more likely to specialize on nectar (Fewell and Page, 2000; Page and Fondrk, 1995; Pankiw and Page, 2001). High- and low-strain bees were raised together in "wild-type" colonies (commercial colonies not derived from the pollen hoarding strains). Workers of each strain were marked with paint on the thorax to identify their strain origins and then were placed into the same wild type test colonies, a type of "common garden," experiment. Colony entrances were examined daily. Marked, returning foragers were captured, and their nectar and pollen loads analyzed. High-strain bees were more likely to collect pollen and collected larger pollen loads and smaller nectar loads than low-strain bees. High-strain bees were also more likely to collect water, and when they collected nectar, accepted nectar with lower sugar content than did bees of the low strain. Low-strain bees were much more likely to return empty from foraging trips (Page *et al.*, 1998).

Differences in pollen load sizes were expected and represented by differences between the strains in their responses to pollen foraging stimuli. Fewell and Winston (1992) showed that colonies respond to changes in quantities of stored pollen by changing the allocation of foraging effort between collecting nectar and pollen. When colonies were presented with additional stored pollen beyond what they had already stored, they responded by reducing the number of pollen foragers and the sizes of the pollen loads. The opposite effect on foraging behavior was observed when stored pollen was removed. Colonies regulate the amount of stored pollen around a homeorhetic set point. Studies by Dreller *et al.* (1999) and Dreller and Tarpy (2000) demonstrated that foragers directly assess the amount of pollen stored in the combs and adjust their foraging behavior accordingly. The mechanism appears to involve the assessment of empty cells near the areas of the nest where larvae and pupae are located. Therefore, the regulatory mechanism involves individual assessment of stored pollen and individual "decisions" with respect to what to collect on a foraging trip (Fewell and Page, 2000). High-strain colonies reach a regulated set point with much larger quantities of stored pollen than do low-strain colonies. Therefore, high-strain bees have a threshold for stored pollen (or empty cells near the brood) that is different from low-strain bees. When cofostered in a wild-type colony, where high- and low-strain bees are much fewer than the resident bees, high-strain bees perceive the amount of stored pollen below their optimal set point, while the low-strain bees perceive it above theirs. As a result, high-strain bees are much more likely to forage for pollen, and low-strain bees are much more likely to forage for nectar.

Young larvae and hexane rinses of young larvae stimulate pollen-specific foraging behavior (Pankiw *et al.*, 1998). Increasing the numbers of larvae in a nest, or augmenting the larvae with larval rinses, results in the recruitment of new pollen foragers and larger pollen loads but does not affect nectar foraging (Dreller *et al.*, 1999; Pankiw *et al.*, 1998). When foragers are not allowed direct contact with larvae, they do not change their foraging behavior with changes in larval quantities (Dreller *et al.*, 1999). Selection for high- and low-pollen hoarding could have resulted in differences in quantities of brood, differences in brood pheromone levels in colonies, or differences in the perception/response systems coupled to pollen foraging stimuli. High- and low-strain bees do not differ in quantities of brood except under space-limited conditions where brood areas are reduced by excess pollen hoarding (Page and Fondrk, 1995).

High- and low-strain bees respond differently to changes in the pollen and brood stimuli in colonies. Pankiw and Page (2001) cofostered high- and low-strain bees in colonies with high- and low-pollen hoarding stimuli. High-stimulus colonies were experimentally manipulated to contain more

larvae and less stored pollen than the low-stimulus colonies. Foragers in the high-stimulus colonies were more likely to collect pollen, collected larger loads of pollen, and smaller loads of nectar. High-strain bees demonstrated a larger difference in foraging behavior between treatments, demonstrating a genotype x–environment interaction where high-strain bees are more sensitive to the foraging stimulus environment. In summary, selection for the colony-level trait—the amount of pollen stored in the comb—resulted in changes in behavior at the individual level. Workers from colonies selected for storing more pollen initiated foraging earlier in life, foraged more successfully, were more likely to collect pollen, collected larger pollen loads and smaller nectar loads, and were more likely to collect water and nectar with lower concentrations of sugar. High- and low-strain bees respond to changes in foraging stimuli. Based on what we know about the regulation of stored pollen, a pollen foraging inhibiting stimulus, and the effects of brood on the release of pollen foraging behavior, it seems likely that high- and low-strain bees differ in their responsiveness to these important stimuli.

C. Sensory Responses

Changes in foraging behavior related to collecting pollen were expected of selection for pollen hoarding. However, high-strain bees are also more likely to forage for water than low-strain bees (Page *et al*., 1998). When high-strain bees forage for nectar, they accept nectar with lower concentrations of sugar than low-strain bees. There was no obvious physiological or behavioral mechanism to explain these relationships until Page *et al*. (1998) looked at the responses of pollen and nectar foragers to sucrose solutions under controlled laboratory conditions. Bees can respond to antennal stimulation with sucrose by extending the proboscis (Kunze, 1933; Marshall, 1935). Page *et al*. (1998) used an increasing concentration series of sucrose solutions to determine the sucrose responsiveness of wild type pollen and nectar foragers. Bees were placed into small tubes to restrict their movement. Then they were sequentially tested at each antenna with a droplet of sucrose solution (Fig. 2A). Sucrose concentrations increased with a logarithmic sequence of 0.1%, 0.3%, 1%, 3%, 10%, and 30%. Their response was recorded as ''yes'' (proboscis extension response, PER) or ''no'' (no PER) for each of the trials, which provided a measure of responsiveness to sucrose. The average proboscis responses of several bees to different sucrose concentrations are represented by the concentration–response curve (Fig. 2B). This curve can be used to estimate bees' sucrose response threshold or their sensitivity for sucrose (Page *et al*., 1998). Bees that are more responsive have lower thresholds and are more sensitive. The results were surprising: pollen foragers were more likely than nectar foragers to respond to water and lower

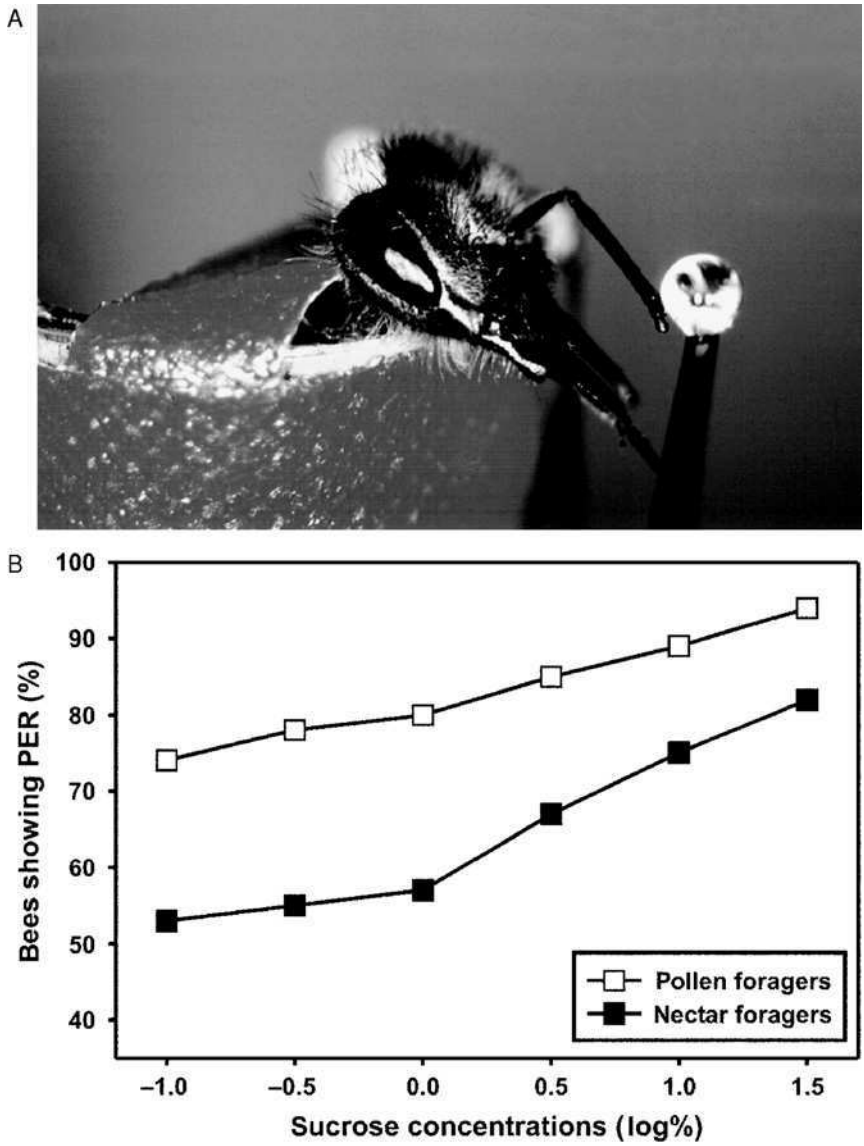

Figure 2 Measuring of sucrose responsiveness in honeybee foragers. (A) Fixed honeybee showing the proboscis extension response (PER). When the antenna of a bee is touched with a droplet of sucrose solution of sufficient concentration, the bee extends her proboscis in expectation of food. This response can be used to measure responsiveness to different sucrose concentrations. (B) Sucrose-concentration response curve of pollen and nectar foragers. The *x*-axis presents the log(%) of the sucrose concentrations tested. The *y*-axis displays the percentage of bees showing the PER. Pollen foragers are more responsive to all sucrose concentrations tested than nectar foragers (Scheiner *et al.*, 2003a).

concentrations of sucrose (Fig. 2B). Apparently, pollen foragers have lower thresholds for water and sucrose and are, therefore, more sensitive for these stimuli.

Responsiveness to sucrose depends on a number of external and internal parameters. Feeding bees, under laboratory conditions with sucrose, generally reduces responsiveness, but the differences between pollen and nectar foragers remain (Page *et al.*, 1998). In free flying bees, responsiveness to sucrose is modulated by feeding and foraging experience (Pankiw *et al.*, 2001). Even the sucrose responsiveness of hive bees changes with changing concentrations of nectar brought back by returning foragers (Pankiw *et al.*, 2004). Sucrose responsiveness varies during the foraging season in pollen and nectar foragers. Nevertheless, pollen foragers consistently show higher sensitivity than nectar foragers (Scheiner *et al.*, 2003a). The effects of genotype on sucrose responsiveness were shown by testing young bees of the high- and low-strain before they initiated foraging (Pankiw and Page, 1999; Pankiw *et al.*, 2002; Scheiner *et al.*, 2001a). In all age groups high-strain bees were more responsive to sucrose solutions and water than low-strains bees. This finding suggests that selection for pollen hoarding behavior had resulted in selection for the gustatory response system, which correlates with foraging behavior. These experiments demonstrate that gustatory sensitivity and foraging behavior are closely related.

If water and sucrose responses are related to nectar and pollen foraging, we should be able to test wild type bees before they start to forage and predict their foraging behavior 2–3 weeks later. Pankiw and Page (2000) tested wild type bees for their responses to water and sucrose within their first week of adult life, before they initiated foraging. Bees were marked for individual identification and placed back into their colony. Colony entrances were observed, returning foragers were collected, and their foraging loads were analyzed. Bees displaying the highest responsiveness to water and sucrose solutions when they were up to 7 days old were most likely to collect water on a foraging trip. The next most responsive group was very likely to collect pollen. Bees with lower responsiveness would later collect nectar or both nectar and pollen. The group with the lowest responsiveness would later in life return empty to the hive (Fig. 3).

The function of gustatory responsiveness for the division of foraging labor is not clear a priori. Why should a pollen forager be very sensitive to sucrose when she is mainly collecting pollen? Why is a water collector simply sensitive to water and insensitive to sucrose stimuli? A number of studies have clarified these questions. Bees who are sensitive to sucrose are also sensitive to stimuli of other modalities, and they show higher stimulus-related motor activity.

Bees that are highly responsive to sucrose are also highly responsive to pollen stimuli (Scheiner *et al.*, 2004a). In these experiments, the gustatory

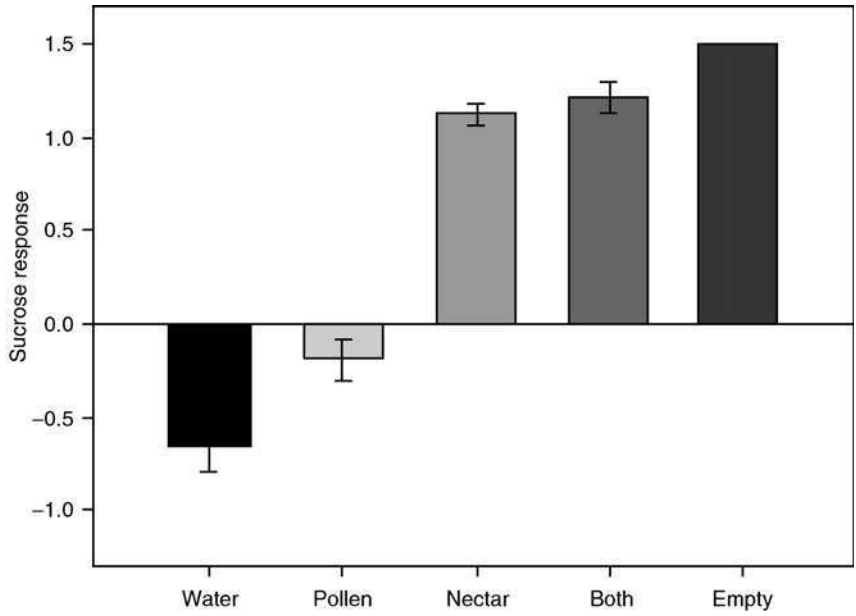

Figure 3 Sucrose responses of 1-week-old bees predict their foraging behavior later in life. The *x*-axis shows the foraging material of the bees tested for their sucrose responses at the age of 1 week, brought back by them when they reached foraging age. The *y*-axis shows the lowest sucrose concentrations ($\log_{10}$) at which 1-week-old bees responded with proboscis extension. Bees with the highest sucrose responsiveness (i.e., the lowest threshold) at young age will later forage for water or pollen. Individuals with low-sucrose responsiveness (i.e., a high threshold) perhaps collect nectar, nectar and pollen, or return empty. From Fig. 1 Pankiw and Page (2000) with the kind permission of Springer Science and Business Media.

responsiveness of bees was measured first. Then the same bees were stimulated with different pollen concentrations, which were produced by mixing pollen with cellulose grains of the same size. Bees that were highly responsive to sucrose, also responded with proboscis extension to the pollen stimulus, provided the concentration of pollen was higher than 6.3%, while bees with low responsiveness to sucrose did not respond to the same pollen concentration. Over 40% of the sensitive animals showed the proboscis response when stimulated with pure pollen, while less than 10% of the sucrose-insensitive bees responded to pure pollen. In another experiment, bees were tested in an olfactometer after measuring their sucrose responsiveness (Scheiner *et al.*, 2004a). Again, bees that were sensitive to sucrose were also more sensitive to olfactory stimuli than were animals that were relatively insensitive to sucrose. Phototactic behavior of bees was tested in a round arena that allowed stimulation of a single bee with small monochromatic light sources (520 nm) of relative intensities between 3% and 100%. Stimulated bees

walked toward the light. Walking behavior was recorded by an infrared camera that was mounted above the arena. Bees with high responsiveness to sucrose were also more sensitive to light stimuli in the arena. All these experiments demonstrate that sucrose responsiveness correlates with sensitivities for other stimulus modalities. Pollen foraging bees are not only sensitive to sucrose but also to pollen, odors, and light stimuli.

The behavioral responses to stimuli that can be measured in honeybees are the result of complex neuronal processes that integrate sensory information and produce motor output. Motor patterns of the proboscis, the antennae, or the legs are controlled by specific motor systems consisting of different types of neurons and often different types of muscles. Therefore, it is important to ask whether the motor system is tuned differently in bees that differ in their sensory responses. Several experiments have shown that sensory input can influence motor output in honeybees. Bees whose eyes are covered by paint scan an object within the range of the antennae with rapid antennal movements. The mechanical stimuli produced during antennal contact with an object initiate motor activity that even shows motor learning (Erber *et al.*, 1997). Antennal scanning activity is significantly higher in bees that are responsive to sucrose compared to animals that are not responsive (Scheiner *et al.*, 2005). This experiment demonstrates that there is a correlation between gustatory responsiveness and stimulus-evoked motor activity.

Responsiveness to sucrose correlates with locomotor activity under ambient light conditions when bees first emerge as adults. Humphries *et al.* (2005) tested locomotion in newly emerged wild type bees by measuring their walking activity in an enclosed arena under ambient light. They then determined their response to sucrose using the proboscis extension response protocols. Bees that were more responsive to sucrose were also more active in the light. A number of independent experiments with wild type nectar foragers have shown that the velocity of walking in the dark is not correlated with gustatory responsiveness but with foraging role (independent unpublished experiments by Hoormann, Erber, and Franz). Pollen foragers walk faster than nectar foragers. High-strain workers were more active than low-strain workers, consistent with the results from wild-type bees (Humphries *et al.*, 2005). Rueppell *et al.*, 2005 tested high- and low-strain males (drones) for locomotor activity under light and dark conditions. High-strain drones were more active under both conditions, which is consistent with the results from workers. Thus, these experiments suggest that wild type pollen and nectar foragers differ in locomotor activity and that the same relations are found in high- and low-strain bees.

In summary, the gustatory responses of bees to sucrose solutions are related to foraging behavior and to sensory responses to odor, pollen, and light. Pollen forages are more sensitive to sensory stimuli than nectar foragers. As a consequence of sensory sensitivities, stimulus evoked motor

patterns are different in sensitive and insensitive animals. Locomotor activity differs between pollen and nectar foragers and also between high- and low-strain bees. Sucrose responsiveness can be used as a robust indicator for general differences of processing sensory information in the central nervous system.

D. Learning and Memory in Wild-Type Bees and Selected Strains

Division of foraging labor correlates with associative learning performance. In different laboratory learning paradigms, pollen foragers were shown to perform better than nectar foragers (Scheiner *et al*., 1999, 2001b, 2003a). In the tactile learning assay which was employed by Scheiner *et al*. (1999) to compare the learning performance of pollen and nectar foragers, bees were trained to associate the characteristics of a small metal plate with a sucrose reward. Returning pollen and nectar foragers were constrained in small tubes and their eyes were occluded with black paint to block visual inputs. The tactile object was brought into the scanning range of a bee. After the bees began scanning the target plate (the conditioned stimulus, CS) a droplet of sucrose solution was touched to one antenna (the unconditioned stimulus, US), eliciting the proboscis extension response (PER). A droplet of sucrose solution was then presented briefly to the tip of the proboscis as a reward (Erber *et al*., 1998). After few trials, most bees learned to respond to the plate without the US (Fig. 4). Pollen foragers learned faster than nectar foragers and reached a higher plateau in their acquisition function. These findings were later also demonstrated for olfactory learning, in which the bees have to associate an odor with a sucrose reward (Scheiner *et al*., 2003a).

Learning differences were not only described for pollen and nectar foragers. High-strain bees perform better in tactile and olfactory learning tests than low-strain bees (Scheiner *et al*., 2001a,b). Because this is true for bees that have not yet initiated foraging, it demonstrates that it is not a function of foraging experience but has genetic determinants.

In general, bees that are more responsive to sucrose learn faster and reach a higher asymptote of learning than bees that are less responsive (Scheiner *et al*., 1999, 2001a,b,c, 2003a, 2004a, 2005). The probability of showing the conditioned response in retrieval tests 24 hours after conditioning is higher in bees that are more responsive to sucrose (Scheiner *et al*., 2004a, 2005).

If bees with a similar responsiveness to sucrose are tested for tactile or olfactory learning, they do not differ in their learning performance, regardless of their genotype or foraging role (Scheiner *et al*., 1999, 2001a,b, 2003a). These findings led to the hypothesis that learning performance is directly related to the evaluation of the sucrose stimulus used during conditioning. If this hypothesis is correct, it should be possible to induce an equal learning

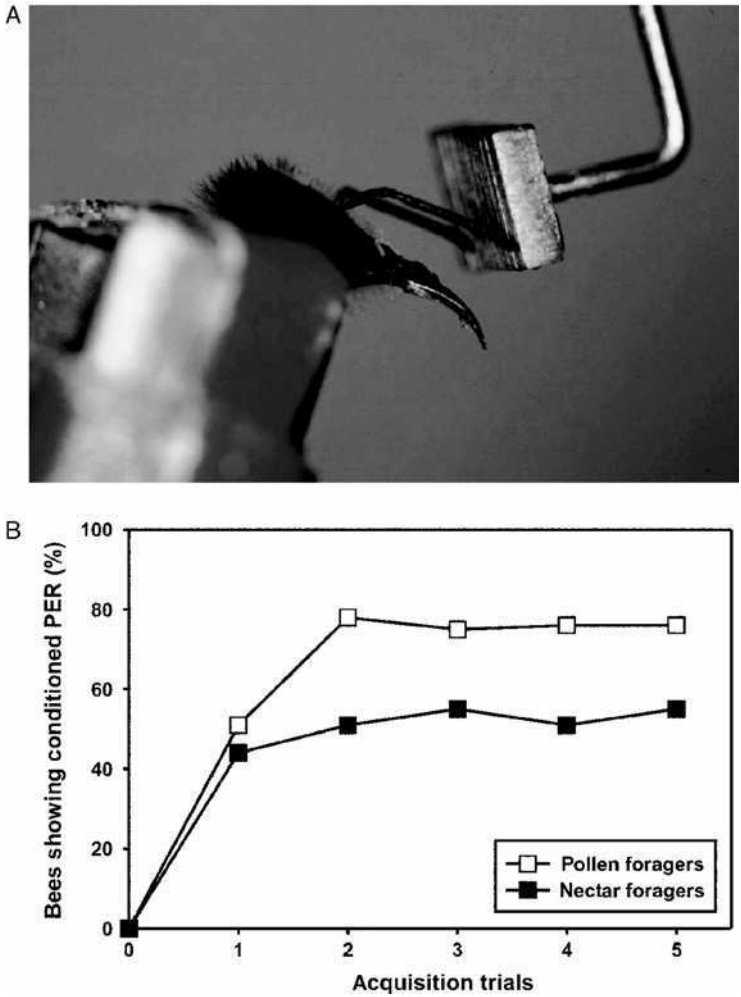

Figure 4 Tactile learning. **(A)** Honeybee showing conditioned proboscis extension response during tactile antennal conditioning. In this learning paradigm, the bee is rewarded for scanning a tactile object. The sucrose reward is briefly presented to the antenna. When the bee extends her proboscis after antennal stimulation with sucrose, she can imbibe a droplet of sucrose solution. After few conditioning trials, the bee shows conditioned proboscis extension while scanning the tactile target plate. **(B)** Acquisition curves of pollen and nectar foragers in tactile antennal learning. The *x*-axis shows the acquisition trials. The *y*-axis shows the percentage of bees displaying the conditioned proboscis extension response (PER). Both groups have reached the plateau of their acquisition function after three acquisition trials. However, the level of acquisition is higher in pollen foragers than in nectar foragers.

performance in bees with very different responsiveness to sucrose by giving them equal *subjective* rewards, based on their individual sucrose responsiveness. For individuals with high-sucrose responsiveness, a low-sucrose concentration should have the same *subjective* reward value as a high-sucrose concentration would have for a bee with low-responsiveness. This hypothesis was tested by Scheiner *et al.* (2005). A mathematical model for the individual reward value of sucrose was developed for bees that differ in gustatory sensitivity. Individuals were placed in classes according to their sucrose responsiveness. Based on their sucrose responsiveness, equal *subjective* reward concentrations were estimated. The performance and memory of all bees during conditioning and in the retrieval tests was very similar.

The correlation between learning performance and individual evaluation of the reward explains why pollen foragers learn better than nectar foragers and why high-strain bees perform better than low-strain bees. Pollen foragers are more responsiveness to sucrose than are nectar foragers; and high-strains bees are more responsive than low-strain bees. Bees with higher responsiveness place a higher reward value on sucrose and, therefore, reach a higher performance level (Page *et al.*, 1998; Pankiw and Page, 2000; Scheiner *et al.*, 1999, 2001b, 2003a, 2005). A similar relationship has been shown for nonassociative learning. Individuals with high-sucrose responsiveness need more trials for habituation of the proboscis extension response and display stronger sensitization by a sucrose stimulus than bees with low-sucrose responsiveness. Because individual sucrose responsiveness increases with age, older bees need more trials for habituation than younger bees (Scheiner, 2004).

E. Transmitter Systems and Neurochemical Signaling Cascades

1. Nervous System Signaling and Sensory Sensitivity

The set of variable correlated traits observed between pollen and nectar foragers, and between high- and low-strain bees, are centered on differences in sensory and motor response (see in an earlier section). As a consequence, differences in signaling cascades affecting sensory and motor response systems are prime candidates for understanding the neurobiochemical and genetic origin of variation in foraging behavior. Central components of nervous system signaling include biogenic amines, protein kinases, and second messengers that interact to affect sensory input, signal processing, and motor response.

a. Biogenic Amines. Biogenic amines modulate sensory and motor responses, traits that vary between pollen and nectar foragers, and bees from the high- and low-pollen hoarding strains. In honeybees, the four biogenic

amines—dopamine, serotonin, octopamine, and tyramine have important functions in nervous system signaling (Blenau and Baumann, 2001, 2003). Their capability to modulate sensory sensitivity makes them candidates for the regulation of foraging behavior. Octopamine, which has been studied most extensively, generally increases sensitivity and related behavioral responses. Responsiveness to gustatory stimuli that are applied to the antenna, for example, is strongly increased after octopamine application (Braun and Bicker, 1992; Menzel *et al.*, 1988, 1990; Scheiner *et al.*, 2002). This amine also increases olfactory sensitivity (Menzel *et al.*, 1991, 1994). Octopamine can also act on the visual system. It enhances, for example, the direction-specific visual antennal reflex (Erber and Kloppenburg, 1995; Erber *et al.*, 1993a). Tyramine has a similar effect as octopamine on gustatory sensitivity (Scheiner *et al.*, 2002). Otherwise, the behavioral role of this amine is less clear because until recently it was mainly considered as the biochemical precursor of octopamine rather than being a neurotransmitter itself. But interest in this amine has been growing since the first tyramine receptor of the bee was cloned (Blenau *et al.*, 2000). Serotonin and dopamine often act antagonistically to octopamine in sensory systems. Dopamine, for example, reduces gustatory responsiveness (Scheiner *et al.*, 2002). Serotonin, which has no effect on gustatory responses, decreases the direction-specific visual antennal reflex (Erber and Kloppenburg, 1995; Erber *et al.*, 1993a,b).

Because sensory sensitivity correlates with different aspects of foraging behavior and because amines can modulate sensory sensitivity, we assume that biogenic amines are involved in division of foraging labor by modulating response-thresholds to foraging-related stimuli (see in a later section).

b. Protein Kinases and Second Messengers. Sensory responses involve complex signaling cascades of which the biogenic amines are only one part. Other important signaling molecules are second messengers, such as cAMP or cGMP, and protein kinases, which activate target proteins by phosphorylation of their threonine or serine residues. Stimulation of the antenna with sucrose, for example, increases the activity of cAMP-dependent protein kinase (PKA) (Hildebrandt and Müller, 1995). Octopamine injections can mimic antennal sucrose stimulation and lead to an increase in PKA activity. This suggests a close interaction of octopamine and PKA during sensing of gustatory stimuli presented to the antenna.

Responsiveness to sucrose correlates with activity of PKA in the antennal lobes (Scheiner *et al.*, 2003b). Bees with high responsiveness to sucrose stimuli applied to the antenna have a higher baseline PKA activity than bees with low-sucrose responsiveness. Activation of PKA by application of 8-Br-cAMP increases responsiveness to sucrose (Scheiner *et al.*, 2003b). High- and low-strain bees differ in their sucrose responsiveness and differ in brain titers for PKA, making cAMP activation of PKA a likely cause of

this difference. Humphries *et al.* (2003) showed that bees selected for high-pollen hoarding have significantly higher titers of PKA than low-pollen hoarding bees at the time they emerge as adults and at 5 days of age. Whether high- and low-strain bees also differ in their PKA activity in the antennal lobes remains to be tested. Together, these findings imply a strong role of PKA in sensory responsiveness to gustatory stimuli applied to the antenna.

cGMP-dependent protein kinase (PKG) also appears to be involved in the perception of sucrose stimuli in insects. The two *Drosophila* variants—sitters and rovers, which differ in their PKG activity (Osborne *et al.*, 1997) also differ in their responsiveness to sucrose (Scheiner *et al.*, 2004b); and we have first indications that feeding of the PKG activator 8-Br-cGMP increases responsiveness to sucrose in honeybees (R. Scheiner and J. Erber, unpublished). Rueppell *et al.* (2004a,b), mapped a quantitative trait locus that affects responsiveness to sucrose close to *Amfor*, the honeybee gene for PKG, suggesting that variation in PKG between the high- and low-pollen hoarding strains may be affecting observed differences in sucrose responses. In addition, Ben-Shahar *et al.* (2003) showed that cGMP increases responsiveness to light. J. Tsuruda and R. E. Page (unpublished data) demonstrated that high-strain bees and wild-type bees with higher sucrose responsiveness are more responsive to light stimuli than low-strain bees and wild-type bees that are less responsive to sucrose. Differences in cGMP signaling provide a plausible explanation for these correlations. It is likely that significant cross talk occurs between the cAMP and cGMP pathways. PKA is activated by cGMP as well as by cAMP (Jiang *et al.*, 2002), and some proteins can act as substrates for both PKA and PKG (Wang and Robinson, 1997).

These examples show the important role of biogenic amines, protein kinases, and second messengers in the modulation of sensory response thresholds. Individual differences in behavioral response thresholds are assumed to be at the basis of division of labor in insect colonies (Beshers and Fewell, 2001; Beshers *et al.*, 1999; Page and Erber, 2002; Robinson, 1992; Theraulaz *et al.*, 1998). Therefore, we can hypothesize that changes in the division of labor profile of a colony are, to some extent, induced by a complex interaction of biogenic amines, second messengers, and protein kinases. A number of studies show how these neuromodulators change division of labor, although the exact mechanisms behind these changes are still poorly understood.

2. Nervous System Signaling and Learning

Pollen and nectar foragers, and bees of the high- and low-pollen hoarding strains, differ in associative learning performance (see in an earlier section).

Sensory system inputs are linked to motor-response systems through learning processes that alter behavior. Associative learning is an important part of honeybee behavior. Foragers, for example, have to remember the location of their hive and different food sources. Once they arrive at a flower, they have to remember how to find and handle the nectar and pollen that is presented. All bees of a colony must remember the odors of their hive and of their nest-mates. These are just a few examples. There are many more situations when bees perform associative learning tasks. Honeybees learn conditioned stimuli of different modalities very fast and establish long-lasting memories under free-flying conditions and in the laboratory (Bitterman *et al.*, 1983; Giurfa, 2003; Menzel and Müller, 1996).

Biogenic amines, especially octopamine (OA) are important modulators of associative learning. Application of this amine improves olfactory acquisition, memory formation, and retrieval traits that distinguish pollen and nectar foragers; and bees from the high- and low-pollen hoarding strains. Octopamine injections into the calyx or the alpha-lobe of the mushroom bodies, which are assumed to be the centers of olfactory learning, enhance memory formation (Menzel *et al.*, 1990), whereas injections of the octopamine-receptor antagonist mianserine into the antennal lobes or downregulation of the expression of the octopamine receptor AmOA1 strongly decrease acquisition and retrieval (Farooqui *et al.*, 2003). Octopamine has also an important function at the cellular level of associative learning. It was shown in associative learning under laboratory conditions that the ventral unpaired median neuron 1 of the maxillary neuromere (VUM_{mx1} neuron; Hammer, 1993) depolarizes in response to the presentation of sucrose rewards to antennae and proboscis. Current injection into the VUM_{mx1} neuron can substitute for the sucrose reward during olfactory conditioning (Hammer, 1993; Hammer and Menzel, 1998). VUM_{mx1} belongs to a group of octopamine-immunoreactive neurons (Kreissl *et al.*, 1994), and it is assumed that VUM neurons release octopamine, which could mediate the reward in some forms of associative conditioning (Hammer, 1997; Hammer and Menzel, 1998).

In contrast to octopamine, dopamine inhibits retrieval of information without affecting acquisition (Bicker and Menzel, 1989; Macmillan and Mercer, 1987; Menzel *et al.*, 1988, 1990, 1994, 1999; Mercer and Menzel, 1982; Michelsen, 1988). Serotonin can reduce both acquisition and retrieval when injected prior to conditioning (Bicker and Menzel, 1989; Mercer and Menzel, 1982; Menzel *et al.*, 1990, 1994). The effect of tyramine on associative learning has not been studied.

These examples imply that biogenic amines are involved in different pathways of associative learning in honeybees. Among the different signaling cascades, the PKA and the PKC signaling pathways are involved in memory formation (Grünbaum and Müller, 1998; Müller, 2000). High- and low-strain bees differ both in their brain content of these protein kinases

(Humphries *et al*., 2003) and in their associative learning performance (Scheiner *et al*., 2001a,b), suggesting that these pathways are involved in foraging division of labor.

It can be assumed that biogenic amines affect associative learning performance by changing the sensory sensitivity for the unconditioned and conditioned stimuli because sensory sensitivity correlates with learning performance (see earlier). However, direct experimental proof is still needed for this hypothesis.

3. Nervous System Signaling and Division of Labor

The titers of dopamine, octopamine, and 5-HT (serotonin) increase as bees' age, with the highest titers being found in foragers (Harris and Woodring, 1992; Schulz and Robinson, 1999; Schulz *et al*., 2004; Taylor *et al*., 1992; Wagener-Hulme *et al*., 1999). Because bees of different ages normally perform different tasks, it is conceivable that biogenic amine titers are part of the regulatory network for age-dependent division of labor. Whether the differences in biogenic-amine titers between bees of different ages are related to age differences or whether they are related to the different tasks the bees perform is often difficult to test. Single-cohort colonies can be very helpful for distinguishing between these alternatives. Thus Schulz and Robinson (1999) showed that differences in the titers of dopamine, octopamine, and 5-HT in mushroom bodies of foragers and nurse bees were related to age, whereas in the antennal lobes the differences were related to different tasks. Another way of studying the role of biogenic amines in division of labor is to manipulate amine titers and to determine the behavioral effects. Thus it was shown that octopamine induced bees to forage precociously, whereas tyramine had the opposite effect (Schulz and Robinson, 2001).

There are also some examples of how biogenic amines affect division of labor among same-aged bees. Božic and Woodring (1998) showed that bees who perform waggle dances after they returned from a foraging bout have higher titers of dopamine, octopamine, and 5-HT throughout the season than bees who followed the dancers. Another example comes from Taylor *et al*. (1992). They showed that pollen foragers had higher titers of dopamine in the optic lobes than in nectar foragers. Whereas pollen foragers also had 5-HT in the optic lobes, no serotonin was found in the optic lobes of nectar foragers.

OA has also been shown to affect response thresholds to brood pheromone. When hive bees were fed with OA for several days, their responsiveness to brood pheromone increased, and the bees subsequently increased their foraging activity (Barron *et al*., 2002). OA treatment did not increase responsiveness to queen mandibular pheromone, which would have resulted in a higher attendance in the queen's retinue (Barron and Robinson, 2005).

This implies that OA can modulate specific olfactory thresholds in different individuals and could thus be a major modulator of division of labor (Schulz and Robinson, 2001; Schulz *et al.*, 2002a).

Because the selected high- and low-pollen hoarding strains of Page and Fondrk (1995) differ systematically in their responsiveness to sucrose and sensitivities for other stimulus modalities, it can be assumed that these strains differ in the titer of biogenic amines. However, Schulz *et al.* (2004) demonstrated that these strains do not differ in their brain titers of octopamine, dopamine, or 5-HT. Apparently, selection for increased pollen hoarding, which led to a suite of traits modulated by these amines, did not result in detectable differences in titers of amines. It is conceivable that different degrees of receptor activation or differences in the signaling cascades downstream the biogenic amines might be responsible for the observed differences in sucrose responsiveness. As discussed in an earlier section, high-strain bees do have higher brain titers of PKA and PKC than that of low-strain bees of equivalent age.

Experimental evidence of the role of second messengers in division of labor in honeybees is rare. Ben-Shahar *et al.* (2002) showed that cGMP-dependent protein kinase (PKG) is involved in the initiation of honeybee foraging behavior. This kinase is encoded by *Amfor*, the so-called "foraging gene". Expression of *Amfor* is higher in foragers than in bees that have not initiated foraging, and application of the PKG activator 8-Br-cGMP induced precocious foraging (Ben-Shahar *et al.*, 2002). Rueppell *et al.* (2004a) demonstrated that differences in *Amfor*, or a gene or genes nearby, explain differences in age of foraging onset between bees of the high- and low-pollen hoarding strains.

F. Hormonal Signaling Cascades

The systemic hormones—ecdysone and juvenile hormone (JH)—are key modulators of insect behavior (Cayre *et al.*, 2000; Hartfelder, 2000). Ecdysone is produced by the prothoracic gland during larval and pupal development, and by the ovary during the adult stage. Putative effects of ecdysone in adult honeybees are currently elusive (Hartfelder *et al.*, 2002; Robinson *et al.*, 1991). JH is a growth hormone produced by the *corpora allata* of insects (Hagenguth and Rembold, 1978). JH has been hypothesized to play an important role in honeybee division of labor by pacing age-related changes in behavior, especially the transition to foraging (Robinson, 1992; Robinson and Vargo, 1997). Many studies have demonstrated elevated blood titers of JH in foragers relative to bees that perform tasks in the nest (Fahrbach *et al.*, 2003; Huang and Robinson, 1992, 1995, 1996; Huang *et al.*, 1994; Jassim *et al.*, 2000; Robinson, 1987; Robinson *et al.*, 1991; Sullivan

et al., 2000, 2003; Withers *et al*., 1995). Treatment with the JH analog methoprene also results in bees initiating foraging behavior earlier in life (Bloch *et al*., 2002). Other evidence suggests that JH affects aspects of adult maturation. Young bees normally do not show associative learning the first 5–6 days after emergence (Ray and Ferneyhough, 1999). When treated topically with JH within 1hour after emergence, however, they show associative olfactory learning when they are 3 days old (Maleszka and Helliwell, 2001). Application of methoprene increases sucrose responsiveness in young bees (Pankiw and Page, 2003), and also elevates responses to alarm pheromones (Robinson, 1987). These roles of JH appear to be closely linked to OA. It is has been suggested that OA and JH regulate each other, and thus modulate the onset of foraging behavior and changes in responsiveness (Kaatz *et al*., 1994; Schulz *et al*., 2002a,b). Foragers have high titers of both JH and OA, particularly in the antennal lobes (Schulz and Robinson, 1999; Spivak *et al*., 2003). When 1-day-old bees are treated with methoprene, their levels of OA in the antennal lobes increase and they forage precociously (Schulz *et al*., 2002b). When the *corpora allata* complex is surgically removed, workers are unable to produce JH. Such bees have been observed to initiate foraging later in life than sham treated controls (Sullivan *et al*., 2000). When the allatectomized workers are treated with methoprene or OA, they forage at an earlier age. These experiments suggest that OA acts downstream of JH. However, OA has also been shown to increase JH release from the *corpora allata in vitro* in a dose-dependent manner (Kaatz *et al*., 1994), suggesting that OA is upstream of JH in the regulatory cascade. Interactions between JH and OA are, therefore, not well understood.

Overall, JH correlates with age-based changes in honeybee behavior and sensory sensitivity, but is it pacing behavioral development? As mentioned in an earlier section, Sullivan *et al*. (2000) removed the *corpora allata* from newly emerged bees. The allatectomized workers initiated foraging, though slightly delayed in time relative to sham treated control bees. This result, which was obtained from observations of workers that returned from presumably successfully foraging flights of more than 15-min duration, was later called into question by data that included information on activities at the entrance of the nest. In this case, the allatectomized bees were observed initiating flight at the same time as controls (Sullivan *et al*., 2003). Worker honeybees from the high- and low-pollen hoarding strains initiate foraging at different ages and also differ in JH titer at adult emergence, however, their JH titer is not different 12 days later (Schulz *et al*., 2004). Thus, it is clear that JH is not necessary for behavioral development, but that treatments with JH and JH analog nonetheless have behavioral effects.

Insights that resolve this paradox emerged with the finding that *vitellogenin* gene activity suppresses the JH titer of worker bees (Guidugli *et al*., 2005).

Vitellogenin is a major yolk precursor in many insects (Babin *et al.*, 1999; Mann *et al.*, 1999) and is also the most abundant hemolymph protein in worker bees that perform tasks in the nest prior to foraging (Engels and Fahrenhorst, 1974; Fluri *et al.*, 1981, 1982). Juvenile hormone is known to suppress the synthesis of honeybee vitellogenin at onset of foraging (Pinto *et al.*, 2000), but the effect of *vitellogenin* gene expression on JH further suggests that these two compounds are linked in a positive feedback loop via a mutual ability to suppress each other. This regulatory relationship is uncommon in insects and was hypothesized by Amdam and Omholt (2003). They argued that the evolution of an unconventional role of honeybee vitellogenin in brood-food synthesis (Amdam *et al.*, 2003) selected for a mechanism that retains bees in the brood nest with high-vitellogenin levels. Foraging behavior, consequently, is triggered when the vitellogenin titer drops below a certain level. The feedback action of JH on vitellogenin is a reinforcing mechanism that causes the workers to become behaviorally and physiologically locked into the forager stage. In accordance with this hypothesis, M. Nelson, K. Ihle, G. Amdam, and R. Page (unpublished data) showed that reduction of *vitellogenin* gene activity by RNA interference (RNAi) causes bees to forage earlier in life. Amdam *et al.* (2006) demonstrated that vitellogenin RNAi increases the sucrose responsiveness of worker bees, and suggested that honeybee vitellogenin is a modulator of behavior and sensory sensitivity that acts via a signaling pathway that includes JH as a downstream feedback element.

Honeybee vitellogenin is produced by the abdominal fat body, but available evidence demonstrates that this protein triggers responses in other cell types (Guidugli *et al.*, 2005), implying that vitellogenin itself can be classified as a hormone. The documented effects of JH and JH analog treatments can be understood as direct results of suppressed vitellogenin action, and predicts that high-pollen–hoarding strain bees, which forage earlier in life than workers from the low-pollen–hoarding strain (see earlier), should demonstrate a precocious drop in vitellogenin hormone titer. Data support this prediction (Amdam *et al.*, unpublished data).

III. Genetic and Phenotypic Architecture of Pollen Hoarding

A. Genetic Architecture

Genetic mapping studies have revealed four major quantitative trait loci (QTL) that explain significant amounts of the phenotypic variance for pollen hoarding and foraging behavior between the high- and low-pollen hoarding strains (Hunt *et al.*, 1995; Page *et al.*, 2000; Rueppell *et al.*, 2004a,b). Three QTL (*pln1*, *pln2*, and *pln3*) were identified by directly mapping the pollen

hoarding trait at the colony level. They were subsequently confirmed by marker association studies of individual foraging behavior. A fourth QTL—*pln4*—was revealed by marker association studies.

Ben-Shahar *et al*. (2002) demonstrated the effects of cGMP on the onset of foraging. They also demonstrated elevated titers of PKG (*Amfor*), a downstream target of cGMP in the signaling cascade in wild-type foragers relative to bees performing tasks in the nest. Genetic variants of PKG of *Drosophila*, the so-called *foraging* gene (*for*), affect the feeding behavior of *Drosophila* larvae and are manifested as variation in their movement (DeBelle *et al*., 1989). The *for* gene also affects the responsiveness of *Drosophila* to sucrose solutions (Scheiner *et al*., 2004b), therefore, it was a likely candidate gene for our studies. We found a polymorphism between our high- and low-strain bees in a non-coding region of *Amfor* and designed a marker (Rueppell *et al*., 2004a). Subsequent studies of bees derived from crosses of the high- and low-pollen hoarding strains demonstrated significant differences in behavior segregating with marker alleles from the two strains; (Rueppell *et al*., 2004a,b). This does not "prove" that *Amfor* itself is responsible for these effects, but it does demonstrate that *Amfor* or something close to it is having an effect. It is interesting that another important signaling gene—*Amtyr1*—mapped to *pln2* (Humphries *et al*., unpublished), making these two signaling genes prime candidates for further research (Fig. 5).

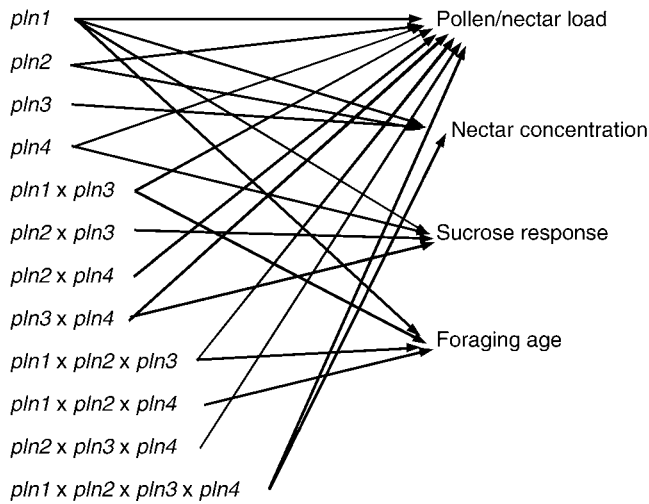

Figure 5 Genetic architecture of traits associated with foraging behavioral differences between the high- and low-pollen hoarding strains (Page and Fondrk, 1995).

The genetic architecture of pollen hoarding and foraging behavior is complex (Fig. 4). All QTL demonstrate pleiotropy, providing an explanation for the association of this set of traits. They are also richly epistatic, which would be expected if they are involved in complex hormonal and neuronal signaling networks. All individual QTL and most of their interactions affect pollen and nectar load sizes. All individual QTL also affect concentration of nectar collected. *pln1* is central. It has a demonstrated direct effect on all behavioral traits. The combination of these QTL studies and the completed honeybee genome sequence and annotation should provide informed candidate genes for future studies of the underlying genetic basis for variation in pollen hoarding and foraging behavior.

B. Phenotypic Architecture

Results discussed in an earlier section, reveal a suite of covarying and interacting phenotypic traits that span behavior to neurobiochemistry that define the architecture of the pollen hoarding trait (Fig. 6). The foraging behavioral traits themselves covary. Pollen and nectar load sizes are

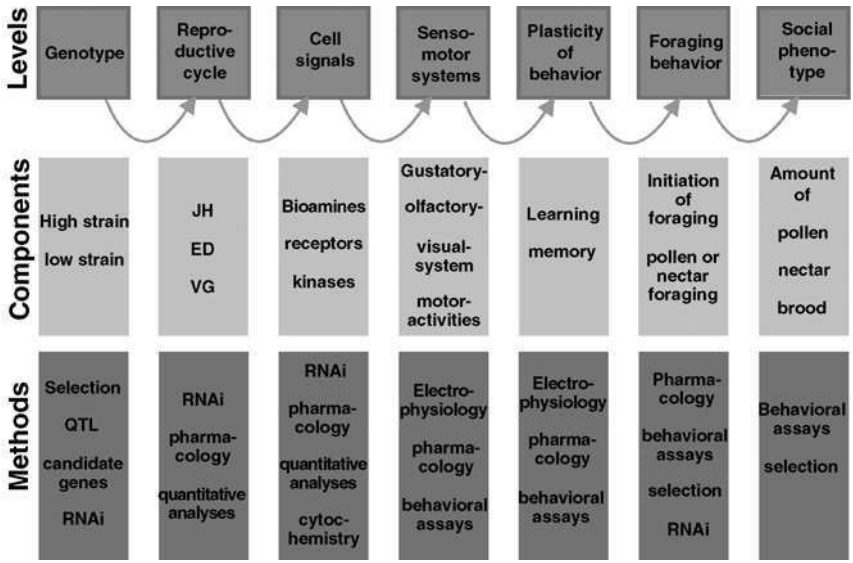

Figure 6 The phenotypic architecture of pollen hoarding behavior in honeybees. Levels of biological organization are shown on the top row spanning genotype to complex social behavior. Phenotypic traits were studied at each level and are shown in the middle row. The bottom row shows the methods that were used to study phenotypic traits at each level.

negatively correlated as a result of constraints on maximum load sizes (Page *et al*., 2000). In addition, nectar load size correlates positively with nectar concentration (Page *et al*., 2000). Bees collect larger loads of nectar and smaller loads of pollen if the nectar has a higher concentration of sugar. Pankiw and Page (2000) showed that newly emerged wild-type bees that are more responsive to sucrose solutions forage earlier in life, collect nectar with lower concentrations of sugar, and collect larger pollen loads than those that are less responsive. This robust result was true for wild-type bees of European origin and Africanized bees (Pankiw, 2003).

Key foci in this architecture are revealed by observed genotypic differences between the high- and low-strain bees, the rich set of correlations of gustatory sensitivity and response revealed by PER sucrose sensitivity assays, and the effects of biogenic amines on the suite of traits revealed by behavioral pharmacology. Collectively the results suggest the involvement of neuromodulatory networks involving cAMP signaling. These neuromodulatory networks affect correlated sensory response systems that include sensitivity to sugar, a central foraging stimulus for honeybees involved in foraging decision making, recruitment behavior, and associative learning. Findings that identify connections between pollen hoarding, vitellogenin hormone dynamics (Amdam *et al*., 2004), and ovary development (Amdam *et al*., 2006) suggest that pollen foraging behavior with the covarying suite of traits associated with it are modulated by a superior hierarchy of regulatory hormones involved in reproduction and reproductive behavior. It is likely that the neuromodulatory networks are themselves modulated by the reproductive hormones.

C. Reproductive Ground Plan

Amdam *et al*. (2004) proposed that the suite of traits associated with foraging behavior and the underlying complex genetic architecture could be explained if foraging specialization was derived from a reproductive regulatory network (West-Eberhard, 1987, 1996). In solitary insects, different stages of the female reproductive cycle (previtellogenesis, vitellogenesis, oviposition, and brood care) are linked and involve coupled physiological and behavioral changes (Finch and Rose, 1995; Lin and Lee, 1998; Miyatake, 2002). Juvenile hormone and ecdysone are key hormones controlling vitellogenesis in many insect species (Brownes, 1994; Hiremath and Jones, 1992; Ismail *et al*., 1998; Sankhon *et al*., 1999; Socha *et al*., 1991). In addition, they regulate behavioral transitions associated with changes in reproductive state such as the shift from foraging for nectar in previtellogenic females to protein foraging in vitellogenic individuals, as it occurs in the mosquito—*Culex nigripalpus* (Hancock and Foster, 2000). JH and

ecdysone also modulate changes in sensory perception, locomotor activity, and reproductive physiology (Lin and Lee, 1998; Zera and Bottsford, 2001)—traits that have been shown to be different in workers from the high- and low-pollen–hoarding strains and in wild-type pollen and nectar foragers (see earlier).

In solitary insects, hormonal effects on reproductive traits typically act in mature adults (Fig. 7), following a prereproductive phase where the animals may enter diapause or aestivate and disperse (Hartfelder, 2000). In honeybees, however, these hormonal signals shifted in time (Amdam *et al.*, 2004), occurring in the late pupal stages where they activate the production of vitellogenin (Barchuk *et al.*, 2002). Differential amplitude of JH titers were observed in newly emerged high- and low-pollen–hoarding strain bees where high-strain workers had higher titers of JH (Schulz *et al.*, 2004). This elevated titer correlates with a higher level of vitellogenin mRNA and a higher vitellogenin hormone titer in the blood (Amdam *et al.*, 2004). Compared to the low-strain bees, workers of the high-pollen–hoarding strain have larger ovaries (they have more ovary filaments) that can show an active previtellogenic ovarian phenotype already at adult emergence (Amdam

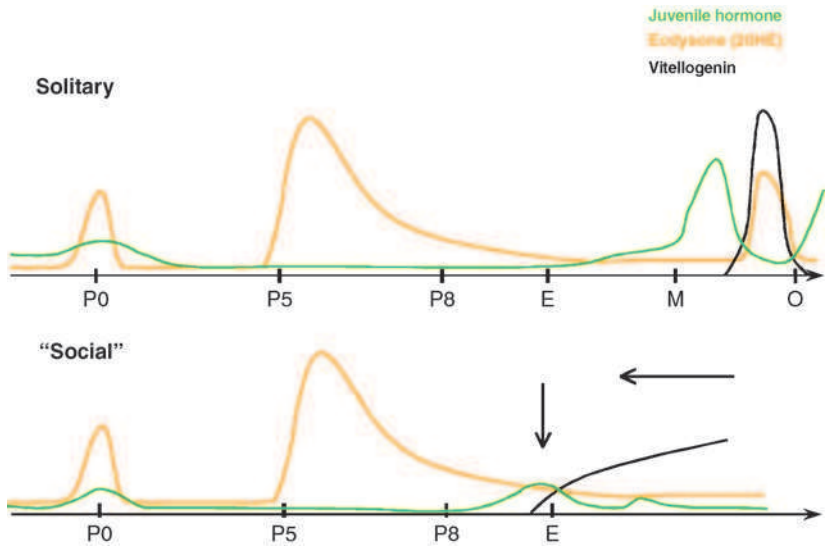

Figure 7 A time course of blood hormone titers from early to late pupal stages (P0–P8) through emergence (E) and into mature prereproductive adults (M) to adults with activated ovaries (O) in solitary and social insects (Barchuk *et al.*, 2002; Pinto *et al.*, 2000). Amdam *et al.* (2006) hypothesized that the spikes of hormone titers seen between M and O in solitary insects have shifted with time in social insects and is homologous with the increases in titer observed just prior to adult emergence.

et al., 2006). It was proposed that if such documented markers of JH and ecdysone action are present early in honeybee adult life, then pleiotropic effects on behavior may have shifted from later life-stages as well (Amdam *et al*., 2004), as demonstrated by the differences in sensory responses and locomotor activity of high- and low-strain bees and the correlation of locomotor and sensory responses in wild-type workers.

Reproductive signaling, early in life, can also explain the observed differences between newly emerged high- and low-strain bees in PKA and PKC titers (Humphries *et al*., 2003). These kinases play key roles in sensory sensitivity and learning. In addition, observed differences in *Amtyr1* mRNA levels (Humphries, unpublished data) can be understood as a pleiotropic effect of a reproductive regulatory network because the tyramine pathway appears to be involved in reproductive tuning of queenless worker honeybees (Sasaki and Nagao, 2002). The finding that ovary size correlates with sensory responsiveness in 5-day-old bees (Tsuruda, unpublished data), and the known association between such sensory responses and foraging behavior 2–3 weeks later suggests that gonotrophic events in young bees have persistent effects on adult behavior. These insights are summarized in the "reproductive ground plan" hypothesis of social evolution (Amdam *et al*., 2004). The hypothesis proposes that the genetic and hormonal networks that govern reproductive development, physiology, and behavior in solitary species represent one fundamental regulatory module with capacity to serve as basis for evolution of social phenotypes.

IV. The Evolution of Division of Labor and Specialization

Division of labor between nest tasks and foraging activity, and foraging specialization on pollen and nectar, likely evolved from the gonotrophic cycle of solitary ancestors of the honeybee. The first step was a shift in the timing of reproductive hormonal signaling events from the mature adult stage into the late pupal stages (Amdam *et al*., 2004). This shift turned on the production of vitellogenin and further caused behavioral traits interlinked with reproductive maturity to be expressed in young bees. These vitellogenic females bypassed the phases of dispersal, diapause, and aestivation that characterized the ancestral prereproductive period. Instead, they expressed a coordinated set of maternal reproductive behaviors, including larval care, nest defense, and foraging (West-Eberhard, 1987, 1996). The second step was the evolution of a feedback interaction between vitellogenin and JH (Guidugli *et al*., 2005), apparently resulting in a regulatory mechanism that enabled vitellogenin to become a pace maker for division of labor. Higher blood titers of vitellogenin keep bees in the nest, performing maternal nonforaging tasks. Blood titers of vitellogenin decrease as a consequence

of vitellogenin consumption in brood rearing (Amdam *et al.*, 2003), and workers with low-vitellogenin levels—a state presumably incompatible with ability to nourish larvae—are triggered to leave the nest to perform foraging tasks. During this transition, a rapid increase in JH strongly suppresses remaining expression of vitellogenin, thereby producing a robust and definite differentiation of the forager phenotype (Amdam and Omholt, 2003; Guidugli *et al.*, 2005). After the transition, bees with active ovaries preferentially forage for protein (pollen), as did their reproductively activated solitary ancestors that were provisioning their brood. Those with inactive ovaries forage primarily for nectar, as do nonreproductive solitary insect females (Dunn and Richards, 2003).

The early initiation of vitellogenesis is the necessary first step in social evolution via the subsocial route to sociality—staying and helping your mother raise siblings at the natal nest (Michener, 1974). Our model thus demystifies this first, essential step in social evolution and shows a simple, plausible mechanism by which it could have occurred. It also demonstrates that behavioral specialization can be an immediate emergent property, conferring a selective advantage to subsocial group living. Foraging specialization is, under our model, an immediate consequence of the ancestral interlinkage between reproductive tuning and foraging-preference for nectar or pollen. Temporal polyethism, furthermore, is an immediate consequence of variation in vitellogenin dynamics caused by developmental and nutritional factors. One factor that converges at the intersection between development and nutrition is worker ovary size: ovary size is determined during honeybee larval development and is influenced by nutrition (Hartfelder and Engels, 1998; Kaftanoglu *et al.*, unpublished). In adult worker bees, ovary size is a documented component of the reproductive network that regulates social behavior (Amdam *et al.*, 2006). Therefore, stochastic feeding events resulting in variation in ovary size could lead to differences in rates of onset of foraging behavior and foraging specialization. Finally, differences in stimulus–response sensitivities resulting from differences in reproductive states could result in a self-organized division of labor as described by Page and Mitchell, 1991, 1998; Fewell, 2003; Fewell and Page, 1999. Patterns of complex division of labor, thereby, emerged without explicit selection for task specialization.

By co-option and compartmentalization of the relationships between gonotrophic state and behavior, which was controlled originally by ancestral developmental programs, social insects evolved a division of labor and task specialization among functionally sterile individuals. Once in place, the social structure of colonies could be adapted by a fine-tuning of the pleiotropic hormonal and neuronal signaling networks that affected the behavior. However, these richly epistatic and pleiotropic networks would impose initial constraints on evolution by decreasing or masking the additive genetic

variance available for natural selection, and by correlated responses to selection for traits not under direct selection. Over two decades, a concerted effort has succeeded in making these correlations transparent for the set of traits observed for the pollen-hoarding syndrome of the honeybee—in sum providing the first direct evidence for an evolutionary origin of complex social behavior.

Acknowledgments

Funding for the research presented here was provided by the National Science Foundation, National Institutes of Health, United States Department of Agriculture, Alexander von Humboldt Foundation, Deutsches Forschungsgemeinschaft, and the Norwegian Research Council.

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Index

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Contents of Previous Volumes

Volume 47

Volume 48

Volume 49

Volume 50

Volume 51

Volume 52

Volume 53

Volume 54

Volume 55

Volume 56

Volume 57

Volume 58

Volume 59

Volume 60

Volume 61

Volume 62

Volume 63

Volume 64

Volume 65

Volume 66

Volume 67

Volume 68

Volume 69

Volume 70

Volume 71

Volume 72

Volume 73