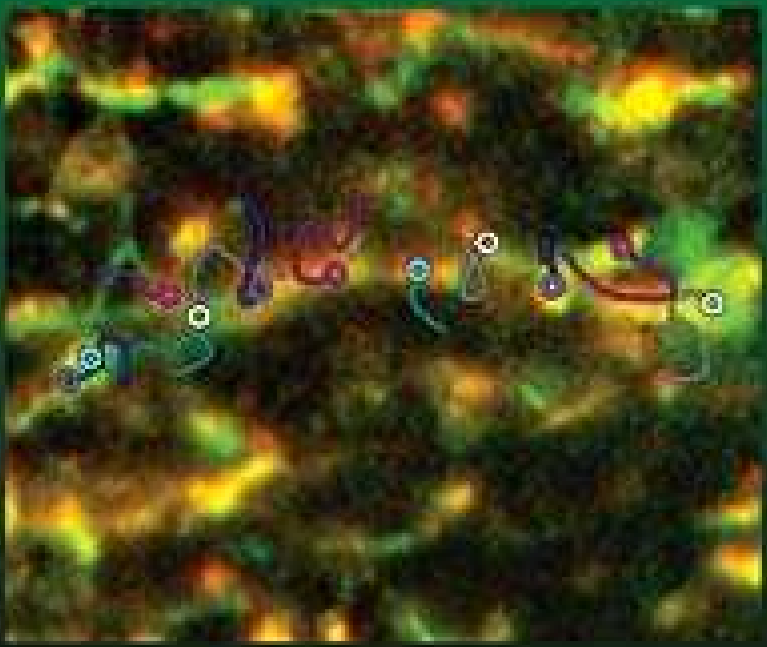
# Current Topics in Developmental Biology



Volume 73

Edited by
Gerald P. Schatten

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Current Topics in
Developmental Biology

Volume 73

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## Volume 73

Edited by

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# Contents

Contributors ix

## 1

**The Molecular Origins of Species-Specific Facial Pattern**
*Samantha A. Brugmann, Minal D. Tapadia, and Jill A. Helms*

I. Introduction 2
II. Molecular and Tissue Interactions that Regulate Craniofacial Patterning 4
III. Conclusions 34
Acknowledgment 35
References 35

## 2

**Molecular Bases of the Regulation of Bone Remodeling by the Canonical Wnt Signaling Pathway**
*Donald A. Glass II and Gerard Karsenty*

I. Introduction 44
II. Canonical Wnt Signaling 45
III. Other Wnt Signaling Molecules 58
IV. Secreted Wnt Inhibitors and Agonists 63
V. Intracellular or Transmembrane Modulators 67
VI. Conclusions and Future Work 69
References 71

## 3

**Calcium Sensing Receptors and Calcium Oscillations: Calcium as a First Messenger**
*Gerda E. Breitwieser*

I. Introduction: Physiological Role of Extracellular $Ca^{2+}$ 86
II. CaR is a Unique Family C G-Protein–Coupled Receptor 88

III. CaR Signal Transduction 96
IV. CaR Translates Extracellular $Ca^{2+}$ Changes into Time-Varying Intracellular $Ca^{2+}$ Signals 97
V. CaR in Development 104
Acknowledgments 106
References 106

# 4

## Signal Relay During the Life Cycle of *Dictyostelium*

*Dana C. Mahadeo and Carole A. Parent*

I. Introduction 115
II. The *Dictyostelium* Life Cycle is Regulated by Five Signal Relay Modules 117
III. Summary 133
IV. Perspectives 134
Acknowledgments 134
References 134

# 5

## Biological Principles for *Ex Vivo* Adult Stem Cell Expansion

*Jean-François Paré and James L. Sherley*

I. Introduction 142
II. Strategies for ASCs Expansion 143
III. Cell Kinetics Symmetry-Based Strategies for ASC Expansion 152
IV. Comparison of SACK-Derived ASC Strains to Conventional Cell Lines 157
V. Future SACK Pathways and Regulators 158
VI. Confronting the Replication Mutation Risk of ASC Expansion 160
VII. Summary 162
References 163

# 6

## Histone Deacetylation as a Target for Radiosensitization

*David Cerna, Kevin Camphausen, and Philip J. Tofilon*

I. Introduction 174
II. Histone Acetylation 176

III. Inhibitors of Histone Deacetylase 179
IV. *In Vitro* Radiosensitization by HDAC Inhibitors 180
V. *In Vivo* Radiosensitization by HDAC Inhibitors 187
VI. Tumor Versus Normal Cells 190
VII. Mechanisms of HDAC Inhibitor-Induced Radiosensitization 191
VIII. Clinical Application of HDAC Inhibitor/Radiotherapy Combinations 196
References 198

## 7

**Chaperone-Mediated Autophagy in Aging and Disease**
*Ashish C. Massey, Cong Zhang, and Ana Maria Cuervo*

I. Introduction: Different Forms of Autophagy 206
II. CMA: A Selective Form of Autophagy 208
III. Physiological Role of CMA 215
IV. CMA and Aging 220
V. Pathologies Related to CMA 221
VI. Concluding Remarks 228
Acknowledgments 229
References 229

## 8

**Extracellular Matrix Macroassembly Dynamics in Early Vertebrate Embryos**
*Andras Czirok, Evan A. Zamir, Michael B. Filla, Charles D. Little, and Brenda J. Rongish*

I. Introduction 238
II. Localization and Function of Select ECM Components 241
III. ECM Position Fate 243
IV. ECM Displacement Mapping 245
V. Tissue Motion Component of ECM Displacement 248
VI. Local ECM Rearrangements 249
VII. Conclusions 252
Acknowledgments 254
References 254

Index 259
Contents of Previous Volumes 267

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# 1

# The Molecular Origins of Species-Specific Facial Pattern

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I. Introduction
  A. Organization of the Face
II. Molecular and Tissue Interactions that Regulate Craniofacial Patterning
  A. First Milestone in Craniofacial Patterning: Specifying the Neural–Nonneural Boundary
  B. Second Step in Craniofacial Patterning: Segregating Tissues into Functional Domains
  C. Third Step in Craniofacial Patterning: Controlling Growth, Differentiation, and Cell Death
  D. Putting it All Together: Species-Specific Craniofacial Morphogenesis
III. Conclusions
Acknowledgment
References

The prevailing approach within the field of craniofacial development is focused on finding a balance between tissues (e.g., facial epithelia, neuroectoderm, and neural crest) and molecules (e.g., bone morphogenetic proteins, fibroblast growth factors, Wnts) that play a role in sculpting the face. We are rapidly learning that neither these tissues nor molecular signals are able to act in isolation; in fact, molecular cues are constantly reciprocating signals between the epithelia and the neural crest in order to pattern and mold facial structures. More recently, it has been proposed that this crosstalk is often mediated and organized by discrete organizing centers within the tissues that are able to act as a self-contained unit of developmental potential (e.g., the rhombomere and perhaps the ectomere). Whatever the molecules are and however they are interpreted by these tissues, it appears that there is a remarkably conserved mechanism for setting up the initial organization of the facial prominences between species. Regardless of species, all vertebrates appear to have the same basic bauplan. However, sometime during mid-gestation, the vertebrate face begins to exhibit species-specific variations, in large part due to differences in the rates of growth and differentiation of cells comprising the facial prominences. How do these differences arise? Are they due to late changes in molecular signaling within the facial prominences themselves? Or are these late changes

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a reflection of earlier, more subtle alterations in boundaries and fields that are established at the earliest stages of head formation? We do not have clear answers to these questions yet, but in this chapter we present new studies that shed light on this age-old question. This chapter aims to present the known signals, both on a molecular and cellular level, responsible for craniofacial development while bringing to light the events that may serve to create difference in facial morphology seen from species to species. 

## I. Introduction

> *The conceivable modifications of the vertebrate archetype are very far from being exhausted by any of the forms that now inhabit the earth, or that are known to have existed here at any period . . .. The discovery of the vertebrate archetype could not fail to suggest to the Anatomist many possible modifications of it beyond those that we know to have been realized in this little orb of ours.*
>
> *Owen, 1849.*

Throughout the millennia, mankind has pondered how the enormous variation in animal form has come into existence. From Owen's concept of a "vertebrate archetype" that served as the foundation on which morphological diversity is generated, to Darwin's theory of evolution, we have been puzzling over the question of how diversity in the Animal Kingdom comes about. Our interest in obtaining answers to this age-old riddle has only increased in the intervening decades since these famous scientists debated the topic. In fact, one might summarize the objective of most current research in developmental and evolutionary biology as having a single goal: to understand the process by which shape and form (i.e., morphogenesis) is regulated. In this chapter, our primary goal is to describe recent study that provides clues into the molecular origins of species-specific *craniofacial* morphogenesis.

We have focused on growth and patterning of the craniofacial complex as a model for species diversity for three reasons. The first rationale is that, despite the fact that animals have such different looking faces, the general organization of vertebrate craniofacial complex is remarkably similar during early embryonic development. This suggests that a fundamental set of patterning genes might initially define the global organization of the facial prominences. From this conserved pattern, the widely divergent variations in facial form might then arise, from the gentle spatiotemporal tweaking of the expression of common genes.

The second reason we have focused on the face as a model system is that the craniofacial region exhibits such extraordinary variation in form, and these variations are closely associated with adaptive radiations into new ecological niches. A prime example is the Galapagos finches, where the principal intraspecies variation is the size and shape of their facial prominences (in birds, facial prominences such as beaks). Thus we propose that understanding the mechanisms regulating craniofacial morphogenesis in any

species holds the potential to understand, at both molecular and cellular levels, the basis for evolutionary diversity.

A third motivation to focus on craniofacial patterning as a window into species-specific morphology is that there are a great number of anatomical landmarks that serve as species-specific characteristics. For example, the dentition was a feature of vertebrates but about 150 million years ago, birds lost this characteristic while mammals retained it. Even within the dentition itself, there are species-specific features: some mammals do not form premolar teeth whereas others (humans included) do. Because of these inherent species-specific differences we can begin to ask questions such as how a particular characteristic (in this case, teeth) might be retained or lost, or how a trait may be modified in such a way to ideally suit an animal. Although the mechanisms responsible for such species-specific morphological differences are still to be discovered, a growing number of studies now show that some answers to these age-old puzzles are within our grasp.

## A. Organization of the Face

Although the vertebrate head exhibits an exceedingly intricate and varied morphology, by the time an animal is born the craniofacial complex from which it arises initially has a much more simple geometry. This arrangement consists of a series of swellings or prominences that undergo fusion and expansion in an orderly and integrated fashion (Fig. 1A–D). There are seven prominences that comprise the vertebrate face: the midline frontonasal prominence, and three paired structures, the lateral nasal, maxillary, and mandibular prominences, which are derived from the first pharyngeal (branchial) arch. The frontonasal prominence contributes to the forehead, middle of the nose, philtrum of the upper lip, and primary palate. The lateral nasal prominence forms the sides of the nose; the maxillary prominences contribute to the sides of the face, lips and the secondary palate; the mandibular prominences produce the lower jaw (Fig. 1E).

The maxillary and mandibular prominences are derived from a single arch, hence one might wonder just how early on in the ontogeny of the craniofacial complex do cells irreversibly segregate into ''maxillary'' and ''mandibular'' subdivisions. That question was recently addressed when two groups of investigators considered this issue from an evolutionary perspective. The question of interest was the extent to which cells destined to occupy the maxillary portion of the first arch were separated from those cells bound to take up residence in the mandibular portion of the first arch. The groups independently examined the contributions of first arch neural crest cells to the maxillary process, in axolotl and chick and found that structures believed to be derived from maxillary condensation (i.e., Meckel's cartilage and the palatoquadrate) are solely derived from the mandibular condensation. Further, these fate-mapping studies proved that the maxillary

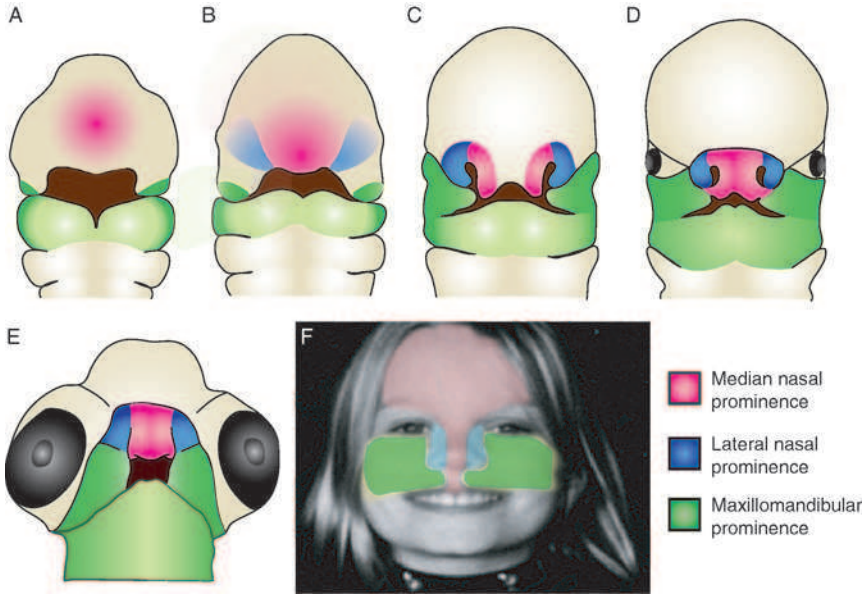


**Figure 1** Development of the craniofacial primordia. (A–D) Representations of frontal views of mouse embryos showing the prominences that give rise to the main structures of the face. The frontonasal (or median nasal) prominence (pink) gives rise to the forehead (A), the middle of the nose (B), the philtrum of the upper lip (C) and the primary palate (D), while the lateral nasal prominence (blue) forms the sides of the nose (B, D). The maxillomandibular prominences (green) give rise to the lower jaw (specifically from the mandibular prominences), to the sides of the middle and lower face, to the lateral borders of the lips, and to the secondary palate (from the maxillary prominences). (E) Frontal view of a chick embryo, also showing which prominences give rise to different facial structures. (F) Frontal view of a human child, with different facial structures color-coded to indicate the prominences from which each structure developed.

process and its skeletal derivatives (the trabecular cartilage) are not derived from the first pharyngeal arch but rather from a condensation located between the eye and the maxillo-mandibular cleft (Cerny *et al.*, 2004; Lee *et al.*, 2004). One might wonder why such information is crucial to the study of craniofacial biology; simply put, when we know the origins of a structure we also gain knowledge about the origins of developmental anomalies affecting that structure. With this in mind, we begin this chapter by exploring new advances into the earliest events in facial patterning.

## II. Molecular and Tissue Interactions that Regulate Craniofacial Patterning

As might be deduced from the initial description of craniofacial morphogenesis, the process by which the face forms requires an elaborate series of intricately linked morphological events involving cell proliferation, differentiation,

programmed cell death, the transdifferentiation of cells from a mesenchymal phenotype to an epithelial one, and vice versa. All of these cellular behaviors are coupled with elaborate cell movement caused by active migration on the part of the cranial neural crest, and passive cell displacement caused during the process of neurulation. How all of these cell behaviors are actually orchestrated has been the subject of intense scrutiny for many years, yet we are only now beginning to understand the molecular and cellular driving forces behind these events.

For the sake of clarity, one might best describe craniofacial development as a comprehensive series of steps that begin with the formation of the earliest tissue domains that separate "anterior" (head) from everything else, continue when these regions become subdivided into craniofacial prominences, and conclude with the differentiation of cells into distinct facial structures (i.e., teeth and palate). We must emphasize, however, that each of these steps is the result of an interplay between multiple tissues and molecules. By dividing the process into patterning "milestones" and examining the events that occur within specific time frames to create discrete facial structures we do not mean to imply that craniofacial morphogenesis is a linear process, only that it is sometimes easier to consider the complex morphological problem in this simpler manner.

## A. First Milestone in Craniofacial Patterning: Specifying the Neural–Nonneural Boundary

### 1. Neural Plate Formation

During embryonic development, one can easily appreciate that the face is erected on a scaffolding of sorts, comprised of the vertebrate forebrain. An adage coined in the middle of the last century to aid clinicians in diagnosing brain anomalies in children stated, "The face predicts the brain" (DeMyer, 1964). In the last decade, we have a much better grasp of how molecular signals emanating from one tissue profoundly affect differentiation within adjacent tissues so one could just as easily say now, "The brain predicts the face" (reviewed in Helms *et al.*, 2005). Therefore, we begin with a review about how these two tissues, the forebrain and the face, begin their close relationship.

The brain arises from anterior ectodermal tissues that adopt a neuroectodermal character in response to signals emanating from an embryonic domain, which in amphibians is commonly referred to as the "organizer". This region constitutes the dorsal lip of the blastopore and as Hilde Mangold and Hans Spemann discovered (Spemann and Mangold, 1924), this organizer has the ability to induce lateral mesoderm to give up its normal fate of becoming muscle and instead adopt a neural fate (reviewed in Harland, 2000). The organizer performs these neural inducing functions by secreting

molecules, such as Noggin (Furthauer *et al.*, 1999; Lamb *et al.*, 1993; Smith and Harland, 1992; Smith *et al.*, 1993), Follistatin (Hemmati-Brivanlou *et al.*, 1994), and Chordin (Sasai *et al.*, 1994, 1995), which interact with and antagonize other molecular signals in the surrounding tissues such as bone morphogenetic proteins (Bmps) (Fainsod *et al.*, 1997; Piccolo *et al.*, 1996; Zimmerman *et al.*, 1996) in the nonneural ectoderm. While it remains widely accepted that the organizer plays a major role in neural induction, ablating the organizer in amphibians, avians, and mammals does not necessarily eliminate neural tissue (Davidson *et al.*, 1999; Sater and Jacobson, 1990; Shih and Fraser, 1996; Smith *et al.*, 1989). This latter finding suggests that other areas of the embryo also possess neural inducing properties. In fact, the most widely accepted model for neural induction is the "default" model, in which embryonic ectoderm is thought to be preprogrammed to a neural fate unless acted on by neural antagonists such as members of the transforming growth factor beta (TGF$\beta$) family (Bmps) (reviewed in Hemmati-Brivanlou and Melton, 1997).

Other vertebrates including birds and mammals have tissues that are equivalent to the amphibian organizer; however the default model has not been as widely accepted as the mechanism that regulates neural induction in these species. Some investigators have carefully scrutinized and compared expression patterns of Bmps and their antagonists in different species and have reevaluated functional data from avian and mammalian systems. Their conclusions provide strong evidence against the default model of neural induction (Streit and Stern, 1999b; Streit *et al.*, 1998). For example, mouse mutants that lack Cerberus, Noggin, or Chordin still develop a nervous system (Bachiller *et al.*, 2000; Belo *et al.*, 2000; McMahon *et al.*, 1998).

There are also inconsistencies in the expression levels of TGF$\beta$s between birds and amphibians; while Bmps are strongly expressed prior to neural induction in frogs, the same molecules are undetectable in avians at this stage of development (Streit *et al.*, 1998). Furthermore, there are species-specific differences in how the surrounding tissues respond to Bmp inhibition: in frogs, blocking Bmp signaling will cause almost any tissue to adopt a neural characteristic (Lamb *et al.*, 1993; and reviewed in Sasai and De Robertis, 1997; Sasai *et al.*, 1995; Weinstein and Hemmati-Brivanlou, 1997). Conversely, in avians, misexpressing Bmp antagonists in competent epiblast does not induce the expression of any neural markers, and a grafted source of Bmp protein does not inhibit neural plate development (Streit and Stern, 1999a,b; Streit *et al.*, 1998).

These experimental differences in amniotes and anamniotes may reflect varying levels of competence in whether tissues adopt a neural or nonneural fate (Streit and Stern, 1999b). They also serve to illustrate how animals have evolved different molecular pathways to achieve similar morphological results. These very early discrepancies in neural plate induction may also be

one of the first mechanisms that animal species employ to create the widely divergent facial features characteristic of their later embryonic development.

## 2. No-Man's Land: The Neural/Nonneural Boundary Region Gives Rise to Placodes and the Neural Crest

Whether by a default mechanism or not, the specification of neural tissues creates a boundary: neural ectoderm occupies the medial part of the embryo and nonneural (epidermal, surface) ectoderm forms in the lateral regions (Fig. 2). Sandwiched between these two tissues is a no-man's land of sorts, called the lateral neurogenic ectoderm (LNE) (Brugmann *et al.*, 2004). The LNE is an especially important region because it gives rise to both cranial neural crest cells, which give rise to the majority of the head skeleton, and the placodes, which make vital contributions to the cranial sensory ganglia. Consequently, considerable effort has gone into studying how the neural crest and placodes are actually generated from this swath of tissue (Bastidas *et al.*, 2004; Brugmann *et al.*, 2004; Glavic *et al.*, 2004; McLarren *et al.*, 2003; Streit, 2002).

The earliest models focused on secreted molecules that formed a gradient of anti-Bmp/Bmp activity at the neural/nonneural boundary. Data to support this

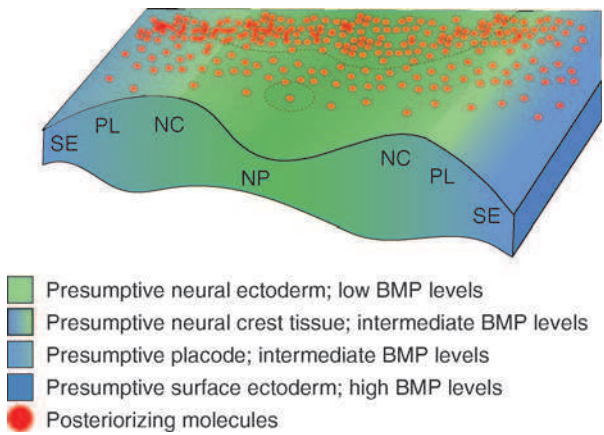


**Figure 2** Default model of neural induction. The embryonic ectoderm can be subdivided into several different domains: presumptive surface ectoderm (SE), pre-placodal (P), neural crest (NC) and neural plate (NP). These different domains arise as a result of a mediolateral gradient of TGF-beta signaling molecules such as Chordin, Noggin and Cerberus, which are secreted from Spemann's organizer and which inhibit BMP signaling. Low levels of TGF-beta molecules prompt lateral ectoderm to form SE, intermediate levels of TGF-beta molecules prompt more medial regions of ectoderm to form PL and NC, and high levels of TGF-beta signaling molecules prompt the medial-most region of ectoderm to form the neural plate (NP) (and later neuroectoderm).

model came from many sources; for example, some investigators showed that an intermediate concentration of anti-Bmp signaling could induce molecular markers of the neural crest and placodes (Aybar and Mayor, 2002; Aybar *et al.*, 2002; Brugmann *et al.*, 2004; Marchant *et al.*, 1998; Mayor and Aybar, 2001). More recent studies propose that a border region, which includes the LNE, is formed by direct physical interactions between neural and nonneural (surface) ectodermal cells. These border regions are thought to be defined in part by the expression of members of the Distal-less Dlx family of transcription factors (McLarren *et al.*, 2003; Woda *et al.*, 2003). For example, overexpression of Dlx5 can imbue cells with the features of LNE cells (McLarren *et al.*, 2003) but by itself cannot induce neural crest or preplacodal ectoderm (PPE) fate (McLarren *et al.*, 2003). Presumably, a combination of transcription factors (Dlx as well as others) is required to generate these cell types. The fact that *Dlx* genes play a role in the specification of the LNE fits in well with the Bmp gradient model of LNE specification, because *Dlx* genes are directly induced by Bmps (Feledy *et al.*, 1999; Luo *et al.*, 2001).

Perhaps a permissive level of Bmp activity is required for the generation of neural crest cells from the LNE (Mayor and Aybar, 2001; Tribulo *et al.*, 2003). Since these same neural crest cells will later form the facial skeleton, one compelling idea is that subtle differences in Bmp activity might be responsible for species-specific variations in facial skeletal form. One test of this theory would be to modulate Bmp activity at the time of LNE specification and, in doing so, alter facial skeletal morphology. Although this experiment has not been undertaken, two groups have shown that modulating Bmp activity at later stages of morphogenesis, after the facial prominences have formed and are occupied by cranial neural crest cells, does alter facial morphology in relatively predictable ways (Abzhanov *et al.*, 2004; Wu *et al.*, 2004).

The question remains, whether variations in Bmp levels *at the time of neural plate formation* can be held accountable for the vastly different facial forms between species. Recent studies have provided indirect evidence that lends credence to this hypothesis. For example, at the time of neural crest induction there are high levels of Bmp activity at the neural plate boundary in frogs and fish while in birds, these Bmp levels are quite low (Endo *et al.*, 2002; Glavic *et al.*, 2004). Since an intermediate level of Bmp activity is necessary for neural crest induction in frogs and birds, this would necessitate that frogs reduce Bmp signaling while birds increased the same in order to achieve the same affect: neural crest induction. New data indicate that both species appear to have used a similar mechanism to regulate their Bmp activity via Notch/Delta signaling (Endo *et al.*, 2002; Glavic *et al.*, 2004). Notch pathway activation can either amplify or repress Bmp signaling at the neural plate boundary, depending on the organism. This bifunctional feature may have evolved in order to compensate for different levels of Bmp activity in various vertebrate species (Meulemans and Bronner-Fraser, 2004).

Other molecules also participate in establishing the neural/nonneural boundary. For example, Msx1 and Pax3 are two transcription factors that work in

concert to generate the neural crest (Monsoro-Burq *et al.*, 2005), and in turn, these transcription factors mediate (and are mediated by) Fibroblast Growth Factor (Fgf) and Wnt signaling (Bach *et al.*, 2003; Bang *et al.*, 1999). Since the proper location and expression levels of these very early signaling molecules control the generation of cranial neural crest cells, which ultimately dictate the pattern of the face, one might easily wonder if the relative levels of Bmps, Wnts, and Fgfs are responsible for species-specific differences in facial morphology. In other words, could tweaking the location and expression levels of these proteins account for the differences between a frog and a fish, a bird and a mouse?

There is growing evidence to support this conjecture. The downstream targets of Bmps, Wnts, and Fgfs are expressed in different patterns among the various species—but whether this is the cause or result of an already-altered facial geometry is still not certain. Foxd3, which mediates delamination of the neural crest from the neural folds (Cheung *et al.*, 2005), and Sox9 and 10, which are responsible for chondrogenic potential of neural crest cells (Barrallo-Gimeno *et al.*, 2004; Cheung and Briscoe, 2003; Honore *et al.*, 2003; Mori-Akiyama *et al.*, 2003; Yan *et al.*, 2002), are expressed in juxtaposed or overlapping regions that are directly adjacent to the neural plate in avian and amphibians—but are expressed in discrete domains in amphioxus (Meulemans and Bronner-Fraser, 2004). These data suggest that subsets of amphioxus neural crest cells had limited skeletogenic potential. The hypothesized merging of these subsets of primitive neural crest cells may explain how modern-day neural crest cells acquired their full potential to influence and regulate craniofacial patterning. Thus, by varying the expression boundaries of transcription factor, the skeletogenic potential of the cranial neural crest is altered; by shifting the boundaries of the LNE, larger or smaller regions of tissue are available for segregation into neural crest and placodal populations (Meulemans and Bronner-Fraser, 2004). Could these small dissimilarities be the earliest basis for variations in neural crest-derived facial features among the species? In other words, might the difference between an elephant's nose and ours be attributable to a subtle shift in the boundary between neural and nonneural ectoderm, and thus the allocation of cells to a cranial neural crest lineage?

## B. Second Step in Craniofacial Patterning: Segregating Tissues into Functional Domains

Once the neural/nonneural boundary is specified, the real work of craniofacial patterning begins: molding the tissues into discrete facial structures and ensuring that those structures are seamlessly woven together. This requires several distinct yet coordinated processes, including the delamination of cells from the neural folds to generate the neural crest; invagination and migration of these cells throughout the head primordia, localized proliferation of epithelial and neural crest cells, and localized regions of programmed cell

death to sculpt the tissues. These processes are under the control of a cocktail of molecules, most of which have been introduced before and include members of the Wnt, Fgf, Bmp, and Hedgehog families, and Hox transcription factors. We begin then with the role that *Hox* genes play in species-specific craniofacial morphogenesis.

## 1. Neural Crest Cells, Hox Genes, and Craniofacial Patterning

Several years ago it was widely believed that neural crest cells contain all the patterning information necessary for the formation of facial structures. A series of experiments by Noden (1983, 1984, 1986) exemplified this concept. Noden (1983) showed that transplanting presumptive first arch neural crest cells into more posterior positions along the neural tube resulted in the transformation of second arch skeletal structures into first arch skeletal structures. If neural crest cells contain sufficient information to transform one pharyngeal arch into another, one might immediately wonder how such "positional information" was encoded. The answer may be by virtue of a Hox code.

Members of the *Hox* gene family are homeobox-containing transcription factors that are expressed in a nested pattern along almost the entire length of the neural tube, and abundant data indicate that the combined pattern of *Hox* genes, referred to as a *Hox* code, an instrumental in establishing the regional identity of cells (reviewed in Capecchi, 1997).

Is a *Hox* code responsible for positional information encoded by cranial neural crest cells as well? Only one *Hox* gene, *Hoxa2*, is expressed in the second (hyoid) arch (Köntges and Lumsden, 1996). When Noden's seminal experiments are considered in light of this information on *Hox* gene expression, we find that *Hoxa2* negative cranial neural crest cells were transplanted into a *Hoxa2* positive environment. Therefore, the question can be framed in a slightly different manner: does the expression of *Hoxa2* provide these transplanted cranial neural crest cells with a regional identity? And is that Hox-encoded regional identity sufficient to explain aspects of species-specific patterning?

Members of Nicole Le Douarin's laboratory addressed these exact questions, and over the course of a number of experiments beautifully demonstrated that cranial neural crest cells maintain their *Hox* code when they are transplanted and their behavior is closely tied to this expression pattern (Fig. 3). For example, if *Hoxa2* negative neural crest cells are transplanted into a *Hoxa2* positive environment, then the cells integrate appropriately and produce a normal hyoid bone (Couly *et al.*, 1998) (Fig. 3D). But if *Hoxa2* expressing neural crest cells are transplanted into a *Hoxa2* negative environment, then the cells still retain their *Hox* negative status—but fail to integrate into the new environment (Fig. 3E). Consequently, the

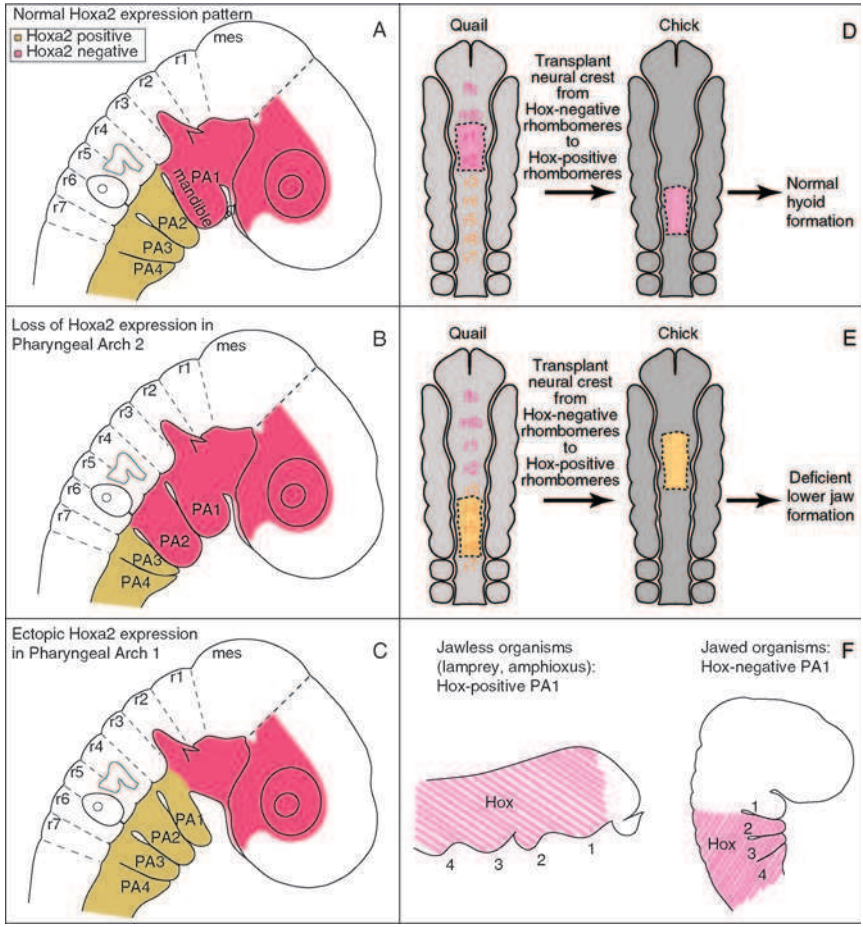


**Figure 3** *Hox* manipulation and transplantation experiments. (A–F) Schematic drawings depicting *Hox* expression and manipulation experiments in both jawed and jawless animals. (A) In jawed animals, *hoxa2* is expressed up to pharyngeal arch 2 (PA2). (B) Loss of *hoxa2* expression from pharyngeal arch 1 (PA1) confers increased plasticity upon neural crest cells in PA1, allowing them to adopt first arch fates. (C) Ectopic expression of *hoxa2* in PA1 results in these cells adopting second arch fates, thereby giving rise to a duplication of the hyoid arch. (D) Experiments by Couly *et al.* demonstrated that transplantation of neural crest cells from anterior, *Hox*-negative rhombomeres to posterior, *hox*-positive rhombomeres resulted in normal hyoid formation from the donated tissue. (E) Parallel experiments by Couly conversely demonstrated that transplantation of neural crest cells from posterior, *hox*-positive rhombomeres to anterior, *hox*-negative rhombomeres resulted in deficient lower jaw formation. (F) Recent experiments have shown that *hox* expression exists as far anterior as PA1 in jawless organisms such as lampreys and amphioxus, unlike jawed vertebrates where *hox* expression is seen only up to PA2 (with no *hox* expression in PA1). Since lampreys and amphioxus are the closest extant relatives to primitive jawless organisms, loss of *hox* expression in PA1 may be tied to the development of jaws in vertebrates.

transplanted cells behave as they would have in their native environment, and the result is ectopic skeletal elements inappropriate to the new location (Couly *et al.*, 1998). Along a similar line, if Hoxa2 positive cells are placed in a *Hox*-negative environment, they retain their *Hox* positive status in the new locale—but once again fail to integrate. The result is a failure in skeletal tissue formation. These experiments collectively suggest that the fate of *some* cranial neural crest cells is restricted by virtue of their *Hox* status (reviewed in Le Douarin *et al.*, 2004).

Gain- or loss-of-function experiments provide further support for the role of *Hox* genes in restricting the fates of cranial neural crest cells. Targeted deletion of *Hoxa2* results in the duplication of first arch skeletal structures in the place of second arch structures (Gendron-Maguire *et al.*, 1993; Kanzler *et al.*, 1998; Rijli *et al.*, 1993; and reviewed in Trainor *et al.*, 2002) (Fig. 3B). Conversely, gain of *Hoxa2* function transforms the cartilaginous elements of the first arch into second arch elements (Grammatopoulos *et al.*, 2000; Pasqualetti *et al.*, 2000) (Fig. 3C).

## 2. Just How Malleable Is the Mandible?

From the data presented above, one might surmise that removing *Hox* expression from a cell confers it with greater plasticity. This concept has not been lost on those evolutionary biologists who seek to understand how vertebrates have gained and lost certain craniofacial structures throughout evolution. Perhaps best studied of these evolutionary events is the development of a hinged jaw.

As embryos, jawless animals (agnathans), such as lampreys, bear a remarkable resemblance to jawed embryos, in that both have pharyngeal arches and a braincase. If jaw-lacking agnathans and jaw-possessing gnathostomes have relatively similar facial features as embryos, then how does one species develop a hinged jaw while the other remains relegated to a jawless existence? One answer might lie in the absence or presence of a Hox code (Fig. 3F). There is a correlation between the lack of *Hoxa2* expression in the first arch and the possession of a hinged jaw joint, but can one infer that the lack of *Hoxa2* allowed the acquisition of a hinged joint? Martin Cohn set out to test this hypothesis by examining Hox expression in jawless animals.

Cohn reasoned that if first arch neural crest cells are *Hox*-positive in a more primitive condition but become *Hox*-negative through evolution, then theoretically, cells would be at liberty to respond to new signals in their changing environment. Such a newly acquired plasticity might then allow for adaptive variations in the jaw structures formed by these neural crest cells. Cohn examined jawless lamprey embryos and found that *HoxL6* was expressed in the first pharyngeal arch, a location which in jawed embryos is *Hox* negative (Cohn, 2002).

While this finding shows a parallel between loss of *Hox* expression and gain of a hinged jaw, it could just as well represent an odd twist of fate for lampreys as opposed to being a molecular feature of a more primitive evolutionary condition. Cohn therefore turned to a more primitive animal to bolster his argument. He used the amphioxus, a vertebrate-like cephalochordate that lacks neural crest but possesses a Hox cluster (Ferrier *et al.*, 2000), and as he had found with lampreys, the Hox homolog *AmphiHox6* was also expressed in the head region of amphioxus (Cohn, 2002). These data also showed that the development of a hinged jaw joint was not related to the acquisition of the neural crest.

Rarely, however, is the answer as straightforward as this; other investigators have examined different species of lamprey and failed to detect *Hox* expression in the first arch (Takio *et al.*, 2004). A resolution to this controversy has not been forthcoming yet.

## 3. *Hox* Gene Regulation by AP2

Although *Hoxa2* is implicated in the agnathan alteration, the protein certainly does not function alone. In fact, some of the most compelling data for the importance of Hoxa2 in craniofacial development has come from studies on the modulator of this modulator: in some neural crest cells, *Hoxa2* expression is controlled by another transcription factor, AP2 (Maconochie *et al.*, 1999). A recent study conducted in the zebrafish *lockjaw* mutant revealed that when *AP2* is lost *Hoxa2* expression is disrupted, causing the second arch to undergo a partial homeotic-like transformation into a first arch (Knight *et al.*, 2004). This morphological conversion bears a strong resemblance to the phenotypes of $Hoxa2^{-/-}$ mice (Gendron-Maguire *et al.*, 1993; Hunter and Prince, 2002; Rijli *et al.*, 1993), which suggests that a primary function of AP2 is to modulate *Hoxa2* expression in the neural crest. Analyses of the *mont blanc* zebrafish mutant bear out this conclusion, since AP2 inactivation arrests neural crest cell differentiation in all but the first pharyngeal arch (Barrallo-Gimeno *et al.*, 2004). The first arch is spared because *Hoxa2* is not normally expressed in this region so loss of the transcription factor has no ill effect on its development. An AP2 binding site has been identified in the *Hoxa2* promoter region, further strengthening the argument that at least one function of AP2 is the regulation of Hoxa2 in neural crest cells. This is not, however, the entire story, since other investigators have shown that deletion of AP2 in Hoxa2 positive neural crest cells produced only a mild phenotype (Brewer *et al.*, 2004). One obvious explanation for the milder-than-predicted phenotype is functional redundancy, since combined null mutations in *AP2* and *Hoxa2* cause early embryonic lethality (Williams, personal communication). Another possibility is that AP2 function in the neural crest may be masked by the fact that AP2 also

has a function in the surface ectoderm. From studies in avian embryos we know that signals emanating from facial ectoderm can control gene expression in the underlying neural crest (Hu *et al.*, 2003), and understanding this type of epithelial-mesenchymal regulation may be the key to unlocking the functions of *Hoxa2* and *AP2*.

The regulatory relationship between *Hoxa2* and *AP2* is a long-standing one, as Marianne Bronner Fraser and her colleagues have shown. *AP2* is expressed in lamprey embryos and even cephalochordates, despite their lack of a neural crest (Meulemans and Bronner-Fraser, 2002). The fact that *AP2* is expressed in the surface ectoderm of amphioxus could mean that its primary and most important function is in this tissue, but experimental evidence for this is still lacking. One other troubling question is related to the coincident expression of *AP2* and *Hoxa2* in many craniofacial tissues except the first arch and the facial ectoderm. If the primary function of AP2 is to induce *Hoxa2* and *AP2* is expressed in the first arch, then how do animals with hinged jaws repress *Hoxa2* in the first arch? This kind of ying/yang issue lies at the heart of understanding how evolutionary advances, such as the development of a hinged jaw joint, come about.

## 4. Species-Specific Differences in Neural Crest Cell Migration

In fish, frogs, and chick, the delamination and migration of cranial neural crest cells are initiated in concordance with neural tube closure. In mice, neural crest cells delaminate and migrate prior to fusion of the neural folds (reviewed in Kulesa *et al.*, 2004). Are these differences in the birthdays and early migration of neural crest cells related in any way to species-specific craniofacial differences? An answer to this relatively simple question has not been forthcoming. Although neural crest transplantations between species demonstrate that grafted donor cells can migrate into host pharyngeal arches (Mitsiadis *et al.*, 2003), the resulting facial architecture is difficult to evaluate. The chimeras do not resemble host chick faces but neither do they bear much resemblance to a mouse. In addition, dissimilarities in facial features cannot merely be ascribed to the differences in birthdays between murine and avian neural crest cells; the axial position of neural crest cells and the structures into which they migrate are also different between the species. In birds, cranial neural crest cells derived from rhombomere 3 contribute to the most proximal region of the first arch, but in mice, frogs, and fish these same cells contribute to the more distal portions of the first arch. In addition, the quantity of neural crest cells arising from the rhombomeres of different species also varies. In frog, fish, and mouse, rhombomere 5 contributes more cells than rhombomere 3, while the opposite is true in chick (reviewed in Kulesa *et al.*, 2004). Could simple differences in the numbers of neural crest cells account for the differences in facial structures

among species? Avian embryos also have elevated levels of cell death in premigratory populations of neural crest in rhombomere 3 and rhombomere 5 (Guthrie and Lumsden, 1991). This patterned cell death is not observed in fish or frogs (Ellies *et al*., 2002). Could these differences be responsible for variations in neural crest migration between amniotes and anamniotes? Perhaps a less restrictive pattern of neural crest migration is one of the reasons that fishes and frogs bear more of a resemblance to one another than they do to lower vertebrates such as lamprey and axolotl.

## 5. Pharyngeal Endoderm and Craniofacial Patterning

Which tissues are responsible for establishing the pattern of the craniofacial features? Are neural crest cells imbued with positional information that allows them to carry out a species-specific pattern of differentiation? Or are the surrounding epithelia—which include the neural ectoderm of the forebrain, the facial ectoderm lining the surface of the pharyngeal arches and frontonasal prominence, and the pharyngeal endoderm coating the inner surface of the pharyngeal arches—responsible for patterning? If we had an answer to this question then understanding species-specific development would be a relatively straightforward proposition. We could meticulously scrutinize the pattern-generating tissue throughout a range of time points and in a variety of species for the subtle variations in gene expression that ultimately lead to differences in facial form, and thus begin to formulate a model by which diversity is generated in the Animal Kingdom.

In that light, we consider a study designed to test the role that neural crest play in pharyngeal arch formation (Veitch *et al*., 1999). The neural tube was ablated prior to neural crest migration and despite the absence of neural crest, pharyngeal arches formed and were properly regionalized (Veitch *et al*., 1999). These surprising findings were some of the first evidence that neither the formation nor the patterning of the pharyngeal arches is absolutely dependent on neural crest cells. Which tissue is then patterning the pharyngeal arch skeleton?

The endoderm has emerged as the most likely candidate for patterning this region of the craniofacial complex. Pharyngeal clefts were in evidence before the evolution of the neural crest (Gans and Northcutt, 1983; Northcutt and Gans, 1983) again suggesting that the endoderm may have played an early role in pharyngeal arch patterning. In more recent years, direct experimental proof has come from analyses of zebrafish mutants. For example, zebrafish *van gogh* (*vgo*) mutants are characterized by an absence of pharyngeal segmentation and a failure of the surrounding mesoderm to pattern correctly (Piotrowski and Nusslein-Volhard, 2000). Although hindbrain segmentation proceeds normally in *vgo* mutants, the endodermal gill slits (i.e., pharyngeal clefts) do not form. Consequently, neural crest cells exiting from the rhombencephalon fuse in the

ventral surface due to the lack of pharyngeal pouches; the end result is a lack of skeletal elements in the pharyngeal region (Piotrowski and Nusslein-Volhard, 2000). This phenotype suggests that the segmentation of the neural crest and the organized differentiation of crest cells to form the pharyneal skeleton are primarily determined by endodermal signaling.

Another zebrafish mutant called *casanova* lacks the entire viscerocranium including the pharyngeal cartilage; the neurocranioum, however, is relatively unaffected (Alexander *et al.*, 1999). When wild type pharyngeal endoderm was grafted into these mutants the defective head skeleton was rescued (Crump *et al.*, 2004a; David *et al.*, 2002), which supports the hypothesis that the pharyngeal endoderm is responsible for patterning the pharyngeal arch skeleton.

Couly and colleagues expanded on these seminal experiments by performing a delicate and extensive set of ablations and transplantations of defined regions of pharyngeal endoderm (Couly *et al.*, 2002). By segregating the endoderm into four transverse stripes that corresponded to the diencephalon (stripe I), anterior (stripe II) and posterior mesencephalon (stripe III), and metencephalon (stripe IV) they were able to show that removal of these regions of endoderm resulted in the reduction or absence of the nasal capsule and upper beak (when stripe I was removed), Meckel's cartilage (removal of stripe II), and the articular; quadrate, and proximal portions of Meckel's cartilage (removal of stripes III and IV) (Couly *et al.*, 2002). The group was able to show that implantation of quail endodermal stripes II and III above the endogenous stripes II and III resulted in supernumerary lower jaws positioned above the host jaw (Couly *et al.*, 2002). Furthermore, rostrocaudal inversion of these grafts resulted in the ectopic lower jaw developing toward the back of the head (Couly *et al.*, 2002). Taken together, these data support the hypothesis that patterning and orientation of the pharyngeal arch skeleton is dependent on the endoderm. In turn, the pharyngeal endoderm is able to instruct the Hox expressing neural crest as to the size, morphology, and orientation of the pharyngeal skeletal elements. The obvious question is then, are there species-specific differences in the molecular composition of the pharyngeal endoderm? To answer that question, we first need to know which signals within the pharyngeal endoderm are responsible for at least some aspect of this patterning information. Once again, the zebrafish provides some of the most persuasive solutions to this inquiry.

In the zebrafish *acerebellar* (*ace*) mutant, the loss of *Fgf8* results in deformed pharyngeal pouches and the reduction of the hyoid cartilage (Draper *et al.*, 2001; Reifers *et al.*, 1998; Roehl and Nusslein-Volhard, 2001). Recently, Chuck Kimmel and his colleagues examined in detail the role of Fgf8 in pharyngeal endoderm patterning (Crump *et al.*, 2004a). They observed deformed pharyngeal pouches and the reduction of the hyoid cartilage. The pharyngeal pouches form from the directed lateral migration

of endodermal cells, and it is this step that is disrupted in *Fgf8*$^{-/-}$ animals (Crump *et al*., 2004a). When Fgf signaling is further reduced by eliminating *Fgf3* the result is a complete failure of pouch formation. In these animals, the pharyngeal endoderm was present but the lateral migration of the endoderm was disorganized and consequently, the hyoid and branchial cartilages were truncated, similar to the *ace* phenotype (Crump *et al*., 2004a).

Experiments conducted in mice offer further support for the role of Fgf signaling in pharyngeal endodermal patterning. *Fgf8* compound heterozygous (*Fgf8*neo/–) mutant embryos exhibited hypoplasia of the first and second pharyngeal arches and their associated clefts (Abu-Issa *et al*., 2002). Although neural crest cells migrated appropriately into the arches, once they arrived they underwent premature programmed cell death, both in areas adjacent and distal to sites of normal *Fgf8* expression (Abu-Issa *et al*., 2002). These data imply that Fgf signaling from the pharyngeal endoderm is critical for the viability of cranial neural crest cells.

Fgfs are not the only molecular signal in the pharyngeal endoderm; endothelin-1 (Edn1) is an intercellular signaling molecule that is expressed in the mesoderm of the pharyngeal arches, as well as in the epithelia of the arches. Both mammalian and teleost data indicate that Edn1 is involved in dorsoventral patterning of the arches (Miller *et al*., 2000; Ozeki *et al*., 2004; Remuzzi *et al*., 2002; Schilling *et al*., 1996), perhaps by establishing a morphogen gradient (Gurdon and Bourillot, 2001; Gurdon *et al*., 1994). Downstream targets of Edn1, such as the bHLH transcription factor Hand2 and the homeobox transcription factor Bapx1, are also involved in dorsoventral patterning in the anterior pharyngeal arches (Miller *et al*., 2003). Specifically, *Hand2* plays a role in specifying the ventral pharyngeal cartilages of the lower jaw and *Bapx1* in specifying the jaw joint (Miller *et al*., 2003; Tucker *et al*., 2004; Wilson and Tucker, 2004).

Recently, yet another zebrafish mutation has shed light on the role of pharyngeal endoderm in craniofacial patterning. *Integrin$\alpha$5* mutants are characterized by the loss of cartilages derived from second arch neural crest cells, as well as the reduction of associated cranial muscles and nerves (Crump *et al*., 2004b). This phenotype most likely results from defects in outpocketing of the pharyngeal endoderm, since the transplantation of wild type endoderm (and not neural crest) can rescue first pouch and second arch cartilage development (Crump *et al*., 2004b).

## 6. Neuroectoderm and Craniofacial Patterning

Specification of subdivisions of the brain, which include the forebrain, midbrain, and hindbrain, is thought to take place during gastrulation, and further revision occurs during neurulation (reviewed in Altmann and Brivanlou, 2001). Following establishment of the subdivisions, each region develops

largely independently from the others, under the influence of local organizing centers like the isthmic boundary, which organizes the midbrain, and rhombomere 4, which organizes the hindbrain (Jaszai *et al.*, 2003). Conditional inactivation of *Fgf8* in the midbrain results in a failure to maintain expression of *Wnt1* as well as *Fgf17, Fgf18*, and *Gbx2*; consequent ectopic cell death results in the loss of the midbrain and cerebellum (Chi *et al.*, 2003).

Similar patterning mechanisms operate during zebrafish brain development. Through misexpression and transplantation studies Kimmel and colleagues found that Fgf8 and Fgf3 emanating from rhombomere 4 were responsible for the initiation and development of more posterior rhombomeres, which ultimately give rise to the hindbrain (Maves *et al.*, 2002). Although blocking translation of either *Fgf8* alone or *Fgf3* alone does not produce severe hindbrain defects, blocking both together inhibited development of rhombomeres 5 and 6 (Maves *et al.*, 2002). On a molecular level, these blockages lead to a complete absence of *Krox-20, Val,* and *Hoxb1* expression, all of which are involved in rhombomere 5/6 development (Maves *et al.*, 2002). Transplantation of rhombomere 4 tissue that contains endogenous Fgf8 promotes development of r5 and r6-associated entities (Maves *et al.*, 2002). When considered together, these data clearly demonstrate a role for Fgf8 emanating from the neuroectoderm in proper craniofacial development.

In birds, indirect evidence suggests that Fgf8 signaling from the neuroectoderm promotes development of the jaws (beaks, in chickens). For example, when *Hoxa2* expressing neural crest cells are removed, the result is the loss of *Fgf8* expression in the forebrain and in the first arch ectoderm, and consequently an open neural tube and arrest in facial skeletal development (Creuzet *et al.*, 2004). Delivering Fgf8 protein appears to rescue development of the facial skeleton, in part because the growth factor promotes cell proliferation in rhombomere 3 (Creuzet *et al.*, 2004). Their data also indicates that the expression of *Fgf8* in the first arch ectoderm is dependent on signals from the neural crest, thereby invoking a positive feedback loop between epithelia and mesenchyme that supports craniofacial morphogenesis (Creuzet *et al.*, 2004). Given its critical role in maintaining cell viability in the craniofacial prominences, one might legitimately wonder if variations in Fgf signaling account for variations in facial form between the species. One obvious mechanism by which the facial primordia could be shaped is by regulating local sites of cell proliferation and death. Such a mechanism operates in sculpting the vertebrate limb bud (Boulet *et al.*, 2004; Cretekos *et al.*, 2001). Perhaps an analogous process is responsible for molding the upper and lower jaws and the frontonasal prominence. To date, this type of careful comparison between species for subtle changes in the expression patterns of Fgf ligands, their receptors, and their effectors has not been undertaken.

In addition to Fgf8, a secreted growth factor in the Hedgehog family also plays a critical role in craniofacial morphogenesis. *Sonic hedgehog* (*Shh*) is first expressed in the midline of the neural plate (Fig. 4A), where it plays a crucial role in establishing mediolateral patterning of this tissue, as the null mutation of the protein demonstrates (Chiang *et al*., 1996). As neurulation

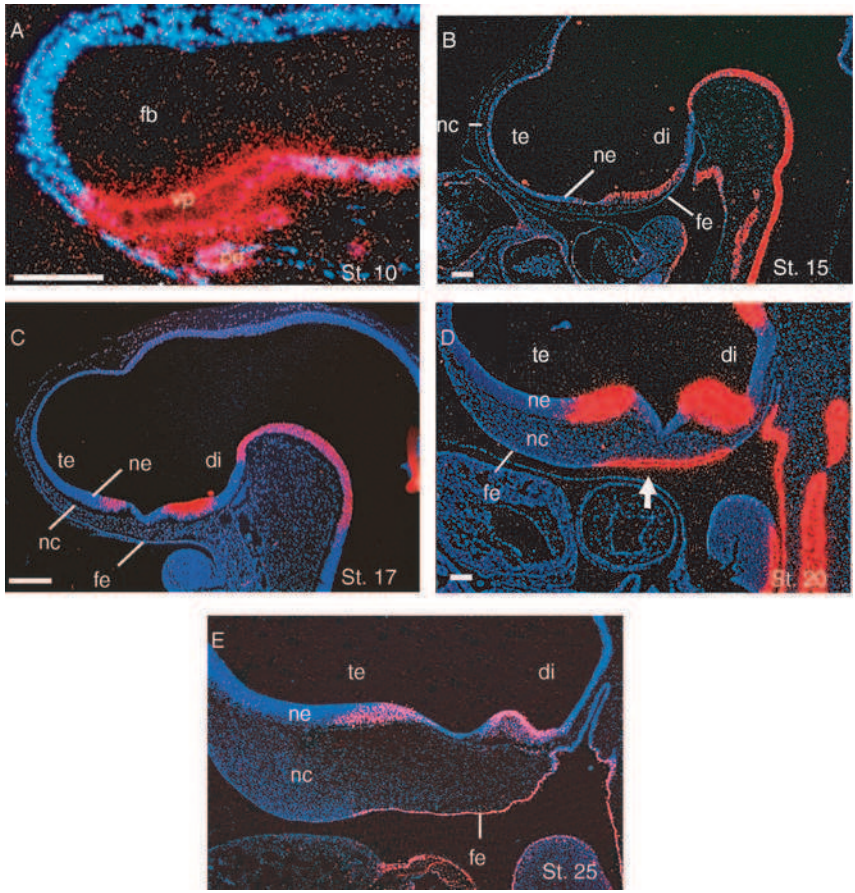


**Figure 4** Ontogeny of *Shh* expression in a developing chick embryo. (A–F) Sagittal sections of chick embryos, where red (pseudocolored using photoshop) represents s*hh* expression as obtained by *in situ* hybridization with $S^{35}$. (A) *Shh* is expressed in the forebrain (fb) at stage 10 in tissues such as the ventral prosencephalon (vp), and pharyngeal endoderm (pe). (B) By stage 15, the prosencephalon has subdivided into the telencephalon (te) and the diencephalon (di); *shh* transcripts are localized to the diencephalic neuroectoderm (ne). (C) At stage 17, *shh* expression is present in the telencephalon. (D) Around stage 20, *shh* is expressed not just in the diencephalic and telencephalic neuroectoderm, but also in the facial ectoderm (fe). (E) From stage 20 onwards, *shh* expression remains constant in the neuroectoderm and facial ectoderm. Panels reproduced courtesy of *Journal of Clinical Investigation*.

proceeds, *Shh* expression is detected in the prosencephalon, which subsequently divides into the telencephalon and diencephalon (Cordero *et al.*, 2004) (Fig. 4B). Later still in development, *Shh* is induced in the ventral telencephalon, which is separated from the diencephalic domain by a *Shh*-negative optic recess (Cordero *et al.*, 2004) (Fig. 4C). Finally, *Shh* is induced in ventral facial ectoderm (Cordero *et al.*, 2004) (Fig. 4D), in a domain that is juxtaposed to an *Fgf8* expression domain in the dorsal ectoderm (Hu *et al.*, 2003). Removing the forebrain domain of *Shh* expression (Cordero *et al.*, 2004; Marcucio *et al.*, 2005) or the facial ectodermal domain of the same (Hu *et al.*, 2003) blocks proper morphogenesis of the frontonasal prominence, which gives rise to the middle and upper face. Although skeletal elements form, their dorsoventral patterning is disrupted and the results are facial clefting and midfacial hypoplasia (Hu and Helms, 1999; Jeong *et al.*, 2004; Rallu *et al.*, 2002).

In zebrafish, a similar role for Shh signaling has been confirmed. Shh, emanating from the same ventral neuroectodermal domain, directly patterned the ventral surface ectoderm without requiring an intermediate signal from the neural crest (Eberhart and Kimmel, personal communication). In fish as well as in birds, loss of neuroectodermal Shh prevents neural crest cells from aggregating into condensations and forming skeletal elements (Cordero *et al.*, 2004, 2005). Collectively, these studies indicate that the forebrain neuroectoderm imparts essential information, in the form of Shh and Fgf signaling, that patterns and guides the differentiation of the frontonasal neural crest.

## 7. Facial Ectoderm and Craniofacial Patterning

For years, we have known that Shh plays an integral role in craniofacial development. Removing, manipulating, blocking, and ectopically expressing *Shh* in the face all profoundly alter normal craniofacial morphogenesis (Cordero *et al.*, 2004; reviewed in Cordero *et al.*, 2005). In addition, humans with defects in Shh signaling exhibit a range of craniofacial malformations (reviewed in Edison and Muenke, 2003; Roessler and Muenke, 2003). Recent studies have begun to parse out how perturbing *Shh* expression in different tissues gives rise to different maladies.

Some of the initial strides toward determining the function of Shh in craniofacial development came from ablation studies, in which removal of *Shh* expressing frontonasal ectoderm led to the arrest of frontonasal outgrowth (Hu and Helms, 1999). Removing non-*Shh* expressing facial ectoderm had no discernible effect on facial morphogenesis (Hu and Helms, 1999), indicating that Shh played a role in directing the patterned outgrowth of the frontonasal prominence. The data also suggested that not all facial ectoderm was equivalent in its capacity to maintain the growth of the facial processes.

This conclusion, while true, proved to be an oversimplification of Shh's participation in craniofacial development. Shh does not act in isolation, but rather in concert with other molecules to produce patterned outgrowth of the middle and upper face. In subsequent study, we identified a molecular boundary in the frontonasal ectoderm, defined by the juxtaposed domains of *Fgf8* and *Shh*, which presaged the initial site of frontonasal process outgrowth (Hu *et al*., 2003) (Fig. 5). Fate maps confirmed that this boundary region, which we referred to as the frontonasal ectodermal zone (FEZ), later demarcated the dorsoventral axis of the upper beak. Ectopic transplantation of the FEZ activated a cascade of molecular events that reprogrammed the developmental fate of frontonasal, non-*Hoxa* expressing mesenchyme, which resulted in duplications of upper and lower beak structures (Hu *et al*., 2003). The dorsoventral polarity of these ectopic structures was entirely dependent on the orientation of the transplanted FEZ (Hu *et al*., 2003).

The location of the FEZ coincides with the location of the major axes of growth in avian beaks. The epithelial domains of *Fgf8* and *Shh* work together to facilitate patterning and beak outgrowth (Abzhanov and Tabin, 2004). Using an *in vitro* approach, Cliff Tabin and his colleagues showed that misexpression of *Shh* alone in neural crest cultures failed to induce chondrogenesis but misexpression of *Shh* with *Fgf8* and *Fgf2* induced chondrogenesis at high rates (Abzhanov and Tabin, 2004). These results were mirrored *in vivo*, in which coinfection with *Shh* and *Fgf8* led to abnormal cartilaginous outgrowths reminiscent of beak tip formation (Abzhanov and Tabin, 2004). These ectopic outgrowths were not patterned properly (Abzhanov and Tabin, 2004), presumably because retroviruses cannot duplicate the endogenous juxtaposition of their gene expression domains. Nevertheless, the data clearly demonstrates that Fgf8 from the FEZ region of the ectoderm acts in concert with Shh to initiate and maintain formation of the upper and lower beak from neural crest derived mesenchyme.

Knowing what a molecule does is quite different, however, from knowing how it is regulated. The question of what maintains the expression of *Fgf8* in facial ectoderm remained unsolved until a recent study by Le Douarin and colleagues, which revealed that *Fgf8* expression is maintained in surface ectoderm by underlying neural crest cells (Creuzet *et al*., 2004). When Le Douarin and colleagues removed neural crest cells from chick embryos, the ectodermal domains of *Fgf8* were abolished and many facial skeletal elements failed to form. These effects could be partially rescued by addition of exogenous Fgf8 (Creuzet *et al*., 2004). What actually induces *Fgf8* in facial ectoderm, however, remains unknown. The expression domain is established around the time of neurulation and persists in the facial ectoderm long before neural crest cells arrive in this location. A series of experiments by

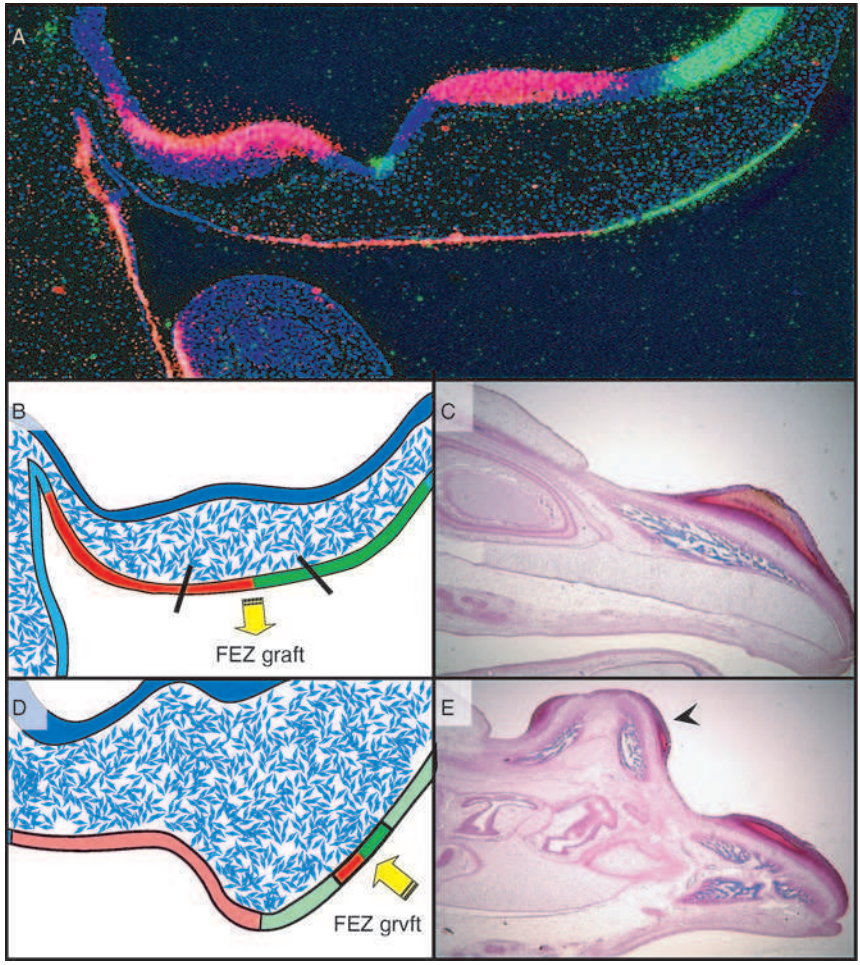


**Figure 5** The facial ectodermal zone (FEZ) is important for outgrowth and patterning of the frontonasal prominence. (A) A sagittal section of a stage 20 chick embryo; red corresponds to a *shh*-expressing domain and green corresponds to a *fgf8*-expressing domain (as obtained by *in situ* hybridization with $S^{35}$, and pseudocolored with photoshop). B and D are schematic drawings of similar sections of stage 20 donor (B) and stage 25 host (D) chick embryos, respectively. (C, E) Sections of stage 36 control and transplanted embryos, respectively, stained with trichrome. Transplantation of the FEZ (yellow arrows) from a stage 20 chick donor (B) to a stage 25 chick host (D) causes an ectopic beak to form by stage 36 (E, black arrowhead). Donor FEZ tissue is indicated by dark red and green, while host FEZ is indicated by light red and green.

Paul Sharpe and coworkers argue that, at least for Fgf8 in the oral ectoderm, the endoderm is responsible for Fgf induction and thus regionalization of the overlying ectoderm (Haworth *et al.*, 2004).

## 8. Ephrins and Craniofacial Patterning

Throughout this chapter, we have tried to present the process of craniofacial patterning as one that occurs as a result of a combination of signaling molecules acting on a variety of tissues. However, till this point, a molecular link between tissue–tissue interactions has been lacking. How do the craniofacial tissues coordinate with one another to direct morphogenesis? A possible answer to this question is via Ephrin/Eph signaling.

Perhaps no other molecular pathway described herein epitomizes the concept of reciprocal signaling like Ephrins and their receptors, the Ephs. Ephrins and Eph receptors are membrane bound proteins that function as a ligand-receptor pair (reviewed in Davy and Soriano, 2005). Upon binding, signaling can occur in both the forward (i.e., in the cell that expresses the Eph receptor) and the reverse (in the cell expressing the Ephrin ligand), thus providing a molecular conduit for cross-talk between and among tissues (Davy *et al*., 2004; Holder and Klein, 1999; Poliakov *et al*., 2004). Ephrin ligands and Eph receptors are expressed in a variety of tissues in complementary patterns and also play a role in craniofacial development, perhaps because of their crucial function in directing neural crest migration.

Neural crest cells migrate from the dorsal neural tube into the prominences of the face and in avians, this migratory stream is divided into three components: those neural crest cells originating from rhombomere 2 that migrate into the first arch; those arising from rhombomere 4 that migrate into the second arch; and those neural crest cells from rhombomere 6 that migrate into the third arch (Lumsden *et al*., 1991) (Fig. 6A). Whereas these data suggest that there is a functional consequence to the separation of the streams of migrating neural crest, in frogs the neural crest cells are not separated—yet they still migrate to specific and separate locations without intermingling (Sadaghiani and Thiebaud, 1987). Therefore, a particular set of guidance cues appears to restrict the migration of neural crest cells into distinctive streams, and based on their expression patterns, the Ephrin/Ephs are prime candidates (Fig. 6B).

To elucidate the role of Eph receptors in neural crest migration, truncated receptors, which acted in a dominant negative fashion, were overexpressed in frog embryos. As a result of this overexpression, neural crest cells were scattered and failed to migrate appropriately (Smith *et al*., 1997). These data indicated that Ephrin/Eph signaling was crucial for the proper migration of the cells (Smith *et al*., 1997). Double *in situ* hybridization analyses showed that EphrinB2 and EphA4 positive neural crest cells were initially in contact with one another then separated after migration began, which suggested that Ephrins might act to restrict the migration of subsets of neural crest cells (Smith *et al*., 1997). Additional experiments revealed that perturbing either Eph or Ephrin expression resulted in the dispersal of neural crest cells, which

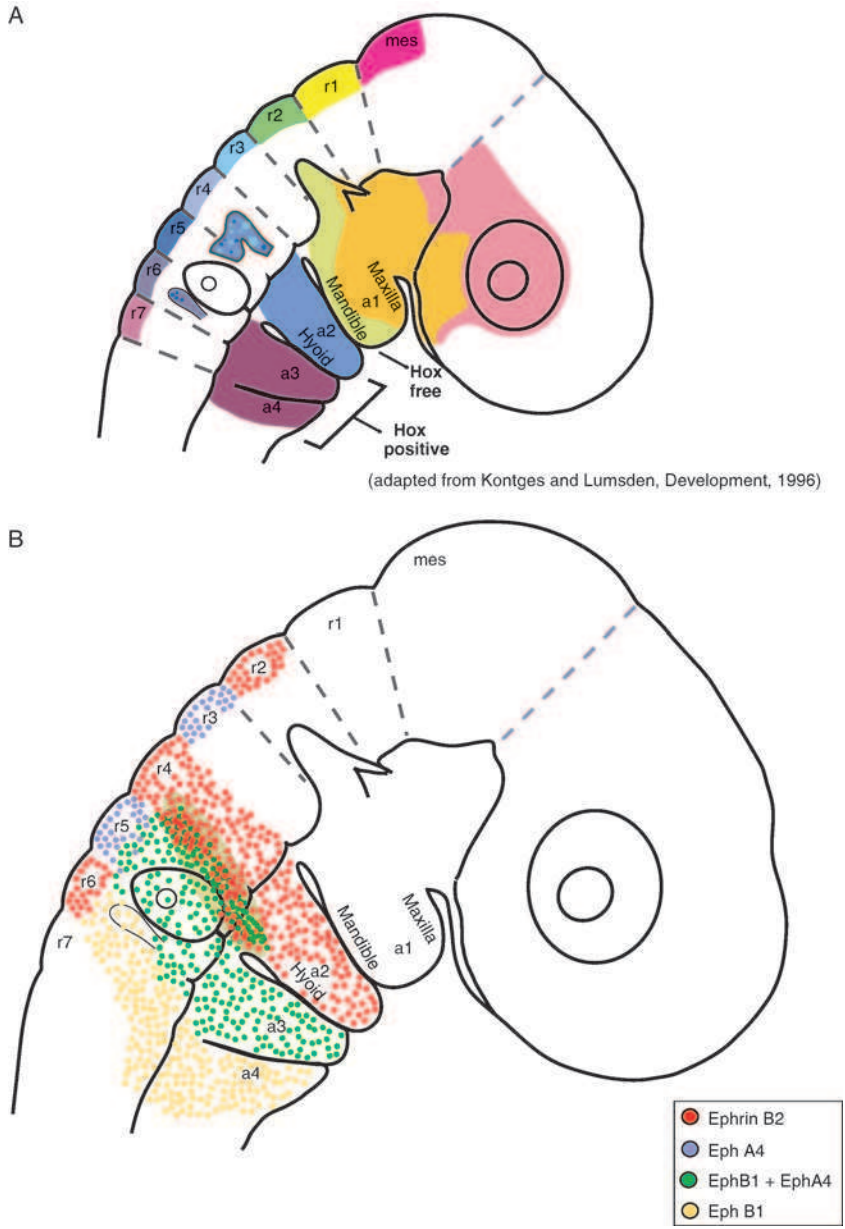


**Figure 6** Ephrin receptor-ligand interactions create boundaries for neural crest migration streams. (A–B) Schematic drawings depicting neural crest migration, and ephrin (eph) expression, respectively. (A) Schematic depicting the migration pattern of neural crest from discrete rhombomeres to corresponding branchial arches. Rhombomeres are color-matched to branchial arches, indicating which rhombomere the neural crest cells in a particular arch or facial prominence hail from. For example, neural crest cells that populate the anterior part of the first arch are colored orange, indicating that the first arch is populated by a mixture of crest

has led to the hypothesis that Ephrin signaling alters the adhesiveness of cells, such that cells destined for one arch may have different adherent properties relative to other neural crest cells. Subsequent experiments in avians indicate that Ephrin/Ephs act as bifunctional guidance cues, responsible for repelling some migrating neural crest cells and stimulating the migration of other cell types (Santiago and Erickson, 2002). If Ephrins and Ephs are necessary and sufficient to dictate neural crest migration patterns, it will be important to elucidate the Ephrin/Eph expression profiles for all arches and determine if an Ephrin/Eph code, similar to that of the Hox code, exists. Regardless, understanding the nature of Ephrin/Eph signaling will surely shed light on the crosstalk that exists between cells, which participate in craniofacial patterning. Further, understanding of molecules necessary for cross-talk at a cellular level will undoubtedly lend itself to elucidating the mechanism of how tissue movements are orchestrated during craniofacial development.

## 9. Something to Sink your Teeth into: Teeth as a Model of Species-Specific Development

Millions of years ago, avians lost their teeth while mammals retained the ability to form a dentition. While sidestepping the issue of what motivated this morphological change, we can still ask the question, what changes had to occur in order for this loss to happen? This question was addressed in the last century by performing tissue recombination experiments, in which avian oral epithelium was combined with mesenchyme from the presumptive molar region of a mouse (Kollar and Fisher, 1980). The result from these experiments was the formation of teeth complete with ameloblasts depositing enamel matrix (Kollar and Fisher, 1980). One recurring problem with these experiments, however, was in proving that the enamel in these chimeric teeth was actually derived from the avian epithelium and was not the result of contamination by mouse epithelium. Nevertheless, the conclusion from this seminal study was that avians lost their teeth not as a consequence of eliminating coding regions for enamel-specific genes from the bird genome but rather because of alterations in the tissue interactions that are required for odontogenesis. And it is precisely this latter point that has been verified in recent years. For example, when avian mesenchyme is combined with

cells from the mesencephalon (pink) and r1 (yellow). (B) Schematic depicting the expression of different eph proteins in rhombomeres, mesoderm, and branchial arches. Interactions between eph ligands and eph receptors may serve to create boundaries between neural crest streams, thereby ensuring that neural crest from a particular rhombomere migrates to the correct arch. For example, ephB2 ligand is expressed by cells in rhombomere 4 prior to migration, while cells expressing eph receptors B1 and A4 populate rhombomere 5 prior to migration. As all of these cells begin to migrate outward from the rhombomeres toward the arches, the eph receptors B1 and A4 interact with eph ligand B2 at the rostral side of the third arch stream. These interactions effectively create a ''barrier'' of sorts that restricts neural crest destined for the third arch from streaming into second arch territory.

mouse odontogenic epithelium, the avian mesenchyme exhibits its odontogenic potential, suggesting once more that avian tissues retain their potential to make teeth if provided with the correct signals (Wang *et al.*, 1998). Paul Sharpe, Tim Mitsiadis, and their colleagues took this study one step further by transplanting into avian embryos mouse neural crest cells destined for the first arch. In those chimeric animals in which neural crest cells populated the arch, they found the induction of tooth-related genes such as *Msx1* and *Pax9* in the mouse mesenchyme, presumably in response to signals emanating from the overlying chick epithelium (Mitsiadis *et al.*, 2003). Thus, these data refine the original conclusion that birds lost their tooth-forming ability because of alterations in tissue interactions that are required for odontogenesis; it now appears that chickens fail to make teeth because their neural crest cells have lost the ability to respond appropriately to signals emanating from the oral epithelium. These data indicate that both neural crest cells and surface ectoderm have species-specific characteristics and both tissues contain information that directs morphogenesis in a species-specific manner.

## 10. Fgf8 and the Concept of the Ectomere

The concept of a molecular tête-à-tête between craniofacial tissues is manifested by the ectomere, a region of epithelium that appears to function as an embryological unit with a well-defined morphological outcome. Similar to rhombomeres, the notion of an ectomere was first presented by Le Douarin and colleagues as a segment of neuroectoderm and surface ectoderm that moved in register toward a final resting place in the facial prominences, all the while carrying on a molecular dialog that was critical to establishing a particular fate (Couly and Le Douarin, 1990). Although no defined ''code'' of expression is yet known to define the ectomere, data from Paul Sharpe's lab as well as from our own lab suggests that Fgf8 may be a molecular component of this domain (Fig. 7).

Le Douarin and Couly defined the ectomere when they created a fate map of the early neurula and denoted which regions along the anteroposterior neural axis were responsible for contributions to the developing face (Couly and Le Douarin, 1990). Using the quail/chick chimeric system they traced the movements of the presumptive facial and hypobranchial ectoderm, which segregated into neuroectodermal and facial ectodermal domains around the time of neurulation. The neural crest which arose from this region of ectoderm moved in accordance with the surrounding neural and surface ectoderm (Couly and Le Douarin, 1990).

Fifteen years later, Paul Sharpe and his colleagues described an ectomere-like concept that operates during tooth development. The regionalization of the oral ectoderm into *Fgf8*-positive (molar) and *Fgf8*-negative (incisor) domains occurs long before the pharyngeal arches have formed; the regionalization is evident as early as neurulation (Haworth *et al.*, 2004). In our own lab we have examined the expression domains of Fgf8 and found that the

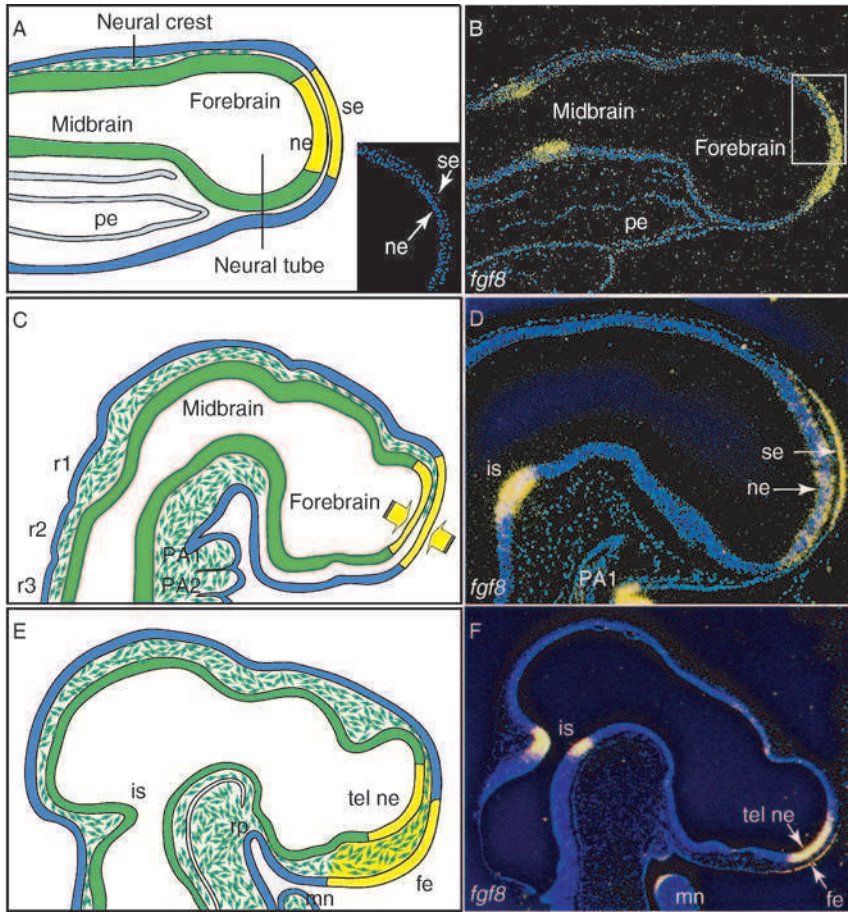


**Figure 7** Fgf8 may delineate an "ectomere." (A, C, E) Schematic drawings of a developing chick embryo, illustrating neural crest migration during craniofacial development. (B, D, F) *Fgf8* expression in the chick craniofacial complex during embryonic development (obtained by *in situ* hybridization with $S^{35}$ and pseudocolored with photoshop). (A–D) As the closed neural tube begins to differentiate into the central nervous system, the neural crest begins to migrate anteriorly from particular rhombomeres (r1–r3) into discrete regions of the face. During this process, the neuroectoderm (ne) and surface ectoderm (se) components of the ectomeres continue to remain aligned (as shown by yellow arrows). (E–F) As neural crest migration nears completion, the neuroectoderm and facial ectoderm (fe; late-stage term for surface ectoderm) components of the ectomere are no longer aligned. C, caudal; is, isthmus; mes, mesencephalon; mn, mandible; PA, pharyngeal arch; pe, pharyngeal endoderm; rp, Rathke's pouch; R, rostral; tel ne, telencephalic neuroectoderm.

neuroectodermal domain of *Fgf8* is aligned with the facial ectodermal domain, until the intervening frontonasal neural crest separate the two epithelia (Helms *et al.*, 2005). As neural crest migration nears completion, the neuroectoderm and facial ectoderm components are no longer aligned, but subsequent experiments show that the facial ectodermal domain of *Fgf8* is

essential to form a border that establishes the dorsoventral boundary of the upper beak (Abzhanov *et al.*, 2005; Hu *et al.*, 2003). We still do not know, however, if Fgf8 signaling is the impetus for the formation of this boundary.

To date, only the functionality of the ectomere is certain. The coupled migration of tissues that eventually give rise to a structure has been established. What remains a hypothesis is the molecular delineation of the region. If Fgf8 is a candidate for this role, then labeling *Fgf8* expressing cells within this area and charting their movement may be necessary to definitively prove its role in defining the ectomere. In addition, studying the concept of ''organizer''-like domains throughout the craniofacial region may in fact be the best mechanism to examine crosstalk between tissues.

## C. Third Step in Craniofacial Patterning: Controlling Growth, Differentiation, and Cell Death

Invariably there are species-specific differences in the early patterning steps described above, but few studies have investigated these stages in detail. Instead, the majority of studies that shed light on the basis for different craniofacial forms have focused on later stages of development, when cell proliferation, differentiation, and death act to shape the craniofacial skeleton. In the following section, we will present an overview of these studies, which elegantly illustrate how studies in mice, fish, and fowl contribute to our understanding of the forces driving species-specific form.

### 1. Species-Specific Differences in Craniofacial Form

To investigate the genetic basis of craniofacial diversity, one would ideally like to have a model organism characterized by enormous variation in facial form. Despite this variability, an ideal model organism would retain the ability to interbreed between the species. This latter feature is especially useful because interbreeding between disparate animals would generate progeny with intermediate phenotypes available for analysis. In addition, an ideal model would have evolved relatively recently so that hybrid incompatibility does not pose a problem. For all of these reasons, the small East African rift fish known as the cichlid has become a favored organism for study (Fig. 8). An astonishing 200 species are estimated to have evolved within the last 10 million years (Kocher, 2004), which certainly places cichlids on the fast track in terms of evolutionary diversity. This rapid diversification also means that interbreeding is possible.

Using the detailed description of cichlid skeletons as a starting point (Barel, 1983), Albertson and colleagues mated two species of cichlids with drastically different facial features, and their progeny were analyzed using Quantitative Trait Loci (QTL) to map locations in the genome that cosegregated with alterations in the jaw skeletons (Albertson *et al.*, 2003a,b). The

traits analyzed reflected general differences in the height, length, and width of the jaws, and the results of the study indicated that modifications in a small number of loci could account for the variations observed in jaw morphology (Albertson *et al.*, 2003a,b). In other words, generating

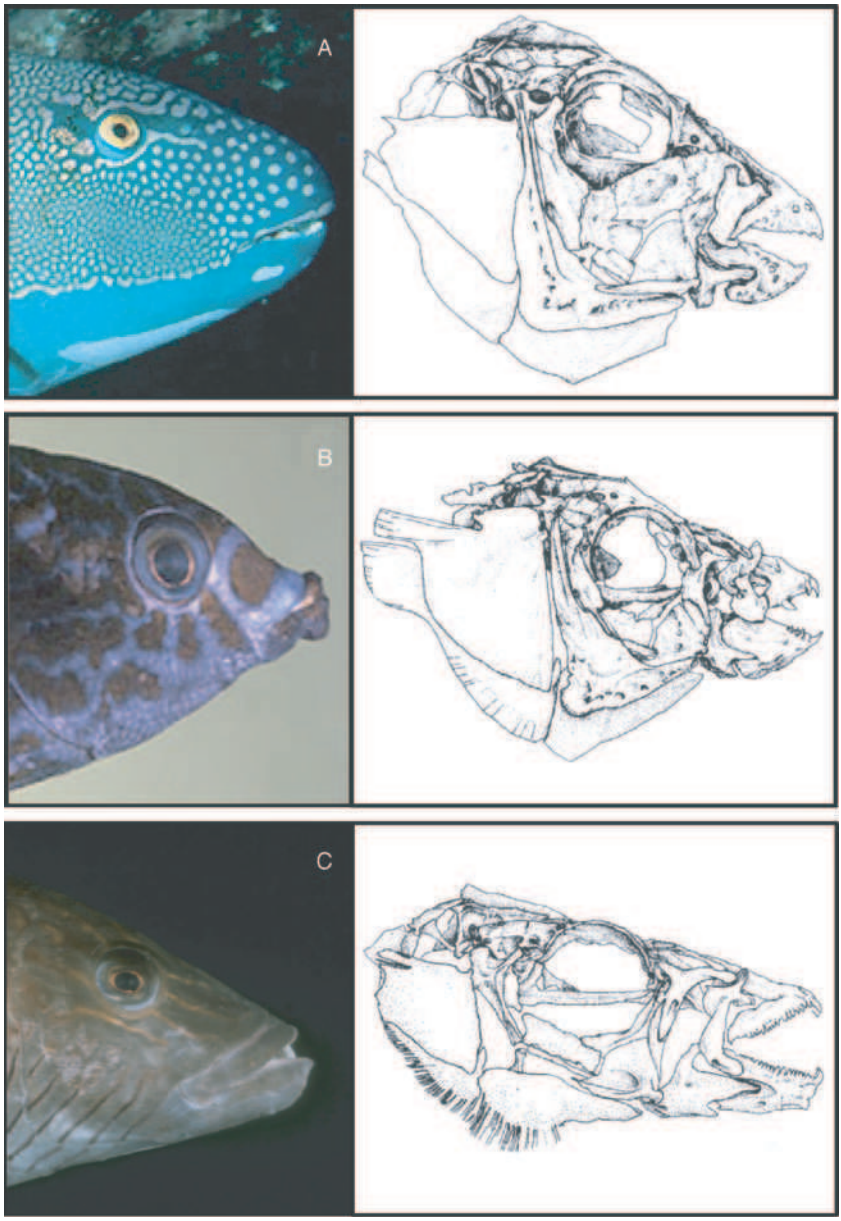


**Figure 8** *Continued.*

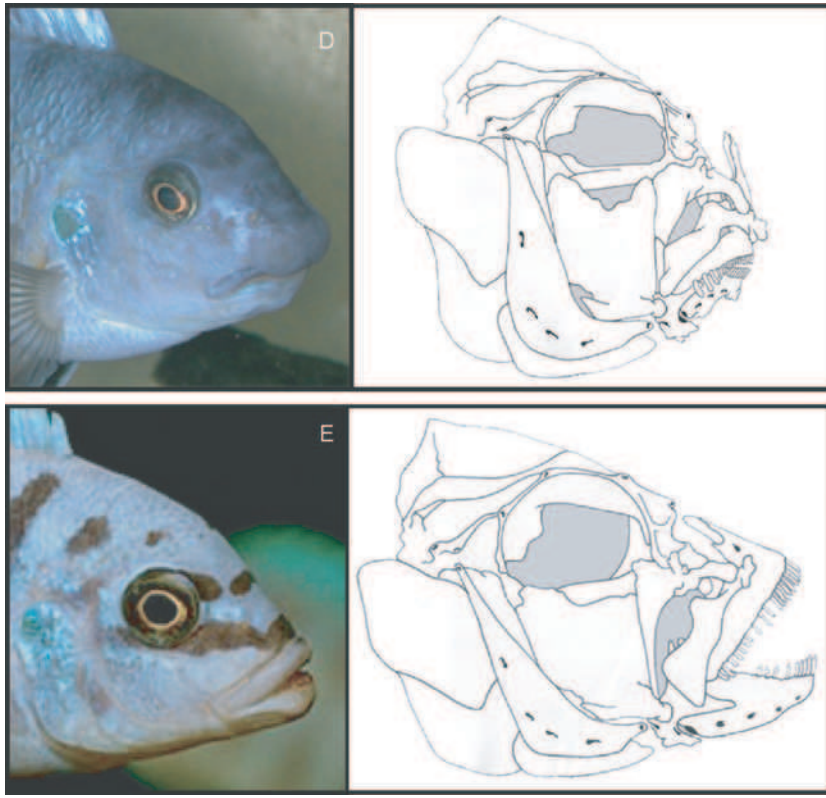


**Figure 8** Bmp4 expression levels control beak depth and height. (A and B) Large ground finches have thick, broad, and long beaks. (C) The embryonic beak of a ground finch exhibits high *Bmp4* expression levels, which promote chondrogenesis and therefore increased beak height, length, and depth (shown by red arrow). (D) Misexpression of *Bmp4* in the frontonasal process mesenchyme of chick embryos produces a noticeably broader and thicker upper beak, paralleling the beak morphology of the ground finch. (E) Alcian staining of chick embryos injected with RCAS-Bmp4 reveals enlarged skeletal elements in the upper beak. (F and G) Cactus finches have thinner, shorter, and narrower beaks. (H) The embryonic beak of a cactus finch exhibits very little *Bmp4* expression, and chondrogenesis of the beak is not as pronounced, which leads to an overall smaller beak. (I) Misexpression of noggin, a Bmp4 antagonist, in frontonasal process mesenchyme of chick embryos produces a noticeably thinner and narrower upper beak, paralleling the beak morphology of the cactus finch. (J) Alcian staining reveals markedly stunted upper beak skeletal elements in chicken embryos injected with RCAS-noggin. Images B–D and G–I from Abzhanov, A., Protas, M., Grant, B. R., Grant, P. R., and Tabin, C. J. (2004) Bmp4 and Morphological Variation of Beaks in Darwin's Finches. *Science* **305,** 1462; Images E, J from Wu, P., Jiang, T. X., Suksaweang, S., Widelitz, R. B., and Chuong, C. M. (2004). Molecular Shaping of the Beak. *Science* **305,** 1465–1466; images reproduced with permission of *Science.* Images A, F courtesy P. Grant, A. Abzhanov and C. Tabin.

enormous variability in facial features does not mean that an enormous number of genes have to change. Rather, the data suggest that as few as a single locus might be responsible for changes in jaw morphology.

## 2. Bmp Signaling in Teleosts: Not the only Fish in the Sea

The next obvious question is whether a single locus contains any candidate genes that might be responsible for such morphological variations. Norihiro Okada and his colleagues focused on one such candidate, Bmp4 (Terai *et al.*, 2002). These scientists took advantage of the fact that cichlids occupying the East African Great Lake exhibit a more varied jaw skeleton relative to the cichlids occupying the nearby rivers (Terai *et al.*, 2002). Okada postulated that the more highly speciated Lake cichlids should exhibit a higher frequency of amino acid substitutions in those genes that were involved in some way with the morphological variations (Terai *et al.*, 2002). Although no significant differences in substitution rates were observed in a number of possible candidates, a different story emerged when the amino acid sequence of Bmp4 was analyzed (Terai *et al.*, 2002). While the coding region for Bmp4 was not changed between the Lake and river cichlids, the group did uncover variations in the prodomain of the molecule (Terai *et al.*, 2002). This finding implied that posttranslational modifications in Bmp4 could account for at least some of the variations in cichlid jaw morphology.

## 3. Bmp Signaling in Fowl: A Tough Nut to Crack

Darwin noted that among the 13 species of finches on the Galapagos, "a nearly perfect gradation may be traced from a beak extraordinarily thick to one so fine that it may be compared with that of a warbler." (Darwin, 1859). These morphological variations are also evident during the embryonic period, but at some point during development, all avian embryos look nearly identical. As Darwin did, one might ask how such variations came about? Cliff Tabin and his coworkers addressed this question and began by scrutinizing embryos from the same finches that Darwin examined (Abzhanov *et al.*, 2004). At a time during embryonic development when the Ground finch and its cactus-dwelling cousin appeared nearly identical, *in situ* hybridization analyses revealed that the *Bmp4* expression pattern was already altered (Abzhanov *et al.*, 2004). Tabin found that even though the frontonasal prominence was roughly equivalent in size and shape, *Bmp4* expression was higher in the Ground finch mesenchyme than in the Cactus finch mesenchyme (Abzhanov *et al.*, 2004) (Fig. 9). To experimentally test whether spatial and temporal changes in *Bmp4* expression could account for the relative size and shape differences in the different finch beaks the investigators turned to chicken embryos and misexpressed *Bmp4* throughout the mesenchyme of the frontonasal prominence (Abzhanov *et al.*, 2004). This process converted the narrow, short chick beak into much broader, bigger beak that bore a resemblance to the large Ground finch (Abzhanov *et al.*, 2004). When considered in light of the cichlid data, one cannot help but wonder if species-specific differences in the regulatory region of the *Bmp4* gene could be responsible for the different expression patterns in the Cactus

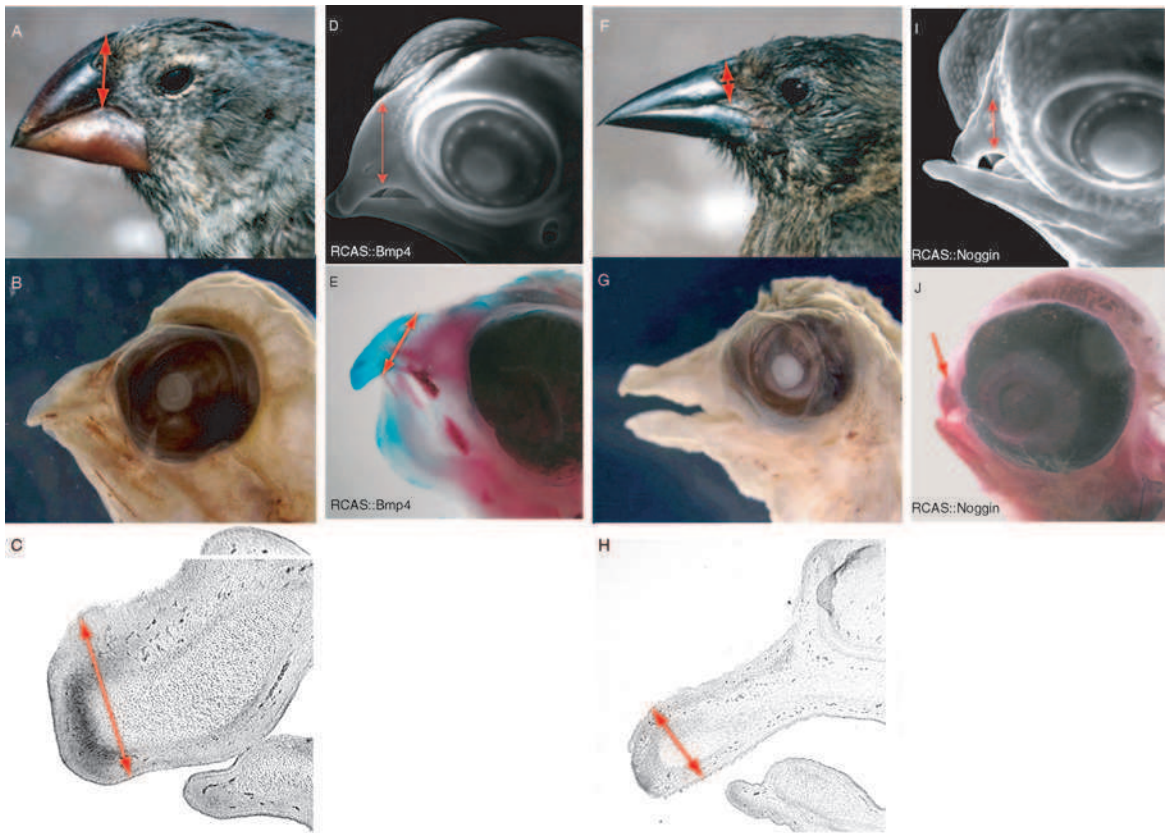


**Figure 9** Morphological differences between jaws of cichlids. (A) The river-dwelling cichlid *Metraclima Zebra* (left) has a jaw structure (right) that is well-suited for sucking. (B) The Great Lakes cichlid *Labeotrophus Fuelleborni* (left) has a jaw structure (right) that is well-suited for biting. Photos of *Labeotrophus Fuelleborni* and *Metraclima Zebra* courtesy of J. Dion and F. Hagblom, respectively. Drawings from Albertson, R. C., Streelman, J. T.,

and Ground finches and whether differences in gene expression domains can account for some of the morphological variation in the bird beaks.

### 4. Three Faces of Aves: Chickens, Ducks, Quails, and Craniofacial Patterning

Like cichlids and labrids, birds have evolved particular facial features appropriate for their ecological niches. Seed-eating avians typically have short, narrow beaks while waterfowl generally have broad bills suited for sieving plant matter and aquatic invertebrates. Cheng-Ming Chuong and coworkers made use of these particular differences in avian beak morphology to address whether spatial variations in *Bmp4* expression coincided with spatial differences in growth between two avian beaks (Wu *et al*., 2004). Chuong and his colleagues analyzed growth zones in the chick's frontonasal prominence at three developmental stages. Using BrdU labeling, they detected two zones of proliferation at the lateral edges of the frontonasal prominence that shifted positions with advancing age and eventually corresponded to the facial organizer, the FEZ (Hu *et al*., 2003). In the duck frontonasal prominence, these proliferating zones were located on the lateral edges of the frontonasal prominence and remained separated even with advancing age (Wu *et al*., 2004). These sites of cell proliferation coincided with sites of *Bmp4* expression, and when Bmp signaling was activated or repressed, the width and breadth increased or decreased, respectively (Wu *et al*., 2004).

Taken together with the Darwin finch and African cichlid data, Bmps are gaining increasing recognition as key regulators of craniofacial form. Could similar posttranslational modifications in the regulatory region of Bmp4 exist in other species with drastically different facial morphologies—say, dogs for instance? And is Bmp4 the mediator or the molecular by-product of morphological change? Answers to these questions will undoubtedly shed some much needed light on the process of species-specific morphological diversity.

## D. Putting it All Together: Species-Specific Craniofacial Morphogenesis

One of the crucial steps toward understanding how unique facial patterns are generated in different animals is to narrow down which cell populations control species-specific patterning. The face is derived from three tissues, and all are important for proper morphogenesis of the face. For example, the forebrain neuroectoderm participates in craniofacial patterning not only by

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and Kocher, T. M. (2003). Directional selection has shaped the oral jaws of Lake Malawi cichlid fishes. *Proceedings of the National Academy of Sciences* **100**(9), 5252–5257; reproduced with permission of *Proceedings of the National Academy of Sciences*.

acting as a structural support on which the middle and upper face develop but also by functioning as a signaling center that controls gene expression and cell behavior in the facial mesenchyme (Chazaud *et al.*, 1996; Cordero *et al.*, 2004; Creuzet *et al.*, 2002, 2004; Hu *et al.*, 2003; Marcucio *et al.*, 2005; Osumi-Yamashita *et al.*, 1994; Schneider *et al.*, 2001; Smith, 2001). The superficial epithelia, which include both facial ectoderm and pharyngeal endoderm, are also sources of instructional cues that also guide the differentiation of neural crest cells (Creuzet *et al.*, 2004; Crump *et al.*, 2004a,b; Graham and Smith, 2001; Haworth *et al.*, 2004; Hu *et al.*, 2003; Miller *et al.*, 2000; Piotrowski and Nusslein-Volhard, 2000; Trokovic *et al.*, 2003). An abundant literature implicates the cranial neural crest in facial patterning as well (reviewed in Heeg-Truesdell and LaBonne, 2004; Helms and Schneider, 2003; Le Douarin *et al.*, 2004; Manzanares and Nieto, 2003; Trainor and Krumlauf, 2001; Trainor *et al.*, 2003).

To directly test the extent to which neural crest cells contain intrinsic information for species-specific facial patterning, we swapped cranial neural crest cells between duck and quail embryos then scrutinized the chimeras to see whether a bill could become a beak and vice versa (Schneider and Helms, 2003). When a large population of quail cranial neural crest cells successfully integrated into the facial prominences of a duck host, the resulting embryos had a quail beak on a duck body. Molecular and cellular analyses of these "qucks" indicated that the transplanted neural crest cells followed the timetable of their original morphogenetic program and they repatterned the host's facial ectoderm to resemble the donor species (Schneider and Helms, 2003).

## III. Conclusions

Generating qucks and duails takes a bit of experimental finagling but Nature performs more dramatic transformations on a recurring basis, throughout evolution and between and among different animal species. Members of a single species have many common characteristics but can show enormous variability in their craniofacial features that can be passed to their progeny during interbreeding. Whatever the origins may be for each trivial difference between offspring and their parents—and a cause for each must exist—the reasoning still holds that the steady accretion of advantageous changes eventually give rise to important modifications in structure, which enable a species to compete for its ecological niche.

Darwin did not show much restraint when he wrote, "Our ignorance of the laws of variation is profound. Not in one case out of a hundred can we pretend to assign any reason why this or that part has varied. But whenever we have the means of instituting a comparison, the same laws appear to have acted in producing the lesser differences between varieties

of the same species, and the greater differences between species of the same genus.''

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# 2

# Molecular Bases of the Regulation of Bone Remodeling by the Canonical Wnt Signaling Pathway

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I. Introduction
II. Canonical Wnt Signaling
  A. Lrp5/Lrp6
  B. $\beta$-Catenin
  C. Lef/Tcfs
III. Other Wnt Signaling Molecules
  A. Wnts
  B. Frizzled
  C. GSK3$\beta$
IV. Secreted Wnt Inhibitors and Agonists
  A. Dkks
  B. Sfrps
  C. Wif-1
  D. Sclerostin
V. Intracellular or Transmembrane Modulators
  A. Kremens
  B. Axin2/Conductin/Axil
  C. Ror2
VI. Conclusions and Future Work
  References

Osteoporosis is a common, prevalent, and debilitating condition, particularly in postmenopausal women. Genetics play a major role in determining peak bone mass and fracture risk, but few genes have been demonstrated conclusively to be involved, much less the signaling pathways with which they are affiliated. The identification of mutations in the gene *Lrp5*, a Wnt coreceptor, as the cause for both osteoporotic and high-bone mass disorders implicated the canonical Wnt signaling pathway in bone mass regulation. Since *Lrp5*, other Wnt components have been identified as

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being regulators of bone mass, and Wnt target genes affecting bone homeostasis have begun to be elucidated. This chapter looks at the various components of the canonical Wnt signaling pathway and the data indicating that this pathway plays a major role in the control of both bone formation and bone resorption, the two key aspects of bone remodeling. 

## I. Introduction

There are two specific cell types responsible for maintenance of a constant bone mass: osteoblasts that are the bone-forming cells, and osteoclasts that are the bone-resorbing cells. The osteoblast, a cell of mesenchymal origin, synthesizes proteins of the extracellular matrix (ECM) and, as such, is responsible for bone formation. It also expresses genes that are necessary and sufficient for the mineralization of this ECM and, thirdly, controls osteoclast differentiation. The osteoclast, on the other hand, has only one main function: bone resorption. Thus, a hierarchy exists within the bone milieu where the osteoblast not only fulfils a greater number of functions than the osteoclast, but also is able to regulate the osteoclast itself.

In vertebrates, a process called bone remodeling takes place, which is required in order to maintain a constant bone mass. There is first a phase of resorption by the osteoclasts, which occurs simultaneously at numerous sites within the skeleton and occurs relatively quickly, that is, about 3 weeks per site. This resorptive phase is followed by the rebuilding of bone by the osteoblasts, which in contrast lasts over 3 months per site. These two phases must remain in balance to achieve homeostasis and a constant bone mass. Any increase or decrease in proliferation or activity of either cell type can lead to aberrant changes in bone mass. Thus, bone remodeling is necessarily dysregulated in an osteoporotic (low bone mass) or osteopetrotic (high bone mass) state.

In young adults and in premenopausal women, bone resorption and bone formation occur at the same rate, therefore bone mass is maintained over time. Whether due to menopause or aging, over time there is a relative increase in bone resorption, leading to bone loss and osteoporosis (Riggs *et al.*, 1998). Both bone formation and bone resorption are subject to regulation, either by local factors secreted by bone cells themselves or systemically by hormones.

At the local level, two positive and one negative regulators of osteoclast differentiation have been identified. *M-Csf* (*macrophage colony stimulating factor*) and the *receptor activator of NF-κB ligand* (*Rankl*) have been demonstrated to be necessary and sufficient for osteoclast differentiation and function (Lacey *et al.*, 1998; Wong *et al.*, 1999; Yasuda *et al.*, 1998; Yoshida *et al.*, 1990). These two factors are secreted by osteoblasts, as is a third factor

that inhibits osteoclast differentiation. *Osteoprotegerin* (*Opg*) encodes a soluble decoy receptor for Rankl, thereby preventing osteoclast differentiation (Simonet *et al.*, 1997). Thus, there are both activators and inhibitors of osteoclast differentiation being secreted by the osteoblast.

At the systemic level a number of signaling pathways have been characterized to influence bone remodeling. The insulin-like growth factor family has been shown to stimulate *Type I collagen* gene expression (Schmid *et al.*, 1989) and stimulate osteoblast proliferation *in vitro* (Canalis, 1993). Meanwhile the bone morphogenetic protein (BMP) family, a subset of the transforming growth factor $\beta$ (TGF$\beta$) superfamily, has been shown to induce ectopic bone formation *in vitro* and indirect evidence links them to bone mass regulation *in vivo* (Mundy *et al.*, 1999; Reddi, 1997; Yoshida *et al.*, 2000). A third signaling family that has been shown to influence bone remodeling is the Wnt signaling pathway.

## II. Canonical Wnt Signaling

The Wnt family of secreted factors is involved in numerous aspects of cellular biology, ranging from cell fate determination, polarity and differentiation to migration, proliferation, and function (Moon *et al.*, 2002). With regards to skeletal biology, Wnts are involved in a variety of processes: from limb patterning and formation to chondrogenesis as well as osteoblast and osteoclast differentiation and function. This chapter will focus mostly on the role of the Wnt signaling pathway in bone remodeling, hence highlighting its effects on the osteoblast and the osteoclast. Reviews on the role of Wnt signaling in limb formation or in chondrocytes can be found in the study by Tuan (2003) and Yang (2003), respectively.

There are at least three distinct intracellular signaling pathways that Wnt proteins utilize to regulate cellular processes once they bind to the seven transmembrane receptors of the Frizzled (Fzd) family on the surface of target cells. The first pathway, known as the *planar cell polarity (PCP)* pathway, mainly occurs during the earlier aspects of embryogenesis. This pathway utilizes Rho/Rac GTPases and c-Jun N-terminal kinase (JNK) to affect cell shape changes and activate *AP-1* target genes (Habas *et al.*, 2003; Shulman *et al.*, 1998). The second intracellular pathway, the *Wnt/*$Ca^{2+}$ pathway, is involved in cell migration. This pathway relies on protein kinase C activation and increases in intracellular $Ca^{2+}$ levels for target gene activation (Kuhl *et al.*, 2000; Slusarski *et al.*, 1997). The third pathway is the most studied and thereby the best characterized and is termed the *canonical Wnt signaling* pathway. The majority of the data concerning Wnt signaling and bone remodeling involve this pathway; hence it will be the focus of this chapter.

The canonical Wnt signaling pathway is involved in the differentiation and proliferation of most organs in mammals (Peifer and Polakis, 2000; Wodarz and Nusse, 1998). These cellular functions are affected by the regulation of the levels and subcellular localization of the key molecule $\beta$-catenin. In the absence of Wnt signaling, the cytoplasmic levels of $\beta$-catenin are kept low; any $\beta$-catenin proteins that are not involved in cellular architecture, which involves the cadherins and the actin cytoskeleton, are ubiquitinated and targeted for proteosomal degradation (Aberle *et al*., 1997). To accomplish this, a variety of kinases, e.g., casein kinase (CK) I, glycogen synthase kinase (GSK) 3$\beta$, and scaffolding proteins, e.g., Dishevelled, Axin, and adenomatous polyposis coli (APC), form a complex that binds $\beta$-catenin and phosphorylates specific residues in the N-terminal region of the protein, targeting $\beta$-catenin for ubiquitination by F-box protein/E2 ligases (Behrens *et al*., 1998; Jiang and Struhl, 1998; Noordermeer *et al*., 1994; Siegfried *et al*., 1992).

To initiate canonical Wnt signaling, a Wnt protein binds to an Fzd receptor and an LDL Receptor-Related Protein 5 or 6 (Lrp5 or Lrp6) coreceptor on the cell surface. This complex leads to phosphorylation of the intracellular aspect of Lrp5/6 (by an as yet unidentified kinase) and allows for the docking of Axin to the complex (Mao *et al*., 2001b; Tamai *et al*., 2004). The docking of Axin to Lrp5/6 is somehow able to prevent the phosphorylation of $\beta$-catenin by GSK3$\beta$, as GSK3$\beta$ cannot be incorporated into the Axin/Lrp5/6 complex. $\beta$-catenin that has not been phosphorylated by CKI and GSK3$\beta$ accumulates in the cytoplasm and then translocates into the nucleus to activate target gene transcription (Behrens *et al*., 1996; Ikeda *et al*., 1998; Orford *et al*., 1997). The most studied nuclear partners of $\beta$-catenin are the lymphoid enhancer-binding factor/T cell factor (Lef/Tcf) transcription factor family (Behrens *et al*., 1996; Molenaar *et al*., 1996; van de Wetering *et al*., 1997). $\beta$-catenin displaces corepressors of Lef/Tcfs, such as Groucho, and forms heterodimers with the Lef/Tcf proteins. With the assistance of transcriptional coactivators, this heterodimer binds DNA and initiates the transcription of target genes (He *et al*., 1998; Tetsu and McCormick, 1999). $\beta$-catenin has a transactivation domain but contains no DNA-binding moieties and thus is unable to bind to DNA. Conversely, the Lef/Tcf transcription factors contain a high mobility group (HMG) DNA-binding domain that enables them to bind to and bend DNA; however, they lack a transactivation domain and are poor transcriptional regulators by themselves. It is only when $\beta$-catenin and Lef/Tcf proteins form a heterodimer that transcriptional activation and expression of canonical Wnt signaling target genes can be achieved (Behrens *et al*., 1996; Molenaar *et al*., 1996; van de Wetering *et al*., 1997). There are instances where canonical Wnt signaling is able to repress gene transcription, although the mechanisms leading to these effects are less clearly understood. One possible explanation is that gene activation or repression may be dependent on the availability of

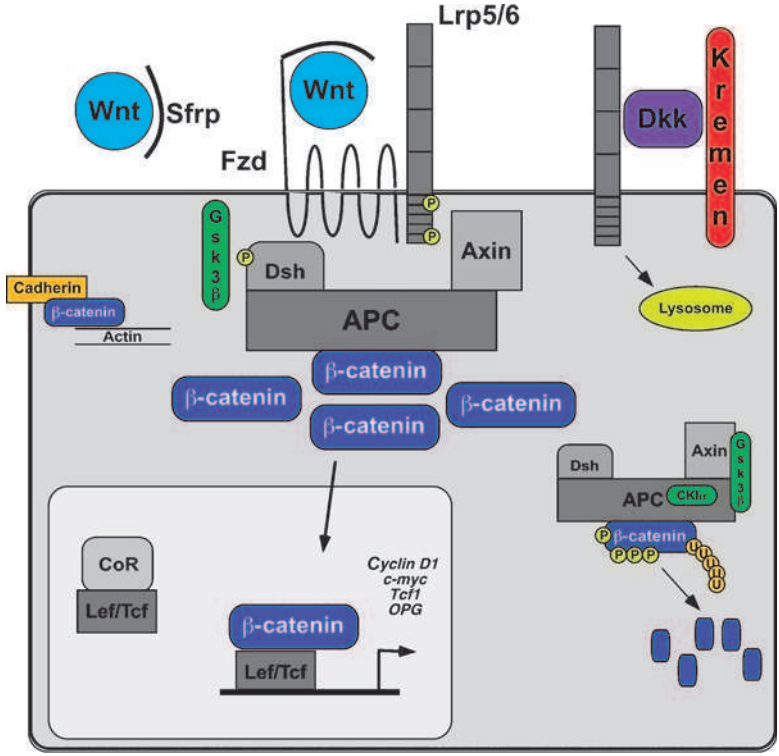


**Figure 1** The canonical Wnt signaling pathway. Canonical Wnt signaling is initiated when Wnts form a complex with Fzd receptors and Lrp coreceptors. This complex formation leads to the phosphorylation of the cytoplasmic tail of Lrp5/6 by an unidentified kinase. Axin, and thereby the entire APC-Dishevelled (Dsh)-$\beta$-catenin complex, interacts with these phosphorylated sites. These interactions prevent GSK3 from joining the complex. $\beta$-catenin thereby escapes phosphorylation and subsequent ubiquitination and proteosomal degradation. $\beta$-catenin accumulates in the cytoplasm and then translocates into the nucleus to interact with Lef/Tcf transcription factors and activate, or repress, target gene transcription. $\beta$-catenin replaces corepressors as the binding partner of Lef/Tcfs. This leads to the activation of a number of known target genes. Lef/Tcf-$\beta$-catenin suppresses the transcription of other genes, which is believed to be due to interactions with other transcription factors. A number of different antagonists inhibit canonical Wnt signaling. Some, such as the soluble frizzled related proteins (Sfrps) bind free Wnts and prevent them from forming a Wnt-Fzd-Lrp complex. Others, such as Dickkopfs (Dkks), decrease the number of Lrp5/6 proteins on the cell surface available to participate in canonical Wnt signaling. Dkks perform this inhibition by binding to Lrp5 and bringing them into close proximity to Kremen proteins. The Lrp5-Dkk-Kremen complex undergoes endocytosis and is transported to lysosomes for protein degradation or recycling.

other coactivators or corepressors within the nucleus (Baker *et al.*, 1999; Cadigan *et al.*, 1998; Jamora *et al.*, 2003; Kahler and Westendorf, 2003; Mansukhani *et al.*, 2005; Prieve and Waterman, 1999) (Fig. 1).

In this chapter, we will start looking at Wnt components on which the majority of data on bone remodeling via Wnt signaling is based (Lrp5/6 and $\beta$-catenin). We will then look at the available information on the other main components of the canonical Wnt signaling pathway, and then finish with new information on a variety of Wnt antagonists and modulators that give new perspective into the roles canonical Wnt signaling plays in the bone milieu.

## A. Lrp5/Lrp6

Until recently, the vast majority of information regarding canonical Wnt signaling and bone remodeling centered on Lrp5. The LDL Receptor-Related Proteins 5 and 6 (Lrp5 and Lrp6) are the mammalian homologs of the *Drosophila* gene arrow (Wehrli *et al.*, 2000). These three proteins are members of the low density lipoprotein receptor (LDLR) family. Members of this family contain 3 major conserved regions: multiple EGF-like motifs that are separated by YWTD spacers, LDLR ligand binding motifs, and a single transmembrane domain (Hey *et al.*, 1998). Unlike the other LDLR proteins, the intracellular domain of Lrp5/6 contains five SH3-like domains (PPPSP) rather than an NPXY internalization motif. When loss of function mutations in arrow gave a phenotype reminiscent of the inactivation of wingless in *Drosophila*, Lrp5/6 became implicated as potential Wnt coreceptors in mammals (Tamai *et al.*, 2000; Wehrli *et al.*, 2000). In response to Wnts, the intracellular tail of Lrp5/6 is phosphorylated by unidentified kinases. This phosphorylation causes the formation of a docking site for Axin, $\beta$-catenin stabilization and accumulation, and Lef/Tcf-mediated transcription (Mao *et al.*, 2001b; Tamai *et al.*, 2004).

### 1. Lrp5

In the human genome Lrp5 is located at chromosome 11q13, where two human diseases, osteoporosis-pseudoglioma (OPPG) and the high bone mass (HBM) syndrome, map. Two groups independently cloned the *Lrp5* gene: one group in a search for novel genes being expressed in osteoblasts (Dong *et al.*, 1998); the other as a candidate gene for susceptibility to insulin-dependent diabetes mellitus at chromosome 11q13 (Hey *et al.*, 1998). Chromosome 11q13 has been mapped to be the locus for several bone abnormalities, including OPPG, HBM, and autosomal recessive osteopetrosis (Gong *et al.*, 1996; Heaney *et al.*, 1998; Johnson *et al.*, 1997; Koller *et al.*, 1998). Several years later, the OPPG collaborative group demonstrated that 12 different mutations in the *Lrp5* gene led to OPPG (Gong *et al.*, 2001). OPPG is characterized by two major findings: an early onset low bone mass

which often leads to fractures during childhood and skeletal deformities, and blindness due to the persistence of the hyaloid vessels during eye vascularization. Trabecular thickness is severely decreased in OPPG children but bone turnover is normal, suggesting that the disease process is osteoblast specific in nature. Obligate carriers for Lrp5 also have a reduced bone mass and an increased likelihood for fractures, indicating that the effects of Lrp5 mutations are dominant in nature (Gong *et al.*, 2001). Three different mouse models for loss of Lrp5 have been generated. Two of them, created via disruption of exon 1 and exon 6 respectively, have a phenotype similar to OPPG in young mice (Holmen *et al.*, 2004; Kato *et al.*, 2002); the third, with disruption of exon 18, has reduced bone thickness in older mice (Fujino *et al.*, 2003). Although there are differences in the phenotypes, these three different models show that loss of function mutations in Lrp5 lead to a decreased bone mass.

Besides osteoporotic mutations in Lrp5, there are other Lrp5 mutations that cause syndromes with HBM. Independent studies of two families with autosomal dominant HBM phenotypes identified a G to T mutation in exon 3 of Lrp5 (Boyden *et al.*, 2002; Little *et al.*, 2002). This mutation leads to a glycine to valine change at amino acid 171 (G171V), which is believed to disturb the first $\beta$-propeller motif in the extracellular domain of Lrp5 and modify its hydrophobic surface (Jeon *et al.*, 2001). A transgenic mouse model overexpressing *Lrp5* with the *G171V* mutation via the rat $\alpha_1(I)$ *collagen* promoter recapitulates the phenotype seen in humans (Babij *et al.*, 2003). These mice have significantly increased bone density and bone strength without any changes in bone morphology. However, our attempt to reproduce these results with transgenic mice was unsuccessful; we saw no deleterious effect on bone mass with overexpression of wildtype *Lrp5* or $Lrp5^{G171V}$ (Glass and Karsenty, unpublished observations).

There is no Lef/Tcf-dependent signaling via $Lrp5^{G171V}$ in the absence of Wnts *in vitro*, which is similar to the finding with wildtype Lrp5 and suggests that the HBM phenotype is not due to constitutive activation of the canonical Wnt signaling pathway. *In vitro* studies show that the G171V mutation disrupts the interaction between Lrp5 and the cell surface receptor chaperone protein Mesd (Zhang *et al.*, 2004). This leads to fewer $Lrp5^{G171V}$ coreceptors on the cell surface such that autocrine Wnt signaling is unaffected but paracrine inhibition of signaling by Dkks, which are canonical Wnt antagonists, is blunted (Boyden *et al.*, 2002; Zhang *et al.*, 2004).

Mutations in Lrp5 lead to a variety of diseases, not just OPPG and HBM. Six different missense mutations in Lrp5 (D111Y, G171R, A214T, A214V, A242T, and T253I) were found in patients with increased bone mass, diagnosed with osteopetrosis type I, van Buchem disease, endosteal hyperstosis, or autosomal dominant osteosclerosis (Kwee *et al.*, 2005; Van Wesenbeeck *et al.*, 2003). All six of these mutations, altered evolutionarily, conserved

residues that are amino-terminal to the first EGF-like domain of Lrp5. These mutations, when studied in COS and 293T fibroblastic cell lines, give similar results as the *G171V* mutation regarding *Lrp5* cell surface expression and Dkk-mediated inhibition (Ai *et al*., 2005). Other missense (*T173M, Y1168H, C1361G*) and frameshift mutations are found in patients with familial exudative vitreoretinopathy with retinal angiogenesis (FEVR). The patients are all blind due to retinal vessel development defects; some of the patients have low bone mass or suffer from fractures (Toomes *et al*., 2004).

Bone remodeling is achieved by the action of bone-forming osteoblasts and bone-resorbing osteoclasts. Lrp5 is detected in regions of bone remodeling; it is not detectable in osteoclasts by *in situ* hybridization but is found in cells of the osteoblast lineage (Gong *et al*., 2001; Kato *et al*., 2002; Little *et al*., 2002). Using the 2.3 kb $\alpha_1(I)$ *collagen* promoter driving green fluorescent protein (GFP) expression to identify and sort fully mature osteoblasts from osteoblasts in earlier states of differentiation, the expression of *Lrp5* was found to be increased in GFP-positive, mature osteoblasts (Kalajzic *et al*., 2005). *Lrp5* can also be detected in osteosarcoma cells and in areas undergoing intramembranous ossification, but it is not detectable in the growth plate (Dong *et al*., 1998; Gong *et al*., 2001; Hoang *et al*., 2004). As expected by this restricted expression pattern, mutations in *Lrp5* only affect bone mass by altering the osteoblast and thereby bone accrual; there are no changes observed in the number of osteoclasts or in bone resorption parameters in patients with OPPG or HBM (Boyden *et al*., 2002; Gong *et al*., 1996, 2001; Kato *et al*., 2002).

The evidence accumulated to date suggests that Lrp5 functions in osteoblasts to enhance proliferation and increase the functional life span of the osteoblast. This idea first developed when a soluble Lrp5 protein, which lacked the transmembrane domain, inhibited the proliferation of calvarial explant cultures (Gong *et al*., 2001). $Lrp5^{-/-}$ mice have a decrease in the number of osteoblasts per bone area, as well as a 50% decrease in matrix apposition rate (MAR) and bone formation rate (BFR) (Kato *et al*., 2002). There is a 50% decrease in the proliferation rate of the osteoblasts with no effect on apoptotic rates. Additionally, $Lrp5^{-/-}$ bone marrow progenitor cells form fewer CFU-Fs with the ability to produce alkaline phosphatase, suggesting that Lrp5 helps promote the proliferation and survival of osteoblast progenitor cells.

Even though a dominant negative model of Lrp5, lacking both C-terminal and transmembrane domains, completely blocks Wnt3a-mediated signaling in osteoblast cell lines (Gong *et al*., 2001), there is still partial Wnt and Lef1-mediated signaling occurring in $Lrp5^{-/-}$ osteoblasts (Kato *et al*., 2002). This suggests that Wnt-mediated signaling may occur in the absence of Lrp5, and that Lrp5 may simply serve as an enhancer of canonical Wnt

signaling (Mao *et al.*, 2001b). There is also evidence that Fzds may be sufficient to activate canonical Wnt signaling (Carron *et al.*, 2003; Gonzalez-Sancho *et al.*, 2004). Lrp6 may also contribute to signaling in osteoblasts and in other cell types within the bone milieu to compensate for the loss of Lrp5.

## 2. Lrp6

The importance of Lrp6 in skeletal biology was discovered via a mouse model when an insertional mutation caused truncation of the Lrp6 protein after residue 321 (Pinson *et al.*, 2000). These mice died at birth; their embryonic phenotype, however, closely resembled that of a combination of various Wnt loss-of-function mutations. *Lrp6*$^{-/-}$ embryos have midbrain and hindbrain defects, similar to those seen in *Wnt1*$^{-/-}$ mice (Pinson *et al.*, 2000; Thomas and Capecchi, 1990). Similar to the *Wnt3a*$^{-/-}$ mice, these mice also have a truncated axial skeleton such that the vertebrae caudal to the lumbar region is absent (Pinson *et al.*, 2000; Takada *et al.*, 1994). The third major phenotype, limb patterning defects, is reminiscent of *Wnt7a*$^{-/-}$ mice (Parr and McMahon, 1995; Pinson *et al.*, 2000; Yang and Niswander, 1995). Although *Lrp6*$^{-/-}$ mice are perinatal lethal, there are hypomorphic Lrp6 mouse models that allow us to look at the function of Lrp6 in adults. The *ringelschwanz* (*rs*) mutant mouse arose due to a naturally occurring point mutation, which lead to an arginine to tryptophan mutation at amino acid 886 (R886W) (Kokubu *et al.*, 2004). This conserved residue lies in a Dkk-binding domain found between the third $\beta$-propeller motif and the third EGF-like region. These mice are thought to be hypomorphic for Lrp6 due to the fact that the mutation prevents efficient transduction of canonical Wnt signaling. These mice have axial skeleton, digit, and neural tube defects similar to those in various Wnt-deficient mice (Kokubu *et al.*, 2004). They also have a delay in the appearance of ossification centers during development and a decreased bone mass in adult mice, both findings similar to those in *Lrp5*$^{-/-}$ mice (Kato *et al.*, 2002; Kokubu *et al.*, 2004). Osteoblast and osteoclast parameters were not measured in the *rs* mutant mice; the similarities to *Lrp5*$^{-/-}$ mice suggest, however, that the phenotype is also osteoblast specific in nature.

Bone mass parameters were also evaluated in mouse models with various degrees of deficiency for both *Lrp5* and *Lrp6*. *Lrp6*$^{+/-}$;*Lrp5*$^{-/-}$ mice were found to be more osteopenic than *Lrp6*$^{+/+}$;*Lrp5*$^{-/-}$ mice (Holmen *et al.*, 2004). Mice which were heterozygous for *Lrp6* and heterozygous or homozygous for *Lrp5* had limb defects of variable expression and incomplete penetrance, suggesting that the limb patterning defects seen in *rs* mutant mice could be worsened by either haploinsufficiency or complete loss of *Lrp5*. *Lrp6*$^{+/-}$;*Lrp5*$^{+/-}$ mice had a higher bone mass than *Lrp6*$^{+/+}$;*Lrp5*$^{-/-}$ mice,

but less bone than wildtype or either heterozygous mouse alone (Holmen *et al*., 2004). Rates of osteoblast and osteoclast proliferation or function were not reported in this study, but presumably only osteoblast parameters would be affected with decreased expression of *Lrp5* and/or *Lrp6*.

In contrast to Lrp5, there are no human diseases or syndromes as yet mapped to mutations in the Lrp6 locus on chromosome 12p13.3-p11.2. Based on the phenotype of the $Lrp6^{-/-}$ mouse, this lack of disease knowledge may stem from embryonic lethality due the loss of Wnt signaling *in utero* from the lack of *Lrp6*. *Lrp6* was found to be expressed in a number of human cancer cell lines and malignant tissues, and was able to drive tumorigenesis in experiments using nude mice, but osteosarcomas were not evaluated in this study (Li *et al*., 2004).

In *Xenopus, Lrp6* is upstream of Dishevelled and $\beta$-catenin in the canonical Wnt signaling pathway; forced expression of *Lrp6* can activate Wnt target genes, cause dorsal axis duplication, and induce neural crest formation when in the presence of Wnts and Fzds (Tamai *et al*., 2000). The extracellular domain of Lrp6 binds Wnt1 and Fzd8 in a Wnt1-dependent manner (Tamai *et al*., 2000). Lrp6 is also a receptor for both Dickkopf1 (Dkk1) and Dickkopf2 (Dkk2), which inhibit Wnt signaling by binding to Lrps at a site different from that of the Wnts (Mao *et al*., 2001a).

The differences in phenotypes between *Lrp5* and *Lrp6* mutations may be due to differences in their affinities for various Wnts or Wnt antagonists. In COS cells, expression of full length *Lrp6*, but not *Lrp5*, was able to increase Lef/Tcf-mediated transcription in the absence of Wnt3a, while both coreceptors potentiated Wnt3a-mediated signaling (Mi and Johnson, 2005). Expression of C-terminal truncated Lrps (transmembrane and intracellular domains, or intracellular domains alone) of *Lrp5* or *Lrp6* was able to activate Lef/Tcf signaling in the absence of Wnt3a, and both potentiated Wnt3a-mediated activation of $\beta$-catenin and Tcf-mediated signaling (Mi and Johnson, 2005). These data suggest that the differences between Lrp5 and Lrp6 regarding canonical Wnt signaling are due to variations occurring in their extracellular domains.

## B. $\beta$-Catenin

The gene for $\beta$-catenin (*CTNNB1*) is located on human chromosome 3p22-p21.3. This 88-kDa protein directly interacts with actin and cadherins and plays a pivotal role in cell shape, adhesion, and migration. $\beta$-catenin is the mammalian homolog of the *Drosophila* gene armadillo, which has profound effects on gene expression and development in the fruitfly (Noordermeer *et al*., 1994). In mammals, $\beta$-catenin is essential for embryonic development

as *β-catenin*$^{-/-}$ embryos die at E7.5 due to gastrulation defects (Haegel *et al.*, 1995). A variety of mouse models looking at tissue-specific inactivation of β-catenin have revealed the importance of this molecule in cell fate determination and cell survival in multiple cells (Brault *et al.*, 2001; Cattelino *et al.*, 2003; Day *et al.*, 2005; Hill *et al.*, 2005; Hu *et al.*, 2005). Wnt1-Cre inactivation of β-catenin resulted in the prevention of prechondrogenic condensations as well as craniofacial abnormalities (Brault, 2001; Huelsken *et al.*, 2001). Two different papers, using *α$_1$(I) collagen-Cre* or *osteocalcin-Cre* mice, show that β-catenin also plays an important role in differentiated osteoblasts by regulating osteoclast differentiation via the Opg-Rankl signaling pathway (Glass *et al.*, 2005; Holmen *et al.*, 2005).

Regarding the expression of *β-catenin* within bone, it was first observed in osteoblastic cell lines at regions of cell-to-cell contact (Cheng *et al.*, 1998). It has also been detected by immunohistochemistry in both the cytoplasm and nuclei of osteoblasts. β-catenin was not detected in mature osteocytes but was found in the osteocytes within active regions of bone remodeling in osteoid osteomas. β-catenin is also detected within osteoclasts (Monaghan *et al.*, 2001), indicating that canonical Wnt signaling could take place within this cell type as well, a proposition that to date has not been extensively studied.

Osteoblasts and chondrocytes, which deposit bone and cartilage, respectively, are derived from the same osteochondroprogenitor cell. β-catenin plays a prominent role in cell fate determination toward osteoblast differentiation. Ectopic Wnt signaling in chondrocytes in *α$_1$(II) collagen-Wnt14* transgenic mice enhances ossification, and suppressed, chondrogenesis in contrast, inactivation of β-catenin using *Dermo1-Cre, α$_1$(II) collagen-Cre* mice, or *Prx1-Cre* mice leads to ectopic chondrocyte formation at the expense of osteoblast differentiation during both intramembranous or endochondral ossification (Day *et al.*, 2005; Hill *et al.*, 2005; Hu *et al.*, 2005). Loss of β-catenin in mesenchymal precursor cells leads to chondrocyte development even in conditions favorable for osteoblastogenesis (Day *et al.*, 2005). Furthermore, β-catenin signaling is both necessary and sufficient to repress mesenchymal cell differentiation in *Runx2* positive, *Sox9* positive skeletal elements (Hill *et al.*, 2005). These results indicate that *β-catenin* expression is crucial for osteoblast fate determination.

As mentioned earlier, β-catenin can promote differentiation and cell survival in osteoblasts; this can be accomplished through both Wnt-dependent and Wnt-independent mechanisms. A gain-of-function mutation in β-catenin (S37A, S45A) introduced into CH310T1/2 cells increased the density of the cells in culture (Bain *et al.*, 2003). This increase in cell number was postulated to be due to an increase in cell survival as cell cycle markers were unchanged; however the rate of apoptosis was not rigorously tested. These cells expressed more alkaline phosphatase but not more osteocalcin (Bain *et al.*, 2003).

Alkaline phosphatase, osteocalcin, and matrix mineralization were induced when another activated β-catenin molecule (*ΔN151*) synergized with *BMP-2* in C3H10T1/2 and C2C12 cells (Mbalaviele *et al.*, 2005). Osteocalcin levels were also found increased in MC3T3 osteoblastic cells with the addition of β-catenin (Kahler and Westendorf, 2003). In the latter study, increasing the levels of Lef1, in the presence of β-catenin, leads to increased repression of the osteocalcin promoter. Hence, the results obtained from *β-catenin* overexpression experiments may be dependent on other factors being at necessary or appropriate levels (Prieve and Waterman, 1999).

β-catenin plays an entirely different role in mature, fully differentiated osteoblasts. Osteoblast-specific activation of β-catenin, using *$\alpha_1$(I) collagen-Cre*, led to an increase in bone mass (Glass *et al.*, 2005). These mice died at 3–5 weeks of age, due to an eruption defect with their lower incisors. Tooth eruption defects are classic signs of a lack of osteoclast-mediated bone resorption and in these mice there was a major decrease in the number of osteoclasts, leading to a functional reduction in bone resorption. The number of osteoblasts was unchanged, as were the expression levels of *Runx2, Osterix*, and *Atf4*, three osteoblast transcription factors. The defect in resorption was due to an increase in the expression of Opg, which functions as an inhibitor of osteoclast differentiation (Glass *et al.*, 2005; Simonet *et al.*, 1997). Conversely, using the same *$\alpha_1$(I) collagen-Cre* to selectively inhibit *β-catenin* expression in osteoblasts led to a decreased bone mass at 1 month of age (Glass *et al.*, 2005). In contrast with the activated β-catenin mice, there was no tooth eruption defect and the mice had a normal life span. Unlike with the loss of *Lrp5*, the low bone mass of these mice was due to an increase in the number of osteoclasts and an increase in bone resorption. The number of osteoblasts was not affected in these mice, nor was there a change in the bone formation rate (BFR). Osteoprotegerin was downregulated with the loss of β-catenin while the levels of Rankl were unchanged (Glass *et al.*, 2005). Coculture experiments using primary osteoblasts and bone marrow monocytes (osteoclast precursors) from the different β-catenin mutant mice showed that the effects on osteoclast differentiation were osteoblast-autonomous and were due to changes in osteoprotegerin (Glass *et al.*, 2005). Wnt3a is able to induce osteoprotegerin expression by a *β-catenin*-dependent mechanism in C3H10T1/2 cells (Jackson *et al.*, 2005). Thus, β-catenin signaling in mature osteoblasts leads to regulation of osteoclast differentiation and function via the Opg-Rankl pathway.

Similar experiments were performed using *Osteocalcin-Cre* to create osteoblast-specific inactivation of *β-catenin* (to inactivate the canonical Wnt pathway) or of *APC* (to constitutively activate the pathway). Similar to the studies using *$\alpha_1$(I) collagen-Cre*, activation of the canonical pathway led to an increase in bone mass and a complete absence of osteoclasts, while inactivation of the pathway led to a low bone mass with an increased

number of osteoclasts (Holmen *et al.*, 2005). However, both mutant mice died by 4 weeks of age; the cause of death was undetermined but was not due to any defects in tooth formation or eruption. There was a delay in osteocalcin expression in the *Δβ-catenin* mutant mice and premature expression seen in the *ΔAPC* mice (Holmen *et al.*, 2005). The number of osteoblasts was decreased in the *Δβ-catenin* mutant mice by 4 weeks of age and completely absent in the *ΔAPC* mice by 2 weeks of age. However, cultured osteoblasts from the *Δβ-catenin* mice showed an increase in *Opg* expression and a decrease in *Rankl* expression, while the converse was observed in *ΔAPC*-cultured osteoblasts (Holmen *et al.*, 2005). Besides the minor discrepancies between the phenotypes observed in the different mouse models, the phenotypes contrast sharply with those observed in Lrp5/Lrp6 mouse models, where only bone accrual is affected without any effects on bone resorption, and they establish that regulation of osteoclast differentiation is a major role for β-catenin-dependent signaling in osteoblasts.

## C. Lef/Tcfs

The Lef/Tcf transcription factors interact with β-catenin in order to activate transcription of Wnt target genes. In both mice and in humans there are four family members: *Lef1* (*Tcf1a*), *Tcf1* (*Tcf7*), *Tcf3* (*Tcf7L1*), and *Tcf4* (*Tcf4L2*). Although there is functional redundancy between these transcription factors *in vitro, in vivo* they have distinct roles both during development and postnatally.

The Lef/Tcf transcription factors are all members of the HMG family. The HMG-binding site allows for these transcription factors to bind to their consensus-binding site $CTTTG^{A}/_{T}{}^{A}/_{T}$ and induce 130° bending of the DNA double helix. This bending allows other transcription factors to bind to their consensus sites and activate gene transcription (Carlsson *et al.*, 1993; Giese and Grosschedl, 1993; Giese *et al.*, 1991, 1995, 1997; Love *et al.*, 1995; van de Wetering and Clevers, 1992). When Wnt signaling is absent, transcriptional corepressors, such as CtBP and TLE/Groucho, and histone deacetylases, bind to the Lef/Tcf proteins (Billin *et al.*, 2000; Brannon *et al.*, 1999; Brantjes *et al.*, 2001; Levanon *et al.*, 1998). When Wnt signaling is activated, β-catenin is able to supplant these corepressors by binding to the N-termini of the transcription factors, improves their interaction with chromatin, and allows other coactivators (such as *p300, Bcl9*, and *Pygopus*) to enhance gene expression (Behrens *et al.*, 1996; Billin *et al.*, 2000; Hecht *et al.*, 2000; Hsu *et al.*, 1998; Huber *et al.*, 1996; Kramps *et al.*, 2002; Miyagishi *et al.*, 2000; Sun *et al.*, 2000; Tutter *et al.*, 2001). There are instances, however, where Wnt signaling and β-catenin can lead to *Lef/Tcf*-mediated repression of gene expression, but the mechanisms

surrounding these circumstances are not yet well understood (Baker *et al*., 1999; Cadigan *et al*., 1998, 2002; Jamora *et al*., 2003; Kahler and Westendorf, 2003; Payre *et al*., 1999; Piepenburg *et al*., 2000; Willert *et al*., 2002; Yang *et al*., 2000).

*Lef1* is detectable at E14.5 in tail prevertebrae, osteogenic cells of the hipbone, and the mesenchymal cells surrounding the cochlea. *Lef1*$^{-/-}$ mice are slightly smaller than their wildtype littermates; they lack teeth, whiskers, and body hair, and die within the first two weeks of life (van Genderen *et al*., 1994). Loss of *Tcf3* leads to embryonic lethality because its expression is required for early anterior-posterior patterning (Merrill *et al*., 2004). Neither of these genes is expressed in osteoblasts at any timepoint during development (Glass *et al*., 2005). In contrast, at E14.5 *Tcf1* is found expressed in cells of the palate, maxilla, mandible, nasal, and basooccipital bones, the thoracic vertebrae, ribs, and femur, and its specific expression in osteoblasts is detectable from this timepoint onward (Glass *et al*., 2005; Oosterwegel *et al*., 1993b). *Tcf1*$^{-/-}$ mice lack early thymocyte progenitors but are immunocompetent; they develop adenomas in the gut and the mammary glands but otherwise appear normal (Roose *et al*., 1999; Verbeek *et al*., 1995). *Tcf4* is expressed in limbs at E10.5 in the mesenchymal cells surrounding areas of chondrogenesis (Cho and Dressler, 1998). *Tcf4* is also expressed in osteoblasts but, unlike *Tcf1*, its expression is not detected until E16.5 (Glass *et al*., 2005). Mice deficient for *Tcf4* appear normal but die shortly after birth due to defects in the epithelial cells and crypt stem cells of their small intestines (Korinek *et al*., 1998).

Of these four mice, only *Tcf1*$^{-/-}$ mice have been analyzed for changes in bone density. These mice have a significant decrease in their bone mass at 1 month of age (Glass *et al*., 2005). This decrease in bone mass is not as severe as that seen with the osteoblast-specific loss of *β-catenin*; presumably, this discrepancy in severity is due to the continued presence of *Tcf4* within the cells. Similar to the osteoblast-specific loss of *β-catenin*, *Tcf1*$^{-/-}$ mice have an increase in the number of osteoclasts without any changes in osteoblast number or function. There is also decreased expression of *Opg* while *Rankl* expression remains unchanged (Glass *et al*., 2005). A genetic interaction between *Tcf1* and *β-catenin* occurs within osteoblasts to regulate bone mass, as mice heterozygous for loss of both *Tcf1* and *β-catenin* have a decrease in bone mass not seen in either the *Tcf1* or the *β-catenin* heterozygous mice. These double heterozygous mice also have increased numbers of osteoclasts and decreased expression of *Opg* (Glass *et al*., 2005). One would predict similar findings on analyzing *Tcf4*$^{-/-}$ and *Tcf4-β-catenin* double heterozygous mice.

There are multiple isoforms of the Lef/Tcf transcription factors, which arise from alternative splicing or different promoter usage. Besides full-length transcripts, some of these isoforms lack the β-catenin interacting domain

and thus serve as dominant negatives, while others lack corepressor binding domains (Hovanes *et al*., 2000, 2001; Van de Wetering *et al*., 1996). With regards to bone, activating isoforms of *Tcf1* have been detected within primary osteoblast cultures, establishing that activation of canonical Wnt signaling target genes can occur within osteoblasts (Glass *et al*., 2005).

In adult mice, Lef/Tcf transcription factors are generally restricted to mitotically active cells such as the skin, hair follicles, lymphoid tissues, intestine, and testis. Within the bone milieu, Lef/Tcf activity is increased in areas of bone remodeling and in proliferating osteogenic cells (de Jong *et al*., 2002; Hadjiargyrou *et al*., 2002; Kahler and Westendorf, 2003; Kato *et al*., 2002; Qi *et al*., 2003). Their expression usually decreases as proliferation slows down and disappears once cells become postmitotic (Kratochwil *et al*., 1996; Mariadason *et al*., 2001; Oosterwegel *et al*., 1993a; Shibamoto *et al*., 2004; Travis *et al*., 1991; Zhou *et al*., 1995). *Tcf1* and *Tcf4* are expressed in adult primary osteoblast cultures (Glass *et al*., 2005), and the expression of *Tcf1* is significantly elevated in mature osteoblasts (Kalajzic *et al*., 2005). Additionally, in $Lrp5^{-/-}$ osteoblasts, the expression level of *Lef1* decreases but that of *Tcf4* remains unchanged after 10 days of culture, further implicating the involvement of Tcf4 in Wnt signaling in osteoblasts (Kato *et al*., 2002).

With regards to the interaction of Lef/Tcfs with other transcription factors, a recent study has shown that *Lef1* physically interacts with *Runx2*, a gene absolutely required for osteoblast differentiation and function (Ducy *et al*., 1997; Kahler and Westendorf, 2003; Komori *et al*., 1997; Otto *et al*., 1997). Lef1, Tcf1, and Tcf4 are also able to repress *Runx2* from activating the *osteocalcin* promoter via binding to a Lef/Tcf consensus site, four base pairs away from the Runx2 binding site, on the promoter (Kahler and Westendorf, 2003; Westendorf *et al*., 2004). Furthermore, an activated $\beta$-catenin mutant enhances Lef1-mediated repression of *Runx2* (Kahler and Westendorf, 2003). In other situations where the binding sites are further apart or coactivators are present, *Runx2* and *Lef/Tcfs* can cooperate to activate target gene expression (Bruhn *et al*., 1997; El-Tanani *et al*., 2004; Giese *et al*., 1995; Hsu *et al*., 1998).

There is controversy over whether canonical Wnt signaling is able to influence *Runx2* expression levels. The prevailing thought was that canonical Wnt signaling did not affect *Runx2* expression (Gong *et al*., 2001; Kato *et al*., 2002), and therefore any effects on Runx2 would be concerning the ability of Runx2 to transactivate target genes through interactions with Lef/Tcfs and/or $\beta$-catenin. Thus, inactivation of the Lef/Tcf proteins would presumably alleviate any inhibition of Runx2-mediated activation of target genes. However, Tcf1 has been shown to bind to a Lef/Tcf regulatory element on the *Runx2* promoter, and Tcf1 has been shown to upregulate *Runx2* promoter activation in MC3T3 and C3H10T1/2 cells (Gaur *et al*., 2005). Thus, the possibility exists that Lef/Tcfs are able to regulate osteoblasts via the

modulation of Runx2 at both the RNA and protein level, as well as through Runx2-independent mechanisms.There is also an increasing amount of data concerning cross talk between the Wnt and the TGF$\beta$ signaling pathways. Both *Smad4* (utilized by all BMPs for signaling) and *Smad3* (TGF$\beta$-specific) are able to synergize with *Lef1* to activate gene expression in response to *BMP-2* or *TGF$\beta$* signaling, respectively (Hussein *et al*., 2003; Labbe *et al*., 2000; Nishita *et al*., 2000). BMP-2 is required for maximal alkaline phosphatase expression and induction of mineralization in Wnt3a-treated C3H10T1/2 cells (Derfoul *et al*., 2004). An activated mutant of $\beta$-catenin ($\Delta$N151) is able to synergize with BMP-2 to induce alkaline phosphatase, osteocalcin, and matrix mineralization of C3H10T1/2 and C2C12 cells, and $\Delta$N151 $\beta$-catenin and BMP-2 stimulated new bone formation *in vivo* after periosteal injections into mouse calvariae (Mbalaviele *et al*., 2005). Thus, BMP/TGF$\beta$ and Wnt signaling are able to intersect within the nucleus, where the interaction between *Smads* and *Lef/Tcfs* are able to facilitate target gene expression.

There is also interaction between BMPs and Wnts via feedback interaction. Data from experiments on C3H10T1/2 cells indicate that BMP-2 is able to induce *Wnt1* and *Wnt3a* in an autocrine fashion, placing BMP signaling upstream of Wnt signaling in osteoblast-like cells (Rawadi *et al*., 2003). There is also data suggestive of BMPs also being downstream of Wnt signaling. BMP antagonists sclerostin and noggin blocked both *Wnt3a* and *BMP-6* induced alkaline phosphatase activity (Winkler *et al*. 2005). Early Wnt3a signaling, as measured by $\beta$-catenin accumulation, was blocked by Dkk1 but not by the BMP antagonists. Moreover, *Wnt3a* induced *BMP-4 mRNA* expression 12 hr before that of alkaline phosphatase in C3H10T1/2 cells (Winkler *et al*., 2005), suggesting that the inhibition of Wnt signaling by sclerostin and noggin was actually due to inhibition of BMP-6 mediated signaling, leading to the loss of alkaline phosphatase expression. Similar effects may be attributed to connective tissue growth factor (CTGF), a BMP antagonist, functioning as a canonical Wnt inhibitor (Luo *et al*., 2004) and transgenic mice overexpressing Gremlin (which is part of the same family of proteins as Cerberus) having low bone mass and a decreased number of osteoblasts (Gazzerro *et al*., 2005).

## III. Other Wnt Signaling Molecules

A plethora of data now exists regarding the roles of Lrp5/6 and $\beta$-catenin/Lef/Tcf signaling in bone remodeling. However, the information regarding Wnt signaling in bone is by no means limited to these proteins. There are a number of components of the canonical Wnt signaling pathway that have also been characterized to play important roles in bone biology, *in vitro* and

*in vivo*. Other components of canonical Wnt signaling will now be discussed regarding their effects on bone remodeling.

## A. Wnts

Wnts are a family of cysteine-rich secreted glycoprotein molecules that are evolutionary conserved from hydra to mammals. They were first identified due to sequence homology to *Drosophila* wingless and the murine *int-1* proto-oncogene. To date there are 19 different Wnts within the human genome (Miller, 2002). This gene family can be subdivided based on their ability to induce axis duplication in *Xenopus* embryos. Those that can induce axis duplication (*Wnt1, Wnt2, Wnt3, Wnt3a, Wnt8,* and *Wnt8b*) comprise the *Wnt1* class of genes; these interact with Fzd and Lrp proteins to activate the canonical Wnt signaling pathway. Those that cannot induce axis duplication (*Wnt4, Wnt5a, Wnt5b, Wnt6, Wnt7a*, and *Wnt11*) belong to the Wnt5a class; rather than the canonical pathway, these bind to Fzds to activate heterotrimeric G proteins and increase intracellular calcium or activate Rho-dependent actin cytoskeletal changes. Even within specific Wnt classes, there is still specificity between the various Wnts, as different Wnts in the same class have different profiles of target gene activation (Jackson *et al*., 2005). The mechanisms behind this specificity have not been fully elucidated. Presumably, each Wnt has different affinities for various Fzds and Lrps, leading to various Wnt-Fzd-Lrp combinations.

Wnts are hydrophobic and highly insoluble, hence their structure has been extremely difficult to solve. The cysteines found in Wnt proteins are presumably involved in intramolecular disulfide bonds. These residues may also be posttranslationally modified as evidenced by palmitoylation being required for Wnt3a activity (Willert *et al*., 2003). Wnts also require an association with glycosaminoglycans for optimal signaling in the ECM (Baeg *et al*., 2001; Lin and Perrimon, 1999; Tsuda *et al*., 1999). All of these factors contribute to the formation of Wnt gradients, which spatially limit the action of Wnts *in vivo*.

As Wnts are secreted molecules, the source of Wnts in the bone milieu still needs to be fully elucidated. Wnts may be found in the ECM, where they would be released during bone remodeling. Osteoblasts are another potential source, as they produce Wnt1, Wnt4, and Wnt14 *in vitro* (Kato *et al*., 2002; Zhang *et al*., 2004); furthermore, *Wnt1* and *Wnt3a* are induced by *BMP-2* in C3H10T1/2 cells (Rawadi *et al*., 2003) and *Wnt5a, Wnt7b*, and *Wnt9* are induced by Hh signaling (Hu *et al*., 2005). A greater knowledge of the sources of Wnts would provide a better understanding of the different cell type or types involved in the regulation of bone remodeling via these signaling molecules.

The majority of the studies looking directly at the action of Wnts on osteoblasts stem from *in vitro* experiments, using osteoblast precursor cell lines. ST2, MC3T3, C2C12, and C3H10T1/2 cell lines can assume an osteoblastic phenotype when treated with BMP-2 or other osteoblast-inducing stimuli. Wnt1 class Wnts are able to induce alkaline phosphatase expression in C2C12, C3H10T1/2, and ST2 cells, while both Wnt1 and Wnt5a class Wnts enhance the growth rate and change the morphology of C3H10T1/2 cells (Bradbury *et al*., 1994; Derfoul *et al*., 2004; Gong *et al*., 2001; Hu *et al*., 2005; Rawadi *et al*., 2003). *Wnt3a* was able to activate *alkaline phosphatase* expression in these cell lines, but not those of *Runx2*, $\alpha_1$*(I) collagen*, or *osteocalcin*. Furthermore, alkaline phosphatase expression was inhibited by Dkk1 or dominant negative forms of *Lrp5* (Rawadi *et al*., 2003). Wnt7b has also been shown to induce alkaline phosphatase expression in C3H10T1/2 cells (Hu *et al*., 2005). *Wnt7b* is expressed in osteoblasts and as its expression level increases during osteoblast differentiation, it may be required in an autocrine manner for osteoblast differentiation (Hu *et al*., 2005; Li *et al*., 2005b). *Wnt7b* is not the only Wnt differentially expressed during osteoblast differentiation. In the 2.3 kb $\alpha_1$*(I) collagen-GFP* transgenic mice, *Wnt3a* was found to be increased in expression in the GFP-positive mature osteoblasts, while noncanonical *Wnt5a* was downregulated (Kalajzic *et al*., 2005). This suggests that canonical Wnt signaling has important implications for both differentiating osteoblasts as well as for fully differentiated osteoblasts.

Besides having a role in osteoblast differentiation, Wnts may also participate in lineage determination of mesenchymal stem cells. Adipocytes, osteoblasts, chondrocytes, and myocytes are all derived from the same mesenchymal precursor, with adipogenesis being the default pathway. Wnt1 and Wnt10b prevent adipocyte differentiation of preadipocyte cells (Ross *et al*., 2000). In contrast, overexpression of *Wnt3a* induces expression of *PPAR*$\gamma$*2* and *FABP*, two adipocyte markers, in C3H10T1/2 cells (Rawadi *et al*., 2003). In mouse models, *Wnt10b*$^{-/-}$ mice have less bone and fewer trabeculae than wildtype littermates, along with decreased levels of *osteocalcin* (Bennett *et al*., 2005). The fact that there was no change in serum tartrate-resistant acid phosphatase (TRAP) levels, a marker for osteoclast resorption, suggests that the effect of Wnt10b on bone mass is solely via the osteoblast. Conversely, Wnt10b transgenic mice, using the adipocyte-specific *FABP4* promoter, have increased bone mass, more trabeculae, and less fat (Bennett *et al*., 2005). Wnt10b seems to be an important regulator of cell fate determination between osteoblastogenesis and adipogenesis, as retroviral infection of *Wnt10b* into ST2 cells led to increased induction of *Runx2*, *Dlx5*, and *Osterix*, all osteoblast transcription factors, while it decreased expression of the adipocyte transcription factors *C/EBP*$\alpha$ and *PPAR*$\gamma$

(Bennett *et al.*, 2005). Hence, Wnts play an important role both in osteoblast differentiation and function.

## B. Frizzled

Frizzled (Fzd) proteins are cell surface receptors for Wnt ligands (Bhanot *et al.*, 1996). There are 10 known family members in humans, with one fewer in mice. Wnts interact with Fzds via a cysteine-rich domain (CRD); this CRD, on the extracellular surface of the proteins, is separated by a region of unknown function from the first of seven transmembrane domains. Although Fzds resemble G-protein coupled receptors, only a few use heterotrimeric G-proteins to increase intracellular calcium levels without activating canonical Wnt signaling (Veeman *et al.*, 2003). With regards to canonical Wnt signaling, Fzds use a KTxxxW motif in their cytoplasmic tails to recruit phosphorylated Dishevelled to the cell membrane, which in turn stabilizes $\beta$-catenin and leads to Lef/Tcf-mediated gene activation (Umbhauer *et al.*, 2000). All human Fzds have the KTxxxW motif, as do all mouse Fzds with the exception of Fzd1 and Fzd5. The importance of this KTxxxW motif may be exemplified by the finding that Fzd1, which lacks this motif, can inhibit canonical Wnt signaling in mesenchymal cells (Roman-Roman *et al.*, 2004).

Regarding Fzd expression in osteoblasts, there is little data to date; what is available suggests that Fzds may function via a feedback mechanism to regulate Wnt signaling in osteoblasts. In analyzing Fzd expression in four different osteosarcoma cell lines, *Fzd6* and *Fzd10* were not expressed and *Fzd3* was found only in one cell line. The other seven Fzds were expressed in either three- or all four-cell lines (Hoang *et al.*, 2004). In contrast, both *Fzd2* and *Fzd6* were expressed in calvarial and primary osteoblasts derived from wildtype and $Lrp5^{-/-}$ mice (Kato *et al.*, 2002). That the expression of these Fzds decreased in both sets of osteoblasts after 10 days of culture conditions favoring mineralization suggests that downregulation of some Fzds may be required to achieve terminal osteoblast differentiation. *Fzd1* and *Fzd8* were both found to be preferentially downregulated in mature osteoblasts (Kalajzic *et al.*, 2005).

Other factors are able to influence Fzd expression in osteoblasts. PTH, EGF, and 1, 25-$(OH)_2D_3$ can increase *Fzd1* and *Fzd2* expressions in rat UMR 106-H5 cells (Chan *et al.*, 1992). PTH can also increase *Fzd1* expression in UMR106 rat osteosarcoma cells; however, in this series of experiments the effect of PTH is partially mediated through PKA-dependent signaling (Kulkarni *et al.*, 2005). BMP-2 stimulates *Fzd1* expression in a variety of mesenchymal cell lines (C2C12, C3H10T1/2, and ST2) (Roman-Roman *et al.*, 2004). Overexpressing *Fzd1* blocked both BMP-2 and

Wnt3a-mediated alkaline phosphatase production in these cells. A similar function has been attributed to *Fzd6* (Golan *et al*., 2004). These data suggest that some Fzds may have an inhibitory role in canonical Wnt signaling, and that the expression of both stimulatory and inhibitory Fzds is self-regulated.

## C. GSK3$\beta$

GSK3$\alpha$ and GSK3$\beta$ are serine-threonine kinases, which are involved in a number of different signaling pathways, such as Wnt, NF-$\kappa$B, sonic hedgehog, and insulin-dependent pathways. These genes are associated with a number of disease processes, including diabetes mellitus, cancer, and neurodegenerative disorders (Ali *et al*., 2001). GSK3$\beta$ is able to phopshorylate multiple Wnt signaling components, including $\beta$-catenin, APC, and Axin. Specifically, the phosphorylation of serines 33 and 37 and threonine 41 by GSK3$\beta$ occurs after serine 45 is phosphorylated by CK1$\alpha$ (Liu *et al*., 2002). This phosphorylation leads to proteosomal-dependent degradation (Jiang and Struhl, 1998). Wnt signaling disperses the GSK3$\beta$-Axin-APC-$\beta$-catenin complex by poorly understood mechanisms. GBP/Frat1 may be involved in displacing Axin from GSK3$\beta$, causing the release and accumulation of $\beta$-catenin (Farr *et al*., 2000; Hay *et al*., 2005; Li *et al*., 1999).

In *Xenopus* and *Drosophila*, expressing kinase-dead GSK3 mutant proteins induces phenotypes similar to Wnt overexpressing models (He *et al*., 1995; Peifer *et al*., 1994; Pierce and Kimelman, 1996; Siegfried *et al*., 1992, 1994), and in mice, increasing brain GSK3$\beta$ levels lead to decreased levels of $\beta$-catenin (Lucas *et al*., 2001). Deficiency for GSK3$\beta$ results in embryonic lethality at E13.5-E14.5 due to defective NF-$\kappa$B signaling and hepatocyte apoptosis (Hoeflich *et al*., 2000). As the limbs in these embryos appeared normal and no other Wnt deformities were seen, GSK3$\alpha$, or some other kinase, must be able to substitute for GSK3$\beta$ until this stage of development.

Lithium chloride is often used to study GSK3$\beta$ function as it is a highly selective inhibitor of the GSK3s and mimics canonical Wnt signaling in cells (Stambolic *et al*., 1996). Lithium causes patterning defects in *Xenopus* that are similar to GSK3 mutant phenotypes and can inhibit the kinase activity of purified GSK3$\beta$ *in vitro* (Klein and Melton, 1996). However, lithium can also affect AKT and receptor-coupled G-proteins, so it is pleiotropic in nature (Avissar *et al*., 1988; Chalecka-Franaszek and Chuang, 1999; May and Gay, 1997). There are other GSK3 inhibitors available for use, such as SB216773, but lithium is better at approximating the expression profile of *Wnt3a*-treated C3H10T1/2 cells (Jackson *et al*., 2005) and, therefore, appears to be the GSK3 antagonist of choice. Lithium should be used in combination with other experiments to confirm that its involvement is via

GSK3-mediated events; however, no change with lithium administration means that GSK3 is not involved.

Lithium has been used in osteoblastic cell line experiments to study GSK3 function in bone formation. Lithium is able to induce *alkaline phosphatase* expression, but not *osteocalcin* expression, in C3H10T1/2 cells, similar to the results obtained from stabilized and overexpressed *β-catenin* (Bain *et al*., 2003). Lithium administration to MC3T3 cells allows for high-density growth *in vitro*, although there were no changes in *β-catenin* levels (Smith *et al*., 2002). However, lithium inhibited serine 9 phosphorylation of GSK3$\beta$ in response to glucocorticoids, which inhibits GSK3$\beta$ activity toward some substrates but not that of Axin (Frame *et al*., 2001; Smith *et al*., 2002; Stambolic and Woodgett, 1994; Wang *et al*., 1994). Dexamethasone is able to inhibit $\beta$-catenin levels and Tcf-mediated transcription. However, not all of this inhibition is done through suppression of canonical Wnt signaling, as dexamethasone can also suppress the PI3K/Akt/GSK3$\beta$/$\beta$-catenin/Tcf1 pathway, thereby also suppressing $\beta$-catenin (Smith and Frenkel, 2005). Hence, GSK3$\beta$ is involved in both Wnt-dependent and Wnt-independent signaling in osteoblasts.

## IV. Secreted Wnt Inhibitors and Agonists

There are a number of different secreted protein families that antagonize the canonical Wnt signaling pathway, which can be subdivided into two different classes. Some Wnt inhibitors bind to the Lrp5/6 receptor and thereby prevent formation of Wnt-Fzd-Lrp complex. Dkks, Wise, and Sclerostin fall into this first category. The second group interacts with either Wnts and/or Fzds and inhibit interactions between these two proteins; this group includes Sfrps, Cerberus, and Wif-1. Studies concerning Wise and Cerberus have been limited to *Xenopus* models (Itasaki *et al*., 2003; Piccolo *et al*., 1999) and thus will not be examined in this chapter. There is evidence, *in vitro* or *in vivo* in many cases, linking the other four inhibitors to roles in bone remodeling.

### A. Dkks

Dickkopfs were first identified in *Xenopus* as being necessary and sufficient for head induction (*dickkopf* is German for big head) (Glinka *et al*., 1998). There are 4 Dickkopf molecules, all of which have a signal peptide sequence and two cysteine-rich domains. Dkk1 and Dkk4 are able to inhibit Wnt signaling by binding to Lrp5/6 and Kremen1/2 (Krm1/2) (Bafico *et al*., 2001; Mao *et al*., 2002). This trimer subsequently undergoes endocytosis; its removal from the surface membrane inhibits activation of the canonical

Wnt signaling pathway. Dkk2 is also able to inhibit Wnt signaling by binding to Lrp5/6 and Krm1/2; however, it is also able to activate $\beta$-catenin signaling in *Xenopus* and promotes Lrp6-mediated axis duplication (Mao *et al*., 2002; Wu *et al*., 2000). As Kremen proteins are able to change Dkks from an agonist to a Wnt antagonist (Mao and Niehrs, 2003), presumably the function of Dkk2 is dependent on the levels of Krm proteins. Unlike the other Dkk proteins mentioned, Dkk3 has no effect on Wnt signaling via Lrp6 or Krm1/2 (Krupnik *et al*., 1999; Mao *et al*., 2002).

*Dkk1*$^{-/-}$ mice are embryonic lethal due to head induction and limb morphogenesis defects (Mukhopadhyay *et al*., 2001). *Dkk2*$^{-/-}$ mice are osteopenic, suggesting that Dkk2 actually functions as a Wnt agonist *in vivo* (Li *et al*., 2005a). *Dkk2*$^{-/-}$ mice have more osteoid and mineralization defects when osteoblasts are cultured *in vitro*, indicating defects in osteoblast function. Dkk2 can be upregulated by canonical Wnt signaling. *Dkk2* overexpression before *Wnt7b* expression leads to suppression of osteogenesis, but expression after peak *Wnt7b* expression induced terminal osteoblast differentiation and mineralization (Li *et al*., 2005a). It is not known how the expression of *Wnt7b* influences Krm levels. *Dkk2*$^{-/-}$ mice also have an increased number of osteoclasts; there is an increase in Rankl, but not Opg, in immature *Dkk2*$^{-/-}$ osteoblasts, but this observation is not found in mature mineralizing osteoblasts (Li *et al*., 2005a). Thus, Dkk2 regulates both osteoblast and osteoclast biology. However, the effects of *Dkk2* deficiency may not all be Wnt related. Dkk2, but not FRP3 (another Wnt antagonist), is able to rescue the phenotypes of *Dkk2* deficiency (Li *et al*., 2005a).

Dkk proteins are implicated in a variety of disease states. In HBM, the glycine to valine mutation in Lrp5 prevents Dkk1 from inhibiting canonical Wnt signaling, at least in fibroblasts (Boyden *et al*., 2002; Zhang *et al*., 2004). In multiple myeloma, the myeloma cells secrete higher amounts of Dkk1, and the increase in *Dkk1* expression correlates with the severity of the skeletal problems (Tian *et al*., 2003). Sera from patients with high levels of *Dkk1*, or recombinant Dkk1, could inhibit alkaline phosphatase expression in C2C12 cells, suggesting that Dkk1 inhibits osteoblast differentiation, which results in the lack of bone accrual seen in multiple myeloma patients.

Glucocorticoid administration can lead to osteoporosis as a side effect. Dexamethasone administration significantly increases expression of *Dkk1* via a glucocorticoid-responsive element-like sequence on the promoter (Ohnaka *et al*., 2004). Dexamethasone is able to repress canonical Wnt signaling, but this is only partially through an increase in *Dkk1* expression (Ohnaka *et al*., 2005). Nonetheless, this upregulation of *Dkk1*, and inhibition of canonical Wnt signaling, may be at least one mechanism underlying glucocorticoid-induced osteoporosis (Ohnaka *et al*., 2004, 2005).

Dkk1 levels are increased in mature osteoblasts (Kalajzic *et al*., 2005). Dkks are also seen in osteosarcoma cells and in osteocytes (Gregory *et al*.,

2003; Zhang *et al*., 2004) however, neutralizing antibodies against Dkk1 actually suppressed cell growth of MG63 osteosarcoma cells (Gregory *et al*., 2003). These results, although contrary to canonical Wnt signaling facilitating cell proliferation, are in agreement with studies from Hartmann and Tabin which suggest that Wnts facilitate exiting proliferation and entering a postmitotic state (Hartmann and Tabin, 2000). It is not known what the level of Kremen protein expression was in these various experiments, hence differences in Kremen levels could explain the discrepancy in these studies. Nonetheless, they establish multiple roles for Dkk molecules in canonical Wnt signaling and bone remodeling.

## B. Sfrps

Secreted frizzled related proteins (Sfrps) are another class of Wnt antagonists, which are encoded by their own separate genes and are not splicing isoforms of the Fzd genes. Sfrps contain two signature domains, a cysteine-rich domain (CRD) that has 30–50% homology to CRDs found in Fzds, and a netrin domain of unknown function. There are five known family members; one of them is also known as FrzB (Frizzled motif associated with bone development). It was initially thought to be expressed in the cartilaginous regions of developing bone (Hoang *et al*., 1996) but recent genetic analysis of osteoblasts at various stages of differentiation was able to detect Sfrp3 (Kalajzic *et al*., 2005) and crossing a *FrzB-Cre* transgenic mouse with the ROSA26 reporter line revealed LacZ staining in the sutures of the developing skull at E14.5 (Tylzanowski *et al*., 2004), suggesting that *FrzB* may be expressed during osteoblast differentiation. Some but not all Sfrps interact with Wnts or Fzds to inhibit Wnt signaling (Bafico *et al*., 1999; Chung *et al*., 2004; Kawano and Kypta, 2003); Sfrp3 (FrzB) inhibits noncanonical Wnt5a-mediated signaling, but is unable to block Wnt3a-mediated activation of alkaline phosphatase (Chung *et al*., 2004; Hoang *et al*., 1996). Sfrp2, on the other hand, is detected in human primary osteoblasts and inhibits canonical Wnt signaling (James *et al*., 2000). Its expression is highest during the late stages of *BMP-2*-induced osteoblast differentiation of C3H10T1/2 and C2C12 cells (Vaes *et al*., 2005). *Sfrp1* is highly expressed during the preosteoblast to preosteocyte transition, and then declines in mature osteocytes (Bodine *et al*., 2005). *Sfrp3* is preferentially expressed in the GFP-positive, mature osteoblasts from the 2.3kb $\alpha_1(I)$ *collagen-GFP* transgenic mice, while *Sfrp1* and *Sfrp2* are found more in GFP-negative, immature osteoblasts (Kalajzic *et al*., 2005).

*Sfrp1*$^{-/-}$ mice have an increase in trabecular but not cortical bone, which is more elevated in females (Bodine *et al*., 2004). Loss of *Sfrp1* leads to decreased apoptosis in osteoblast lineage cells *in vivo* and *in vitro* with

increased proliferation, alkaline phosphatase expression, and matrix mineralization of calvarial cells *in vitro* (Bodine *et al.*, 2004, 2005). *Sfrp1* expression in preosteoblasts was increased by $PGE_2$ treatment with an increase in cell death, and was decreased in preosteocytes by TGF$\beta$1 with a decrease in cell death (Bodine *et al.*, 2005). *Sfrp1*$^{-/-}$ mice have increased levels of Tcf1, Runx2, and osteocalcin (Gaur *et al.*, 2005). Hence, Sfrp1 may be an inhibitor of bone accrual by negatively regulating canonical Wnt signaling and increasing the rate of apoptosis in osteoblasts.

Sfrp1 may also function as an inhibitor of osteoclast formation. *In vitro*, recombinant Sfrp1 dose dependently inhibited osteoclast formation in Rankl + mCSF-treated splenic cultures, indicating a direct effect on hematopoietic cells. Sfrp1 is able to bind to Rankl in ELISAs (Hausler *et al.*, 2004). Thus, Sfrp1 could inhibit endogenous Wnt signaling and also bind to Rankl in the prevention of osteoclast formation. Sfrp1 may also inhibit canonical Wnt signaling by blocking the expression of *Ror2* (Billiard *et al.*, 2005).

Sfrps may also play a role in the low bone mass associated with multiple myeloma (MM). Conditioned media from BMP-2-treated RPMI8226 and U266 MM cell lines and primary MM cells contained *Sfrp2* (Oshima *et al.*, 2005). Multiple myeloma patients with advanced bone destructive lesions also expressed *Sfrp2*. *Sfrp2* suppressed *BMP-2* induced osteoblast differentiation and Sfrp2 immunodepletion restored mineralized nodule formation *in vitro*. Thus, in addition to enhanced osteolysis, myeloma cells also suppress bone formation at least in part through Sfrp2-mediated inhibition of the canonical Wnt pathway (Oshima *et al.*, 2005). Dkk1 was not found to be increased in multiple myeloma cells in this study, in contrast with other published studies on Wnt inhibitors involved with multiple myeloma (Tian *et al.*, 2003). Although there are some discrepancies, these results are not mutually exclusive; therefore there may be at least two inhibitors (Dkk1 and Sfrp2) that myeloma cells use to inhibit bone accrual once they invade the bone milieu.

Sfrps may also have a role in tissue mineralization. *Sfrp4* is highly expressed in tumors associated with hypophosphatemia, osteomalacia, renal phosphate wasting, and abnormal vitamin D metabolism (Berndt *et al.*, 2003). Sfrp4 inhibits canonical Wnt signaling in renal cells and inhibits the renal reabsorption of inorganic phosphate independently of PTH. These data suggest that Sfrps may also influence bone strength by regulating phosphate levels.

### C. Wif-1

Wnt inhibitory factor-1 (*Wif-1*) is highly conserved in vertebrates and interacts with a number of Wnts, although it has no homology to Sfrps or Fzds (Hsieh *et al.*, 1999). *Wif-1* is detected in ossification centers in E16.5

embryos in a pattern that coincides with that of *Runx2* (Vaes *et al*., 2002). It is found preferentially expressed in trabecular rather than cortical bone in mouse-tail vertebrae, suggesting it plays a role in osteoblast differentiation (Vaes *et al*., 2005). After treatment with BMP-2, *Wif-1* expression was greatly increased in C2C12, C3H10T1/2, and MC3T3 osteoblastic cell lines, but not in fibroblasts (Vaes *et al*., 2002, 2005). However, the role of Wif-1 in bone biology has not been determined via knockout mouse models nor *in vitro* bone differentiation models. Shifted, the Drosophila homolog of Wif-1, is able to regulate the accumulation of Hh protein and its movement in the fly wing, hence it is possible that besides functioning as a Wnt inhibitor, Wif-1 may also affect Shh signaling in osteoblasts (Glise *et al*., 2005).

### D. Sclerostin

Sclereosteosis is an autosomal disease that is very similar to HBM diseases, which is due to mutations in the *SOST* gene (Balemans *et al*., 2001; Beighton *et al*., 1976; Hamersma *et al*., 2003; Semenov *et al*., 2001). $SOST^{-/-}$ mice (which are null for sclerostin) have HBM (Li *et al*., 2005b). Sclerostin binds to the first two YWTD-EGF repeat domains of Lrp5/6 and prevents Lrp-Fzd interaction (Li *et al*., 2005b; Semenov *et al*., 2005). Sclerostin is expressed when osteocalcin expression occurs (during late osteoblast differentiation, after expression of *Wnt7b*) in primary osteoblast differentiation experiments (Li *et al*., 2005b). However, sclerostin may not function purely as a direct Wnt antagonist. Sclerostin can block *Wnt3a* and BMP-6 mediated activation of alkaline phosphatase in C3H10T1/2 cells, while Dkk1 can only block that induced by *Wnt3a* (Winkler *et al*., 2005). In addition, early Wnt3a signaling, as measured by $\beta$-catenin accumulation, was not affected by sclerostin but was blocked by Dkk-1. As *Wnt3a* induced the appearance of *BMP-4* mRNA 12 hrs prior to that of alkaline phosphatase in C3H10T1/2 cells (Winkler *et al*., 2005), it suggests that sclerostin can inhibit Wnt signaling by impeding downstream signaling via BMPs.

## V. Intracellular or Transmembrane Modulators

### A. Kremens

Kremens (Kringle-coding genes marking the eye and the nose; Krms) are single transmembrane receptors that interact with Dkk and Lrp6 to inhibit canonical Wnt signaling (Mao *et al*., 2002; Nakamura *et al*., 2001). A trimer complex formed between Krm, Dkk, and Lrp6 is removed from the cell

surface via endocytosis and Lrp6 is either recycled or degraded. This Krm-Dkk-Lrp6 interaction, therefore, removes Wnt coreceptors from the cell surface and inhibits canonical Wnt signaling (Mao *et al.*, 2002). Presumably, Lrp5 undergoes a similar fate with Krms but this has not been verified. Krm1 and Krm2 have both low- and high-affinity binding sites for Dkk1, Dkk2, and Dkk4, but do not interact with Dkk3 (Mao and Niehrs, 2003; Mao *et al.*, 2002). Krm proteins are able to convert Dickkopf proteins from agonists of Wnt signaling to antagonists (Mao and Niehrs, 2003) and, therefore, may be required for Dkk-mediated inhibition of Wnt signaling. There are three extracellular motifs (Kringle, WSC and CUB domains), all of which are required for binding to Dkk, while there are no motifs in the intracellular domain, which is not required for Krm internalization (Mao *et al.*, 2002; Nakamura *et al.*, 2001). Although Krms are established as playing an important role in canonical Wnt signaling, their direct effects on bone remodeling have not been elucidated to date.

## B. Axin2/Conductin/Axil

Axin2/conductin/Axil is expressed in the osteogenic fronts and periosteum of developing sutures. *Axin2*$^{-/-}$ mice have skull malformations resembling craniosynostosis, with premature suture fusion (Yu *et al.*, 2005). These mice have an enhanced expansion of osteoprogenitor cells, accelerated ossification, stimulated expression of osteocalcin and osteopontin, and increased mineralization, and are mediated through activation of $\beta$-catenin. Loss of *Axin2* promotes osteoblast proliferation and differentiation *in vivo* and *in vitro*. Hence *Axin2* serves as an inhibitor of bone accrual *in vivo* (Yu *et al.*, 2005).

## C. Ror2

Ror2 is an orphan receptor tyrosine kinase, containing an Fzd-like CRD and a Krm-like Kringle domain on its extracellular surface. Mutations in Ror2 lead to Robinow syndrome and brachydactyly type B (Afzal *et al.*, 2000; Oldridge *et al.*, 2000; Schwabe *et al.*, 2000; van Bokhoven *et al.*, 2000). Ror2 interacts with Wnt5a and Fzd2 via its CRD and was initially shown to be able to activate JNK and the planar cell polarity pathway, similar to Wnt5a (Oishi *et al.*, 2003). *Ror2*$^{-/-}$ mice have several skeletal abnormalities, attributable to cartilage and growth plate defects (DeChiara *et al.*, 2000).

*Ror2* is expressed in chondrogenic regions of developing bone (DeChiara *et al.*, 2000) and was thought to be specific to regions of cartilage in developing bone. However, recent study has detected *Ror2* at low levels in stem cells and high expression in preosteoblasts (Billiard *et al.*, 2005). *Ror2*

expression is suppressed by Sfrp-1 (Billiard *et al.*, 2005), suggesting that Ror2 may have a role in both canonical and noncanonical Wnt signaling. Ror2 inhibits both Wnt1- and Wnt3-mediated stabilization of $\beta$-catenin, but Ror2 potentiates only Wnt1-mediated activation of a Wnt-responsive reporter; it also enhances Wnt1 but represses Wnt3 activity in osteoblastic cells (Billiard *et al.*, 2005). However, the mechanism by which Ror2 is able to potentiate Wnt1 signaling in a $\beta$-catenin independent manner remains unknown.

## VI. Conclusions and Future Work

Wnt signaling controls fate determination, differentiation, proliferation, survival and function, as well as osteoclast differentiation, within osteoblasts. The regulation of both cell types by canonical Wnt signaling exemplifies the diversity of target genes this signaling pathway possesses. Lrp and $\beta$-catenin-dependent signaling show how dysregulation of two components of the same pathway can give rise to the same results through completely different etiologies. The roles of other components of the canonical Wnt signaling pathway have been explored, along with how they influence bone remodeling. Finally, the secreted antagonists and the transmembrane/intracellular regulators shed new light on how canonical Wnt signaling functions, and open new doors through which canonical Wnt signaling in bone cells can be studied.

There are still a number of unanswered questions regarding canonical Wnt signaling and its regulation of bone remodeling. How is the specificity of Wnt signaling achieved within the bone milieu? What Wnts interact with what Fzds and Lrps to activate Wnt signaling within osteoblasts? Are these three components sufficient, or are there other extracellular modifiers that further regulate specificity? What are the differences between Lrp5/6-dependent and Lrp5/6-independent signaling within osteoblasts? Do Tcf1 and Tcf4 regulate the same set of genes, or is there specificity regarding gene regulation, and if so, how is it decided? These are some of the questions that are currently being addressed to achieve a better understanding as to how Wnt signaling affects bone formation.

Clearly, the most urgent question is to understand the reasons for the discrepancies found within the various mouse models for aberrant canonical Wnt signaling. While Lrp5 and Lrp6 mutations primarily affect osteoblasts directly and thereby bone accrual, manipulation of $\beta$-catenin and Tcf proteins in those same cells primarily influences osteoclasts and bone resorption. What accounts for the differences in phenotypes seen in these different models? Are they due to Lrp5 and Lrp6 signaling via noncanonical pathways? As other signaling pathways can also influence GSKs and $\beta$-catenin,

are the mouse models truly representative of canonical Wnt signaling activation or inhibition? The findings from these mouse models may seem contradictory as we try to elucidate the role of Wnt signaling in bone regulation, but this viewpoint presumes that Wnt signaling may not be multifactorial in its effects. It is possible that Wnt signaling is capable of influencing both bone formation and bone resorption concurrently through the osteoblast. The *Dkk2*$^{-/-}$ mice suggest that the phenotypes seen in the Lrp and the $\beta$-catenin mouse models may be two halves of the same coin. Even if this is the case, how these models are able to influence only one aspect of bone remodeling remains to be determined.

There is also little known on the role of canonical Wnt signaling within osteoclasts. To date, the vast majority of the data regarding canonical Wnt signaling and its effects on the osteoclast hinges on the osteoblast via the Rankl-Opg pathway. $\beta$-catenin is present in osteoclasts (Monaghan *et al.*, 2001) and canonical Wnt signaling is observed in osteoclasts (Hens *et al.*, 2005), yet we do not know its function in these multinucleated cells. This could initially be examined by constitutively activating or deleting $\beta$-catenin in osteoclast progenitor cells or in mature osteoblasts. *Pu.1-Cre* or *NF-κB-Cre* mouse lines would express *Cre* and cause gene alterations in osteoclast precursor cells. The use of *TRAP-Cre, Cathepsin K-Cre*, or *Cd11b-Cre mice* to express *Cre* in differentiated osteoclasts may prove invaluable; these mouse lines have already been generated (Chiu *et al.*, 2004; Ferron and Vacher, 2005). Similar to what has been performed in osteoblasts, the systematic identification of Wnts, Fzds, and Lef/Tcfs within osteoclasts will help focus our efforts to the analysis of cell lines *in vitro* and mouse models *in vivo* for aberrations in differentiation, proliferation, survival, or function.

Finally, what other transcription factors and signaling pathways affect canonical Wnt signaling? Do Atf4 and Osterix, two other transcription factors necessary for osteoblast differentiation and function (Nakashima *et al.*, 2002; Yang *et al.*, 2004), interact with Lef/Tcfs or $\beta$-catenin? Are Hedgehog and Notch signaling able to serve as upstream, downstream, and synergetic components of canonical Wnt signaling, as has been identified with BMP/Smad regulation? Will additional Wnt inhibitors be found that also contribute to the pathogenesis of multiple myeloma-associated osteoporosis? What roles, if any, do known Wnt components and antagonists such as Wif-1, Cerberus, Wise, Kremens, and Ror2 play in regulating bone remodeling, and how will they exert their effects? Are any other Wnt signaling components, besides Lrp5 and sclerostin, altered in other disease processes leading to osteoporosis or HBM? Continued analysis of the Wnt signaling pathway and its effects on bone, as well as discoveries concerning the genetic etiologies of various bone abnormalities and syndromes, will bring in the future greater insight into how canonical Wnt signaling regulates bone remodeling.

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# 3

# Calcium Sensing Receptors and Calcium Oscillations: Calcium as a First Messenger

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I. Introduction: Physiological Role of Extracellular $Ca^{2+}$
  A. Inverse Relation Between Serum $Ca^{2+}$ and Parathyroid Hormone Secretion
  B. Expression Cloning of a "CaR" from Bovine Parathyroid
II. CaR is a Unique Family C G-Protein–Coupled Receptor
  A. Structural Domains of CaR
  B. CaR Integrates a Variety of Context-Sensitive Metabolic Signals
III. CaR Signal Transduction
IV. CaR Translates Extracellular $Ca^{2+}$ Changes into Time-Varying Intracellular $Ca^{2+}$ Signals
  A. $Ca^{2+}$ Chronically Regulates Cells Which Express CaR
  B. Increases in Extracellular $Ca^{2+}$ Produce Intracellular $Ca^{2+}$ Oscillations
  C. Allosteric Agonists Facilitate CaR-Induced $Ca^{2+}$ Oscillations by Distinct Mechanisms
  D. Consequences of Intracellular $Ca^{2+}$ Oscillations
V. CaR in Development
  Acknowledgments
  References

Calcium sensing receptors (CaR) are unique among G-protein–coupled receptors (GPCRs) since both the first (extracellular) and second (intracellular) messengers are $Ca^{2+}$. CaR serves to translate small fluctuations in extracellular $Ca^{2+}$ into intracellular $Ca^{2+}$ oscillations. In many cells and tissues, CaR also acts as a coincidence detector, sensing both changes in extracellular $Ca^{2+}$ plus the presence of various allosteric activators including amino acids, polyamines, and/or peptides. CaR oscillations are uniquely shaped by the activating agonist, that is, $Ca^{2+}$ triggers sinusoidal oscillations while $Ca^{2+}$ plus phenylalanine trigger transient oscillations of lower frequency. The distinct oscillation patterns generated by $Ca^{2+}$versus $Ca^{2+}$ plus phenylalanine are the results of activation of distinct signal transduction pathways. CaR is a member of Family C GPCRs, having a large extracellular agonist binding domain, and functioning as a disulfide-linked dimer. The CaR dimer likely can be driven to distinct active conformations by various $Ca^{2+}$ plus modulator combinations, which can drive preferential coupling to divergent signaling pathways. Such plasticity with respect to both agonist and signaling outcomes allows CaR to uniquely contribute to the physiology of organs and tissues where it is expressed. This

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chapter will examine the structural features of CaR, which contribute to its unique properties, the nature of CaR-induced intracellular $Ca^{2+}$ signals and the potential role(s) for CaR in development and differentiation.



## I. Introduction: Physiological Role of Extracellular $Ca^{2+}$

Cytoplasmic $Ca^{2+}$ varies in a dynamic manner as a result of $Ca^{2+}$ release from intracellular stores (endoplasmic/sarcoplasmic reticulum, mitochondria) or regulated $Ca^{2+}$ influx from the extracellular space. Variations in cytoplasmic $Ca^{2+}$ represent potent regulatory signals, activating enzymes, and altering myriad protein interactions. Multiple cellular mechanisms have evolved to control the magnitude and duration of cytoplasmic $Ca^{2+}$ alterations. While cytoplasmic $Ca^{2+}$ can vary over a greater than five-fold range from $\approx$100 nM, extracellular $Ca^{2+}$ in human serum varies in the range of 1.1–1.3 mM (with minor daily fluctuations). Extracellular $Ca^{2+}$, therefore, represents both a constant threat and a crucial source of intracellular $Ca^{2+}$. Recent studies suggest that, in addition to providing a reservoir for replenishment of intracellular $Ca^{2+}$ via influx through transporters and/or ion channels, extracellular $Ca^{2+}$ can serve as a first messenger, activating the plasma membrane-localized calcium sensing receptor (CaR) (reviewed by Tfelt-Hansen and Brown, 2005). The properties of CaR are uniquely suited to sensing small changes in extracellular $Ca^{2+}$: (1) CaR has a low affinity for $Ca^{2+}$ ($EC_{50}$ from 2.5 to 4 mM depending on cell type) (reviewed in Breitwieser *et al.*, 2004); (2) CaR exhibits high cooperativity with respect to $Ca^{2+}$ activation (Hill coefficient in the range of 3–4) (reviewed by Riccardi, 2002); and (3) CaR is able to sense small changes in extracellular $Ca^{2+}$ in the presence of a significant baseline concentration of $Ca^{2+}$ (Breitwieser and Gama, 2001). Interestingly, CaR activation by extracellular $Ca^{2+}$ induces complex, time varying intracellular $Ca^{2+}$ signals (reviewed by Ward, 2004).

### A. Inverse Relation Between Serum $Ca^{2+}$ and Parathyroid Hormone Secretion

Parathyroid chief cells secrete parathyroid hormone (PTH) in response to fluctuations in serum $Ca^{2+}$ with an inverse dependence on the $Ca^{2+}$ concentration, that is, as serum $Ca^{2+}$ increases, PTH secretion decreases (reviewed in Ambrish and Brown, 2003). The inverse relationship between serum $Ca^{2+}$ and PTH release is highly cooperative, with a set-point, or concentration at which PTH release is half-maximally inhibited, of

approximately 1.0 mM $Ca^{2+}$ (Brent *et al.*, 1988; Brown, 1983). Abrupt lowering of serum $Ca^{2+}$(hypocalcemia) causes an immediate increase in PTH release as well as increases in production, transcription, and stability of preproPTH mRNA (Sela-Brown *et al.*, 1999; Yamamoto *et al.*, 1989). Prolonged hypocalcemia (often seen in patients with renal insufficiency) results in parathyroid cell hyperplasia and increases PTH secretory capacity (Silver *et al.*, 1994). Increases in serum $Ca^{2+}$ (hypercalcemia) have the opposite effect, increasing intracellular PTH degradation, and reducing transcription/stability of preproPTH mRNA (Yamamoto *et al.*, 1989).

## B. Expression Cloning of a "CaR" from Bovine Parathyroid

Several critical studies in isolated parathyroid cells suggested the existence of an extracellular $Ca^{2+}$ sensor (reviewed in Brown *et al.*, 1990). First, the potency of a variety of divalent cations for inhibition of PTH release correlated with their effects on parathyroid cell membrane potential but could not be explained by the electromotive force generated by their concentration gradients across the membrane (Lopez-Barneo and Armstrong, 1983). Second, step changes in extracellular $Ca^{2+}$ induced a complex intracellular $Ca^{2+}$ response, which is characteristic of receptor-mediated stimulation of intracellular $Ca^{2+}$ release followed by a sustained component of plasma membrane influx, similar to that elicited by other $Ca^{2+}$-mobilizing hormones (Nemeth and Scarpa, 1986). The intracellular $Ca^{2+}$ response induced by extracellular divalent cations was accompanied by increases in inositol 1,4,5-trisphosphate (Ins(1,4,5)$P_3$) and diacylglycerol (DAG) (Brown *et al.*, 1987) products of hormone receptor-mediated stimulation of phospholipase C. Finally, extracellular divalent cations were capable of inhibiting accumulation of parathyroid cell cAMP, an effect that was prevented by pretreatment with pertussis toxin, suggesting the involvement of heterotrimeric GTP binding proteins (Chen *et al.*, 1989). The accumulated evidence in favor of a $Ca^{2+}$ sensor led to the expression cloning of an extracellular $Ca^{2+}$-sensing receptor from bovine parathyroid cells (Brown *et al.*, 1993). The unique clone (5.3 kb, encoding a 120-kDa protein) was a glycosylated G-protein–coupled receptor with a large extracellular domain (ECD) and the canonical heptahelical domain, which showed 25–30% homology with metabotropic glutamate receptors (mGluR). CaR is expressed not only in the cells/tissues associated with maintenance of organismal $Ca^{2+}$ homeostasis (parathyroid, kidney, intestine, and bone) but also in many epithelial cell types and the nervous system (neurons and glial cells) (reviewed by Riccardi, 2002).

## II. CaR is a Unique Family C G-Protein–Coupled Receptor

The last 15 years have seen the emergence of Family C of G-protein–coupled receptors (GPCRs) that includes eight metabotropic glutamate receptors (Houamed *et al.*, 1991; Masu *et al.*, 1991), two $\gamma$-aminobutyric acid ($GABA_BR$) (Kaupmann *et al.*, 1997), and multiple pheromone and taste receptors (Herrada and Dulac, 1997; Matsunami and Buck, 1997; Ryba and Tirindelli, 1997). CaR represents a unique (single gene) member of this family (Brown *et al.*, 1993). Agonists for this family of receptors are simple molecules present in the extracellular milieu, including amino acids and ions.

### A. Structural Domains of CaR

CaR shares several unique structural features with other members of Family C GPCRs, including a large ECD that contains the agonist binding site(s) and a heptahelical transmembrane domain that couples to and activates heterotrimeric G proteins (reviewed by Bai, 2004). CaR and the mGluRs share, in addition, a cysteine-rich domain (CysRD) between the agonist binding domain and the transmembrane domain, which presumably participates in coupling the agonist-binding event to transmembrane domain activation (reviewed in Conn and Pin, 1997). In common with other members of Family C, CaR has a large intracellular carboxyl terminus that participates in signaling and interaction(s) with scaffold proteins. Members of Family C function as dimers. Figure 1 illustrates a model for the structural domains of CaR and potential alterations resulting from receptor activation.

#### 1. Venus Flytrap Domain in the ECD

A novel feature of Family C GPCRs immediately discernible on cloning of the initial members was the large amino-terminal ECD (Conn and Pin, 1997; Houamed *et al.*, 1991; Masu *et al.*, 1991). Conservation of the ECD structural motif extends to CaR (Brown *et al.*, 1993; Conklin and Bourne, 1994; Jiang *et al.*, 2004), $GABA_B$ receptors (Galvez *et al.*, 1999; Kaupmann *et al.*, 1997), and pheromone receptors (Herrada and Dulac, 1997; Matsunami and Buck, 1997; Ryba and Tirindelli, 1997). Identification of the ECD as the primary site for agonist binding has come from several types of experiments. First, chimeric receptors produced between distinct subtypes of mGluRs have affinities for subtype-selective agonists that correlate with the source of the ECD (Takahashi *et al.*, 1993; Wroblewska *et al.*, 1997). Chimeras made between mGluRs and CaR exhibit agonist sensitivities that are conferred by the ECD (Brauner-Osborne *et al.*, 1999; Hammerland *et al.*, 1999).

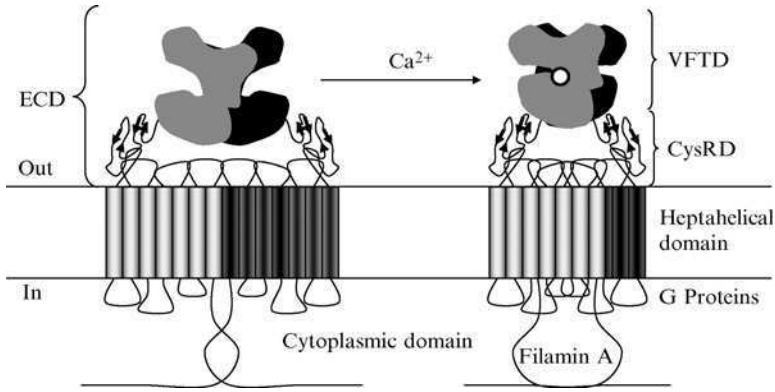


**Figure 1** Domain structure of CaR. CaR functions as a disulfide-linked dimer. $Ca^{2+}$ binds to the VFTD within the ECD of CaR. The activating conformational change is transmitted to the transmembrane, heptahelical domain by the CysRD, also part of the ECD. The cytoplasmic face of CaR interacts with heterotrimeric G proteins through the i2 and i3 loops and the proximal carboxyl terminus. The scaffold protein filamin A interacts with CaR at a distal region of the carboxyl terminus. A tentative model for CaR activation, based on studies with the related receptor mGluR1, is illustrated. The VFTD has a bilobed structure that can exist in an open (inactive) or closed (active) conformation, as has been shown for mGluR1 (Kunishima *et al.*, 2000; Tsuchiya *et al.*, 2002); closure is favored by agonist binding. Closure of the VFTD causes movement of the transmembrane domains relative to each other, increasing the contacts between monomers of the dimer, as has been shown for mGluR1 (Tateyama *et al.*, 2004).

Second, expression of a truncated secreted form of the ECD of mGluR1 or 4 (Han and Hampson, 1999; Okamoto *et al.*, 1998) produces a dimeric soluble protein having rank order potencies for mGluR agonists comparable to the full-length receptor. Finally, sequence analysis identified a structural similarity between the amino-terminal portion of the ECD of mGluRs and bacterial periplasmic binding proteins that bind amino acids (O'Hara *et al.*, 1993). The ECD of mGluRs (and CaR) can therefore be further subdivided into a large, amino-terminal agonist binding domain, termed the Venus flytrap domain (VFTD) defined by its bilobed structure (Conklin and Bourne, 1994) and a small cysteine-rich domain (CysRD) that is localized between the VFTD and the transmembrane helical domain.

Crystallization and structural analysis of the VFTD of mGluR1 confirmed the structural similarity to the bacterial periplasmic binding proteins, localized the glutamate binding site, and illustrated the conformational changes that occur on agonist binding to the VFTD (Kunishima *et al.*, 2000; Tsuchiya *et al.*, 2002). Amino acid modulators of CaR bind to a homologous site within the CaR VFTD (Mun *et al.*, 2004; Zhang *et al.*, 2002), and the $Ca^{2+}$ binding site(s) have been localized to a pocket adjacent to the amino acid binding site (Silve *et al.*, 2005). It must be noted, however,

that numerous point mutations, which alter the apparent affinity of CaR for $Ca^{2+}$ (identified in patients with hypo- or hyperparathyroidisms), are localized throughout the receptor at the ECD (Bai *et al.*, 1996; Pearce *et al.*, 1996; Spiegel, 1996) within the transmembrane domain and/or intracellular domains (reviewed by Hu and Spiegel, 2003), suggesting caution is required in equating alterations in apparent agonist affinity with direct contribution (s) to the binding site(s) for $Ca^{2+}$. The cooperativity evident for both $Ca^{2+}$-mediated activation of signaling pathways and the sigmoidicity of the $Ca^{2+}$-dependence of PTH secretion in parathyroid cells argues for the involvement of multiple $Ca^{2+}$ or divalent cations in the redistribution of the CaR ECD from the inactive to the active conformation(s) (reviewed by Chang and Shoback, 2004). Chimeras containing the CaR ECD fused to the mGluR1 transmembrane domain/carboxyl terminus exhibit a sigmoidal activation response to $Ca^{2+}$ and an affinity for extracellular $Ca^{2+}$ consistent with wt CaR (e.g., Hu *et al.*, 2000).

## 2. CysRD in the ECD

$Ca^{2+}$ binding to the CaR VFTD initiates a conformational change; receptor activation must result from coupling of this conformational change to conformational changes within the transmembrane domain, initiating heterotrimeric G protein activation at the cytoplasmic side of the membrane. The primary sequences of CaR and the mGluRs include a CysRD of approximately 60 amino acids between the VFTD and transmembrane helix 1 (Conn and Pin, 1997). The CysRD is not present in the more distantly related $GABA_B$ receptors (Galvez *et al.*, 1999) but present in pheromone and taste receptors (Herrada and Dulac, 1997; Matsunami and Buck, 1997; Ryba and Tirindelli, 1997). Alignments of mGluRs and CaR demonstrate complete conservation of the locations of the nine cysteine residues within the CysRD, implying a similar domain structure and mechanism of coupling to transmembrane domain activation (Hu *et al.*, 2000).

All nine cysteine residues of the CaR CysRD are critical for receptor activation (Fan *et al.*, 1998; Hu *et al.*, 2000); intramolecular disulfide bonds within the CysRD likely contribute to formation of a rigid link between the VFTD and the transmembrane domain. There are no direct disulfide bonds linking the VFTD and CysRD or the CysRD and the transmembrane domain (Hu *et al.*, 2001). Deletion of the CysRD of CaR generates a nonfunctional receptor (Hauache *et al.*, 2000). A chimeric CaR containing the VFTD and transmembrane domain of the human sequence but the CysRD from the puffer fish *Fugu ruprides* (which differs from human CaR at 20 residues within the CysRD) is fully functional, with an $EC_{50}$ and maximal response for activation by $Ca^{2+}$ comparable to wt human CaR, while replacing the human CaR CysRD with that from mGluR1 (which

differs at 40 residues) generated a chimera that was severely impaired with both a significant increase in $EC_{50}$ for $Ca^{2+}$ and a decrease in maximal signaling (Hu *et al.*, 2000). Truncations that eliminate the CaR ECD (VFTD plus CysRD) generate an active form of the CaR transmembrane domain (Ray *et al.*, 1997, 2005), suggesting that the CysRD acts as an inhibitory domain preventing transmembrane domain activation in the absence of agonist. Alignment of sequences for the CysRD of CaR and members of family C and 3D modeling identified a structural domain that incorporates four $\beta$-strands and three disulfide bridges (termed NCD3G, for nine-cysteine domain of family 3 GPCRs) (Yu *et al.*, 2004). The NCD3G domain is identifiable in eukaryotes but not present in microbes or plants (Yu *et al.*, 2004). A number of disease-causing mutations in CaR map to the NCD3G domain suggesting a critical role in receptor function/activation (Pearce *et al.*, 1995).

## 3. Heptahelical Domain

Most families of GPCRs bind agonist within a pocket defined by the transmembrane helices with some contributions from the extracellular loops (Bockaert and Pin, 1999; Strader *et al.*, 1994), but Family C members bind agonist at the VFTD, requiring transduction of the activating conformation change from the VFTD to the transmembrane domain. For CaR, transduction of the signal from the VFTD to the transmembrane domain involves not only the CysRD (see previous section) but also the extracellular loops connecting helices of the heptahelical transmembrane domain, particularly the e2 loop. Truncations that remove the entire ECD (VFTD plus CysRD) can be expressed and targeted to the plasma membrane by incorporation of the amino-terminal 20 amino acids of rhodopsin (Ray and Northup, 2002; Ray *et al.*, 1997) or the signal sequence from CaR (Pace *et al.*, 1999). The CaR transmembrane domain can be activated by cations in the absence of the ECD with an $EC_{50}$ of 2 mM (compared with an $EC_{50}$ of 3.5–4 mM for wt CaR), although the activation does not display the high cooperativity characteristic of full length CaR (Ray *et al.*, 1997). Interestingly, a variety of other cations, including spermine, poly-L-arginine, the allosteric agonist NPS R-568, and $Gd^{3+}$, were also able to activate the amino terminal-truncated CaR, and synergistic interactions were observed between the activating cations (Ray and Northup, 2002). The second extracellular loop of CaR contains five negatively charged residues; mutation of these residues to alanine decreased the ability of most cations to activate the receptor and eliminated synergistic interactions between NPS R-568 and the other agonists (Ray *et al.*, 2004). While these studies point to independent regulation of CaR activation at cation binding sites within the ECD and transmembrane domain, it is possible that the absence of the

ECD may make available site(s) not normally involved in cation binding. Mutations generated in the full length CaR suggest that the e2 loop acidic residues are not directly involved in binding cationic agonists, since mutations (either single or multiple) to alanine cause a decrease in the $EC_{50}$ for $Ca^{2+}$ activation, presumably acting via the VFTD (Hu *et al.*, 2002). It is likely, therefore, that acidic residues within the e2 loop are involved in stabilizing the inactive conformation of the transmembrane domain (Hu *et al.*, 2002).

An emerging novel feature of Family C GPCR members, including CaR, mGluRs, and $GABA_BRs$, is their modulation by allosteric ligands that bind at sites other than the VFTD (reviewed in Pin *et al.*, 2004). CaR presents a unique difficulty with respect to the development of drugs since the physiological ligand at the VFTD is an inorganic ion. The identification of drugs that act at the transmembrane domain provides a means of modulating CaR activity in the presence of baseline $Ca^{2+}$ concentrations. Screening of phenylalkylamine-type compounds that have some efficacy against other $Ca^{2+}$-binding proteins identified fendiline ($EC_{50} \approx 12$ μM) as a prototype for the development of higher affinity CaR modulators (Nemeth *et al.*, 1998). The first compounds with calcimimetic (agonist) effects were NPS R-568 and NPS R-467, having $EC_{50}$s in the 10–50 nM range (Nemeth *et al.*, 1998). Subsequent screening efforts have yielded additional allosteric modulators of CaR, including Calhex 231 (Petrel *et al.*, 2003), calindol (Kessler *et al.*, 2004a), and others (Kessler *et al.*, 2004b). Interestingly, none of the calcimimetic compounds are capable of modulating CaR in the absence of $Ca^{2+}$. The binding site for NPS R-568/NPS R-467 has been localized to the transmembrane domain by chimera studies (Hammerland *et al.*, 1999; Nemeth *et al.*, 1998). Using a combination of molecular modeling and mutagenesis/functional studies, the binding site(s) for several distinct allosteric modulators of CaR have been identified; NPS R-568, NPS 2143, Calhex 231 and calindol all bind to a residue at the extracellular face of helix 7 (E837), with additional distinct salt bridge or hydrophobic contacts within the pocket between transmembrane helices at the extracellular interface (Miedlich *et al.*, 2004; Petrel *et al.*, 2004; Ray *et al.*, 2005). Binding of allosteric activators increases the apparent affinity for $Ca^{2+}$ activation, likely by stabilizing the activated conformation of the transmembrane domain (Miedlich *et al.*, 2004), while allosteric antagonists (e.g., NPS 2143) presumably stabilize the inactive conformation (Hu *et al.*, 2005; Miedlich *et al.*, 2004).

Transduction of the signal from the transmembrane domain to G proteins involves both intracellular loops and the proximal carboxyl terminus of CaR. The i2 loop of Family C members is longest and exhibits the greatest sequence diversity, while the i3 loop is short and has a highly conserved "signature sequence" (...PENFNEAK...) common to CaR, mGluRs, and

$GABA_BRs$ (Chang *et al.*, 2000). Chang *et al.* (2000) have explored the importance of the i2 and i3 loops of CaR in signaling to phospholipase C (PI-PLC) through $G_q$. Alanine substitution at two hydrophobic residues at the amino terminal end of the i2 loop modulates PI-PLC activation by CaR (L704 and F707), while a larger portion of the i3 loop, containing both hydrophobic and acidic residues, is required. The i3 loop residues are within the Family C "signature sequence" shared with GPCRs that activate a range of G protein subtypes, and thus the i3 loop residues are most likely important for G protein coupling rather than subtype specificity (Chang *et al.*, 2000). Systematic alanine scanning mutagenesis at the proximal carboxyl terminus alters signaling to phospholipase C, suggesting that residues within this region also contribute to CaR-mediated $G_q$ activation (Gama and Breitwieser, 1998; Miedlich *et al.*, 2002).

## 4. Cytoplasmic Carboxyl Terminus

The carboxyl terminus of CaR is large (≈216 residues) and serves several different functions. The proximal carboxyl terminus is critical for CaR signaling through $G_q$ to PI-PLC (Gama and Breitwieser, 1998). Residue T876 facilitates highly cooperative G protein activation and induction of intracellular $Ca^{2+}$ oscillations (Gama and Breitwieser, 1998; Miedlich *et al.*, 2002), while the residues between 868 and 886 are involved in determining the rate of desensitization of CaR-mediated $Ca^{2+}$ responses (Gama and Breitwieser, 1998). Two independent yeast two hybrid screens have implicated a distal region, residues 907–997 (Awata *et al.*, 2001) or 972–1031 (Hjalm *et al.*, 2001) of the CaR carboxyl terminus in direct interaction(s) with the cytoskeletal/scaffold protein filamin A. The high affinity filamin A interaction domain has been further localized to residues 962–981 of the CaR carboxyl terminus (Zhang and Breitwieser, 2005). High affinity interactions of CaR with filamin A stabilize CaR by slowing degradation (Zhang and Breitwieser, 2005). In addition, activation of MAPK signaling by CaR requires interaction(s) with filamin A, although the mechanism of scaffold-induced signaling is not well characterized. Filamin A organizes CaR signaling to ERK1/2 as well as RhoA (Pi *et al.*, 2002), but whether signaling components of both pathways are simultaneously present in the complex of CaR with filamin A is not yet known. CaR also interacts with caveolins, presumably through the carboxyl terminus, but the residues involved have not been identified (Jung *et al.*, 2005; Kifor *et al.*, 2003). Thus CaR interacts with a number of critical scaffold/signaling proteins via the carboxyl terminus; these interactions may be differentially regulated in distinct cell types or at different stages of development and/or differentiation.

### 5. Obligate Dimerization of CaR

Members of family C GPCRs function as obligate dimers, stabilized by either disulfide bonds between agonist binding domains within the ECD (CaR and mGluRs) (Ray *et al.*, 1999; Tsuji *et al.*, 2000) or via coiled–coil interactions between subunits ($GABA_B1R$ and $GABA_B2R$) (Kammerer *et al.*, 1999). Dimerization is critical to trafficking to the plasma membrane ($GABA_BRs$) and has also been implicated in receptor activation.

Studies of CaR mutations identified in patients exhibiting hypo- or hypercalcemia (recently reviewed by Tfelt-Hansen and Brown, 2005) have underscored the fundamental importance of dimerization in CaR function. CaR mutations fall into two functional classes: (1) inactivating mutations, which exhibit an apparent decrease in affinity for agonists and/or reduced plasma membrane localization and/or inability to activate G proteins, and (2) activating mutations, which increase the apparent affinity of the receptor for agonists. Most revealing with respect to dimer function are studies in which pairs of receptors bearing distinct inactivating mutations are coexpressed (Bai *et al.*, 1999). Results suggest that each monomer can be divided into two distinct domains, the ECD and the transmembrane/carboxyl terminal domain. Cotransfection of two receptors bearing distinct mutations within the same domain are inactive or severely compromised, while pairs of receptors bearing mutations within complementary domains exhibit near normal activity (Bai *et al.*, 1999). $GABA_BRs$ ($GABA_BR1a$ plus $GABA_BR2$) function exclusively as heterodimers, with agonist binding confined to one partner and G protein interaction/activation confined to the other partner (reviewed by Pin *et al.*, 2004). CaR may represent a more general case of allosteric cooperativity in which either monomer may bind agonist and/or interact with G proteins but conformational coupling alters both monomers (Bai *et al.*, 1999; Miedlich *et al.*, 2002). Receptors bearing complementary inactivating mutations maintain function via their ability to transmit/accept conformational information from the wild type domain of their partner.

## B. CaR Integrates a Variety of Context-Sensitive Metabolic Signals

While $Ca^{2+}$ is the defining agonist for CaR ($EC_{50}$ ranges from 2.5 to 4 mM depending on cell type), CaR is activated or allosterically regulated by a wide array of polycations in a variety of distinct physiological contexts. Additional di- and trivalent cations, which can activate CaR, include $Mg^{2+}$ ($EC_{50}$ ranges from 4–7 mM) (Ruat *et al.*, 1996), $Gd^{3+}$ ($EC_{50}$ 20–50 μM) (Bai *et al.*, 1996), protamine ($EC_{50}$ 0.2 μM) (Brown *et al.*, 1991), poly-L-arginine ($EC_{50}$

0.2 μM) (Brown *et al.*, 1991; Gama *et al.*, 1997), -amyloid protein ($EC_{50}$ 1 μM) (Ye *et al.*, 1997), spermine (0.2–0.5 mM depending on the concentration of $Ca^{2+}$) and spermidine (1–2 mM) (Quinn *et al.*, 1997) and additional di- and trivalent ions at low affinity (>5 mM) (reviewed in Breitwieser *et al.*, 2004). Recent studies suggest that amino acids, particularly the aromatic amino acids and histidine (reviewed in Conigrave *et al.*, 2002), act as allosteric agonists, binding within the VFTD. It should be emphasized that all affinities determined for CaR agonists and modulators represent $EC_{50}$ values for activation of particular signal transduction pathways (most commonly release of inositol phosphates, changes in intracellular $Ca^{2+}$ or activation of the MAPK pathway), since their affinities are too low to be measured by direct binding.

The primary agonist activating CaR is $Ca^{2+}$ in many cellular contexts, but local environmental influences, such as pH (Quinn *et al.*, 2004), ionic strength/osmolality (Chen *et al.*, 1987; Quinn *et al.*, 1998) and localized production of polyamine metabolites (Cheng *et al.*, 2004), and/or the presence of amino acids (Conigrave *et al.*, 2004), may strongly influence the responsiveness of CaR to ambient $Ca^{2+}$ concentrations. The *in vitro* $EC_{50}$ of CaR for $Ca^{2+}$ is higher (3–4 mM) than that generally observed for CaR-regulated processes *in vivo* (e.g., $EC_{50}$ for CaR-mediated inhibition of PTH secretion by parathyroid cells is approx. 1.2 mM) (Ambrish and Brown, 2003). While these differences may be attributed to differential regulation of CaR (by phosphorylation, interacting proteins, etc.), it is also possible that the cellular context provides allosteric modulators, which alter CaR $Ca^{2+}$ affinity. CaR may "sense" a complex set of metabolic variables in distinct cellular environments, for example, in kidney, CaR may sense ionic strength/osmolality as well as $Ca^{2+}$; in intestine, CaR may sense both amino acids and polyamines, while in the parathyroid glands, $Ca^{2+}$ may predominate. A major challenge in defining CaR contributions to organismal physiology and development is to dissect the relevant metabolic regulatory factors controlling CaR activation in each specific cellular context.

The absence of identified antagonists has not only limited the biochemical and pharmacological characterisation of CaR (making impossible traditional binding studies for characterization of receptor affinities, G protein interactions, and quantitation of cell expression), but also hampered dissection of the role(s) of CaR in various cells and/or tissues. Functional confirmation of CaR's role in cell physiology is often derived from the effects of various allosteric modulators, including clinically important aminoglycoside antibiotics like neomycin, as well as the allosteric agonists (NPS R-568 or NPS R-467) or antagonists (NPS 2143), which bind within the transmembrane domain.

## III. CaR Signal Transduction

CaR couples changes in extracellular $Ca^{2+}$ (or $Ca^{2+}$ plus metabolites) to a variety of immediate intracellular responses, including activation of phospholipases (PI-PLC, $PLA_2$, PLD), generation of $IP_3$, DAG, arachidonic acid and metabolites, time-varying changes in intracellular $Ca^{2+}$, changes in protein phosphorylation (protein kinase C and calmodulin-dependent protein kinase), activation of ion channels, and regulation of hormone secretion (reviewed by Ward, 2004).

CaR activation mediates long-term changes in cell function by initiating changes in gene expression through a variety of distinct signaling pathways. CaR activation by physiologically relevant changes in extracellular $Ca^{2+}$ concentrations or amino acids induces long-lived (>30 min) intracellular $Ca^{2+}$ oscillations (Breitwieser and Gama, 2001; Young and Rozengurt, 2002). In a number of cell types, intracellular $Ca^{2+}$ oscillations have been shown to be potent activators of gene expression, and CaR activation may alter gene expression and/or proliferation by these mechanisms (Dolmetsch *et al.*, 1998; Hu *et al.*, 1999; Li *et al.*, 1998). CaR also activates various MAPK signaling cascades dependent on cell type and interacting proteins (reviewed by Rodland, 2003). In the parathyroid, CaR activation increases ERK1/2 phosphorylation by both $G_q$ and $G_i$-dependent pathways (Corbetta *et al.*, 2002; Kifor *et al.*, 2001); disruption of these pathways and high levels of ERK1/2 activation are observed in parathyroid adenomas, in part because of a decrease in the expression of a CaR-interacting protein, caveolin-1, which facilitates cytoplasmic retention of phosphorylated ERK1/2 (Kifor *et al.*, 2003). CaR has been shown to activate the ERK1/2 pathway (via either PKC phosphorylation of MEK1/2; Corbetta *et al.*, 2002; src activation of ras/c-Raf-1; McNeil *et al.*, 1998; or PI3 kinase activation; Bilderback *et al.*, 2002), as well as the p38 kinase (Tfelt-Hansen *et al.*, 2003; Yamaguchi *et al.*, 2000), JNK/SAPK (Tfelt-Hansen *et al.*, 2003) pathways, and the Akt pathway (Ward *et al.*, 2005) for cell survival. As with many GPCRs, cellular context is critical for establishing the dominant pathway that will be activated. Overall, however, a consistent response to CaR activation in a variety of cell types is enhanced proliferation and cell survival. While CaR activation may stimulate normal pathway(s) for cell proliferation and cell survival in many cell types, it can contribute to the consequences of malignancy in others. In cells that metastasize to bone, including keratinocytes, breast, and prostate epithelial cells, CaR-mediated activation of MAPK signaling pathways enhances synthesis and secretion of parathyroid hormone related peptide (PTHrP), a mediator of hypercalcemia of malignancy (Rodland, 2004; VanHouten *et al.*, 2004; Yano *et al.*, 2004). Specific pharmacological modulation of CaR may have a role in limiting these signaling outcomes.

## IV. CaR Translates Extracellular $Ca^{2+}$ Changes into Time-Varying Intracellular $Ca^{2+}$ Signals

CaR activation results in changes in intracellular $Ca^{2+}$ in every cell type in which it has been studied, contributing to both acute changes in cell state (activation of protein kinases and other $Ca^{2+}$-dependent proteins) as well as long-term changes in cell proliferation and/or differentiation (likely mediated in part by $Ca^{2+}$ oscillations). This raises several issues unique to CaR. First, extracellular $Ca^{2+}$ is critically required for cell function, signaling, and survival, and thus cells expressing CaR are chronically exposed to agonist. Serum $Ca^{2+}$ is in the range of 1.1–1.3 mM, while most tissue culture medium contains 1.5–1.8 mM $Ca^{2+}$, a range that can result in significant activation of CaR (recall that the $EC_{50}$ for CaR activation by $Ca^{2+}$ is in the range of 2.5–4 mM). Many GPCRs are desensitized and/or downregulated on chronic exposure to agonist (reviewed by Ferguson, 2001; Gainetdinov *et al.*, 2004), and thus CaR may be regulated by mechanism(s) that permit $Ca^{2+}$ sensing in the constant presence of agonist. Second, serum $Ca^{2+}$ concentration is tightly controlled, with minor daily fluctuations on a constant baseline (reviewed in Ambrish and Brown, 2003). CaR must sense these small changes in extracellular $Ca^{2+}$ and translate them into a robust intracellular $Ca^{2+}$ signal that can acutely regulate cell physiology and secretion as well as initiate long-term changes in cell proliferation or differentiation. As discussed later, CaR exhibits robust signaling under physiological conditions, despite the considerable constraints imposed by chronic exposure to serum (extracellular) $Ca^{2+}$.

### A. $Ca^{2+}$ Chronically Regulates Cells Which Express CaR

One of the constraints on CaR function is chronic exposure to potentially desensitizing concentrations of serum/extracellular $Ca^{2+}$ (generally in the range of 1.1–1.3 mM). HEK-293 cells, which are transfected with CaR, are chronically regulated by CaR signaling having a reduced content of thapsigargin-sensitive stores (Breitwieser and Gama, 2001; Miedlich *et al.*, 2002) and intracellular (cytoplasmic) $Ca^{2+}$ concentrations, which are a function of extracellular $Ca^{2+}$ over the range from 0.5 to 3 mM (Breitwieser and Gama, 2001). Reductions of extracellular $Ca^{2+}$ result in a rapid monotonic decrease in cytoplasmic $Ca^{2+}$ in cells expressing CaR, while intracellular $Ca^{2+}$ stores and cytoplasmic $Ca^{2+}$ concentrations are relatively insensitive to small changes in extracellular $Ca^{2+}$ (from 0.5 to 3 mM) in untransfected HEK-293 cells (Breitwieser and Gama, 2001). Chronic exposure of CaR to a range of extracellular $Ca^{2+}$ concentrations at or below the $EC_{50}$ resets the CaR response threshold but does not eliminate CaR responses (Breitwieser and Gama, 2001).

## B. Increases in Extracellular $Ca^{2+}$ Produce Intracellular $Ca^{2+}$ Oscillations

Small, physiologically relevant increases in extracellular $Ca^{2+}$ (0.5 mM increments) induce intracellular $Ca^{2+}$ oscillations in cells expressing CaR. The threshold for induction of intracellular $Ca^{2+}$ oscillations is influenced by the starting extracellular $Ca^{2+}$ concentration, that is, oscillations generally begin 0.5 mM above the baseline $Ca^{2+}$ concentration (over the range from 0.5 to 3 mM) (Breitwieser and Gama, 2001). The $Ca^{2+}$ oscillation frequency is temperature-dependent, ranging from 1.3/min (25°C) to 4/min (37°C) (Breitwieser and Gama, 2001; Young and Rozengurt, 2002), and independent of the extracellular $Ca^{2+}$ concentration. Intracellular $Ca^{2+}$ oscillations induced by many GPCRs vary as a function of agonist concentration (e.g., Young *et al.*, 2003). It is possible that the limited range of $Ca^{2+}$ concentrations over which CaR oscillations are elicited (2.5–8 mM from a threshold of 2 mM $Ca^{2+}$) coupled with the alterations in driving force for $Ca^{2+}$ influx through receptor regulated pathways limit possible variations in oscillation frequency. Oscillations induced by step increases in extracellular $Ca^{2+}$ are long-lived, decaying after 30–40 min to a new steady state intracellular $Ca^{2+}$ (Breitwieser and Gama, 2001). The cessation of intracellular $Ca^{2+}$ oscillations is not the result of complete desensitization of plasma membrane-localized CaR (since a supra-activating concentration of $Ca^{2+}$ of 10–20 mM can still elicit a response) (Breitwieser and Gama, 2001) but rather represents a resetting of the threshold for oscillatory responses. Sustained oscillations are inhibited by PKC-dependent phosphorylation of CaR (Young *et al.*, 2002). The mutant CaR(T888A) has overall CaR responsiveness comparable to wt CaR but does not exhibit $Ca^{2+}$ oscillations, suggesting that CaR-induced intracellular $Ca^{2+}$ oscillations may be the result of cyclical phosphorylation/dephosphorylation of T888 (Young *et al.*, 2002). CaR is thus uniquely capable of "sensing" small changes superimposed on a steady baseline of extracellular $Ca^{2+}$. Figure 2A illustrates the key features of CaR-mediated intracellular $Ca^{2+}$ responses activated by extracellular $Ca^{2+}$.

CaR-induced alterations in intracellular $Ca^{2+}$ are produced by a combination of release from thapsigargin-sensitive intracellular stores (Breitwieser and Gama, 2001; Miedlich *et al.*, 2002) and influx through plasma membrane-resident pathways, which are dependent on cell type, for example, HEK-293 cells express a variety of store- or receptor-operated calcium influx channels (Bugaj *et al.*, 2005) that can contribute to $Ca^{2+}$ oscillatory responses (Wu *et al.*, 2002). Under physiological conditions, elevations in intracellular $Ca^{2+}$ invariably trigger activation of pathways responsible for restoration of intracellular $Ca^{2+}$, including reuptake into intracellular stores and/or mitochondria, as well as plasma membrane-localized efflux pathways,

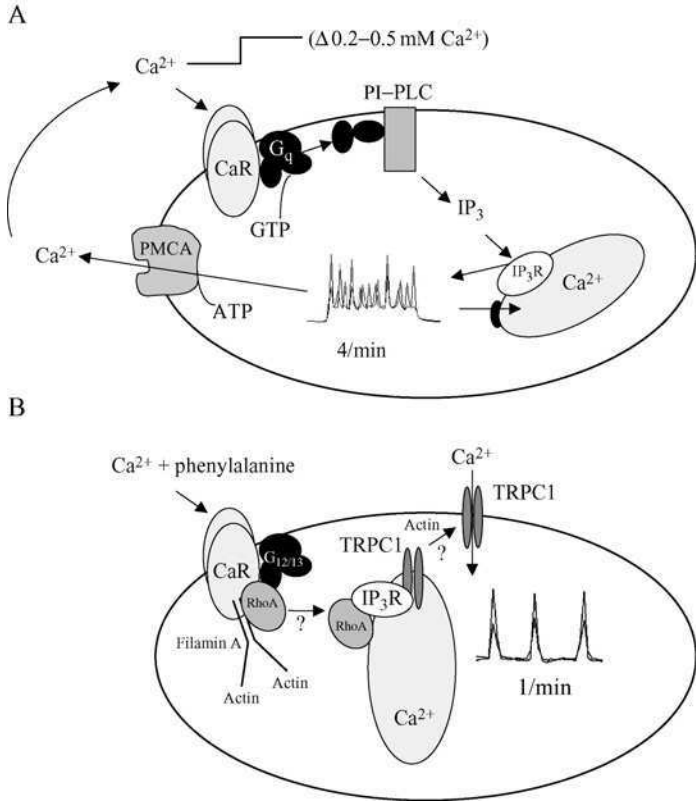


**Figure 2** Pathways for CaR-mediated induction of intracellular $Ca^{2+}$ oscillations. (A) $Ca^{2+}$ activates CaR, inducing coupling to and activation of the heterotrimeric GTP binding protein $G_q$, which activates phospholipase C (PI-PLC), leading to production of Ins(1,4,5)$P_3$ ($IP_3$), which can activate $IP_3$ receptors located on intracellular membranes, releasing store $Ca^{2+}$. Increased intracellular $Ca^{2+}$ is either pumped back into stores via a thapsigargin-sensitive pump, or out of the cell via the plasma membrane-localized $Ca^{2+}$-ATPase (PMCA). In restricted extracellular spaces, extracellular $Ca^{2+}$ may increase as a result of PMCA activity, potentiating CaR activation. $Ca^{2+}$-induced oscillations are sinusoidal, on an elevated baseline of intracellular $Ca^{2+}$. Oscillations may be the result of PKC-mediated phosphorylation of CaR and cyclical production of $IP_3$ (see text). (B) Phenylalanine in the presence of $Ca^{2+}$ activates CaR and induces coupling to and activation of the heterotrimeric GTP binding proteins $G_{12/13}$, leading to RhoA activation. Activation of RhoA by CaR requires interaction with the cytoskeleton scaffold protein filamin A, which also interacts with actin via carboxyl terminal actin binding domains (Stossel *et al.*, 2001). We hypothesize that RhoA-mediated induction of $Ca^{2+}$ oscillations may occur via RhoA interaction with $IP_3$ receptors and/or TRPC1 channels, targeting them to the plasma membrane in an actin-dependent process, as described for endothelial cells activated by thrombin (Mehta *et al.*, 2003). Question marks indicate interactions or parts of the hypothetical pathway which have not been established for CaR. Activation of CaR by phenylalanine generates low frequency, transient $Ca^{2+}$ oscillations (see text).

including the plasma membrane calcium pump (PMCA) and $Ca^{2+}$ exchangers (reviewed by Berridge *et al.*, 2003). Because an increase in extracellular $Ca^{2+}$ activates CaR and generates an increase in intracellular $Ca^{2+}$, it is possible, particularly in tissues with restricted interstitial spaces, for CaR signaling to result in a further increase in extracellular $Ca^{2+}$ as a result of $Ca^{2+}$ efflux from stimulated cells. Activation of CaR can thus result in autocrine or paracrine potentiation of CaR-mediated cell activation in a highly localized tissue compartment. This possibility has been explicitly tested in HEK-293 cells cultured on special coverslips treated to provide restricted spaces for growth of cell clusters (De Luisi and Hofer, 2003). CaR-expressing cells exhibited robust, long-lived intracellular $Ca^{2+}$ oscillations when stimulated with $Ca^{2+}$ or spermine, and clusters of cells with robust intracellular $Ca^{2+}$ oscillations showed an increase in extracellular $Ca^{2+}$ as well, monitored with fura-$C_{18}$ doped into the extracellular plasma membrane leaflet (De Luisi and Hofer, 2003). Blockers of the PMCA inhibited oscillations, suggesting that extrusion of intracellular $Ca^{2+}$ by PMCA potentiated CaR-mediated $Ca^{2+}$ signaling. Figure 2A illustrates the essential features of autocrine/paracrine regulation of CaR $Ca^{2+}$ signaling by PMCA-mediated $Ca^{2+}$ efflux. It is not difficult to envision such a mechanism operating in many cells and tissues; the challenge is to characterize the extent of its contributions by direct *in vivo* measurements of both intracellular and extracellular $Ca^{2+}$.

## C. Allosteric Agonists Facilitate CaR-Induced $Ca^{2+}$ Oscillations by Distinct Mechanisms

$Ca^{2+}$-dependent CaR activation can be facilitated by allosteric agonists that bind to either the VFTD or within the transmembrane domain. NPS R-568 binds to a defined pocket within the transmembrane domain and causes a rightward shift in the dose response relation for $Ca^{2+}$-dependent activation of CaR, as well as a decrease in the threshold for induction of intracellular $Ca^{2+}$ oscillations (Miedlich *et al.*, 2004). While $Ca^{2+}$-induced oscillations generally occur upon an increase in $Ca^{2+}$ concentration above the chronic threshold, addition of NPS R-568 can generate oscillations without a change in extracellular $Ca^{2+}$. $Ca^{2+}$ oscillations induced by NPS R-568 are initiated at extracellular $Ca^{2+}$ concentrations as low as 0.5 mM; the peak response (maximal number of oscillating cells) shifts to lower extracellular $Ca^{2+}$ concentrations as the NPS R-568 concentration is increased (Miedlich *et al.*, 2002). In contrast to $Ca^{2+}$-induced oscillations, NPS R-568-induced oscillations exhibit variable frequency, from 2/min at 10 mM NPS R-568 (3 mM extracellular $Ca^{2+}$) to 0.85/min at 0.1 mM NPS R-568 (at either 1 or 3 mM extracellular $Ca^{2+}$) (all experiments at 25°C) (Miedlich *et al.*, 2002).

Allosteric agonists, such as NPS R-568, are able to enhance CaR activation at the threshold extracellular $Ca^{2+}$ concentration, allowing these compounds to alter CaR-mediated signaling without simultaneously altering the driving force for $Ca^{2+}$ influx (which is an unavoidable consequence of CaR activation by extracellular $Ca^{2+}$ alone). CaR-mediated intracellular $Ca^{2+}$ oscillations occur over a narrow range of extracellular $Ca^{2+}$ concentrations (from 2–5 mM) in the absence of NPS R-568; addition of NPS R-568 shifts the range of extracellular $Ca^{2+}$ conducive to induction of intracellular $Ca^{2+}$ oscillations to 0.1–3 mM, well within the range of normal serum $Ca^{2+}$, suggesting that clinically relevant doses of allosteric agonists are likely to induce intracellular $Ca^{2+}$ oscillations in cells expressing CaR. Activation of CaR by addition of NPS R-568 at constant extracellular $Ca^{2+}$ triggers alterations in intracellular $Ca^{2+}$ by the same mechanism as alterations in extracellular $Ca^{2+}$ alone, that is, both responses are blocked by thapsigargin-mediated release of intracellular stores (Breitwieser and Gama, 2001; Miedlich *et al.*, 2002). Titration of CaR-mediated intracellular $Ca^{2+}$ oscillations by combinations of $Ca^{2+}$ plus NPS R-568 results in predictable levels of CaR activation (Miedlich *et al.*, 2002, 2004), suggesting that NPS R-568 acts by stabilizing the active conformation generated by $Ca^{2+}$.

Aromatic amino acids also allosterically facilitate $Ca^{2+}$-dependent CaR activation (Young and Rozengurt, 2002) and induce intracellular $Ca^{2+}$ oscillations, although the characteristics of the oscillations differ from those produced by either $Ca^{2+}$ alone or $Ca^{2+}$ plus NPS R-568. First, oscillations produced by $Ca^{2+}$ plus phenylalanine (the amino acid most frequently used in these studies) result in low frequency (1/min at 37°C) transient oscillations from the baseline intracellular $Ca^{2+}$ (Rey *et al.*, 2005; Young and Rozengurt, 2002). In contrast, $Ca^{2+}$ or $Ca^{2+}$ plus NPS R-568 generate sinusoidal oscillations with an elevated baseline (Miedlich *et al.*, 2002; Young and Rozengurt, 2002). Second, the oscillations produced by $Ca^{2+}$ plus phenylalanine are blocked by the PKC inhibitor Ro-31–8220 and 2-aminoethoxydiphenylborane (2-APB), a membrane-permeant inhibitor of $Ins(1,4,5)P_3$ receptors and capacitative $Ca^{2+}$ entry channels (Young and Rozengurt, 2002); in contrast, $Ca^{2+}$ activation of CaR was not blocked by 2-APB, although the oscillatory response was converted to a transient response by Ro-31–8220. Thus, addition of phenylalanine in the presence of baseline $Ca^{2+}$ generates a CaR-mediated intracellular $Ca^{2+}$ oscillatory response that has a reduced maximal frequency, differential sensitivity to blockers, and a distinct oscillatory profile, suggesting that a distinct signaling pathway may be responsible.

Dissection of the two signaling pathways that lead to distinct CaR-induced intracellular $Ca^{2+}$ oscillations utilized translocation of fluorescent markers of cell signaling, including GFP-PHD (a fusion of the pleckstrin

homology domain of PLC-$\delta$1 with GFP, sensing production of Ins(1,4,5)$P_3$), PKC$\alpha$-YFP (an indirect sensor of $Ca^{2+}$ oscillations), and PKD-RFP (sensing production of DAG) (Rey *et al.*, 2005). CaR activation by $Ca^{2+}$ elicited oscillatory translocations of GFP-PHD and PKC$\alpha$-YFP to the membrane and translocation of PKD-RFP to the membrane, indicative of CaR-mediated activation of $G_q$ and PI-PLC, with the attendant production of Ins(1,4,5)$P_3$ and DAG (Rey *et al.*, 2005). Furthermore, the results suggest that CaR elicits oscillatory increases in Ins(1,4,5)$P_3$, consistent with PKC-dependent cycling of CaR between phospho- and dephosphorylated states at position T888 (Young *et al.*, 2002). In stark contrast, phenylalanine-induced CaR oscillations did not cause redistribution of either GFP-PHD or PKD-RFP, suggesting that PI-PLC was not activated by CaR under these conditions (Rey *et al.*, 2005). PKC$\alpha$-YFP did cyclically redistribute, as would be expected if $Ca^{2+}$ oscillations were generated by phenylalanine. Phenylalanine induced oscillations require an intact actin cytoskeleton; disruption of the actin cytoskeleton with either cytochalasin D or latrunculin A inhibited phenylalanine—but not $Ca^{2+}$-induced oscillations (Rey *et al.*, 2005). The small GTP binding proteins of the Rho family participate in actin cytoskeleton organization (Burridge and Wennerberg, 2004), and the involvement of RhoA in phenylalanine-induced oscillations was confirmed by *Clostridium difficile* toxin B-mediated inhibition. Again, $Ca^{2+}$-induced oscillations were not sensitive to toxin B inhibition, supporting divergent signaling pathways (Rey *et al.*, 2005). High affinity interactions with filamin A, a scaffold protein linked to the actin cytoskeleton (Stossel *et al.*, 2001), were required for phenylalanine-induced intracellular $Ca^{2+}$ oscillations (Rey *et al.*, 2005), while deletion of the high affinity filamin A interaction site of CaR carboxyl terminus does not affect $Ca^{2+}$-induced oscillations (Gama and Breitwieser, 1998). Phenylalanine-mediated oscillations required the heterotrimeric G-protein $\alpha$ subunits $G_{12/13}$, while $Ca^{2+}$-mediated oscillations utilized $G_q$ (Rey *et al.*, 2005). How might these protein interactions lead to transient intracellular $Ca^{2+}$ oscillations? While we do not yet know the answer for CaR, thrombin-mediated $Ca^{2+}$ entry in endothelial cells is also dependent on RhoA, actin polymerization and $G_{12/13}$ (Mehta *et al.*, 2003). In endothelial cells, RhoA activation leads to translocation of Ins(1,4,5)$P_3$ receptors and TRPC1 channels to the plasma membrane, leading to $Ca^{2+}$ influx (Mehta *et al.*, 2003). Translocation of Ins(1,4,5)$P_3$ receptors and TRPC1 channels is blocked by actin depolymerization (latrunculin) or inhibition of RhoA (C3 toxin, or transfection with a dominant negative RhoA mutant) (Mehta *et al.*, 2003). HEK-293 cells express TRPC1 channels (Bugaj *et al.*, 2005), and thus a mechanism comparable to that characterized in endothelial cells is possible for phenylalanine-induced transient $Ca^{2+}$ oscillations through CaR. Figure 2B illustrates a potential pathway for CaR-mediated activation of transient $Ca^{2+}$ oscillations on activation with phenylalanine.

The divergence of signaling pathways from CaR leading to a common intracellular signal, that is, $Ca^{2+}$ oscillations, is indicative of the existence of multiple distinct active conformations of the CaR dimer. As discussed in section II, $Ca^{2+}$ and amino acids, including phenylalanine, bind at the VFTD domain, while NPS R-568 binds within the heptahelical domain. Substantial evidence supports the notion that GPCRs have agonist-specific conformations that can result in divergent signaling outcomes (Perez and Karnik, 2005), and Family C GPCRs in particular have been shown to exhibit additional degrees of variability in conformational coupling between monomers within a dimer (Pin *et al.*, 2005). The challenge in the future will be to define and target the distinctive CaR conformations, which will permit differential modulation of distinct CaR-activated signaling pathways.

## D. Consequences of Intracellular $Ca^{2+}$ Oscillations

The distinctive patterns of $Ca^{2+}$ oscillations produced by $Ca^{2+}$, $Ca^{2+}$ plus phenylalanine, or $Ca^{2+}$ plus NPS R-568 may produce divergent outcomes for both acute activation of protein kinases and enzymes in response to elevated intracellular $Ca^{2+}$, and long-term changes in gene expression. While our understanding of CaR-mediated changes in gene expression is in its infancy, it is clear that many cell functions are regulated by $Ca^{2+}$ oscillation frequency (De Koninck and Schulman, 1998; Dolmetsch *et al.*, 1998; Li *et al.*, 1998; Negulescu *et al.*, 1994; Oancea and Meyer, 1998). Protein kinase C is repeatedly targeted to the plasma membrane in response to $Ca^{2+}$ oscillations, providing repeated access to membrane-localized DAG required for kinase activation (Oancea and Meyer, 1998). Such a mechanism serves as a "coincidence detector," allowing maximal enzyme activation only under conditions which generate two independent activating stimuli, that is, $Ca^{2+}$ and DAG. Nuclear factor of activated T cells (NFAT) translocates to the nucleus in response to a range of $Ca^{2+}$ oscillation frequencies (Dolmetsch *et al.*, 1998), via a mechanism based on $Ca^{2+}$-dependent dephosphorylation. In unstimulated cells, phosphorylated NFAT resides in the cytoplasm; receptors that induce intracellular $Ca^{2+}$ oscillations activate $Ca^{2+}$-sensitive protein phosphatases that dephosphorylate NFAT, allowing translocation to the nucleus and NFAT-mediated gene transcription (Tomida *et al.*, 2003). Since the rate limiting step is nuclear translocation of dephosphorylated NFAT, sensitivity to $Ca^{2+}$ oscillation frequency arises as a result of a frequency-dependent buildup of dephosphorylated, cytoplasmic NFAT (Tomida *et al.*, 2003). Similar $Ca^{2+}$ oscillation frequency dependence has been observed for NF-$\kappa$B (Hu *et al.*, 1999). Synergistic interactions and convergence between $Ca^{2+}$ and mitogen-activated protein kinase cascades have also been noted (reviewed in Gawler, 1998; Mellstrom and Naranjo,

2001). Note that both phenylalanine-induced transient $Ca^{2+}$ oscillations and CaR-mediated activation and p42/44 kinases require interaction with filamin A (Rey *et al.*, 2005; Zhang and Breitwieser, 2005), whereas $Ca^{2+}$-induced $Ca^{2+}$ oscillations do not (Miedlich *et al.*, 2002). The functional and developmental consequences of CaR-induced $Ca^{2+}$ oscillations must ultimately depend on the nature of the CaR activating signal ($Ca^{2+}$ and/or amino acids, and/or polyamines) and the presence and relative dominance of particular signaling pathways, and must therefore be systematically dissected in each cell type of interest.

## V. CaR in Development

A CaR knockout mouse ($Casr^{-/-}$) was generated shortly after cloning of the *Casr* gene (Ho *et al.*, 1995). Homozygous knockout mice exhibit a severe phenotype consistent with the hallmarks of neonatal severe hyperparathyroidism, NSHPT (markedly elevated serum $Ca^{2+}$ and PTH, parathyroid hyperplasia, bone abnormalities, retarded growth, and premature death), while heterozygous mice ($Casr^{+/-}$) recapitulate familial hypocalciuric hypercalcemia, FBHH (modest serum $Ca^{2+}$, $Mg^{2+}$ and PTH elevations, hypocalciuria) (Ho *et al.*, 1995). $Casr^{-/-}$ mice are hypercalcemic in utero, with elevated PTH levels (Kovacs *et al.*, 1998). $Casr^{-/-}$ mice are severely compromised by hyperparathyroidism resulting in perinatal lethality (Ho *et al.*, 1995), making it difficult to dissect the contributions of CaR to development and function of tissues other than the parathyroid.

Two groups have crossed the $Casr^{-/-}$ mice with knockouts that minimize the contributions of the parathyroid gland, that is, the PTH gene ($PTH^{-/-}$) (Kos *et al.*, 2003) or the glial cells missing-2 gene ($Gcm2^{-/-}$), the master regulatory gene for parathyroid gland development (Tu *et al.*, 2003). In both $Casr^{-/-}/PTH^{-/-}$ and $Casr^{-/-}/Gcm2^{-/-}$ mice, the severe skeletal abnormalities and perinatal lethality seen in the $Casr^{-/-}$ mice is rescued, suggesting that this phenotype is a secondary consequence of hyperparathyroidism and hypercalcemia. The $Casr^{-/-}/PTH^{-/-}$ mice exhibit a wider range of both serum $Ca^{2+}$ concentrations and renal $Ca^{2+}$ clearance, suggesting that CaR is involved in fine-tuning of these functions (Kos *et al.*, 2003). In addition, CaR may control parathyroid cell proliferation independent of its effects on PTH secretion, since $Casr^{-/-}/PTH^{-/-}$ mice have parathyroid gland hyperplasia (Kos *et al.*, 2003). The generation of two double knockout models, which survive to adulthood without severe abnormalities, will permit study of the contributions of CaR to normal physiology.

Disease phenotypes result not only from mutations, which compromise CaR function (NSHPT or FBHH, recapitulated in $Casr^{-/-}$ or $Casr^{+/-}$ mice, respectively), but from gain-of-function mutations in the

*CaR* gene (autosomal dominant hypocalcemia, i.e, ADH or a Bartter's-like syndrome) (recently reviewed by Tfelt-Hansen and Brown, 2005; mutations and polymorphisms updated on the CasRdb, http://www.casrdb.mcgill.ca/). An alternate approach showing promise for dissection of CaR contributions to development and physiology is the study of the effects of defined activating or inactivating mutations. One such model, identified from a screen of mouse mutants generated with a mutagenic alkylating agent, is the *Nuf* mouse (so named for the "*nu*clear *f* lecks," i.e., cataracts, in the lens) (Hough *et al.*, 2004). The mouse phenotype (hypocalcemia, hyperphosphatemia, and reduced plasma PTH levels) is consistent with human gain-of-function mutations in CaR generating autosomal dominant hypocalcemia. Analysis of the *Casr* gene in *Nuf* mice identified a point mutation, L723Q, in a residue conserved across species (Hough *et al.*, 2004). CaR(L723Q) has an increased sensitivity to activation by extracellular $Ca^{2+}$ when expressed in HEK-293 cells, consistent with the mouse phenotype. Examination of additional loss- or gain-of-function *Casr* mutations in mice will help define the role(s) of CaR in normal physiology and development and provide models for study of the pathophysiological consequences of *Casr* mutations.

CaR expression is developmentally regulated in kidney, with little expression during the prenatal period. There is a rapid increase in expression during postnatal week 1, with constant expression through adulthood after postnatal day 14 (Chattopadhyay *et al.*, 1996). The postnatal rise in CaR expression parallels the increase in renal responsiveness to vasopressin and PTH (Chattopadhyay *et al.*, 1996). Similar developmental regulation of CaR expression is observed in rat hippocampus, with little expression of CaR through postnatal day 5, followed by a marked increase in expression (mRNA and protein) by day 10. Expression remains high for 30 days and then decreases three-fold to adult levels (Chattopadhyay *et al.*, 1997). Interestingly, the time period of high level CaR expression coincides with the most rapid period of brain development, suggesting that CaR may play a role in development of the hippocampus. CaR is also expressed in oligodendrocytes, suggesting a role in myelination; as with CaR expression in neurons, expression peaks during rapid brain development and declines to a lower level of expression in the adult (Ferry *et al.*, 2000). The results of temporal studies of CaR expression point to possible contributions of CaR to the development of neurons and glia, and maturation of the kidney, but considerable additional studies are required to ascribe a deterministic role to CaR. Given the survival of double knockout mice ($Casr^{-/-}/PTH^{-/-}$ and $Casr^{-/-}/Gcm2^{-/-}$) to adulthood without severe defects (Kos *et al.*, 2003; Tu *et al.*, 2003), and the survival of humans with NSHPT following parathyroidectomy (e.g., Fugimoto *et al.*, 1990), it is likely that CaR may play a critical, but modulatory role in the development and function of these and other tissues and organs.

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# 4

# Signal Relay During the Life Cycle of *Dictyostelium*

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I. Introduction
II. The *Dictyostelium* Life Cycle is Regulated by Five Signal Relay Modules
  A. Nutrient Sensing
  B. Quorum Sensing
  C. Gradient Sensing
  D. Morphogen Sensing
  E. Environmental Sensing
III. Summary
IV. Perspectives
  Acknowledgments
  References

A fundamental property of multicellular organisms is signal relay, the process by which information is transmitted from one cell to another. The integration of external information, such as nutritional status or developmental cues, is critical to the function of organisms. In addition, the spatial organizations of multicellular organisms require intricate signal relay mechanisms. Signal relay is remarkably exhibited during the life cycle of the social amoebae *Dictyostelium discoideum*, a eukaryote that retains a simple way of life, yet it has greatly contributed to our knowledge of the mechanisms cells use to communicate and integrate information. This chapter focuses on the molecules and mechanisms that *Dictyostelium* employs during its life cycle to relay temporal and spatial cues that are required for survival.

## I. Introduction

Individual *Dictyostelium* cells are found growing autonomously on soil bacteria. As the food source becomes steadily depleted and the population increases, cells initiate a coordinated developmental program that ultimately leads to the formation of a fruiting body composed of dormant spores, which survive harsh environmental conditions, atop a stalk of vacuolated


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cells (Fig. 1). The coordination of the developmental program occurs when the population reaches a threshold or quorum, and individual cells start behaving cooperatively, integrating signals from the environment and each other. In response to chemoattractants, cells aggregate and differentiate into a motile multicellular slug composed of prespore and prestalk cells. Within the slug, signals regulate the sorting of prespore and prestalk cells to different compartments and environmental factors trigger movement away and

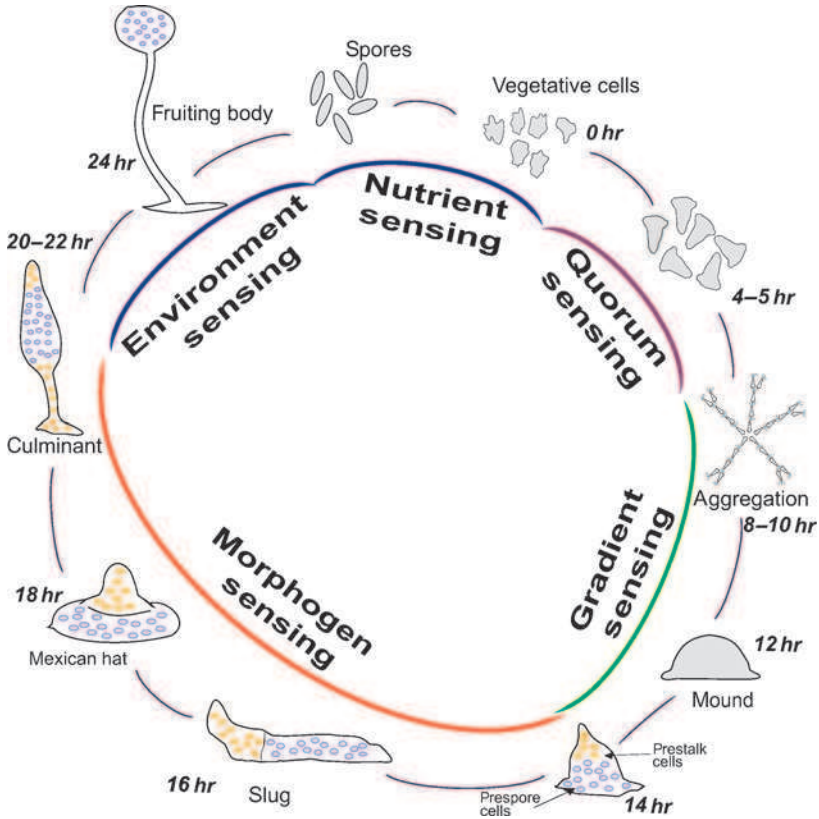


**Figure 1** Schematic representation of the life cycle of *Dictyostelium* divided into sensing modules. Developmental checkpoints within each signaling module correspond to significant morphological changes in the organism (see text). Next to each stage of development, the corresponding time after starvation (hours) is indicated. The developmental program begins with vegetative cells that respond to factors by forming a multicellular organism in which they differentiate to become resistant spores (blue) or supportive stalk (yellow) cells. Once favorable conditions have returned, spores will germinate to release the next generation of vegetative cells.

toward different stimuli. At this time, cells take a decision to either become resistant spores or supportive vacuolated stalk cells. The spores retain the ability to sense their environment, germinating when conditions become favorable. Signal relay is required at all these developmental checkpoints (Fig. 1).

The signal transduction pathways used throughout the developmental program of *Dictyostelium* are highly conserved. The similarities expand beyond signaling modules to actual components, many of which are highly homologous to their mammalian counterparts. Processes, such as cell–cell communication via G-protein–coupled receptors (GPCRs), chemotaxis, and cytokinesis, have been extensively characterized in *Dictyostelium* with the goal of understanding the common mechanisms present in other eukaryotic cells. Indeed, the highly accessible genetics and biochemistry of *Dictyostelium* have rendered it a key model organism in the study of fundamental biological processes. Axenic strains, capable of growing on defined media, are easily maintained in the lab, and the ability to grow cultures to a density of $1 \times 10^7$ cells/ml allows for rapid production of biochemical material. The genetic tractability of *Dictyostelium* has recently been bolstered by the sequencing of the 34 Mb genome predicted to contain 12,500 proteins (Eichinger *et al.*, 2005). Functional inactivation of genes by homologous recombination is facilitated by the haploid state of the genome. Ectopic expression of genes via constitutive, inducible, and cell type–specific promoters allow for the precise control of gene expression. In addition, the use of fluorescent reporters, such as the Green Fluorescent Protein (GFP), has facilitated the localization of proteins during live cell imaging (Tsien, 1998; Tsien and Miyawaki, 1998). Many genes have been identified by screens of random mutants, generated chemically or by restriction enzyme mediated integration (REMI) (Guerin and Larochelle, 2002; Kuspa and Loomis, 1992), and complementation of chemically generated mutants by high copy expression of gene libraries has recently been developed (Robinson and Spudich, 2000).

## II. The *Dictyostelium* Life Cycle is Regulated by Five Signal Relay Modules

The *Dictyostelium* life cycle (Fig. 1) can be broken into five signal relay modules: *Nutrient Sensing, Quorum Sensing, Gradient Sensing, Morphogen Sensing, and Environmental Sensing*. The cycle begins with individual cells that feed on bacteria. As cells act autonomously, they each have the ability to sense folic acid, a bacterially derived metabolite. As long as a food source is present, cells will grow and divide. As the population begins to rise, cells secrete quorum-sensing factors that reflect the size of the population. Unlike mammalian organisms that grow and differentiate at the same

time, *Dictyostelium* cells stop growing when they enter development and must therefore have adequate cell numbers to achieve the proper size and proportion for the subsequent multicellular stages. Once the quorum checkpoint has passed, cells become synchronized through the coordinated secretion of the chemoattractant 3′,5′-cyclic adenosine monophosphate (cAMP), the master controller of development. The binding of cAMP to specific GPCRs called cAMP receptors (cARs) initiates a series of signal transduction events that lead to chemotaxis and gene expression as well as to the synthesis of additional cAMP to relay the signal to distal cells. The signal transduction cascades activated by cAMP triggers cells to move together in a process called aggregation, which initiates the formation of a multicellular structure called a mound.

After all of the 100,000 or so cells have aggregated into the mound stage during the first 6 hr of development, cells begin to differentiate into subpopulations and sort to separate compartments. The *Morphogen Sensing* module allows the clonal population to subdivide into prestalk and prespore cells at the 10–12 hr stage of development. This cell proportioning is controlled by extracellular morphogens, such as cAMP, which regulate distinct gene expression patterns in the two subpopulations. The prestalk cells migrate to occupy the apex or tip of the mound, while the prespore cells are excluded from this area. The apex elongates upwards and falls over on the stratum to form the motile slug. Within the slug, prestalk cells position in the anterior while the prespore cells are present only in the posterior region. The prestalk cell population can be further subdivided into pstA, pstO, and pstAB cells based on the expression of extracellular matrix proteins (Williams *et al.*, 1989). These subsets of prestalk cells form different supportive regions of the terminal fruiting body. Within the prespore region of the slug, cells prepare to become dormant spores that will give rise to the next generation. The tip of the slug remains sensitive to positive and negative environmental cues. Positive cues include moisture, light, and $O_2$, while negative cues include ammonia and nonoptimal pH. Slugs move to an optimal environment for the final stages of development.

The slug stage is followed by a process called culmination, which involves the terminal differentiation of the multicellular organism into a mature fruiting body. The slug tip rises off the stratum, while the remainder of the slug surrounds it forming a shape reminiscent of a Mexican hat. This transient Mexican hat stage signifies the establishment of the stalk tube that begins to form with vacuolated dead stalk cells. The spore mass is lifted off the stratum to the top of the stalk. Spores have the ability to assess the survivability of their surroundings through the *Environmental Sensing* module that allows integration of factors that are required to support growth. If growth conditions exist, such as a food source and proper

temperature, spores will germinate to release vegetative amoebae and the cycle begins again.

## A. Nutrient Sensing

### 1. Folic Acid

In their vegetative state, cells grow and divide independently of each other, yet this autonomous behavior still requires cells to detect signals received from their environment. One of the key factors that vegetative cells respond to is the bacterially produced folic acid. In this context, folic acid acts as a chemoattractant generating a spatial cue that cells use to hunt bacteria (Pan *et al.*, 1972). Interestingly, the gradient of folic acid released by bacteria and sensed by the vegetative cells is further enhanced by the action of the extracellular folate deaminase. This enzyme inactivates folic acid and therefore increases the sensitivity of the cell to the gradient of newly deposited chemoattractant (Bernstein *et al.*, 1981; Pan *et al.*, 1975). *Dictyostelium* secrete most of the folate deaminase enzyme they generate, however, a portion remains bound to the extracellular face of the plasma membrane, thus adding another level of enhancement of the attractive signal gradient of folic acid.

The chemoattractant responsiveness of cells to folic acid has been shown to be G protein-dependent, but the identity of the receptor(s) remains elusive (De Wit and Snaar-Jagalska, 1985; Segall *et al.*, 1988). Interestingly, cells do demonstrate different sensitivities to folate analogues, which suggest the presence of at least two distinct receptor classes defined as $\alpha$ and $\beta$. The $\alpha$ folic acid receptor is detected in vegetative cells, while the $\beta$ folic acid receptor is detected in vegetative and differentiated cells (Hadwiger *et al.*, 1994). The folic acid signal is transduced via the $\alpha$ subunit of the heterotrimeric G protein G$\alpha$4. Cells lacking G$\alpha$4 do no exhibit chemotaxis to folic acid. In addition, folic acid mediated responses, such as activation of adenylyl and guanylyl cyclase, are absent in *g$\alpha$4*$^{-}$ cells (Hadwiger *et al.*, 1994). Based on these observations, it appears that G$\alpha$4 mediates signal relay through both $\alpha$ and $\beta$ folic acid receptors.

## B. Quorum Sensing

As the *Dictyostelium* cells grow and divide, largely unaware of each other, a set of compounds is used to assess the threshold of cell numbers required to form a complete multicellular organism. Prestarvation Factor (PSF), Conditioned Media Factor (CMF), and Counting Factor (CF) are the three

molecules that monitor the population size within an area and control the choice between growth and differentiation.

### 1. Prestarvation Factor

PSF is an autocrine factor that is secreted during growth and early development in which it accumulates in the extracellular environment (Clarke *et al.*, 1988). PSF is not only a density-sensing factor but also determines the ratio of nutrients to *Dictyostelium* cells. PSF acts to "prime" the cells for the oncoming process of aggregation by inducing the expression of a subset of genes required for sensing cAMP. Although the PSF receptor has not been identified, the response has been shown to partly depend on signaling to G proteins. In the G protein-dependent pathway, the folate released from bacteria downregulates PSF signaling, presumably via the folate-signaling cascade discussed earlier.

Two of the well-characterized PSF-responsive genes are the extracellular lectin discoidin I and the extracellular cAMP phosphodiesterase (ePDE). Discoidin-I is a lectin that is important for aggregation, while the ePDE is responsible for resetting the gradient sensing machinery and allowing cells to respond to additional chemotactic signals by degrading extracellular cAMP (Crowley *et al.*, 1985; Faure *et al.*, 1988). The expression of these genes was established to be G protein-independent as in $g\beta^-$ cells, a mutant that lacks the G$\beta$ subunit required for signaling, expression of Discoidin I and ePDE occurs normally in response to PSF (Burdine and Clarke, 1995; Lilly *et al.*, 1993).

Studies on the signal transduction pathways regulated by PSF have focused on the role of cAMP and protein kinase A (PKA). Mutants lacking the catalytic subunit of PKA (*pkacat*$^-$) fail to aggregate when starved (Mann *et al.*, 1992). However, when conditioned media containing PSF is added, *pkacat*$^-$ cells respond, albeit in a severely reduced fashion (Burdine and Clarke, 1995). Remarkably, the prestarvation response was not dependent on the presence of the adenylyl cyclase Adenylyl Cyclase A (ACA), the enzyme primarily responsible for generation of cAMP during early development, as the PSF response in *aca*$^-$ cells is normal (Burdine and Clarke, 1995). At the time, ACA was the only adenylyl cyclase known to be expressed early in development. Later work showed that another adenylyl cyclase, Adenylyl Cyclase B (ACB), is also expressed at low levels during early development and may be involved in mediating the ACA independent activity by generating low levels of cAMP sufficient to activate PKA (Burdine and Clarke, 1995; Meima and Schaap, 1999; Soderbom *et al.*, 1999).

The relationship between PSF and PKA was confirmed by the involvement of the YakA–PufA signaling components in the translational control of PKA (Souza *et al.*, 1998). YakA was identified as a homolog to the yeast

Yak1P, a key serine/threonine kinase that regulates growth attenuation. Likewise, *Dictyostelium* cells lacking YakA have defects in growth and development. These cells divide faster than wild type cells, suggesting that YakA regulates the timing of the cell cycle. Moreover, when *yakA*$^-$ cells are starved, they fail to initiate development, a phenotype that is rescued by the ectopic expression of the catalytic subunit of PKA (Souza *et al.*, 1998). In a second site suppression screen of *yakA*$^-$ cells, another factor, PufA was discovered. PufA is a homolog of the Puf proteins studied in *Drosophila* and *C. elegans*. These proteins all share a Pumilio homology domain (Pum-HD) that is responsible for repressing translation via an element encoded in the 3′-UTR of the cognate mRNA (Souza *et al.*, 1999). It has been established that the binding of PufA to the PKAcat mRNA inhibits translation. On starvation, cells lacking PufA, therefore, lose regulation of PKAcat, rapidly initiate development and overexpress key proteins required for aggregation. Overexpression of the recognition motif of PufA in wild type cells phenocopies the loss of PufA. Through a series of very elegant studies, it has been demonstrated that YakA negatively regulates the expression of PufA, thereby contributing to the stringent control of PKAcat expression, one of the key mediators of developmental progression (Souza *et al.*, 1999). The molecular relationship to PSF is currently inferred by timing of YakA expression. The peak of induction of YakA transcription coincides with the peak of PSF production. Genetic perturbations of the signaling events between PSF, YakA, and PufA, have yet to be identified.

## 2. Conditioned Media Factor

Mehdy and Firtel demonstrated that conditioned media harvested from developing wild type cells contains components aside from cAMP that promote the induction of both prestalk and prespore genes (Mehdy and Firtel, 1985). This CMF is an 80-kDa glycoprotein that is secreted by starving cells and crucial for development (Jain *et al.*, 1992).

CMF mediates both G protein-dependent and -independent effects within cells. The CMF receptor CMFR1, which is predicted to have two or three transmembrane segments and has no significant homology to known proteins, accounts for 50% of CMF binding to cells. Cells *cmfr1*$^-$ lack the induction of prespore and prestalk genes normally expressed in wild type cells (Deery and Gomer, 1999). In contrast, *cmfr1*$^-$ cells retain the G protein-mediated activation of adenylyl and guanylyl cyclases in response to added CMF. Based on the residual binding and retained signaling in *cmfr1*$^-$ cells, the existence of a second receptor that is responsible for the G protein-mediated CMF responses has been proposed (Deery *et al.*, 2002). It has been shown that CMF inhibits the GTPase activity of G$\alpha$2, the

subunit that mediates signaling from the cAMP receptor, cAR1 (Brazill *et al.*, 1998). The resulting sustained G$\alpha$2 activation could account for the ability of CMF to trigger low-density aggregation of cells. Interestingly, cells lacking PldB, an isoform of the phosphatidylcholine-specifc lipase, mimic the low-density aggregation observed in cells treated with exogenous CMF (Chen *et al.*, 2005).

## 3. Counting Factor

The search for other quorum sensing molecules leads to the identification of CF in 1996 by Brock and Gomer (Brock *et al.*, 1996). In a screen for mutants that are aberrant in aggregation size, one such mutant formed normal sized aggregates of $10^5$ cells that later break up into smaller aggregates of about $5 \times 10^3$ cells. The mutated gene responsible for this defect was isolated and named *SmlA* for small aggregates. *SmlA* encodes a novel 35-kDa cytosolic protein that is expressed during growth and early development. To establish the mechanism of action of SmlA, chimeric aggregates composed of wild type and *smlA*$^-$ cells were studied. Interestingly, a mixture containing as little as 5% mutant cells was enough to confer the *smlA*$^-$ phenotype to a chimeric population. It was, therefore, proposed that *smlA*$^-$ cells overproduce a secreted component that alters the behavior of neighboring cells. While the exact role of SmlA in the secretion of this factor still remains to be elucidated, CF has been purified and is composed of a very large 450-kDa protein complex containing at least six different proteins (Brock and Gomer, 1999). A mutant lacking one of the six polypeptides (Countin) cannot regulate aggregate size and gives rise to very large structures that cannot develop properly. The terminal structures formed by this mutant are so large that the sorus housing the spores cannot remain atop the stalk and invariably falls over onto the stratum (Brock and Gomer, 1999). When exogenously expressed, Countin exhibits most of the activity of the larger CF complex (Gao *et al.*, 2002). Countin affects developing cells in many ways to promote efficient regulation of organism size. This factor decreases cell–cell adhesion by down-regulating the expression of adhesion proteins (Roisin-Bouffay *et al.*, 2000). It also enhances cell motility by increasing the level of F-actin and decreasing the extent of myosin assembly within the cell and potentiates the response of cells to the chemoattractant cAMP (Gao *et al.*, 2002; Tang *et al.*, 2001, 2002).

As cell numbers increase, *Dictyostelium* employs an intricate system to establish population size, nutrient status, and differentiation control using various factors. PSF, CMF, and CF act in concert through diverse signaling pathways to establish the optimal population size and timing of developmental program progress.

## C. Gradient Sensing

### 1. cAMP: The Master Regulator of Development

The cAMP signal relay system employed during aggregation is essential for the development of *Dictyostelium*. This is a process by which 100,000 cells come together by migrating directionally and relaying signals to each other. The regulation of this crucial stage of development revolves around the production, secretion and inactivation of cAMP. Since the 1960s, when this small diffusible molecule was identified as the chemoattractant, the modulation of cAMP levels inside and outside cells has been under intense investigation by many groups (Konijn *et al.*, 1969; Meima and Schaap, 1999; Parent and Devreotes, 1996). This process begins with "pacemaker cells", a small subpopulation of cells that spontaneously begin to produce and secrete cAMP. The neighboring cells sense the cAMP from the pacemaker cells and respond by moving up the cAMP gradient in a head to tail fashion, and propagating the cAMP signal to neighboring cells (Fig. 2). The migration of the cells through the cAMP waves is readily observed using darkfield microscopy. The optical properties of stationary and motile cells are very different, and concentric "waves" of cells can be viewed as cells respond to periodic pulses of cAMP (Dormann and Weijer, 2001). This periodic pulsing of cAMP occurs every 6 min and is dependent on the exquisite regulation of the synthesis and degradation of cAMP.

### 2. cAMP Mediates its Effects by Binding Specific Membrane Receptors

A series of GPCRs shown to bind cAMP were designated cAR1-4 for cAMP binding receptors. The high affinity cAMP receptors cAR1 and cAR3 are required for aggregation as deletion of either gene result in cells that cannot aggregate (Johnson *et al.*, 1991, 1993; Klein *et al.*, 1988; Saxe *et al.*, 1991; Sun and Devreotes, 1991). Mutants of cAR2 arrest at the mound stage, while mutants of cAR4 have altered cell type–specific gene expression, demonstrating their requirement later in development (Louis *et al.*, 1994; Saxe *et al.*, 1993). Coupled to the cAMP receptors are the heterotrimeric G proteins composed of $\alpha$, $\beta$, and $\gamma$ subunits. In *Dictyostelium*, G protein complexes may contain 1 of 11 $\alpha$ subunits coupled to a single $\beta\gamma$ subunit (Lilly *et al.*, 1993; Wu *et al.*, 1995; Zhang *et al.*, 2001). During aggregation, cAR1, the aggregation-specific receptor, coupled to G$\alpha 2\beta\gamma$, mediates many of the cAMP-dependent effects (Sun and Devreotes, 1991). cAMP mediates both G protein-dependent and -independent events via the cAR1 receptor. Activation of adenylyl and guanylyl cyclase, PLC, and modulation of the actin and myosin cytoskeletons are G protein-dependent effects of cAMP, whereas receptor phosphorylation, $Ca^{2+}$ mobilization, and ERK2 activation

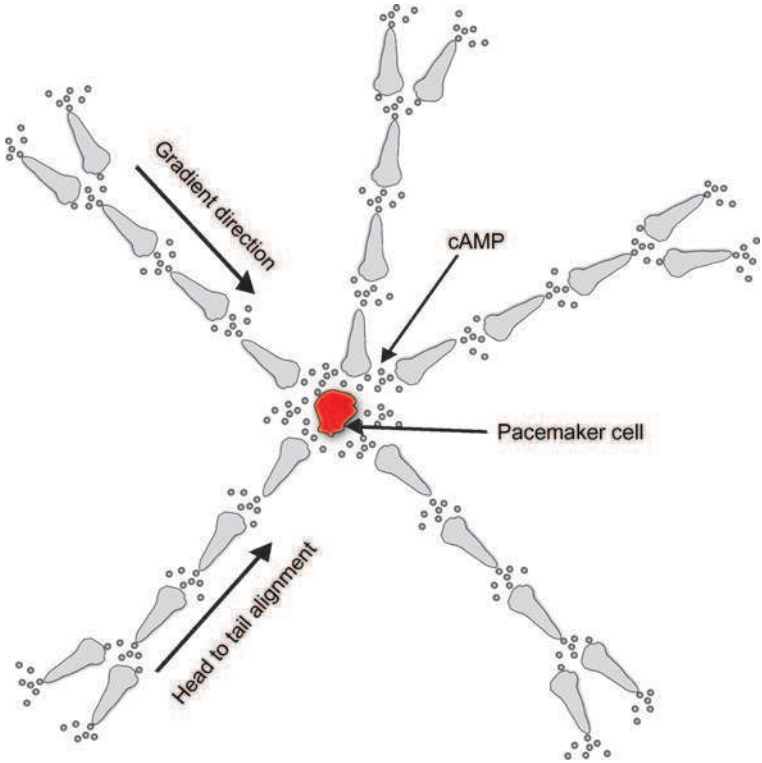


**Figure 2** Signal relay during *Dictyostelium* aggregation. During the gradient sensing module, pacemaker cells begin to secrete the chemoattractant cAMP. On binding of cAMP to receptors (cARs), cells orient and migrate in a head-to-tail fashion toward the source of chemoattractant. In addition, the binding of chemoattractant stimulates the production and secretion of additional cAMP. The spatial confinement of ACA, the adenylyl cyclase responsible for cAMP production, to the rear of cells facilitates the delivery of cAMP to neighboring cells (see text).

are all events that are activated independently of G proteins (Maeda *et al.*, 1996; Milne *et al.*, 1997; Segall *et al.*, 1995).

## 3. Synthesis of cAMP Is Highly Regulated

There are three distinct adenylyl cyclases expressed throughout the *Dictyostelium* developmental program. ACA shares homology with the mammalian G protein-coupled adenylyl cyclases containing two sets of six transmembrane segments each followed by a highly conserved catalytic domain. ACA is expressed during early development and provides the majority of the

cAMP that controls gene induction and aggregation. Mutants lacking ACA are not able to autonomously enter development when starved (Pitt *et al.*, 1992, 1993). However, in response to exogenously added cAMP, *aca*$^-$ cells are able to induce gene expression and to chemotax to a point source of cAMP (Kriebel *et al.*, 2003). In contrast to wild type cells, which align in a head to tail fashion during chemotaxis, *aca*$^-$ cells migrate independently of each other (Kriebel *et al.*, 2003). Remarkably, in addition to the catalytic activity of ACA, the localization of enzymatic activity may be important for signal relay. Live cell imaging has shown that ACA is enriched at the rear of chemotaxing cells suggesting that the mechanism of signal relay may involve restriction of activity to the posterior of cells (Kriebel *et al.*, 2003). ACA activity is tightly regulated by the action of the G$\alpha 2\beta\gamma$ heterotrimeric complex and two cytoplasmic proteins: Cytosolic Regulator of Adenylyl Cyclase (CRAC) and Pianissimo (Fig. 3). CRAC is a 78-kDa protein containing a N-terminal Pleckstrin Homology (PH) domain that mediates recruitment to the plasma membrane on chemoattractant stimulation (Insall *et al.*, 1994; Lilly and Devreotes, 1994, 1995). The *Pia* gene encodes

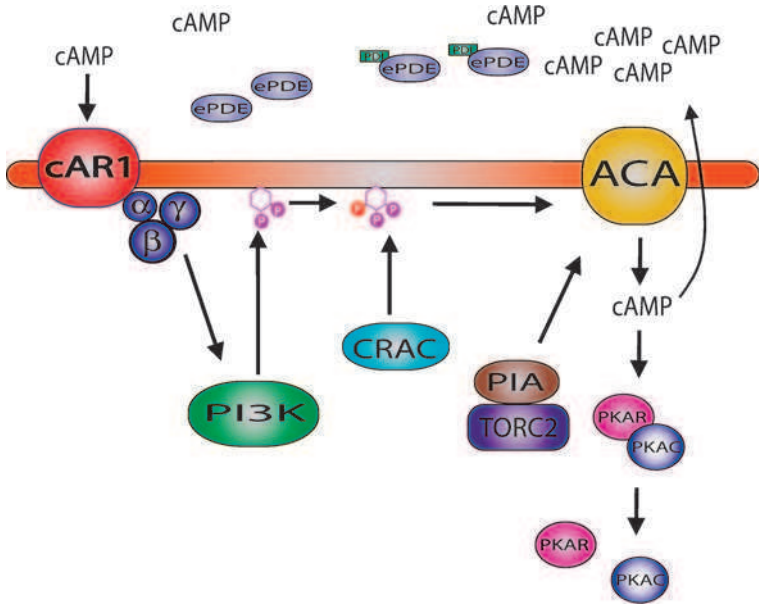


**Figure 3** Illustration depicting the signal transduction events that take place during *Dictyostelium* aggregation. cAMP binding to cAR1 leads to the activation of PI3K and the recruitment of the cytoplasmic, PH-domain containing protein, CRAC. CRAC and another cytoplasmic protein Pianissimo act in concert to allow the G protein-dependent stimulation of the adenylyl cyclase ACA (see text).

the *Dictyostelium* homolog of Rictor, a key member of the TORC2 (Target of Rapamycin Complex 2) in mammals and *Drosophila* (Chen *et al.*, 1997; Sarbassov *et al.*, 2004). Cells lacking CRAC or Pianissimo are unable to activate adenylyl cyclase and do not aggregate when starved. In addition, although $aca^-$ cells can chemotax, $crac^-$ and $pia^-$ cells have impaired ability to chemotax towards cAMP (Chen *et al.*, 1997; Comer *et al.*, 2005; Lee *et al.*, 2005).

ACB is large multidomain protein containing two hydrophobic domains near the N-terminus, a pseudo-histidine kinase domain, and a single catalytic domain at the C-terminus (Meima and Schaap, 1999; Soderbom *et al.*, 1999). ACB is expressed at low levels during growth but is required at the culmination stage, providing much of the cAMP to drive terminal differentiation. $acb^-$ cells aggregate normally but produce terminal structures that are aberrant with unstable spores, demonstrating the requirement for cAMP generation late in development (Meima and Schaap, 1999; Soderbom *et al.*, 1999). Adenylyl Cyclase G (ACG) is a unique adenylyl cyclase as it resembles membrane bound guanylyl cyclases in topology (Pitt *et al.*, 1992). ACG contains a large extracellular loop connected to a transmembrane region followed by a single cytoplasmic catalytic loop. The regulation of ACG occurs through an intramolecular switch in the extracellular loop in response to elevated osmolarity in the fruiting body (Saran and Schaap, 2004). ACG is only expressed at the terminal stages of spore formation and its activity helps to maintain spores in a dormant state (Cotter *et al.*, 1999; van Es *et al.*, 1996). Furthermore, $acg^-$ spores lack the ability to return to dormancy when challenged with high osmolarity during germination as seen in wild type spores.

## 4. cAMP Signal Relay Is Spatially Confined

Chemotaxis, crucial to form an aggregate, is remarkably precise and differences in receptor occupancy between the front and the back of cells of as little as 2% are enough to evoke a directional response. This sensitivity is achieved by the polarization of key components of the cAMP signal relay system. As cells polarize and align in the chemoattractant gradient, the cARs trigger the reciprocal recruitment of phosphatidylinositol-3 kinase (PI3K) at the front and the phosphotidylinositiol 3 phosphatase PTEN (Phosphatase and Tensin Homolog Deleted on Chromosome Ten) at the back and sides (Funamoto *et al.*, 2002; Iijima and Devreotes, 2002). This results in sustained levels of PtdIns(3,4)$P_2$ and PtdIns(3,4,5)$P_3$ (PIP2/3) at the leading edge of cells, and the recruitment of PH domain-containing proteins like PKB and CRAC (Meili *et al.*, 1999; Parent *et al.*, 1998). Recruited PH domain-containing proteins then serve as nucleation sites to activate various downstream effectors, including the acto-myosin contractile apparatus

(Parent, 2004). The spatial confinement of intracellular components amplifies the extracellular gradient sensed by cells and is required for cells to migrate directionally towards cAMP.

## 5. Removal of cAMP Is Tightly Controlled

In addition to the transient refractory period the cAMP signaling system undergoes after stimulation, *Dictyostelium* cells use an intricate removal system to degrade the extracellular cAMP and prevent the loss of directional information and gene expression resulting from saturation of the receptors. Work done by Malchow and Gerisch demonstrated the presence of phosphodiesterase activity on the cell membrane and in the extracellular space (Gerisch *et al.*, 1972; Roos *et al.*, 1975). Gerisch and colleauges also showed that cells secrete an ePDE inhibitor called PDI (Riedel *et al.*, 1972). The genes encoding ePDE and its inhibitor, PDI were later cloned and extensively characterized (Franke and Kessin, 1981; Franke *et al.*, 1991; Orlow *et al.*, 1981). The regulation of ePDE expression is complex with both the secreted and cell associated forms encoded by a single gene (PdsA) controlled by a promotor with three distinct developmental profiles (Hall *et al.*, 1993; Podgorski *et al.*, 1989).

Cells lacking PdsA fail to aggregate when starved showing that timely inactivation of the chemotactic signal is crucial for signal relay (Sucgang *et al.*, 1997). It was demonstrated that ePDE and cAR1 cosediment in detergent-resistant fractions suggesting that their close proximity may be required for the efficient modulation of signal relay (Xiao and Devreotes, 1997). In addition, overexpression of wild type ePDE leads to rapid development, presumably by reducing the refractory time the cells endure because of the low levels of cAMP in the media (Faure *et al.*, 1988). Later in development, the role of ePDE was assessed by ectopic expression of the inhibitor, PDI under control of the late promoter portion of the *PdsA* gene, which resulted in severe defects in stalk cell differentiation and failed to produce normal structures (Wu *et al.*, 1995).

The diffusible, heat-stable glycoprotein PDI is secreted as the cells reach the stationary growth phase (Franke *et al.*, 1991; Riedel *et al.*, 1972; Wu and Franke, 1990). The regulation of PDI expression is controlled by the levels of extracellular cAMP, thus cells can modulate the activity of ePDE by monitoring cAMP concentrations (Franke *et al.*, 1991). The binding of the secreted PDI to the ePDE is essentially irreversible with a $K_d$ of less than $10^{-10}$ M. The PDI interacts with the ePDE to increase the $K_m$ of the enzyme from 5 μM to 2 mM, while having no effect on the $V_{max}$ of the enzyme (Franke and Kessin, 1981). Cells lacking the secreted inhibitor have a delayed onset of development and do not propagate waves of cAMP (Palsson *et al.*, 1997). Although they cannot manipulate and propagate

periodic waves of cAMP signaling, *pdi*$^{-}$ cells proceed through development to form small fruiting bodies. The mode of action of the inhibitor is unique, as no similar component has been identified for other types of phosphodiesterases.

## D. Morphogen Sensing

### 1. cAMP Controls Morphogenesis

The periodic pulses of cAMP allow for the coordination of cell movement to the aggregation center. Once in a tight aggregate, cells coalesce into a mound structure that continues to send out radial waves of oscillating cAMP pulses (Tomchik and Devreotes, 1981). At this point, cells begin to sort to specific regions of the mound that are dependent on their fate. The prestalk cells move to the center of the mound and form an apical structure called the tip. This tip elongates upwards and then falls over thereby forming a structure called the slug (Fig. 1). Within the slug, cells have already taken up residence in specific regions. Prestalk cells are located primarily at the tip, whereas prespore cells populate the posterior of the slug. Remarkably, the choice of cell fate has been directly correlated with the status of progression through the cell cycle during growth. Cells that just finished S phase or were in early G2 phase predominantly become prestalk cells whereas cells that were in late G2 phase become prespore (there is no detectable G1 phase in *Dictyostelium*) (Wang *et al.*, 1988). In addition, other factors, such as nutrient history of the cell, also contribute to the choice of cell fate (Gomer and Firtel, 1987).

### 2. Cell Fate Is Influenced by Differentiation Inducing Factor

Differentiation Inducing Factor–1 (DIF-1), a small, chlorinated, hexaphenone compound was identified by its ability to induce stalk cell maturation in cells plated at very low density (Kay *et al.*, 1983; Morris *et al.*, 1987). The control of DIF-1 signaling in the slug demonstrates the intercellular communication between cell types in *Dictyostelium*. Kay and Thompson (2001) established that prespore cells coordinate differentiation of prestalk cells by producing DIF (Kay and Thompson, 2001). The cell type specific production of DIF sets up a gradient of the freely diffusible compound from the rear of the slug towards the front. The polarity is further strengthened by the presence of the DIF dechlorinase, the enzyme that inactivates DIF, at the anterior of the slug, most notably in the tip (Kay *et al.*, 1993). The balance of DIF signaling may therefore allow for the proper proportioning of prespore and prestalk cells in the developing organism. The biosynthetic pathway of DIF has been extensively studied, with the final rate-limiting step performed

by a methyltransferase, DmtA (Kay and Thompson, 2001). Surprisingly, cells lacking DmtA develop normally except that they lack the Pst-O stalk cell type. This suggests that there must be additional factors that contribute to the maturation of the other stalk cell subtypes (Maeda *et al.*, 2003; Thompson and Kay, 2000).

## 3. Ammonia Controls Culmination

Much of the energy required for development is generated by protein catabolism, in which the organism utilizes the ammonia released to signal various developmental checkpoints (Schindler and Sussman, 1977a,b). For example, the choice between slug migration and culmination is governed by the presence of ammonia. High ammonia levels maintain slug migration whereas removal promotes fruiting body formation (Davies *et al.*, 1993). A series of mutants that affect sensitivity to ammonia shed light on the mechanism for this developmental checkpoint. Cells lacking the histidine kinase DhkC form fruiting bodies irrespective of ammonia levels, while wild type cells remain as slugs if high ammonia is present (Singleton *et al.*, 1998). Remarkably, the expression of a truncated protein containing only the kinase domain but not the putative receptor domain results in cells that preferentially remain in slugs. Exogenous addition of cAMP restores this slugger phenotype (Singleton *et al.*, 1998). DhkC is part of a larger family of *Dictyostelium* histidine kinases that have been shown to influence activity of the intracellular phosphodiesterase RegA (Loomis *et al.*, 1997). The receptor domain of DhkC may sense levels of ammonia, which lead, via the phosphotransfer protein RdeA, to lowered intracellular cAMP levels and thus, lower PKA activity within the cell (Thomason *et al.*, 1999). The PKA activity required to drive the final events during development can be controlled by removal of ammonia when required through the inactivation of RegA.

The removal or inactivation of ammonia has been assigned to the recently identified ammonia transporters in *Dictyostelium* (Follstaedt *et al.*, 2003). Three putative ammonia transporters (amtA,B,C) were identified and characterized. *In situ* hybridization showed that AmtA expressed in the anterior prestalk region of the slug. AmtB is only expressed in the prespore region. AmtC is expressed primarily in the prespore region but shows expression in the anterior tip of the slug. Cotter *et al.* (1992) had proposed a "source and sink" model for ammonia movement through the developing organism. In this model, protein catabolism and ammonia accumulation up in the prestalk cells could be alleviated by the transfer of the ammonia to the prespore cells, which would incorporate the ammonia into amino acid synthesis in preparation for spore dormancy. With this proposed model, Singleton *et al.* (1998) assigned functional duties to the ammonia transporters as follows: AmtA could be an ammonia exporter in prestalk cells and the AmtB and C

components importers in prespore cells. The phenotype of *amtc*$^{-}$ cells closely resembles the cells that have overexpression of the DhkC kinase, which remain as slugs irrespective of ammonia concentrations (Singleton *et al.*, 1998). This suggests that the AmtC present at the tip of slugs may be responsible for rapidly removing ammonia when the decision to move from migratory slug to stationary culminant is required (Follstaedt *et al.*, 2003).

### 4. Folate

Another contributor to specialization of cells within the developing organism is folate. G$\alpha$4 has been linked to a folic acid receptor during development and required for morphogenesis and spore production (Hadwiger *et al.*, 1994). *G$\alpha$4*$^{-}$ cells arrest at the mound stage with a severely depleted prespore population. Intriguingly, G$\alpha$4 is not expressed in prespore cells, but only in prestalk like cells present in the prespore region of the slug called anterior like cells (ALCs). Thus G$\alpha$4 must drive prespore differentiation through intercellular communication from the ALCs. In addition, exogenous addition of folate or overexpression of G$\alpha$4 delays prestalk differentiation (Hadwiger and Srinivasan, 1999; Srinivasan *et al.*, 1999). The influence of folate through G$\alpha$4 on the cell-type proportioning underscores the importance of intercellular communication in terminal differentiation.

## E. Environmental Sensing

### 1. Role of Spore Differentiation Factors in Spore Formation

Elevation of intracellular cAMP and the resultant rise in PKA activity is required for spore encapsulation (Virdy *et al.*, 1999). Furthermore, a gradient in gene expression has been observed from the tip of the culminant to the base of the fruiting body (Balint-Kurti *et al.*, 1998; Richardson *et al.*, 1994). This gradient of gene expression suggests that diffusible signals are required to mediate the final steps of spore formation. Two diffusible distinct peptides purified with spore promoting activity were named SDF-1 and SDF-2 for Spore Differentiation Factors. SDF-1 is produced *de novo* and phosphorylated by PKA in prestalk cells and subsequently released in nanomolar concentrations to trigger the encapsulation of spores (Anjard *et al.*, 1997, 1998b).

SDF-2 was identified by the ability to promote spore encapsulation in cell culture. It differs from SDF-1 in that it is not phosphorylated by PKA and does not require *de novo* protein synthesis for activity (Anjard *et al.*, 1998a). SDF-2 works through the histidine kinase DhkA to elevate PKA activity

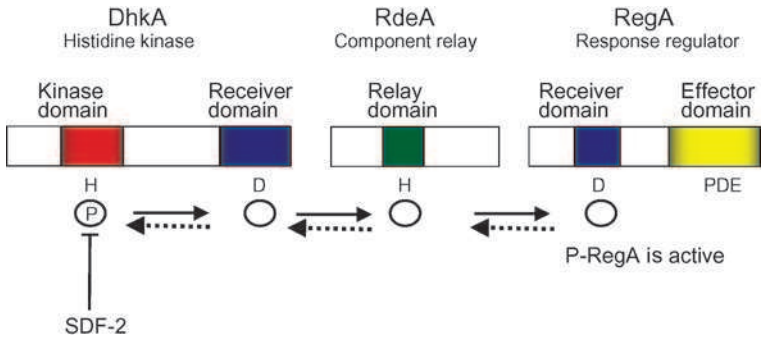


**Figure 4** Schematic representation of the regulation of the intracellular phosphodiesterase RegA by histidine kinases. In the absence of ligand, the histidine kinase, DhkA, is an active kinase facilitating the transfer of phosphate to RdeA and ultimately to RegA. Phosphorylation of RegA activates the phosphodiesterase activity, which results in lower intracellular cAMP levels. On binding of SDF-2, the constitutive phospho-transfer from DhkA to RegA reverses, thereby inactivating RegA and allowing cAMP levels to rise. The resultant high levels of cAMP lead to elevated PKA activity required for sporulation (see text).

(Wang *et al.*, 1999). SDF-2 binds to DhkA and alters the constitutive phospho-transfer to the RegA resulting in an increase in intracellular cAMP due to the inactivation RegA (Anjard and Loomis, 2005) (Fig. 4). Recently, the source of SDF-2 has been traced to the cytoplasmic acyl-CoA binding protein AcbA. AcbA is produced in prespore cells and secreted and processed by the serine protease TagC to release peptides, one of which is SDF-2 (Anjard and Loomis, 2005). Interestingly, TagC is a protein expressed in stalk cells and may represent another level of intercellular communication by coordinating development between these two cell types (Good *et al.*, 2003). Moreover, the homolog of AcbA in mammalian systems functions in a similar fashion to modulate the activity of the $GABA_A$ receptor; in fact the $GABA_A$ receptor agonist diazepam induces spore encapsulation in *Dictyostelium* (Anjard and Loomis, 2005).

## 2. Spore Encapsulation

One of the hallmarks of the developmental cycle of *Dictyostelium* is the generation of resilient spores that can survive harsh environmental conditions. As mentioned in the previous sections, the signal transduction pathways mediating spore differentiation emanate very early in the developmental program of the organism. With such an investment in the next generation of cells, *Dictyostelium* has developed many checkpoints governing spore formation and the maintenance of dormancy. These external cues for dormancy are present in a viscous matrix that bathes the spore within the

fruiting body. Although there are many different inputs governing spore dormancy, they all revolve around the maintenance of high levels of cAMP and PKA activity within the spore. For germination to proceed, a drop in cAMP levels and the resultant drop in PKA activity are required (Cotter *et al.*, 2000).

### 3. Ammonium Phosphate Keeps cAMP Levels High

As the fruiting body is formed, there is a remarkable increase in the osmolarity in the fluid that bathes the spores within the sorus. The high osmotic pressure maintains spore dormancy through the activation of the adenylyly cyclase ACG (Cotter *et al.*, 1999). Through an intramolecular sensing mechanism, ammonium phosphate, the component responsible for the osmolarity, drives the synthesis of cAMP (Saran and Schaap, 2004). The resultant intracellular cAMP increase activates PKA and suppresses germination. Germinating spores lacking ACG cannot revert to dormancy when challenged with high osmotic pressure as seen with wild type spores (van Es *et al.*, 1996).

### 4. Discadenine Is an Autoinhibitor

In addition to high osmolarity and ammonia concentrations, the adenine derivative Discadenine also controls spore dormancy. The production of Discadenine is highly regulated with the enzymes responsible for its synthesis only present after spores have formed (Taya *et al.*, 1978). Discadenine acts to maintain spore dormancy at nanomolar concentrations. More specifically, it is postulated that the hisitidine kinase DhkB is the discadenine receptor, as cells lacking this component are able to complete the developmental time course and produce spores that germinate within the fruiting body. The unstable spore phenotype can be rescued by the addition of 8-Br-cAMP or ectopic expression of the catalytic subunit of PKA in *dhkb*$^{-}$ cells (Zinda and Singleton, 1998). The direct link between discadenine and DhkB remains to be demonstrated.

### 5. Germination Signals Overcome Environmental Checkpoint

Spores held atop the fruiting body are poised to germinate when conditions are favorable for growth, that is, food source, correct temperature, and pH. To allow for germination, spores must be removed from all of the inhibitory molecules present in the sorus. This dispersal of molecules allows for the progermination signals to act. Once dispersed, spores test the environment for the proper conditions for growth. For example, extracellular glucose is a

trigger to mobilize the cytoskeleon, however spores remain in their cellulose coat (Kishi *et al.*, 2000). Peptides and presumably metabolic by-products from bacteria, such as folic acid, are also required to promote spore germination (Dahlberg and Cotter, 1978). Spores retain a "fail-safe" point, a germination checkpoint that involves the sensing of the external environment for potential hazards (Kishi *et al.*, 1998). If conditions are not favorable, spores may retreat from the active state back to dormancy (Gauthier *et al.*, 1997). If all the signals are in place, namely the removal of inhibitors and the presence of favorable conditions, spores will germinate to release amoebae in a synchronous fashion (Cotter *et al.*, 2000).

*Dictyostelium* spores have an optimal lifespan of about 14 days, after which the viability of individual spores begins to drop. In a last ditch effort to cheat death, spores acquire the ability to "auto-activate" (Dahlberg and Cotter, 1978). This process allows for the coordinated release of nascent amoebae that are "primed" to enter the developmental program and form a migratory slug that can move to another location. This autoactivation process is mediated through intercellular communication between dispersed spores. A small, low molecular weight autoactivator is produced and triggers the rapid and highly synchronous release of amoebae. Spores that have been autoactivated for 5 hr acquire the ability to aggregate within 30 min, while exogenously activated spores require an additional 6 hr to aggregate similar to cells removed from feeding conditions. This implies that through the assembly of the cAMP signaling machinery, the spores have committed to enter the developmental program before emergence.

## III. Summary

The simple life led by *Dictyostelium* exhibits a remarkable diversity of signal relay modules that incorporate inputs from neighbors as well as the environment. As cells grow and divide, cells sense the nutrient status of the environment. As the population increases, mechanisms are in place to track growth in cell numbers. When the switch to enter development is made, signal relay governs the number and directionality of cells to come together and form a multicellular organism. Within this organism, clonal cells respond and trigger cues that differentiate the population into distinct cells types that will either support or survive and make it to the next generation of cells. As the spores are held on top of the stalk, they retain the ability to sense the environment and cues from each other to control the decision to germinate. Once the germination process has begun, cells can again signal to each other and trigger synchronous germination to release cells. At all of these developmental checkpoints, signal relay between cells is crucial.

## IV. Perspectives

*Dictyostelium* exhibits many different modes of signal relay during its life cycle. For example, during gradient sensing, cells respond and produce a single chemoattractant, cAMP, to control the action of a clonal population of cells. In mammalian systems, neutrophils are among the first responders to sites of infection, and the signal transduction mechanisms they use to leave blood vessels and travel to wound sites is highly analogous to the gradient sensing signals operational during *Dictyostelium* aggregation. Neutrophils sense the presence of formylated peptides released by bacterial metabolism and extravasate and travel through tissues by following the peptide gradients. Once at the site of infection, neutrophils kill the bacteria and relay chemotactic information to neighboring neutrophils as well as accessory immune cells. Neutrophils respond to primary chemoattractants by synthesizing secondary signals such as leukotriene $B_4$ ($LTB_4$) and Interleukin-8 (IL-8) (Cassatella, 1999; Soberman and Christmas, 2003). IL-8 may act in a paracrine fashion on neighboring neutrophils by facilitating the release of reactive oxygen species to combat infection (Brechard *et al.*, 2005). The study of signal relay in *Dictyostelium* has had a remarkable impact on our understanding of neutrophil behavior, and future studies will undoubtedly uncover more unexpected similarities.

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# 5

# Biological Principles for *Ex Vivo* Adult Stem Cell Expansion

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I. Introduction
II. Strategies for ASCs Expansion
  A. Transgenesis
  B. Growth Factor Supplementation
  C. Embryonic Stem Cell-Based Strategies for ASC Expansion
III. Cell Kinetics Symmetry-Based Strategies for ASC Expansion
IV. Comparison of SACK-Derived ASC Strains to Conventional Cell Lines
V. Future SACK Pathways and Regulators
VI. Confronting the Replication Mutation Risk of ASC Expansion
VII. Summary
  References

Adult stem cells (ASCs) are the engines that drive the renewal of adult mammalian tissues. They divide continuously, throughout life, to produce new progeny cells that undergo a robust development program of differentiation and maturation to replace older expired tissue cells. The same cell turnover program may function to provide limited repair and regeneration of adult tissues in some cases. The regenerative potential of ASCs drives the current intense interest in adapting them for applications in cell replacement therapy. However, research to explore this potential has been blunted by an unyielding biological problem. ASCs have proven highly refractory to expansion of their numbers and long-term propagation in culture. A review of reported strategies to overcome this problem reveals that many studies focus on traditional cell culture factors that may not apply to ASCs and overlook a special property of ASCs that may be universally critical for successful expansion, asymmetric cell kinetics (ACK). This property is reflected by the different kinetics fate of the two sister cells resulting from an ASC division: one cell remains an ASC and keeps the potential to divide for the entire life span of the tissue, while the other cell's progeny eventually differentiates and undergoes terminal division arrest. This unique property of ASCs may prove to be the obligatory factor that must be breached by any method that will succeed in accomplishing routine expansion of ASCs of diverse tissue origin. 

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## I. Introduction

Two undertakings drive the demand for expanded populations of adult stem cells (ASCs), basic research and the quest for new cell replacement therapies. Although the existence of adult tissue stem cells was established experimentally nearly a half century ago (Becker *et al.*, 1963), for the most part, the cellular identification and production of these cells in large quantities for research has remained an elusive aspiration. Even the best-defined ASC, the hematopoietic stem cell (HSC), is still wanting for unique markers for its identification and methods for its expansion in culture (Kiel *et al.*, 2005; Kondo *et al.*, 2003). Because these two challenges have not been met, research to develop a detailed, molecular understanding of ASCs has lagged. Given this history, it is no surprise that efforts to develop new therapies based on ASCs are dammed up behind the slow progress of ASC research.

Solving the ASC expansion problem may also solve their identification problem. Several molecular markers have been described that show respective preferential expression in mouse and/or human HSCs (Chen *et al.*, 2002; Christensen and Weissman, 2001; Kiel *et al.*, 2005; Kondo *et al.*, 2003; Spangrude *et al.*, 1988). Some of these markers are also selectively expressed in rare cells in other stem cell compartments, for example CD34 in hair follicle (Morris *et al.*, 2004; Trempus *et al.*, 2003). However, none of these markers show exclusive expression in HSCs or any other ASC. Lineage-committed early progenitor cells also express many of them. Efforts to identify unique stem cell molecular profiles in gene micro-array studies have been thwarted by the low purity of ASC-enriched populations and the technical variability of micro-array analyses (Easterday *et al.*, 2003; Evsikov and Solter, 2003; Fortunel *et al.*, 2003; Ivanova *et al.*, 2002; Ramalho-Santos *et al.*, 2002). If genes that are expressed exclusively in ASCs exist, the availability of large numbers of ASCs in high purity will enable their discovery.

An understanding of key biological principles that govern proliferation of ASCs is essential for success in developing methods for their expansion. Thus far, the rationales considered for expansion efforts have been largely hypothetical, without actually supporting experimental results. Among these previous rationales are loss of viability factors, loss of growth factors, disruption of essential cell-matrices contacts, disruption of essential cell–cell contacts, and, to a lesser extent, retention of asymmetric cell kinetics (ACK). Of course, based on general concepts in cellular and developmental biology, it is understandable why each of the first four factors has been consistently targeted for manipulation in expansion studies. The fifth factor, ACK, has only recently been considered as an important barrier to the expansion of ASCs in culture (Lee *et al.*, 2003; Merok and Sherley, 2001; Rambhatla *et al.*, 2001; Sherley, 2002).

In the past few years, numerous methods based on the first four proposed limiting factors have been described to facilitate the expansion of ASCs (Jiang *et al*., 2002; Park *et al*., 2003; Reya *et al*., 2003; Toma *et al*., 2001). The most promising among them and their limitations are considered in this chapter. First, transgenic approaches used to develop ASCs genetically modified for improved expansion are discussed (Fig. 1A). Second, studies that utilize exogenous growth factors to stimulate ASC proliferation are evaluated from the critical perspective of what cell type is actually being expanded (Fig. 1B). Third, a related, currently highly touted approach, derivation of ASCs from embryonic stem cell (ESC) cultures, is considered (Fig. 1C). The final treatment is a critical evaluation of methods that are based on regulating the cell kinetics symmetry of ASCs to overcome their inherent ACK (Fig. 1D). This property, which is a defining property of ASCs, may also be the key restriction to their expansion in culture.

## II. Strategies for ASCs Expansion

### A. Transgenesis

Transgenic approaches involve the modification of the genome of either stem cells or their supporting cells. ASCs can be isolated from an animal expressing the transgene ubiquitously or under the control of spatio-temporal regulatory elements. Viral infection of explanted cell populations, containing ASCs in gene therapy protocols, is also considered a transgenic approach. The goal is to transduce ASCs with a transgene whose expression promotes their expansion. One important limitation is the need to control the expression of the transgene. Many systems have been developed to activate or inactivate a transgene with exogenous compounds, but background expression is always a concern (Corbel and Rossi, 2002; No *et al*., 1996). When effective in all cells, deletion of a transgene flanked by two *loxP* sites following action of Cre recombinase is the only method that can extinguish the expression of the transgene after ASC expansion (Rossant and McMahon, 1999; Sauer, 2002).

Another problem occurring with the transgenic approaches is the risk of disrupting gene regulation. Integration of a transgene in the genome can disrupt the regulation of neighboring genes. If one of these genes corresponds to an oncogene or a tumor-suppressor gene, then the risk of cellular transformation can increase. Examples of this phenomenon occurred in patients undergoing gene therapy for X-linked severe combined immunodeficiency disorder (SCID-Xl). Two of eleven children who were treated subsequently developed leukemia, a likely consequence of oncogene activation following transgene integration (Hacein-Bey-Abina *et al*., 2003). Site-specific

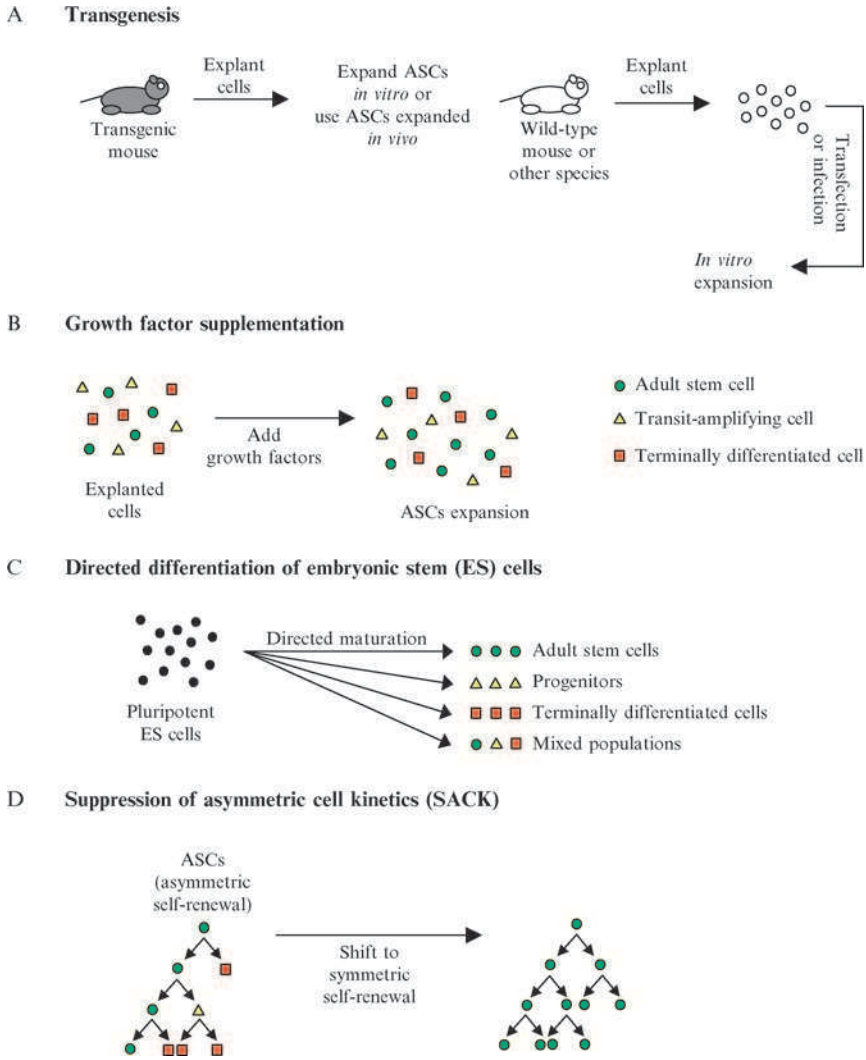


**Figure 1** Experimental approaches used for the expansion of adult stem cells. (A) Cells are explanted from a transgenic mouse expressing a transgene to promote ASC expansion. Expansion is attempted *in vitro* or may have already occurred *in vivo* before explantation. An alternative approach consists in explanting cells from a wild-type animal and integrating of a transgene by transfection or viral infection before proceeding to *in vitro* ASCs expansion. (B) Cells are explanted from the tissue of interest and specific growth factors are added to the culture medium to stimulate ASCs expansion. Explanted cells may be enriched for ASCs before expansion. (C) Undifferentiated pluripotent embryonic stem cells are directed to a specific differentiation program. The resulting cells are either ASCs, transit-amplifying cells, terminally differentiated cells, or a mixed population of the three. (D) Explanted ASCs that naturally undergo asymmetric self-renewal may have their cell kinetics symmetry switched to symmetric self-renewal by a method called suppression of asymmetric cell kinetics (SACK), allowing the number of ASCs to increase exponentially.

integration via homologous recombination or other mechanisms would be the safest way to generate transgenic ASCs with minimal disturbance of gene expression. However, a large number of ASCs would be needed for such an approach; of course, lack of a method for obtaining large numbers of ASCs is the problem.

Despite these limitations, research with diverse transgenic models elucidated molecular pathways that can be manipulated to induce the expansion of ASCs. Features of the ASC expansion accomplished in these transgenic mouse models for the antiapoptotic gene *Bcl-2*, the homeotic gene *HoxB4*, and the *Notch* gene are discussed in the following sections.

### 1. Bcl-2

One way to favor the expansion of ASCs is to suppress their apoptosis, a highly regulated process that supports tissue homeostasis by removing defective and potentially disease-causing cells (Meier *et al.*, 2000; Rathmell and Thompson, 2002). In many tissues, the ASCs appear extremely sensitive to apoptosis following minimal exposure to DNA damaging agents (Cai *et al.*, 1997; Marshman *et al.*, 2002; Roberts *et al.*, 1995). Increasing the resistance of ASCs to apoptosis might tip the balance toward better survival and, thereby, more effective expansion of ASCs.

*Bcl-2* is a gene that encodes for a well-known inhibitor of apoptosis. Bcl-2 exerts its function by maintaining mitochondrial integrity by association with the mitochondrial outer membrane (Borner, 2003). Overexpression of the Bcl-2 protein makes cells resistant to cell death following diverse stimuli that normally trigger apoptosis (Domen *et al.*, 1998). Relevant to this discussion, transgenic mice overexpressing *Bcl-2* in HSCs show an increase in the number of HSCs in their bone marrow. HSCs isolated from these mice exhibit a better plating efficiency *in vitro* and more effective bone marrow repopulation than nontransgenic HSCs (Domen *et al.*, 2000).

Though these results are encouraging, expansion of ASCs by inhibition of apoptosis must be viewed with caution. It is well established that prevention of normal programmed cell death of tissue cells favors the development of cancers (Johnstone *et al.*, 2002). To this effect, transgenic mice with ubiquitous overexpression of *Bcl-2* show a higher frequency of malignancies (Cory *et al.*, 1994; McDonnell *et al.*, 1989). Even if *Bcl-2* overexpression were targeted to specific ASC populations, the induced retention of cells that would normally be programmed for cell death and removal poses a significant risk for neoplastic transformation. Thus, although investigations with *Bcl-2* transgenic mice may provide biological insights to HSC cell kinetics mechanisms, these studies do not provide a practical method for expansion of ASCs that would be safe for cell therapy applications.

## 2. HoxB4

The *Hox* family genes encode homeodomain transcription factors that regulate many early developmental processes (Hombria and Lovegrove, 2003). Many *Hox* genes are expressed in primitive HSC-enriched populations and downregulated in differentiated hematopoietic cells (Sauvageau *et al*., 1994). Moreover, overexpression or downregulation of some *Hox* genes affects hematopoiesis in specific lineages (Izon *et al*., 1998; Lawrence *et al*., 1997; Sauvageau *et al*., 1995, 1997; Thorsteinsdottir *et al*., 1997, 1999). These observations suggested that *Hox* genes were involved in events leading to HSC specification and/or self-renewal.

In HSCs, the most widely studied *Hox* gene has been *HoxB4*. It was first shown that retroviral expression of *HoxB4* significantly enhanced *in vivo* HSC repopulation in primary and secondary recipients (Antonchuk *et al*., 2001). In repopulation experiments, HoxB4-induced expansion of HSCs was attenuated when normal levels of HSCs were achieved, showing that the homeostatic controls that regulate HSC pool size were still effective on *HoxB4*-overexpressing HSCs. In later studies, *HoxB4*-transduced cells were reported to be capable of achieving rapid and extensive expansion *in vitro* while maintaining full lympho-myeloid repopulation capability (Antonchuk *et al*., 2002).

Like any transgenic approach, the necessity to integrate the transgene into the target cell genome and the permanence of this transgene make this method less convenient for clinical applications. An original approach was recently designed to circumvent these problems: a stromal cell line was transduced with a lentiviral vector that encoded for a HoxB4 protein fused to a plasma membrane permeabilization sequence (Amsellem *et al*., 2003). This allowed the modified HoxB4 protein to be secreted from the stromal cells and taken up by surrounding cells (hematopoietic cells in this case), in which HoxB4 could activate its target genes. By using this methodology, they showed that HoxB4 delivery could expand the number of human long-term culture-initiating cells (LTC-ICs) by 20-fold and the *in vivo* repopulating cells by 2.5-fold after 5 weeks.

Despite these experimental successes, like *Bcl-2*, the safety of the *HoxB4* overexpression approach in long-term clinical applications is also likely to be problematic. Overexpression of other *Hox* genes, like *HoxB8* and *HoxA10*, causes leukemia in mice (Perkins and Cory, 1993; Thorsteinsdottir *et al*., 1997). Also, some abnormalities in myeloid differentiation have been linked to high levels of *HoxB4* expression (Brun *et al*., 2003; Schiedlmeier *et al*., 2003). These outcomes are foreboding for adoption of *HoxB4*-induction for expansion of HSCs for clinical applications.

### 3. Notch

Notch signaling is a highly conserved pathway that controls cell fate choices in both invertebrates and vertebrates. *Notch* genes encode for transmembrane receptors and are composed of four members (*Notch1-4*) in mammals. A special property of Notch receptors is that their intracellular domain is translocated to the nucleus after a cleavage event induced by ligand binding. The translocated domain of Notch then interacts with DNA-binding proteins to modulate the transcription of Notch target genes (Artavanis-Tsakonas *et al*., 1999).

Notch activation is involved in the maintenance of self-renewal in some types of ASCs from worms to mammals. In culture, murine HSCs, retrovirally transduced with a modified *Notch1* gene encoding for a constitutively active protein, generate immortal cell lines from which lymphoid and myeloid progeny can be derived (Varnum-Finney *et al*., 2000). These cell lines can repopulate all the hematopoietic compartments after transplantation, but with a lower level of contribution to the B-cell compartment. This finding was consistent with previous studies showing inhibition of B-cell differentiation by Notch-1 signaling (Pui *et al*., 1999). The *Notch-1* ligand, *Jagged-1*, is expressed by the osteoblasts in the proposed HSC niche (Pereira *et al*., 2002). When activated by parathyroid hormone, these osteoblasts produce high levels of Jagged-1; and this is associated with a simultaneous increase in the number of HSCs induced by intrinsic Notch-1 activation (Calvi *et al*., 2003).

The Notch pathway is also implicated in the maintenance of neural stem cells (NSCs) (Shen *et al*., 2004). Coculture of NSCs with endothelial cells activates Notch signaling in the NSCs, and this stimulates their self-renewal and expansion, as shown by the increase in the production of secondary neurospheres (the best known indicator of NSC expansion) when compared with NSCs grown without endothelial cells.

Another indicator of the importance of Notch activity in ASCs is the expression of the gene *musashi-1* in NSCs (Kaneko *et al*., 2000; Sakakibara *et al*., 1996) and intestinal epithelial stem cells (Potten *et al*., 2003). This gene encodes for an RNA-binding protein that inhibits Numb, a repressor of Notch signaling (Imai *et al*., 2001).

However, Notch-1 activation appears to favor lymphoid differentiation over myeloid differentiation from HSCs (Radtke *et al*., 2004), and Notch activation promotes glial differentiation from NSCs under some circumstances (Ge *et al*., 2002). One explanation for these results would be that the Notch pathway simply slows the differentiation of daughter cells following ASC division without fully suppressing their ACK while promoting one differentiation fate over another. Another explanation could be a

requirement for Notch signaling to be active only in the stem cell stage. A persistence of Notch activity in early progenitors possibly commits them preferentially toward a differentiation program at the expense of others. Full understanding of the molecular processes responsible for these phenomena will be necessary for progress toward medical applications.

One advantage of *Notch* over *HoxB4* and *Bcl-2* for expansion of ASCs is that it is possible to activate the Notch pathway by supplementing culture media with Notch ligands. Therefore, there is no need to genetically modify the target ASCs, if they express Notch receptors. Examples of this strategy have been described in recent years for other signaling pathways, and they are reviewed in the next section.

## B. Growth Factor Supplementation

The use of growth factors that stimulate the expansion of ASCs is more convenient than genetic modification for expansion. Media supplementation is far less time consuming and does not pose the gene disruption risks associated with transgene integration. However, the growth factors reviewed in this section have worrisome pleiotropic effects. The activation of unwanted signaling pathways is a significant risk associated with the growth factor supplementation approach, especially when modulated pathways are involved in the development of neoplasia.

### 1. Fibroblast Growth Factors

*Fibroblast Growth Factors* (*FGF*) genes encode for growth factors with a wide range of biological activities. Over 20 *FGF* genes have been identified in mammals (Itoh and Ornitz, 2004; Ornitz and Itoh, 2001). Their study led to the determination of functions for many of them in the processes governing stem cell maintenance and expansion. For example, FGF-7 (keratinocyte growth factor) protects intestinal epithelial stem cells against cell death induced by low-dose irradiation (Potten *et al.*, 2001). It also stimulates the proliferation of pancreatic duct cells (a possible location for adult pancreatic stem cells) when secreted by insulin-producing cells in the islets (Krakowski *et al.*, 1999).

Other FGFs influence stem cell behavior in other systems. FGF-2 is essential for the survival and proliferation of NSCs *in vitro*, and is routinely used for neurosphere cultures (Ray *et al.*, 1993; Weiss *et al.*, 1996). In the mouse, FGF-10 acts as a survival factor for the maintenance of the stem cell population residing in developing incisor germs (Harada *et al.*, 2002). And in the mouse embryonic liver, FGF-1 and FGF-4 enrich explant cultures for bipotential hepatic progenitors (Sekhon *et al.*, 2004).

So far, the most promising results have been obtained by supplementing culture media with FGF-1 in *in vitro* HSC cultures. FGF receptor expression levels in primitive bone marrow cells were found to be correlated with the stem cell activity of cultures. Prolonged culture of bone marrow cells in serum-free medium supplemented with FGF-1 leads to the expansion of HSC activity (de Haan *et al.*, 2003). However, this method has not been able to expand pure populations of HSCs, suggesting that FGF-1 may act indirectly or with a combination of other factors released by other bone marrow cell types. Whether or not FGF-1 can suppress the ACK of HSCs remains unknown. Further studies will be required to elucidate the full range of cellular functions affected by FGFs.

## 2. Wnt Ligands

*Wnt* genes encode secreted glycoproteins that act as ligands for receptors from the *Frizzled* family of receptors and correspond to the mammalian homologs (19 in total) of the *Drosophila wingless* gene (Willert *et al.*, 2003). In the canonical Wnt pathway, Wnt molecules bind to their receptor and activate a signaling pathway that inhibits the degradation of $\beta$-catenin and leads to its accumulation in the cytoplasm. Cytoplasmic accumulation of $\beta$-catenin promotes its translocation into the nucleus, where it activates its target genes by cooperating with the TCF/LEF family of transcription factors. The canonical Wnt pathway controls multiple biological processes, including cell-fate determination and proliferation. Wnt proteins also act through a noncanonical pathway mediating cadherin-dependent cell–cell interactions (Nelson and Nusse, 2004). For this chapter, discussion will focus on Wnt function through the canonical pathway.

The requirement for Wnt signaling in stem cell maintenance is strongly conserved in multiple tissues throughout evolution. For example, the *Drosophila* homolog *wingless* is essential for ovarian stem cells' maintenance (Song and Xie, 2003). In mammals, Wnt signaling is required to maintain the stem cells residing in the intestinal crypts. It promotes their proliferation and coordinates the expression of adhesion molecules necessary for the migration of their progeny out of the niche (He *et al.*, 2004; Kuhnert *et al.*, 2004). Following inhibition of Wnt signaling by the antagonist Dickkopf-1, cell proliferation ceases in the intestinal crypts (Kuhnert *et al.*, 2004). In the skin, self-renewal, proliferation, and migration of the epidermal stem cells are also regulated by Wnt signaling. Transgenic mice expressing a stabilized form of *$\beta$-catenin* in their epidermis develop excess fur (Lo Celso *et al.*, 2004), while those whose epidermal stem cells have had their *$\beta$-catenin* gene deleted have their hair cycle blocked as a consequence of the loss of epidermal stem cell activity (Huelsken *et al.*, 2001).

Recent studies about the role of Wnt signaling in the maintenance and expansion of HSCs gave promising results. Overexpression of a constitutively active *β-catenin* transgene in HSCs lead to their expansion in *in vitro* culture and increased their repopulating ability. Conversely, inhibition of Wnt signaling in HSCs blocked their growth and made them ineffective for bone marrow repopulation (Reya *et al*., 2003). Moreover, treatment of HSC cultures with soluble Wnt3A resulted in rapid expansion of the HSCs (Reya *et al*., 2003; Willert *et al*., 2003). However, despite these transient effects, Wnt ligand supplementation has not proven effective for long-term maintenance of HSCs. The cellular basis for this failing remains unclear.

An important consideration with the manipulation of elements from the Wnt-β-catenin signaling pathway is the risk of carcinogenesis (Reya and Clevers, 2005). For example, hyper-activation of this pathway in intestinal stem cells is known to lead to neoplasia. Familial adenomatous polyposis (FAP) is a hereditary predisposition to the development of colon cancer due to mutations in the *adenomatous polyposis coli* (*APC*) gene that lead to inappropriate stabilization of β-catenin and constitutive activation of Wnt-responsive genes (Rubinfeld *et al*., 1996). Aberrant Wnt activation is also involved in the development of epidermal cancer (Chan *et al*., 1999) and leukemia (Jamieson *et al*., 2004; Muller-Tidow *et al*., 2004). It is thus possible that prolonged treatment of ASCs with molecules activating the Wnt pathway will promote their neoplastic transformation. More studies focused on the long-term effects of continuous activation of the Wnt canonical pathway in ASCs will be clearly needed for a sound assessment of whether this can be a viable approach to producing therapeutic quantities of ASCs.

## 3. Bone Morphogenetic Proteins

Bone Morphogenetic Proteins (BMPs) are signaling molecules belonging to the transforming growth factor β (TGFβ) superfamily that signal through binding to specific BMP receptors. Binding to their receptors leads to the nuclear translocation of SMAD proteins, which can then exert their action by regulating the transcription of specific target genes (Balemans and Van Hul, 2002). In *Drosophila*, overexpression of the *BMP* homolog *decapentaplegic* (*dpp*) expands the germline stem cell (GSC) niche and it leads to expansion of GSC number (Xie and Spradling, 1998, 2000). In mice, BMP molecules are necessary to induce the proliferation of embryonic liver precursors after their specification (Rossi *et al*., 2001). In human bone marrow, primitive hematopoietic cells express BMP type I receptors, and in culture high concentrations of BMP-4 extend the lifetime of their repopulation capability (Bhatia *et al*., 1999). However, net expansion

and long-term propagation of ASCs *in vitro* by BMP supplementation has not been convincingly demonstrated. For example, BMPs appeared to act on HSCs by primarily controlling the HSC niche size in the bone marrow (Zhang *et al*., 2003). Proof of net ASC expansion by direct action of BMP molecules on ASCs is still lacking.

### 4. Sonic Hedgehog

Signaling through the hedgehog (Hh) family of secreted proteins has been implicated in ASC cell kinetics in evolutionarily distant organisms. In *Drosophila*, ovarian GSCs cannot proliferate in the absence of Hh, whereas an excess of it produces supernumerary GSCs (Zhang and Kalderon, 2001), suggesting that Hh stimulates their proliferation. In the mouse nervous system, *Sonic hedgehog* (*Shh*), a vertebrate homolog of drosophila Hh, is essential for the maintenance of NSCs and their proliferation (Machold *et al*., 2003; Palma and Ruiz i Altaba, 2004). Shh is also able to induce the proliferation of human HSCs in culture. Human bone marrow cells enriched in primitive hematopoietic cells show survival of the repopulating cells after a 7-day culture period when the medium is supplemented with Shh. Moreover, the number of repopulating cells is increased approximately 2-fold after the culture in Shh-supplemented medium. Interestingly, the BMP-4 inhibitor *noggin* blocks this Shh-induced expansion, suggesting that Shh acts *via* downstream BMP signaling in this system (Bhardwaj *et al*., 2001).

Like other proliferation-inducing signaling pathways, constitutive Hh pathway activity is associated with the development and progression of neoplasia. Hh pathway activation increases the frequency of tumors in diverse tissues (Berman *et al*., 2002, 2003; Borzillo and Lippa, 2005). For example, continuous Hh activity transforms prostate progenitor cells and induces prostate cancers (Karhadkar *et al*., 2004), and constitutive Hh activity is required for medullobalstoma growth (Berman *et al*., 2002). Therefore, given the broad tissue range of Hh involvement in carcinogenesis, activation of the Hh pathway to expand ASCs for therapeutic applications will require approaches to safeguard against these detrimental effects of chronic pathway activation.

## C. Embryonic Stem Cell-Based Strategies for ASC Expansion

The recent development of methods for successful culture of ''human embryonic stem cells (hESCs)'' (Thomson *et al*., 1998) engendered the concept of a developmental approach to expansion of ASCs. Although studies with the goal of learning how to control the development of specific types of adult cells in ESC cultures were already underway in laboratories working with mouse

ESCs, the announcement of similar technology for human cells set off an explosion of new interest. The expression of this interest mainly appeared in the form of efforts to develop and grow mature adult tissue cells from human ESC cultures (Bjorklund *et al.*, 2002; Hoffman and Carpenter, 2005; Kehat *et al.*, 2001; Kim *et al.*, 2002; Reubinoff *et al.*, 2000; Zhan *et al.*, 2004). The focus of these studies has largely been on developing cultures of mature tissue cells with particular desired functions (e.g., hepatocyte metabolism), without attention to the requirement for ASCs for long-term cell replacement therapy (Sherley, 2004).

HSCs are the one type of ASCs whose production has been actively pursued in ESC cultures. HSCs are of particular interest because of their well-established effectiveness for bone marrow replacement therapies and their potential as donor cells for gene therapies. Despite their proven therapeutic effectiveness, HSCs in both rodents and humans have proven highly refractory to expansion in culture. Thus far, attempts to recover HSCs from differentiating ESC cultures have also relied on transgenic strategies. Genes like *HoxB4* (as described above) have been introduced by retroviral transduction into differentiating mouse ESC cultures prior to or during times when hematopoietic cell types arise. The transduced cells were then able to reconstitute the hematopoietic compartment of adult mice (Kyba *et al.*, 2002; Rideout *et al.*, 2002).

The ESC strategy for producing mature cells for repair of damaged or diseased tissues has also received a lot of attention. However, because of the requirement for ASCs for long-term restoration of tissues, the ESC strategy must ultimately address the same issues as other strategies for expanding ASCs (Sherley, 2004). Unless a sufficient subpopulation of the cells present in the ES-derived tissue are ASCs, periodic reinfusion of new functional cells will be required to maintain the newly generated tissue fully functional. That problem could be manageable in the case of slowly renewing tissues, but it will have to be solved in order to apply ESC-based therapies in tissues with a fast rate of renewal (i.e., hematopoietic compartment, skin, digestive tract, lung). In addition, it will require a significant advance in knowledge of cellular processes that control the production of ASCs from embryonic/fetal precursors (Mayhall *et al.*, 2004; Sherley, 2002a). Currently, there is a daunting dearth of knowledge in this area of stem cell biology.

## III. Cell Kinetics Symmetry-Based Strategies for ASC Expansion

All of the approaches described above for adult tissues are based on the hypothesis that ASCs have lost an important factor(s) required for their proliferation when explanted to culture. A few approaches, which are not

treated here, adopt the same hypothesis with respect to loss of critical interactions with other cells (e.g., niche cells) or extracellular components (e.g., basement membrane components) (Garcion *et al.*, 2004; Grueterich *et al.*, 2003). A more recent strategy differs from all others in this respect. It postulates that an intrinsic property of ASCs is primarily responsible for difficulty in expanding their numbers in culture. This property is asymmetric self-renewal that is manifested as ACK (Sherley, 2002a).

A defining property of ASCs *in vivo* is asymmetric self-renewal. ASCs are both the engine that drives tissue cell renewal and turnover and the memory for the tissue's cellular differentiation architecture. They must undergo asymmetric self-renewal to meet these opposing roles. Thus, ASC divisions produce differentiating progeny cell lineages and new ASCs at the same rate, hence self-renewal that is asymmetric (Potten and Morris, 1988). Asymmetric self-renewal is mirrored by ACK. Whereas ASCs have the potential to divide for the entire lifespan of their tissue, their nonstem cell differentiated progeny undergo terminal division arrests with eventual death and removal from the tissue. Thus, the cell kinetics fates of ASC cell division are asymmetric.

There is an ongoing debate regarding the exact mathematical form of ACK in adult mammalian tissues (Lajtha, 1979; Matioli *et al.*, 1970; Ro and Rannala, 2001). Stochastic models invoke that ASC divisions can produce two, one, or zero ASCs with zero, one, or two nonstem cell sisters, respectively, with the net rate of production of different cell types from the division of multiple ASCs per tissue unit insuring life-long maintenance of ASC number. Deterministic models invoke that, in general, ASC divisions produce one ASC and one nonstem progeny cell that is a precursor for differentiated cells in the tissue. Deterministic models allow that under specific physiological conditions (e.g., adult maturation, wound repair), ASCs undergo limited symmetric cell kinetics (producing two ASCs) to establish new tissue units. However, at no time do ASCs undergo differentiation in deterministic models (Sherley, 2002b).

No matter which mathematical form accurately describes the ACK of ASCs *in vivo*, if maintained in culture, they pose a major barrier to expansion. In fact, deterministic ACK have been demonstrated in diverse mammalian cell cultures, including presenescent human diploid lung fibroblasts (Rambhatla *et al.*, 2001; Sherley *et al.*, 1995), presenescent human intestinal epithelial cells (Sherley *et al.*, 1995), precrisis mouse fetal fibroblasts (Rambhatla *et al.*, 2001), HSC-enriched human blood cell preparations (Huang *et al.*, 1999), adult rat hepatocytic stem cells, and adult rat cholangiocytic stem cells (Lee *et al.*, 2003). DNA methylation lineage patterns in the epithelial cells from intestinal crypts also support deterministic asymmetric self-renewal (Yatabe *et al.*, 2001). Rambhatla and others (Lee *et al.*, 2003; Rambhatla *et al.*, 2001; Sherley, 2002a) proposed that after

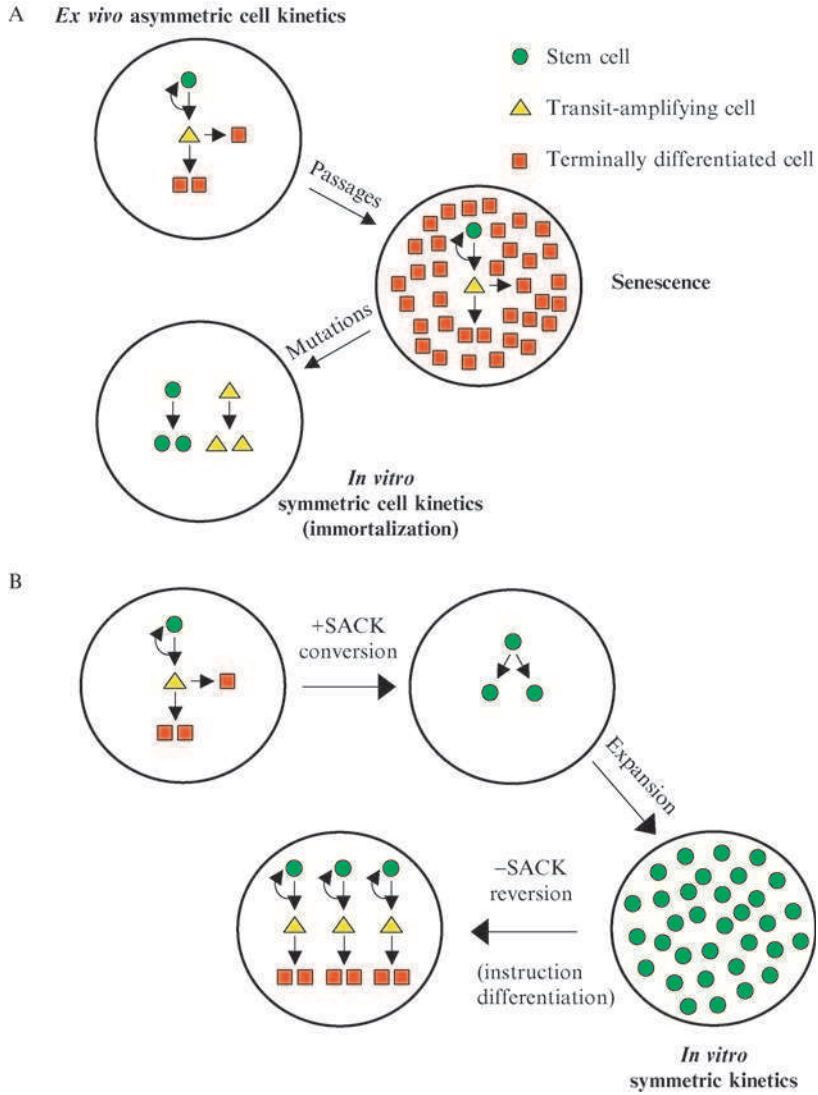


**Figure 2** Suppression of asymmetric cell kinetics provides a means for mutation-free expansion of ASCs. (A) ACK model for cellular senescence. Explanted ASCs (green circle) divide with ACK to generate one daughter stem cell that will divide continuously with ACK, and another daughter cell that will undergo a limited number of cell cycles (transit-amplifying cell; yellow triangle), or stop dividing (terminally differentiated cells; red square). Following successive passages, the stem cells become diluted in the growing pool of nondividing differentiated cells. Conventional cell lines are eventually established following mutations that confer symmetric cell kinetics to stem cells or self-renewal properties to transit-amplifying cells. Routine protocols for the derivation of cell lines thus promote the selection of cells that acquired these growth-promoting mutations. (B) "Suppression of asymmetric cell kinetics

ASCs were explanted to culture, the continuation of their ACK promoted their dilution among differentiating progeny cells (Fig. 2A). Serial passage of cultures, with a reduced ASC fraction, due to ACK in culture accelerated the eventual loss of ASCs from later cultures. Passaging is accomplished by transferring a fraction of the cells from a replete culture to start the next culture in the series. Since ACK hold the number of stem cells constant, the combination of reduced fraction with general culture dilution causes a progressive loss of ASCs from subsequent cultures until none remain. At this point, serial cultures have no long-term division potential and are eventually populated by cells that all undergo terminal division arrest. Thus, persistent ACK were put forth as a new hypothesis for mammalian cell culture senescence and the prime factor limiting ASC propagation in culture (Merok and Sherley, 2001; Rambhatla *et al*., 2001; Sherley, 2002a).

The recognition that ACK might be the key barrier to ASC expansion, led to the concept of suppression of ACK (SACK) for expansion of ASCs in culture (Lee *et al*., 2003; Sherley, 2002a). If ASCs could be induced to divide with symmetric cell kinetics instead of ACK, the SACK theory dictated that they could be expanded and propagated in culture. Ideally, the SACK method should be reversible, so that when needed, expanded ASCs could resume asymmetric self-renewal for production of functional, differentiated progeny cells (Fig. 2B).

The SACK method was possible because of knowledge derived from previous studies of the molecular and biochemical basis of ACK in engineered cell models that mimic ASC asymmetric self-renewal. These novel cell lines were engineered for experimentally controlled expression of the wild-type p53 tumor suppressor protein (Fig. 3A) (Liu *et al*., 1998a,b; Rambhatla *et al*., 2001). The p53 protein is well known for its functions in cell growth regulation, DNA damage response mechanisms, and carcinogenesis (Bargonetti and Manfredi, 2002; Giaccia and Kastan, 1998; Hofseth *et al*., 2004; Oren, 2003). Restoration of wild-type *p53* in a *p53*-null, immortalized, mouse fetal fibroblast line induced ACK. The kinetics were characterized by cell divisions that continuously produced a cycling cell (ASC-like) and a cell that underwent cell cycle arrest. Prior to restoration of wild-type p53 function, the *p53*-null parent cells divided with symmetric cell kinetics. In later studies, inspection of primary mouse fetal fibroblast cultures from wild-type mouse revealed ACK; whereas similar cultures from *p53* knockout mice displayed only symmetric cell kinetics (Rambhatla *et al*., 2001).

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(SACK)" for the derivation of ASC strains. After plating, ASCs are grown in medium supplemented with SACK agents in order to convert them to symmetric cell kinetics reversibly. This allows their expansion and, following withdrawal of the SACK agents from the culture medium, their return to normal ACK for the generation of differentiating progeny.

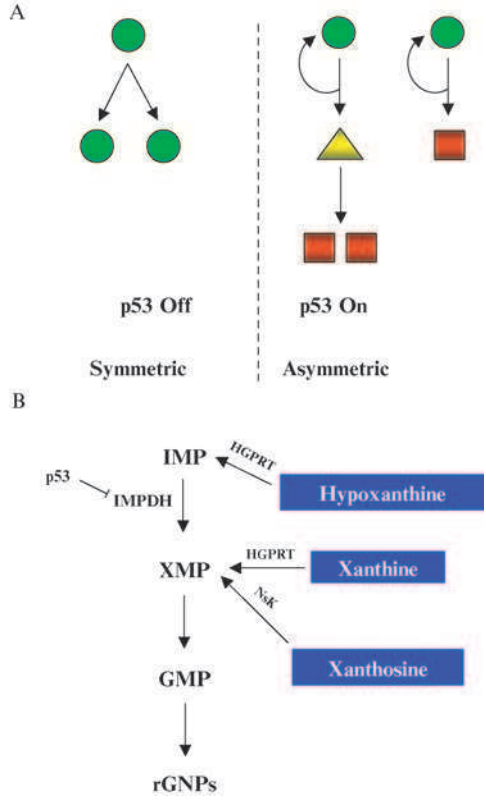


**Figure 3** (A) Model cell lines for the study of ASCs kinetics. p53-null cells divide with symmetric cell kinetics (i.e., daughter cells continue to divide). When *p53* expression is restored to basal physiological levels, cells adopt ACK. One daughter cell keeps dividing, while the other undergoes an immediate cell cycle arrest or divides only once more. (B) Biochemical pathway controlling ACK. Conversion of inosine-monophosphate (IMP) into xanthosine-monophosphate (XMP) by the enzyme inosine-monophosphate dehydrogenase (IMPDH) is the rate-limiting step for rGNP synthesis in mammalian cells. Downregulation of *IMPDH* expression by p53 is required for ACK. It causes a decrease in the cellular concentration of rGNPs. Exogenous compounds (xanthosine, xanthine, hypoxanthine) that promote rGNP biosynthesis can prevent ACK and are called SACK agents (blue boxes). GMP, guanosine monophosphate; HGPRT, hypoxanthine-guanine phosphoribosyl transferase; NsK, nucleoside kinase.

Investigations with the engineered *p53*-expressing cell lines revealed a cellular pathway that controlled cell kinetics symmetry (Fig. 3B) (Rambhatla *et al*., 2005; Sherley, 2002a). *p53* expression was shown to cause the down-regulation of *inosine-5′-monophosphate dehydrogenase* (*IMPDH*) expression (Liu *et al*., 1998a,b). IMPDH is the rate-limiting enzyme for guanine ribonucleotide (rGNP) biosynthesis in mammalian cells. Though the exact

mechanism of regulation has not been determined, restoration of normal levels of p53 in cells results in reduction in IMPDH mRNA, protein, and activity. In its role as rate-limiting enzyme for rGNP biosynthesis, reduction in IMPDH activity causes decrease in rGNP production and pools. The p53-dependent regulation of this pathway is required for acquisition of the ACK state.

Consistent with the requirement for reduced rGNP biosynthesis to maintain ACK, compounds that promote rGNP biosynthesis were found to prevent ACK. Such compounds became the first proposed SACK agents (Sherley, 2002a). They included xanthosine, xanthine, and hypoxanthine, three purine nucleotide precursors that promote expansion of rGNP pools (Fig. 3B). As such, they each counter the repressive effect of p53 expression on IMPDH and rGNP biosynthesis. Hypoxanthine and xanthosine were shown to induce engineered model cells to shift from ACK to symmetric cell kinetics in a reversible fashion (Sherley, 1991; Sherley *et al.*, 1995). This activity against the engineered cells predicted that these agents might also induce the same reversible cell kinetics shifts in ASCs and promote their expansion in culture.

The first test of the SACK method was performed using xanthosine with primary adult rat liver cells (Lee *et al.*, 2003). Cells that remained in the supernatant after low-speed centrifugation of preparations from collagenase-perfused livers (process that eliminates many large mature hepatocytes) were clonally derived in medium supplemented with xanthosine. The cell strains derived exhibited properties indicative of ASCs, including Xs-dependent ACK and production of differentiated progeny cells (Lee *et al.*, 2003; Semino *et al.*, 2003). ASC strains for hepatocytes and biliary epithelium cells were discovered to have emerged independently from the same liver cell preparation. This observation may be a first indication that the SACK method will have general application for expansion of ASCs from diverse tissues. Certainly this is highly probable because asymmetric self-renewal and ACK are universal properties of vertebrate ASCs; and the underlying molecular (p53) and biochemical (IMPDH) elements are well conserved among vertebrate species.

## IV. Comparison of SACK-Derived ASC Strains to Conventional Cell Lines

The SACK-derived, clonal ASC cultures are referred to as "strains" to reflect that their long-term propagation is predicted not to require immortalizing or transforming mutations that are typical for previously described cell "lines." Such spontaneous genetic alterations seem to be required for establishment of previously described cultured cell lines. Characteristically, the critical mutations occur in the *p53* gene (Rogan *et al.*, 1995; Whitaker *et al.*,

1995; Wynford-Thomas, 1996). The essential importance of *p53* mutations in establishment of stable cell lines is confirmed by a multitude of early studies showing that introduction of wild-type *p53* into established cell lines results in growth suppression. However, only more recently was it recognized that this characteristic growth suppression occurred as a result of a shift from symmetric cell kinetics to ACK (Sherley *et al*., 1995). The cell variants with mutated *p53* genes, which are responsible for establishment of immortalized cell cultures, often lose the ability to produce differentiated progeny. This finding suggests that ACK may be obligatory for induction of differentiation programs in some cell types.

When maintained in xanthosine-supplemented medium, SACK-derived ASC strains exhibit long-term culture without loss of their stem cell properties (Lee *et al*., 2003). By promoting symmetric ASC divisions, xanthosine is postulated to reduce the selective advantage that engenders the outgrowth of mutated cell variants, their increased cell division frequency. In cell culture, cell variants that acquire a greater frequency of division under routine culture conditions will accumulate and, given sufficient time, come to be predominant in the culture. By keeping ASCs in a state of active symmetric division, they are better able to compete against the cell variants that most commonly take over cell cultures, cells with mutations in the growth-suppressive *p53* gene.

## V. Future SACK Pathways and Regulators

The early success of the SACK method demonstrates the advantage of knowledge of experimentally accessible biochemical targets that are barriers to ASC expansion. The main advantage of the SACK strategy over transgenic and growth factor supplementation approaches is its basis on a mechanism predicted to be shared by all types of ASCs, namely ACK. SACK could thus be used to expand ASCs from diverse types of tissues. This strategy primarily suppresses one function of p53 (inhibition of IMPDH) while maintaining others. This combination of effects lowers the risk of cellular transformation by reducing, in culture, the selective growth advantage brought by p53 inactivation.

Even if ACK is a universal property of ASCs, SACK agents might not be 100% effective for ASC expansion; and despite having common properties ASCs from different tissues may show distinct requirements for *in vitro* maintenance and proliferation. Therefore, in addition to the SACK agents, it may be necessary to supplement expansion media with survival factors and compounds that activate the cell cycle while not committing ASCs toward one differentiation program at the expense of another. For this purpose, integration of data accumulated from the studies describing the effects of growth factors on ASC behavior may prove advantageous.

A promising application of the SACK strategy is recovery of ASCs that have not been physically identified (e.g., HSCs). Already, there is one example of this potential in the derivation of an adult rat biliary epithelium stem cell line by SACK (Lee *et al.*, 2003). By explanting and culturing cells with SACK agents, without fractionation, selective expansion of diverse ASCs should be possible (Fig. 4). Ideally, cells from a targeted tissue would be initially grown at a density sufficiently low to allow single cell clones to be expanded. ASC clones would be identified by virtue of their SACK-responsiveness. If a cell clone were an expanded ASC, its cell kinetics would revert to ACK after withdrawal of SACK agents. In contradistinction,

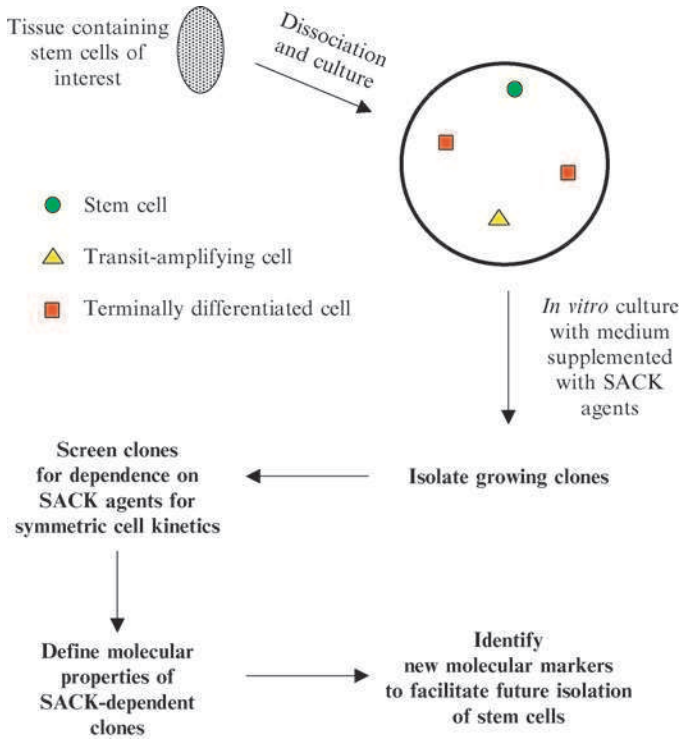


**Figure 4** Future application of the SACK strategy. Cells from tissues of interest can be explanted with or without enrichment for ASCs, before being grown *in vitro* in culture medium supplemented with SACK agents. Cells can be plated at low density to allow subsequent isolation of individual clones. After their isolation and growth, the selected clones are screened for their dependence on SACK agents for symmetric cell kinetics. ASC clones correspond to the ones showing dependence. Investigations of their molecular properties will enable the discovery of new molecular markers to optimize future isolation of ASCs from the tissue of origin.

non-ASC clones would not show dependency on SACK agents for symmetric cell kinetics.

Subsequent studies to define cellular molecules uniquely associated with SACK-responsive clones may yield new molecular markers to identify and isolate ASCs directly from the tissues of interest. Even in tissues where the identity and location of ASCs are better characterized, the SACK methodology could be advantageous for optimization of their isolation and expansion. For example, in the bone marrow, markers used for the enrichment of HSCs are not comprehensive, because HSCs have a heterogeneous profile for the cell-surface proteins used to enrich for them. Therefore, SACK-responsiveness could improve HSC isolation and expansion by providing a more inclusive means for their identification.

## VI. Confronting the Replication Mutation Risk of ASC Expansion

Any effective method for expansion of ASCs for future use as cell replacement therapies must address the concern of cell mutagenesis. Even with highly efficient repair mechanisms, each S-phase results in the acquisition of several mutations that result from unrepaired or misrepaired DNA replication errors (i.e., spontaneous mutations). The main source of cell mutations is predicted to be this mechanism (Cairns, 1975). Estimates made for general physiological conditions project that mutations due to replication errors predominate over mutations from damaged DNA bases by 100- to 1000-fold (Sherley, 2005). With an estimated four spontaneous mutations per S phase, long-term propagation of ASCs in culture may yield cells that pose a significant cancer risk on transplantation.

Endogenous ASCs must have a mechanism to address the spontaneous mutation problem; and they seem to have evolved a mechanism for reducing its impact on their natural history. During mitosis, the conventional view is that chromosomes randomly segregate between sister cells (Meselson and Stahl, 1958). This leads to an equal probability for sister cells to inherit mutations resulting from replication errors. Because they are the only dividing cells to persist in an animal over the course of its entire life span, ASCs pose a higher risk for carcinogenic transformation as a result of accumulating mutations. However, the rate of mutagenesis in humans is predicted to lead to a higher rate of cancer than is observed (Cairns, 2002). This finding inspired the hypothesis that ASCs must have a special mechanism for mutation avoidance.

To explain the difference between predicted and observed cancer rates, the immortal DNA strand hypothesis was proposed (Cairns, 1975). It stipulated that following asymmetric cell divisions ASCs repeatedly cosegregate to

themselves the set of chromosomes bearing the oldest DNA strands and discard to their differentiating sisters the chromosomes that contain newer DNA copies. This age-specific DNA strand cosegregation is accomplished by nonrandom chromosome segregation. With this mechanism, ASCs could avoid accumulating mutations due to replication errors by restricting mutated DNA strands to differentiating cells that have a limited life span. Thus, an immortal DNA strand mechanism would decrease the risk for the accumulation of sufficient mutations to trigger carcinogenesis (Cairns, 1975; Marshman *et al*., 2002; Sherley, 2005).

The immortal DNA strand hypothesis is supported by *in vivo* studies (Potten *et al*., 2002; Smith, 2005) and by studies with cultured cells (Karpowicz *et al*., 2005; Merok *et al*., 2002; Rambhatla *et al*., 2005). The p53-inducible cultured mouse cell model for conditional asymmetric self-renewal, described earlier in this chapter, provided the first experimentally accessible model for investigation of molecular mechanisms that control immortal DNA strand cosegregation. Studies with these cells showed that p53/IMPDH-dependent reduction in rGNP pools and the associated ACK are required for nonrandom chromosome cosegregation (Rambhatla *et al*., 2005).

The existence of immortal DNA strand cosegregation has important implications for expansion of ASCs. With SACK, it is predicted that the mechanism controlling immortal DNA strand cosegregation in ASCs will be lost, and the resulting symmetrically dividing ASCs will accumulate mutations at a significantly higher rate than *in vivo*. Even if immortal DNA strand cosegregation were maintained during symmetric divisions, an expanding subpopulation of ASCs would accumulate mutations at a higher rate because at each division, mutations would be transmitted to one of the two symmetric ASC sisters. Thus, it appears that the surest way to reduce the risk of harmful mutations during expansion of ASCs would be to limit the number of generations of expansion. Such a limitation might preclude attainment of the full potential of the SACK approach or, for that matter, any other successful expansion approach. This critical issue is explored below.

From a single cell, 50 cell-generations of symmetric division would produce $2^{50}$ cells, which correspond to approximately 450 kg of cell mass. On an average, the cells composing this mass would have (4 mutations per generation/2 cells)(50 generations) = 100 mutations per cell (Sherley, 2005). The authors are not aware of a reported calculation of the probability that a cell with a 100 mutations will have sufficient genetic alterations for human cancer cell formation. Assuming that combined point mutations in any 5 of about 100 different specific genes (Hahn and Weinberg, 2002a,b; Hanahan and Weinberg, 2000), with an average length of 50,000 basepairs, can cause carcinogenesis, allows a very rough estimate of this probability.

With this modeling, the probability can be approximated as $[(100 \times 50{,}000)/(3 \text{ billion})]^5 \sim 1 \times 10^{-14}$. Each cell has 100/5 opportunities to acquire the five required mutations, reducing the probability to $2 \times 10^{-13}$. There will be $2^{50}$ cells in the final 450 kg mass. Thus, the probability that *many* will have sufficient mutations for carcinogenesis approaches certainty. Reducing the number of cell generations to $2^{40}$ (corresponding to approximately 450 g of cell mass, which is still in a range of amount sufficient for cell therapy applications) reduces the overall estimate of carcinogenic risk to about 20%, which, empirically, is still far from acceptable. Therefore, the mutation limit for ASC expansion raises considerable concern for any successful method for ASC expansion.

It is important to keep in perspective that the previous treatment was not based on quantitative measurement. Clearly, direct quantification of mutation rates and cell transformation rates of expanded ASCs will be essential in the evaluation of the safety of ASC replacement therapies. A recent report tempers the mutation limit concern somewhat. An adult rat hepatocyte stem cell strain showed no sign of cellular transformation after >80 cell generations of continuous culture (Lee *et al.*, 2003). The normal cell phenotype of this strain was confirmed by the absence of *in vitro* cell transformation properties and its failure to form tumors in immunodeficient mice. In culture, cell transformation rates of human cells are significantly lower than those of rodent cells (Hahn *et al.*, 2002; Rangarajan *et al.*, 2004). Thus, the success with SACK-based expansion of rat liver ASCs is a first empirical indicator that it will be possible to engineer expansion of therapeutic quantities of ASCs while avoiding the risks associated with replication mutagenesis.

## VII. Summary

The future of ASC-based medical therapies rests on the successful breach of biological barriers to *ex vivo* propagation of these remarkable tissue cells. Bringing down the expansion barrier should also advance parallel efforts to discover molecular markers that uniquely identify ASC and develop tools for their rapid purification. The challenge of expanding ASCs fills one pan of long immobile scales in ASC research. This challenge is balanced by a dearth of knowledge of the biological properties of ASCs. Success in reducing either of these weights in the field will rapidly move the other. Developing methods to procure high-purity ASCs in large numbers will support advanced investigations of their function and features. Such knowledge is essential for successful development of ASC-based biomedical applications. The void of experimentally determined knowledge, relevant to expanding ASCs has been filled with theoretical ideas based on general concepts for tissue cell

proliferation. Thus, many reported attempts to expand ASCs are based on conventional ideas of increasing proliferation by activation of growth-stimulatory molecular pathways. Methods differ in the specific strategy for activation (e.g., transgenic animal production, gene transfer, growth-factor supplementation), but all adhere to this same biological principle. None of these methods have proven successful as yet, and all are limited by significant potential shortcomings like inconvenient procedures, insertional mutagenesis, and carcinogenic risk.

A recent approach targets an ASC-specific property, ACK. In initial studies targeting rodent ASCs, this method, called SACK for suppression of ACK, shows promise as a general method for expansion and long-term propagation of ASCs in quantities sufficient for research investigations and development of ASC-based therapies. However, thus far, the SACK method has not shown ideal efficiency. It may be possible to achieve overall more efficient ASC expansion by integrating the SACK approach with other ASC expansion strategies that have not proven effective alone. Such integrated studies have not been reported to date.

In theory, all methods that succeed in expanding ASCs will need to address the risk of producing cancer-prone cell variants. Cycling cells are predicted to accumulate spontaneous mutations that result primarily from DNA replication errors. Although there is good evidence that a unique, nonrandom chromosome segregation mechanism evolved to limit ASC mutagenesis *in vivo*, this mechanism is predicted to be lost in ASCs expanded in culture as a result of induced symmetric cell divisions. Although the risk of rare cancer-prone cells in expanded ASC preparations should not compromise their use for research investigations, it will surely complicate their use for human cell therapies. The limited experimental data available suggests that actual mutation rates in ASCs may not reach a prohibitive level. However, these data are too preliminary and too limited in scope for any degree of comfort now. Of course, ASCs surely have many new secrets to reveal, and one of more of these may yield a solution to the replication mutation problem as well as other ASC challenges. Successful, routine expansion of ASCs is the essential step to unlocking these secrets.

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# 6

# Histone Deacetylation as a Target for Radiosensitization

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I. Introduction
II. Histone Acetylation
III. Inhibitors of Histone Deacetylase
IV. *In Vitro* Radiosensitization by HDAC Inhibitors
V. *In Vivo* Radiosensitization by HDAC Inhibitors
VI. Tumor Versus Normal Cells
VII. Mechanisms of HDAC Inhibitor-Induced Radiosensitization
VIII. Clinical Application of HDAC Inhibitor/Radiotherapy Combinations
References

Due to an increase in the understanding of molecular radiobiology, strategies for enhancing tumor radiosensitivity have begun to focus on targeting the molecules and processes that regulate cellular radioresponse. Toward this end, histone acetylation has begun to receive considerable attention as a potential target for radiosensitization. Histone acetylation, which is determined by the competing actions of histone acetylases (HATs) and histone deacetylases (HDACs), plays a role in regulating chromatin structure and gene expression—two parameters that have long been considered determinants of radioresponse. As a means of modifying histone acetylation status, considerable effort has been put into the development of inhibitors of HDAC activity, which is often aberrant in tumor cells. This has led to the generation of a relatively large number of structurally diverse compounds that inhibit HDAC activity and result in histone hyperacetylation, and importantly, are applicable to patient treatment. Whereas a number of these HDAC inhibitors have antitumor activity in preclinical cancer models when delivered as single agents, recent studies have indicated that these compounds also significantly enhance tumor cell radiosensitivity. A structurally diverse set of HDAC inhibitors have been shown to enhance the *in vitro* radiosensitivity of human tumor cell lines generated from a spectrum of solid tumors. Moreover, HDAC inhibitors increased the radiosensitivity of human tumor xenografts. Although the mechanism responsible for this radiosensitization has not been definitely elucidated,

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data suggest that inhibiting the repair of radiation-induced DNA damage may be involved. Whereas HDAC inhibitors are currently in clinical trials as single modalities and in combination with chemotherapeutic agents, recent results suggest that these compounds may also enhance the antitumor effectiveness of radiotherapy. 

## I. Introduction

Approximately 60% of cancer patients will receive radiotherapy sometime during their treatment. Given that in 2005 it is estimated that 1.4 million nonskin cancers will be diagnosed in North America (www.cancer.org), it would appear that the development of agents that selectively enhance tumor radiosensitivity should positively impact a significant number of cancer patients. Attempts to increase the radiosensitivity of tumor cells have traditionally focused on combining radiation with standard cytotoxic chemotherapeutic agents. This approach typically lacks a mechanistic explanation for any increase in tumor response and is often limited by unacceptable levels of normal tissue toxicity. Consequently, whereas often effective in experimental models, the results obtained when such combinations were applied in a clinical setting have been generally less than expected (Willett *et al.*, 2003). With advances in molecular radiobiology, strategies for enhancing radiosensitivity have begun to focus on targeting the molecules and processes that regulate cellular radioresponse. Toward this end, a wide variety of molecules have been shown to influence radiosensitivity in one or more experimental models affecting such fundamental processes as cell cycle checkpoints, DNA repair, gene expression, and apoptosis. With respect to clinical relevance, however, a potential molecular target must not only serve as a determinant of radiosensitivity, but should also be susceptible to pharmacological manipulation and importantly, selectively influence the radiosensitivity of tumor cells over that of normal tissue.

With these criteria in mind, histone acetylation has begun to receive considerable attention as a potential target for radiosensitization. Histone acetylation is determined by the opposing actions of histone acetyltransferases (HATs) and histone deacetylases (HDAC), with each containing a number of family members (Ruijter *et al.*, 2003; Thiagalingam *et al.*, 2003). Whereas oncogenesis has been associated with HAT inactivation, it is aberrant HDAC activity leading to the transcriptional repression of tumor suppressor genes that is generally considered a common event contributing to tumor formation (Johnstone, 2002). Given this molecular insight, and because it is easier to inhibit an enzyme rather than stimulate its activity, the inhibition of HDAC activity has generated considerable interest as a potential form of cancer treatment. There are currently a relatively large number

of compounds encompassing a variety of structural and pharmacological classifications that have been shown to inhibit HDAC activity and result in histone hyperacetylation (Dokmanovic and Marks, 2005; Drummond *et al.*, 2005). In a number of *in vitro* and *in vivo* experimental models HDAC inhibitors have been reported to induce tumor cell differentiation, apoptosis and/or growth arrest (Biade *et al.*, 2001). Moreover, consistent with aberrant acetylation being characteristic of neoplastic cells (Marks *et al.*, 2004), initial data suggests that the cytotoxic effects of HDAC inhibition are tumor selective (Almenara *et al.*, 2002; Amin *et al.*, 2001; Richon *et al.*, 2000; Vrana *et al.*, 1999). Indeed, for tumors originating from hematopoietic or lymphoid tissue, HDAC inhibitors typically induce differentiation to a nonmalignant phenotype or apoptotic cell death, suggesting potential as a single modality cancer treatment (Almenara *et al.*, 2002). However, for most cell lines generated from solid tumors, the primary effect of HDAC inhibition is one of reversible cytostasis, as is the case for most target directed chemotherapeutic agents.

This apparent inability to eradicate most nonhematopoietic/lymphocytic neoplastic cells, however, does not eliminate the clinical potential of HDAC inhibitors in the treatment of solid tumors. As described below, histone acetylation plays a critical role in regulating chromatin structure and gene expression—two parameters that have long been associated with regulating radioresponse. Consistent with these effects, early work done in the 1980s by J. T. Leith and colleagues demonstrated that the HDAC inhibitor sodium butyrate at relatively nontoxic concentrations increased the radiosensitivity of human colon carcinoma cell lines (Arundel *et al.*, 1985). However, at that time butyrate was considered a "differentiating agent" and its mechanism of action, let alone its HDAC inhibitory activity, had not been fully recognized. Butyrate-induced radiosensitization was simply attributed to cell differentiation, whether histone acetylation status was affected at the radiosensitizing dose or over the treatment time in the cell models examined was not addressed. Moreover, because of its very short half-life and low achievable serum concentration, sodium butyrate has limited clinical applicability (Kruh, 1982; Miller *et al.*, 1987; Novogrodsky *et al.*, 1983; Perrine *et al.*, 1993). In the last couple of years, a number of clinically relevant agents that inhibit HDAC activity have been shown to enhance the radiosensitivity of a variety of human tumor cell lines grown *in vitro* and/or as solid tumor xenografts in immuno-compromised mice. However, whereas in some cases the radiosensitization induced by an HDAC inhibitor was correlated with histone hyperacetylation, the actual mechanism responsible remains unclear. In addition, the putative selective sensitization of tumor cells over normal tissue, although suggested, has not been fully substantiated. This chapter will present the evidence suggesting that histone deacetylation is a relevant target of tumor cell radiosensitization, speculate regarding the mechanisms

involved, and discuss the critical questions/issues that remain to be addressed in order to facilitate the clinical application of HDAC inhibitors as radiosensitizing agents.

## II. Histone Acetylation

Histone proteins play an intimate role in maintaining and regulating chromatin structure from nucleosomes to higher-order packaging and chromosome organization. Critical to the dynamic modulation of chromatin structure are posttranslational histone modifications, which include acetylation, phosphorylation, methylation, ubiquitinylation, and ADP-ribosylation. These covalent modifications form the basis of the histone code hypothesis (Jenuwein and Allis, 2001), which posits that specific combinations of such modifications govern chromatin structure to dictate a variety of functions, including the expression of particular genes. One of the most thoroughly studied of these modifications is histone acetylation, which involves the $\varepsilon$-$NH3^+$ group of conserved lysine residues located usually at the N-terminal tails of core histones (H2A, H2B, H3, and H4).

Histone acetylation is mediated by histone acetyltransferases (HATs), which transfer an acetyl group from acetyl coenzyme A to the specific lysine residue. HATs are grouped according to families: Gcn5-related *N*-acetyltransferase (GNAT), which includes mammalian GCN5, HAT1, and PCAF (Dutnall *et al.*, 1998); MYST-related, which includes MOZ, YBF2/SAS3, SAS2, and TIP60; nuclear hormone-related HATs, which includes SRC1 and ACTR (SRC3), and p300/CBP (Carrozza *et al.*, 2003; Roth *et al.*, 2001). The families share homology mostly within the catalytic HAT domain with little or no other sequence homology (Kuo and Allis, 1998). All HATs can acetylate histones, yet are part of multicomponent complexes, which regulate their substrate preference and activity. In addition, GNAT family members can function directly as coactivators of transcription and p300, CBP, and PCAF have been shown to acetylate transcription factors enhancing DNA binding. In contrast, some members of the MYST family are involved in gene silencing (Ogryzko, 2001; Sterner and Berger, 2000).

Histone deacetylation is mediated by a series of histone deacetylases (HDACs). To date, 19 human genes that encode HDACs have been identified and are classified into three subfamilies (Gray and Ekstrom, 2001; Voelter-Mahlknecht *et al.*, 2005; Zhou *et al.*, 2000). Class I HDACs (HDAC1, 2, 3, 8, and 11) are closely related to the *Saccharomyces cerevisiae* transcriptional regulator RPD3, are generally nuclear and associate with transcriptional repressors and cofactors (Marks *et al.*, 2004). Class II HDACs (HDAC4, 5, 6, 7, 9, 10, and HDRP/MITR) share similar domains with the yeast protein HDA1, are larger proteins, and migrate between the

cytoplasm and nucleus (Bjerling *et al.*, 2002). The active site of class I and II HDACs is evolutionarily conserved containing a catalytic pocket with a zinc ion and a hydrophobic pocket allowing for the insertion of a lysine side chain. Class III HDACs or sirtuins (SIRT1-7) are the most recently discovered HDACs, are NAD-dependent, and share homology with the yeast *Sir2* gene. Yeast *Sir2* has been implicated in chromatin silencing, cellular metabolism, and aging (Guarente, 2000). As HATs, these enzymes do not act in isolation, but are components of multiprotein complexes. It should be noted that there is significant redundancy in the activities of class I and II HDACs, which has implication regarding strategies for their inhibition. That is, while it is possible to inhibit a specific HDAC (e.g., using siRNA), with respect to inducing a cellular response, it is likely more effective to use agents that inhibit multiple HDACs (see the following section).

Histone acetylation status, which is determined by the competing actions of HATs and HDACs, plays a critical regulatory role in general transcription as well as the transcription of specific genes. Acetylation of the $\varepsilon$-$NH_2$ group on lysine residues within histone tails correlates with nucleosome remodeling (Marmorstein and Roth, 2001) and is thought to result by the disruption of interactions between nucleosomes and DNA, between neighboring nucleosomes, and/or between nucleosomes and other proteins (Luger *et al.*, 1997; Rhodes, 1997; Wolffe and Pruss, 1996). The acetylation of histones weakens DNA-histone contacts by neutralizing the positive charge of the histone tails and decreasing their affinity for negatively charged DNA. Among the consequences of this charge neutralization is the disruption of interactions between nucleosomes and DNA, between neighboring nucleosomes, and/or possibly between nucleosomes and other proteins (Luger *et al.*, 1997; Rhodes, 1997; Wolffe and Pruss, 1996). Hyperacetylation of histones has also been correlated with transcriptional activity. More specifically, the net effect is relaxation of the nucleosome structure, creating an environment that is more accessible to transcription factors. Thus histone hyperacetylation typically correlates with an increase in general transcriptional activity (Allfrey *et al.*, 1964; Brown *et al.*, 2000). However, HATs or HATs containing complexes can also regulate the expression of specific genes through the interactions with sequence-specific transcription factors, resulting in their recruitment to a given promoter site (Brown *et al.*, 2000).

Conversely, histone deacetylation mediated by HDACs results in more condensed chromatin and the formation of more stable nucleosomes, which generally leads to an inhibition of general transcription (Glass and Rosenfeld, 2000; Kouzarides, 1999; McKenna *et al.*, 1999). With respect to the transcription of specific genes, transcriptional corepressors, such as SMRT and N-CoR (Privalsky, 2004), interact with HDACs forming a complex that limits the accessibility of chromatin to transcription factors

(Chen and Evans, 1995; Chen *et al.*, 1996). Because histone deacetylation is readily inhibited by pharmacological agents, the consequences of the resulting hyperacetylation on gene transcription has been frequently demonstrated. Inhibition of HDAC activity has been reported in a cell type specific manner to increase the expression of such genes as those corresponding to p53 (Kim *et al.*, 2001), p21 (Richon *et al.*, 2000), c-myc (Sasakawa *et al.*, 2003), caspase 3 (Bernhard *et al.*, 2001), and gelsolin (Saito *et al.*, 1999). However, inhibitors of HDAC have also been shown to decrease the expression of *Her2* (Scott *et al.*, 2002), *thioredoxin* (Butler *et al.*, 2002), *Raf-1* (Mitsiades *et al.*, 2004), and *Cyclin* (Fournel *et al.*, 2002), again in a cell type specific manner. The mechanism responsible for the reduced expression remains undefined, perhaps reflecting the complexity of the gene regulatory processes. It should be noted that the effects of HDAC inhibition on transcriptional regulation are highly dependent on other forms of histone posttranslational modifications (i.e., other components of the histone code), as well as DNA methylation (Fischle *et al.*, 2003; Spotswood and Turner, 2002; Zhang and Reinberg, 2001). The interactions between acetylation and other histone and DNA modifications that are involved in transcription suggests that the role of histone acetylation in the regulation of other cellular processes, such as radioresponse, may also be dependent on such interactions (Horn and Peterson, 2002; Mutskov *et al.*, 1998; Tse *et al.*, 1998).

Given the effects of histone acetylation on the dynamics of chromatin structure, it is not surprising that it has been also implicated in the regulation of DNA replication (Zhou *et al.*, 2005), site-specific recombination (Tamburini and Tyler, 2005) and, of specific relevance to this chapter, DNA repair (Hassa and Hottiger, 2005; Masumoto *et al.*, 2005). With respect to histone acetylation status and DNA repair, the vast majority of information has been generated using yeast as a model system. For example, Masumoto *et al.* showed that GCN5 yeast knockouts were unable to acetylate the N-terminal lysines on histones H3 and H4 that flank double-strand break, induced by the site-specific homothallic (HO) endonuclease (Masumoto *et al.*, 2005). The result was an inability to repair the HO-created lesions. This suggested that acetylation by GCN5 marks regions of chromatin corresponding to DNA damage and implicates HAT activity in the repair of double strand breaks, which is the critical lesion leading to radiation-induced cell death. With respect to HDACs, Rpd3 complexed with Sin3 is required for efficient nonhomologous end joining (NHEJ) as yeast strains lacking in these complexes are deficient in NHEJ repair (Bird *et al.*, 2002). There is also some indication that HDAC activity may play a role in DNA repair in mammalian cells. Ataxia telangectasia mutated (ATM) is a critical molecule in the initiation and regulation of the DNA damage response (Lavin *et al.*, 2005). In coimmuno-precipitation experiments, ATM was found to be associated with HDAC1 after irradiation (Kim *et al.*, 1999). Moreover,

Kao *et al.* reported that HDAC4 interacts with 53BP1, another protein involved in the DNA damage response (Kao *et al.*, 2003).

## III. Inhibitors of Histone Deacetylase

The association of aberrant histone acetylation patterns with the silencing of tumor suppressor genes has suggested that inhibition of HDACs in general could serve as a target for cancer therapy. Thus, considerable effort has been put into the development of clinically applicable HDAC inhibitors. Although new inhibitors are being generated, at present, there are six general classes of HDAC inhibitors, most of which target the catalytic domains of class I and II HDACs. These inhibitors for the most part are not specific for a given HDAC but show slight preferences to either class I or II HDAC (Drummond *et al.*, 2005). HDAC inhibitors, in essence, increase histone acetylation by allowing HAT activity to continue unabated without the deacetylation that normally occurs under controlled conditions. The hydroxamic class of HDAC inhibitors includes suberoylanilide hydroxamic acid (SAHA), and trichostatin A (TSA). These compounds chelate zinc in the active site and contain a hydrophobic backbone that spans the active site of the hydrophobic pocket (Yoshida *et al.*, 2001). The short chain fatty acids, which include sodium butyrate and valproic acid (VA), comprise another class of HDAC inhibitors. These compounds simply target the active site zinc and due to their smaller size and lack of hydrophobic backbone, are considerably less potent than the hydroxamic acids (Remiszewski, 2002). The cyclic peptide class includes depsipeptide and apicidin (Jose *et al.*, 2004). The apicidin cyclic ring attaches to the outer surface of the HDAC, while aliphatic chain binds to the active site catalytic ion (Jose *et al.*, 2004). In contrast, depsipeptide requires intracellular reduction to activate aliphatic groups that bind the zinc ion (Miller *et al.*, 2003). Trapoxin and 2-amino-8-oxo-9,10-epoxydecanoic acid belong to the epoxyketones class, which have an epoxyketone group that binds irreversibly to the catalytic site of the HDAC. Benzamides (MS-275 and CI-994) also attach to the catalytic zinc ion (Saito *et al.*, 1999). MS-275 is a potent HDAC inhibitor and has been shown to inhibit HDAC1 more than HDAC3 (Hu *et al.*, 2003). The final classification includes synthetic combinations of known compounds, specifically hydroxamic-acid-containing peptides having features of both hydroxamic acids and cyclic tetrapeptides (Coffey *et al.*, 2001). M-carboxycinnamic acid bis-hydroxamide (CBHA) is an example of a hybrid-polar inhibitor in this class (Coffey *et al.*, 2001; Furumai *et al.*, 2001). These hybrid compounds have been shown to selectively inhibit HDACs 1 and 4 compared to HDAC6 (Furumai *et al.*, 2001). Although these HDAC inhibitors all inhibit class 1 and 2 HDACs to various degrees, there are also

additional molecular actions that are HDAC inhibitor specific. For example, most HDAC inhibitors induced the expression of p21, however, only TSA results in the acetylation of tubulin (Blagosklonny *et al.*, 2002). Importantly, compounds from each of these classes of HDAC inhibitors have been clinically evaluated for antitumor activity (Vigushin and Coombes, 2002).

## IV. *In Vitro* Radiosensitization by HDAC Inhibitors

In general, inhibition of HDAC activity results in histone hyperacetylation, which relaxes chromatin structure and modifies gene expression; each of these processes individually has been implicated in the regulation of radiosensitivity. However, whereas modification of these parameters are consistent with the potential for an effect on radiosensitivity, direct support of a role for HDAC in regulating radioresponse was initially provided by the HDAC inhibitors sodium butyrate and trichostatin A (Arundel *et al.*, 1985; Biade *et al.*, 2001). Early work done in the 1980s demonstrated that sodium butyrate at relatively nontoxic concentrations increased the radiosensitivity on human colon carcinoma cell lines (Arundel *et al.*, 1985). However, because of its very short half-life and low achievable serum concentration, sodium butyrate has limited clinical applicability (Miller *et al.*, 1987; Perrine *et al.*, 1993). As mentioned above, the radiosensitization induced by sodium butyrate was not directly correlated with changes in histone acetylation status. Similarly, trichostatin A was initially shown to produce a significant increase in the *in vitro* radiosensitivity of a human colon carcinoma cell line (Biade *et al.*, 2001). However, trichostatin A has excessive cytotoxicity apparently due to actions involving the acetylation on nonhistone proteins (Hubbert *et al.*, 2002) and is unstable under *in vivo* conditions (Blagosklonny *et al.*, 2002; Saito *et al.*, 1999). Moreover, trichostatin A-induced radiosensitization was attributed to chromatin compaction, not relaxation, and correlated with histone phosphorylation (Biade *et al.*, 2001). Whereas more recent studies have correlated the trichostatin A-induced histone hyperacetylation with enhanced radiosensitivity (Kim *et al.*, 2004; Munshi *et al.*, 2005; Zhang *et al.*, 2004b), the reported induction histone phosphorylation along with the accumulation of cells in mitosis (Blagosklonny *et al.*, 2002), a radiosensitive phase of the cell cycle, complicates the mechanistic interpretation of the observed radiosensitization. Thus, although these HDAC inhibitors were shown to enhance the level of radiation-induced cell killing, neither of these agents is generally applicable to clinical use, sodium butyrate because of pharmacokinetic parameters and trichostatin A because of excessive toxicity (Miller *et al.*, 1987; Saito *et al.*, 1999).

With advances in drug discovery, there have been a number of new HDAC inhibitors developed with more favorable *in vivo* pharmacokinetic

and toxicity profiles. One of the new HDAC inhibitors undergoing clinical investigation is the benzamide MS-275. It is a potent HDAC inhibitor and has been reported to have *in vivo* anti-tumor activity in a number of preclinical models (Saito *et al.*, 1999). Building on the initial work of Leith and colleagues (Arundel and Leith, 1987; Arundel *et al.*, 1985; Hallows *et al.*, 1988; Leith, 1988), MS-275 was the first of the clinically relevant HDAC inhibitors to be evaluated as a radiosensitizing agent (Camphausen *et al.*, 2004a). For this study, two human tumor cell lines of different histological origins were used: DU145 (prostate carcinoma) and U251 (glioma). Because the hypothesis was that the HDAC inhibitory action of MS-275 modifies tumor cell radiosensitivity, as an indicator of HDAC inhibition, initial experiments determined the effects of MS-275 on histone acetylation status in each cell line. In these experiments, cells were exposed to MS-275 for 6–48 hr and, as expected, there was an increase in the levels of acetylated histones H3 and H4 by 6 hr after the addition of MS-275 reaching a maximum from 24 to 48 hr in both cell lines. However, histone acetylation and deacetylation is a dynamic process with some species of acetylated histones having a half-life on the order of minutes (Duncan *et al.*, 1983; Vidali *et al.*, 1978). Therefore, to determine the dependence of the elevated acetylation levels on the continued presence of MS-275, cultures were first exposed to the HDAC inhibitor for 48 hr to induce maximal acetylation; the media was then removed, and fresh MS-275-free media added. Following this protocol, the induced histone hyperacetylation was significantly reduced by 6 hr after MS-275 removal approaching control levels by 16–24 hr. These results indicate that the hyperacetylation of histones H3 and H4 depends on the continuous exposure to MS-275. This rapid decline in histone hyperacetylation after the removal of the HDAC inhibitor proved, as described below, to play a significant role in its radiosensitizing activity.

To determine whether MS-275-induced histone hyperacetylation was associated with changes in tumor cell radiosensitivity, a standard protocol for evaluating potential radiosensitizers was followed. DU145 and U251 cultures were exposed to MS275 for 48 hr (time of maximum histone acetylation), irradiated, trypsinized into single cell suspensions, and plated into MS-275-free media for determination of colony-forming efficiency and the generation of radiation cell survival curves. It should be noted that a colony-forming efficiency assay, which quantifies clonogenic survival, reflects the contribution of all forms of radiation-induced cytotoxicity including mitotic catastrophe, apoptosis, senescence, permanent growth arrest, and any other as of yet unidentified modes of cell death. Thus, it is considered the ''gold standard'' for evaluating radiosensitivity. Following this MS-275 treatment protocol, and according to the clonogenic survival analyses, there was only a minor increase in DU145 cell radiosensitivity and no effect on the radioresponse of U251 cells. The minor increase in DU145 radiosensitivity

was actually similar to that initially reported for colon carcinoma cells exposed to sodium butyrate before irradiation (Arundel *et al.*, 1985).

Whereas the hypothesis was that histone hyperacetylation resulted in radiosensitization, no specific mechanism had been proposed. Therefore, it was possible that an enhancement in radiation-induced cell killing required the hyperacetylation to be maintained after irradiation. As described above, it had been established that histone hyperacetylation begins to decrease by 6 hr after removal of MS-275. Therefore, to maintain acetylation levels postirradiation, cultures were exposed to MS-275 for 48 hr, trypsinized into single cell suspensions, and seeded into MS-275-containing media. After allowing 6 hr for cell attachment (but no division), cells were then irradiated with the MS-275 remaining in the culture media. This somewhat nontraditional protocol was followed in an attempt to eliminate effects of trypsinization on postirradiation signaling/recovery processes and yet provide a continual exposure to MS-275, and thus continual histone hyperacetylation, pre- and postirradiation. Moreover, this protocol allows for the irradiation of single cells and not microcolonies, which eliminates the confounding parameter of multiplicity and its effects on apparent radiosensitivity. This continuous MS-275 exposure protocol resulted in a significant enhancement in the radiosensitivity of DU145 and U251 cells with dose enhancement factors at a surviving fraction of 0.1 of 1.9 and 1.3 for DU145 and U251, respectively. The results suggested that perhaps the addition of MS-275 immediately after irradiation could be sufficient to enhance radiation-induced cell killing. However, when MS-275 was added only after irradiation, without a preirradiation exposure, there were only minor increases in radiosensitivity for both cell lines. These initial results, although far from establishing a causal relationship, were the first to demonstrate a correlation between MS-275-induced hyperacetylation and the enhancement of radiosensitivity. Moreover, they provided mechanistic insights in that they suggested that to obtain maximal enhancement in radiosensitivity exposure to an HDAC inhibitor is required both before and after irradiation.

To determine whether radiosensitization was a property of other HDAC inhibitors and the requirement for a pre- and postirradiation exposure was unique to MS-275, these studies were extended to other clinically applicable HDAC inhibitors. Of particular interest was VA, which has well-established efficacy in the treatment of epilepsy and other seizure disorders (Pinder *et al.*, 1977). Its broad-spectrum anticonvulsant activity has been suggested to result from a combination of mechanisms including the increase in $\gamma$-aminobutyric acid, a decrease in $\gamma$-hydroxybutyric acid, and a direct interaction with the neuronal membrane blocking voltage dependent sodium channels (Loscher, 1999). Because of its effectiveness, oral bioavailability, and generally low toxicity profile, VA has been frequently used as a chronic antiepileptic therapy (Perucca, 2002). More recently, VA was shown to be an

effective inhibitor of HDAC (Gottlicher *et al*., 2001; Phiel *et al*., 2001). Using VA and VA analogs, Phiel *et al.* showed that the teratogenic but not the antiepileptic activity of VA is likely due to HDAC inhibition (Phiel *et al*., 2001). These results, along with previous reports (Gottlicher *et al*., 2001), indicated that the antitumor effects of VA are likely the result of HDAC inhibition. The chemical structure of VA is that of an eight-carbon branched–chained fatty acid, similar to that of another HDAC inhibitor sodium butyrate. In contrast to the 30 min serum half-life of sodium butyrate, VA has a serum half-life of 9–18 hr and can be administered orally (Perucca, 2002). Moreover, as demonstrated by its antiseizure medication and in contrast to most other HDAC inhibitors, VA penetrates the blood–brain barrier and can be chronically administered with minimal toxicity (Perucca, 2002). Thus, given VA's 30-year history of safe clinical use in the treatment of CNS disorders, the rationale for investigating the combination of this HDAC inhibitor with radiation lies in the potential contribution to brain tumor therapy.

Thus, the effects of VA on tumor cell radiosensitivity were evaluated using two human brain tumor cell lines SF539 and U251 (Camphausen *et al*., 2005). Initial experiments characterized the histone acetylation status in each cell line as a function of VA exposure showing that the levels of acetylated histones H3 and H4 increased in a concentration-dependent manner with maximum levels reached by 24 hr for both cell lines. As shown for MS-275, removal of VA from the culture media resulted in the rapid loss of histone hyperacetylation. Whereas MS-275-induced hyperacetylation began to decrease by 6 hr after drug removal, in both of the human brain tumor cell lines histone hyperacetylation was essentially at control levels by approximately 3 hr after VA removal. Thus, comparing chemically disparate MS-275 and VA suggests that histone hyperacetylation is dependent on the continued presence of the HDAC inhibitor. However, these results also suggested that the half-life of the hyperacetylation induced by an HDAC inhibitor is at least somewhat agent specific.

To determine whether VA exposure enhanced radiosensitivity and whether, like MS-275, it was dependent on pre- and postirradiation exposure, SF539 and U251 cultures were subjected to the same two combination protocols. For pretreatment, cells were exposed to a VA concentration sufficient to induce maximal histone hyperacetylation for 24 hr, trypsinized into single cell suspensions, and seeded into VA-free media. After allowing 6 hr for cell attachment (but no division), cells were irradiated and colony-forming efficiency determined. This VA treatment protocol enhanced the radiosensitivity of SF539 cells with a dose enhancement factor at a surviving fraction of 0.1 (DEF) of 1.3 and had only a minor, if any, effect on the radiosensitivity of U251 cells (DEF 1.1). However, when SF539 and U251 cultures were exposed to VA for 24 hr, trypsinized into single cell

suspensions, and seeded into VA-containing media, which allowed for the continual exposure to VA, DEFs of 1.6 and 1.5, respectively, were obtained. Thus these data indicate that VA exposure, both before and after irradiation, results in an increase in tumor cell radiosensitivity, similar to the results obtained for MS-275.

The majority of compounds that have been reported to enhance radiosensitivity (i.e., radiosensitizers) involve treating the cells only before irradiation. Thus, the requirement for pre- and postexposure for the maximum sensitization induced by the 2 HDAC inhibitors described above is unusual and, consequently, is not only of mechanistic significance, but also has implications regarding potential clinical applications. To further explore whether this exposure requirement is a general characteristic of these compounds we evaluated three additional HDAC inhibitors for their radiosensitizing potential (Fig. 1). For these studies, three structurally disparate HDAC inhibitors were evaluated that are currently in clinical trials: SAHA, a hydroxamic acid; CI-994, a benzamide; and depsipeptide, a cyclic tetrapeptide. Shown in Fig. 1A is exposure of the pancreatic cell line MiaPaca to SAHA before radiation resulted in a slight increase in radiation-induced cell killing. However, the pre- and postirradiation exposure resulted in a significantly greater increase in radiosensitivity. Other groups have evaluated the radiosensitizing ability of SAHA (Chinnaiyan *et al.*, 2005; Zhang *et al.*, 2004b), however, different protocols were used and neither involved pre- and postirradiation exposure. Zhang *et al.* reported that exposure of human squamous cell carcinoma cell lines to SAHA for 24 hr before irradiation induced only a minor increase in radiosensitivity (Zhang *et al.*, 2004b), consistent with the results presented in Fig. 1A. It is unclear as to whether a more significant increase in the radiosensitivity of the squamous carcinoma cell line would have been obtained with the addition of a postirradiation SAHA exposure. Chinnaiyan *et al* showed that exposure of a prostate carcinoma cell line and a glioma cell line to SAHA for 3 days prior to irradiation also enhanced radiosensitivity (Chinnaiyan *et al.*, 2005). However, the consequences of a 3-day SAHA exposure period on cell cycle distribution and other cellular characteristics, including histone acetylation, were not addressed. CI-994 is a benzamide that crosses the blood–brain barrier (Suzuki *et al.*, 1999) and thus of interest with respect to brain tumor treatment. As shown in Fig. 1B, this HDAC inhibitor also enhances the radiosensitivity of the U251 glioma cell line, if exposure is pre- and postirradiation. Finally, pre- and postirradiation exposure to depsipeptide results in a significant radiosensitization of U251 cells (Fig. 1C). This HDAC inhibitor was also reported to enhance the radiosensitivity of squamous cell carcinoma cell lines, however, the complete radiation survival curves were not shown (Zhang *et al.*, 2004b).

With respect to other HDAC inhibitors, Munshi *et al.* showed that exposure of human melanoma cell lines to the HDAC inhibitors NaB, PB,

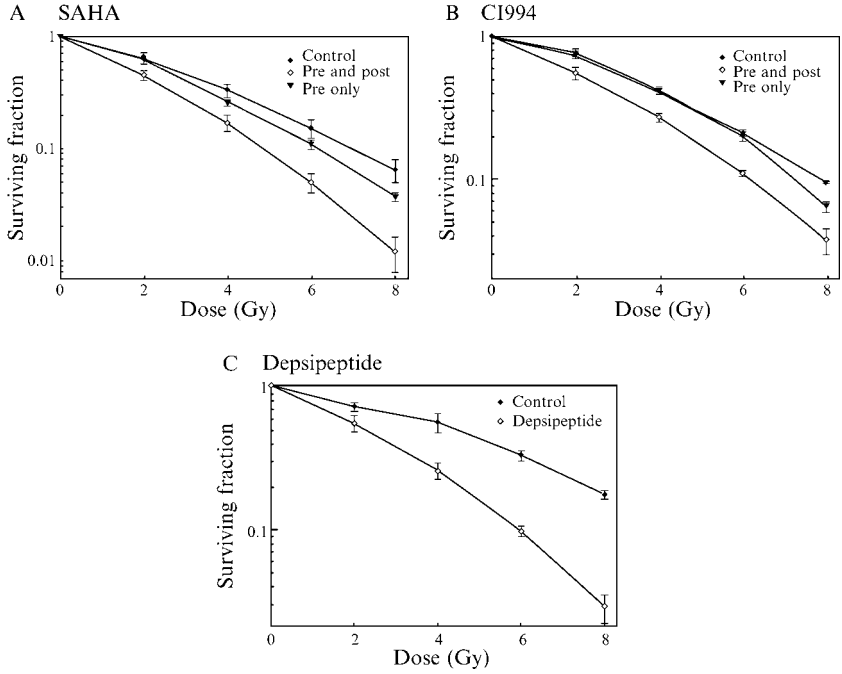


**Figure 1** The effects of HDAC inhibitors on tumor cell radiosensitivity. For each HDAC inhibitor cells were exposed for 24 hr and then trypsinized, plated as single cells in media with or without inhibitor, and irradiated 6 hr later. Colonies were determined 12–14 days later and survival curves constructed after normalization for cell killing induced by drug only. Values represent the mean ± SE of 3–4 independent experiments. Control refers to exposure to vehicle only. (A) MiaPaca cells treated with SAHA (1 μM), (B) U251 cells treated with CI994 (2 μM), and (C) U251 cells treated with depsipeptide (2nM), only pre- and postradiation is shown.

tributyrin, and TSA for 24 hr before radiation resulted in significant increases in the levels of radiation-induced cell killing. These investigators also correlated histone hyperacetylation at the time of irradiation with the radiosensitization induced by NaB. Whereas the effects of pre- and postirradiation exposure were not reported, a clear radiosensitization was detected using the preirradiation treatment only; it is unclear whether, as we have seen in our studies, that additional sensitization will be induced by adding postirradiation exposure. However, SAHA, CI994, and depsipeptide each required pre- and postirradiation for maximum radiosensitization (Fig. 1), which is consistent with a previously reported results for MS-275 and VA. Thus, data generated to date from our group using five structurally disparate HDAC inhibitors and a variety of solid tumor cell lines suggest that maximum radiosensitization requires that these agents be present before and after

irradiation. However, in the absence of a well-defined mechanism of the radiosensitization, and given the use of different HDAC inhibitors and just as important, different cell lines, more confidence in the necessity for a pre- and postirradiation exposure protocol would be engendered through further evaluation by other research groups.

Although the details of the optimal protocol for each compound remained to be determined, a characteristic of the clinically applicable HDAC inhibitors, in general, appears to be the ability to enhance radiosensitivity. A summary of the specific HDAC inhibitors that have been shown to enhance the radiosensitivity of tumor cells using *in vitro* and *in vivo* experimental models is shown in Table I. It should also be noted that TSA and SAHA have been reported to increase the sensitivity of tumor cells to DNA damaging chemotherapeutic agents (Kim *et al.*, 2003). Such results have provided a rational basis for combining HDAC inhibitors with standard

**Table I** HDAC Inhibitors and Radiosensitization

| Agent | Class | *In Vitro* | *In Vivo* | Active Clinical Trials Alone/With Chemo[a] |
|---|---|---|---|---|
| Sodium butyrate | Short chain fatty acid | X[b] | | |
| Phenylbutyrate | Short chain fatty acid | X[b] | | 1/1 |
| Valproic acid | Short chain fatty acid | X[c] | X[c] | 6/4 |
| Tributyrin | Short chain fatty acid | X[b] | | |
| MS-275 | Benzamide | X[d] | X[e] | 3/2 |
| CI994 | Benzamide | X[j] | | |
| Trichostatin A | Hydroxamic | X[b,f,g] | | |
| SAHA | Hydroxamic | X[g,h,j] | | 16/5 |
| Depsipeptide | Cyclic Peptide | X[g,j] | | 20/2 |
| CBHA | Hybrid | X[i] | | |

Table I provides a list of the HDAC inhibitors that have been shown to enhance tumor cell radiosensitivity. An "X" indicates detected radiosensitization with the corresponding reference. The *in vitro* and *in vivo* refer to monolayer cultures and xenograft tumors grown in immunocompromised mice, respectively. The currently active clinical trials of HDAC inhibitors used as single modalities (alone) or in combination with chemotherapeutic agents as listed in www.cancer.gov are also provided.

[a]www.cancer.gov.
[b]Munshi *et al.*, 2005.
[c]Camphausen *et al.*, 2005.
[d]Canphausen *et al.*, 2004a.
[e,f]Kim *et al.*, 2004.
[g]Zhang *et al.*, 2004b.
[h]Chinnaiyan *et al.*, 2005.
[i]Zhang *et al.*, 2004a.
[j]Table I, this chapter.

chemotherapy in clinical trials, a number of which are currently ongoing (Table I).

## V. *In Vivo* Radiosensitization by HDAC Inhibitors

The *in vitro* data have clearly shown that HDAC inhibitors can enhance the intrinsic radiosensitivity of tumor cell and have suggested a correlation between this enhancement and the induction of histone hyperacetylation. Moreover, these studies suggested that histone hyperacetylation could be used as a marker in the design of *in vivo* antitumor protocols combining HDAC inhibitors and radiation. The availability of such markers indicative of drug action, that is, radiosensitization, is one of the putative advantages of the molecular targeting strategy for radiosensitizing tumor cells. Thus, to further evaluate the antitumor potential of an HDAC inhibitor/radiation combination, it was necessary to extend these *in vitro* results to an *in vivo* xenograft model. The first studies involved MS-275 delivered to mice bearing DU-145 (a prostate carcinoma) tumors grown in the hind leg (Camphausen *et al.*, 2004b).

To evaluate histone hyperacetylation *in vivo* as an indicator of the radiosensitizing activity of HDAC inhibitors, which would provide the basis for the rational design of an *in vivo* combination protocol, the effects of MS-275 on the histone acetylation status of DU145 xenografts were determined. In these studies mice were injected with MS-275 given as a 6 mg/kg/injection at 12 hr intervals for up to 3 days. Mice were then sacrificed daily 6 hr after 2, 4, or 6 injections and the level of acetylated histone H4 in each of DU145 tumors determined by immunoblot analysis. The maximum histone acetylation in DU145 xenografts was achieved after 4 to 6 injections of MS-275, after the last injection of MS-275 histone acetylation rapidly decreased by 24 hr, which is similar to what was observed *in vitro* (Camphausen 2004b). These data were generated using a standard *in vivo* protocol of postmortem evaluation of tumor lysates. However, to simulate a more clinically relevant situation, serial biopsies were also performed on individual DU145 tumors before and 6 hr after six doses of MS-275. This protocol generated similar results as those obtained in the evaluation of individual tumor lysates. That is, tumor biopsies obtained from mice before treatment contained no detectable histone acetylation, yet in sampling after treatment with MS-275 tumor specimens contained significant levels of hyperacetylated histone. Thus, analysis of individual mice or through serial biopsies suggested that to increase tumor radiosensitivity, radiation should be delivered after four doses of MS-275 and followed by the two additional injections.

Based on the histone hyperacetylation results and taking into account *in vitro* data indicating that the hyperacetylation had to be present before

and maintained after irradiation, the *in vivo* protocol was designed such that MS-275 was delivered for 2 days before and 1 day after a single dose of radiation. Comparison of tumor growth rates revealed that MS-275 treatment clearly enhanced the radiation-induced tumor growth delay with a dose enhancement factor of approximately 2.8. Thus, consistent with previous *in vitro* experiments, these data indicate that MS-275 enhances the radiosensitivity of DU145 xenografts.

Similar *in vivo* results were obtained using a combination of VA and radiation against U251 glioma xenografts (Camphausen *et al*., 2005). In this study, mice bearing xenografts received 150 mg/kg of VA delivered at 12 hr-intervals for 3 days for a total of six injections. This treatment regimen was previously shown to provide a serum VA concentration roughly equivalent to that needed to provide antiseizure activity in humans (Pinder *et al*, 1977). Tumor histone hyperacetylation, although not detected after two injections, was clearly present after three and, consistent with the *in vitro* results, was rapidly reduced in the U251 xenografts after the cessation of VA treatment. These VA-induced changes in tumor histone acetylation status were also detected in the serial biopsies of the U251 xenografts, as described for MS-275. Based on these data and taking into account the *in vitro* results indicating that histone hyperacetylation had to be present before and maintained after irradiation, an *in vivo* combination protocol was designed to evaluate the effects of VA on the radiation-induced growth delay of U251 xenografts. Specifically, animals with subcutaneous U251 leg tumor xenografts were given VA in six injections of 150 mg/kg delivered at 12-hr intervals over 3 days, with a single dose of radiation (4 Gy) delivered 6 hr after the third VA dose (time of increased histone acetylation), which was followed by three additional VA injections. In terms of tumor growth delays, VA treatment only had essentially no effect on tumor growth rate, yet significantly enhanced the growth delay induced by radiation with a dose enhancement factor of 2.6.

Thus, data available to date indicate that MS-275 and VA, HDAC inhibitors of disparate chemical structures, can enhance the *in vivo* radiosensitivity of experimental tumor models. These results, combined with *in vitro* data showing that a variety of HDAC inhibitors act to enhance radiation-induced tumor cell death, suggest that this general class of compounds may comprise a clinically relevant group of radiosensitizing agents. The putative advantage of using such molecularly targeted radiosensitizers is the availability of markers indicative of drug activity. Whereas the *in vivo* studies suggested that tumor histone acetylation status may provide such markers, in a clinical setting, it is not always possible to obtain tumor biopsies before, during, and after treatment. Thus the availability of a surrogate tissue that could provide relevant information regarding a putative marker would be of considerable benefit in protocol design. In a treatment

situation, one of the most easily obtained cell types is the lymphocyte, which would be an ideal candidate as a surrogate tissue for evaluating the effects of an HDAC inhibitor on histone acetylation. However, in the studies described here, DU145 xenografts were grown in SCID mice, which lack a significant lymphocyte population (Camphausen *et al*., 2004b). Therefore, to investigate the potential of lymphocytes to serve as readily available tissue for evaluation of histone acetylation status it was necessary to use nontumor bearing C57BL/6 mice. These mice were injected with four doses of MS-275, delivered at 12 hr intervals, and lymphocytes collected 6 hr after the last injection. This protocol resulted in the maximal increase in histone H4 acetylation in the DU145 xenografts after treatment of SCID mice. The lymphocytes isolated from C57BL/6 mice, treated with MS-275, had a significantly greater level of histone H4 acetylation than lymphocytes isolated from vehicle-injected mice. As a control for the change in mouse strain, the histone acetylation status of other normal tissue in SCID mice were evaluated after MS-275 treatment. MS-275 was injected at 12 hr intervals for up to 3 days; mice were sacrificed at 6 hr after the last injection, liver and spleen tissue were then evaluated for histone H4 acetylation. Histone H4 hyperacetylation was clearly evident after four injections of MS-275 treatment in both the liver and spleen of SCID mice. Thus, based on these studies, it appeared that tumor tissue, liver, and spleen from SCID mice, as well as lymphocytes from C57BL/6 mice all had similar temporal responses in histone H4 acetylation status after MS-275 treatment. These data thus suggest that patient lymphocytes may serve as a surrogate tissue for determining tumor histone hyperacetylation and thus aid the clinical design of HDAC inhibitor/radiotherapy combination protocols.

It should be noted, however, that whereas the enhancement of *in vivo* tumor radiosensitivity by both MS-275 and VA has been shown to correspond to histone hyperacetylation as detected in postmortem tumors, serially biopsied tumors and normal lymphocytes, liver and spleen, there has not been a rigorous evaluation of the putative utility of histone hyperacetylation as a marker for radiosensitization. The *in vivo* combination protocols were simply designed to deliver radiation at the time of maximal histone acetylation and to maintain the hyperacetylation for 24 hr after irradiation. Tumor growth delay assays have not been performed combining radiation with an HDAC inhibitor dose that does not induced tumor histone hyperacetylation or at a time when the induced histone acetylation has returned to untreated levels. The prediction would be that under these conditions, there would be no enhancement in tumor radioresponse. However, this remains an assumption based on *in vitro* results. Clearly, to more accurately evaluate the clinical potential of histone hyperacetylation as a marker for HDAC inhibitor-induced radiosensitization, these types of control experiments need to be performed.

## VI. Tumor Versus Normal Cells

Whereas the ability to predict tumor histone acetylation status from lymphocytes may be of value in the design of clinical protocols combining HDAC inhibitors and radiation, the induction of histone hyperacetylation in normal lymphocytes, as well as liver and spleen suggests that the radiosensitivity of normal tissues may also be enhanced. Clearly, if this is the case, then the therapeutic application of HDAC inhibitors as radiosensitizers will be limited. However, with respect to HDAC inhibitors as a single modality, it is generally considered that because of the aberrant histone deacetylase activity in tumor cells as compared to normal cells that, at least in theory, tumor cells and not normal cells would be susceptible to the cytotoxic/cytostatic effects of HDAC inhibitors. For the most part this has been consistent with experimental studies (Cress and Seto, 2000; Miller *et al.*, 2003). In addition, *in vivo* administration to experimental animals of clinically relevant HDAC inhibitors using antitumor drug doses are generally without significant toxicities (Atadja *et al.*, 2004; Kelly *et al.*, 2003; Patnaik *et al.*, 2002).

Similarly, because of the difference in histone acetylation patterns between normal and tumor cells, it has been suggested that the radiosensitizing actions of HDAC inhibitors would be selective for tumor cells. Indeed, Munshi *et al* reported that following an NaB exposure protocol that induced the radiosensitization of two melanoma cell lines, no effect on the radiosensitivity of a normal human fibroblast cell line was detected (Munshi *et al.*, 2005). These same investigators found that a tumor radiosensitizing treatment of SAHA delivered to normal fibroblasts also had no effect on their radiosensitivity (A. Munshi and R. E. Meyn, personnel communication). Consistent with these results, Kim *et al.* found that whereas HDAC inhibitors enhanced the *in vitro* sensitivity of tumor cells to DNA damaging drugs, they had no effect on the drug sensitivity of normal breast or intestinal cells (Kim *et al.*, 2003). Whereas the relationship of radiation-induced death of normal cells as detected in monolayer culture to the actual normal tissue injury induced by radiation is unclear, Chung *et al.* evaluated the effects of HDAC inhibitors on radiation-induced skin injury using a rat model (Chung *et al.*, 2004). They found that PB, TSA, and VA each suppressed the skin fibrosis associated with cutaneous radiation syndrome, which was then attributed to the suppression of aberrant expression of *TGF$\beta$*.

Thus, results to date suggest that not only do HDAC inhibitors have no effect on the intrinsic radiosensitivity of normal fibroblasts *in vitro*, but may also actually protect against radiation-induced injury *in vivo*. However, Stoilov *et al* reported that NaB treatment of normal human lymphocytes inhibited the repair of radiation-induced DNA double strand breaks as

measured by premature chromosome condensation (Stoilov *et al.*, 2000). There were no cell survival results reported in this study and, consequently, it is not possible to conclude that NaB enhanced lymphocyte radiosensitivity. In addition, lymphocyte death after irradiation primarily occurs via apoptosis, in contrast to radiation-induced death of most solid tumor cells, which occurs primarily via mitotic catastrophe. This difference in the mode of cell death complicates extrapolating the lymphocytes data to solid tumor cells. Regardless, these data raise questions as to possible effects of HDAC inhibitors on the radioresponse of normal tissue. If there is an enhancement in the apoptosis of acutely responding tissue, like lymphocytes, then such information will be critical to the possible clinical application of HDAC inhibitors as radiosensitizers. Thus, in the absence of well-defined mechanistic information, it remains necessary to continue to evaluate other HDAC inhibitors on the *in vitro* response of other types of normal cells and investigate their effects on radiation-induced normal tissue injury—both early and late forms.

## VII. Mechanisms of HDAC Inhibitor-Induced Radiosensitization

Initial investigations into the mechanism responsible for the radiosensitization induced by HDAC inhibitors focused on the benzimide MS-275, which had been shown to enhance the radiosensitivity of both the DU145 prostate carcinoma cell line and the U251 glioma cell line (Camphausen *et al.*, 2004a). There are a number of cellular parameters that can influence radiosensitivity that potentially could account for the MS-275-induced radiosensitization. Cell cycle phase distribution affects radiosensitivity in which cells are least sensitive to radiation-induced death in S-phase and most sensitive in mitosis. MS-275 has been reported to modify cell cycle distribution decreasing the number of cells in S phase (Saito *et al.*, 1999). Moreover, exposure to another HDAC inhibitor TSA can result in the accumulation of cells in mitosis (Blagosklonny *et al.*, 2002). Because such effects can impact radiosensitivity, it was necessary to determine cell cycle phase distribution after MS-275 exposure (Camphausen *et al.*, 2004a). Following the treatment protocol shown to enhance radiosensitivity, DU145 and U251 cells were exposed for 48 hr to MS-275, trypsinized, and seeded into MS-275 containing media. Six hours after seeding, that is, the time of irradiation, cells were collected for flow cytometric analysis. As reported, there was essentially no difference in cell cycle phase distribution patterns for vehicle and MS-275-treated DU145 or U251 cells. These results indicated that simply the redistribution of cells into a radiosensitive phase of the cell cycle does not account

for the MS-275-mediated enhancement in radiation-induced cell killing. It should be noted that the MS-275 concentration used to enhance radiosensitivity was 300 nM, whereas the concentration that affected the cell cycle distribution of a human ovarian carcinoma cell line was 1 μM (Saito *et al.*, 1999).

With respect to the form of radiation-induced cell death, where apoptosis is considered a sensitive mode of death, most cell lines generated from solid tumors die via mitotic catastrophe, which generally occurs at higher doses. Thus, the ability to switch the mode of radiation-induced cell death from mitotic catastrophe to apoptosis can account for radiosensitization. MS-275, as for HDAC inhibitors in general, has been reported to induce apoptosis in some tumor cells lines but not others (Jaboin *et al.*, 2002). However, apoptosis did not account for the radiosensitization induced by MS-275. Exposure of DU145 and U251 cells to the individual treatments, with MS-275 or radiation, resulted in less than 10% apoptosis and the combination resulted in simply an additive level (Camphausen *et al.*, 2004a). In contrast, Munshi *et al.* in their study of NaB-mediated radiosensitization of melanoma cell lines reported an apparent greater than additive increase in apoptosis by the combination of NaB and radiation as measured by DNA fragmentation (Munshi *et al.*, 2005). However, DNA fragmentation was increased from approximately 6% to 12% and 10% to 14% for MeWo and A375 cells, respectively, as a result of the NaB/radiation combination; the significance of relatively minor changes in apoptosis in a background of over 90% cell death according to the clonogenic survival is unclear. Thus, whereas the role of apoptosis may be cell line and/or HDAC inhibitor dependent, at this point converting the mode of cell death from mitotic catastrophe to apoptosis does not appear to be a primary mechanism mediating the radiosensitization induced by HDAC inhibition.

A critical event in determining radiosensitivity is the repair of DNA double strand breaks (DSBs). Recently, γH2AX expression has been established as a sensitive indicator of DSBs induced by clinically relevant doses of ionizing radiation (Rogakou *et al.*, 1998). At sites of radiation-induced DNA DSBs, the histone H2AX becomes rapidly phosphorylated (the phosphorylated form is referred to as γH2AX) forming readily visible nuclear foci (Rogakou *et al.*, 1998). Although the specific role of γH2AX in the repair of DSBs has not been defined, recent reports indicate that the dephosphoryation of γH2AX and dispersal of γH2AX foci in irradiated cells correlates with the repair of DNA DSBs (Nazarov *et al.*, 2003; Rothkamm *et al.*, 2003). Moreover, Macphail *et al* in their study of 10 cell lines reported that the loss of γH2AX correlates with clonogenic survival after irradiation (MacPhail *et al.*, 2003). Thus, to determine the effects of MS-275 on the induction and repair of DNA DSBs after irradiation, foci of γH2AX were used as an indicator of DNA damage. In both DU145 and U251 cell lines, the number

γH2AX foci was significantly increased by 1 hr after exposure to 5 Gy, remaining constant out to about 6 hr after irradiation; by 24 hr the percentage of positive cells began to approach control levels. This time course of foci induction and dispersion was typical for mammalian tumor cells (MacPhail *et al.*, 2003). In cultures exposed to MS-275 before and after irradiation, the percentage of cells expressing foci were essentially the same at the 6-hr time point as for 5 Gy only. However, at the 24-hr time point the number of cells expressing γH2AX foci in cultures receiving the combined MS-275/radiation treatment was significantly greater as compared to the radiation only group. Thus the maintenance of γH2AX foci levels suggested that the MS-275-mediated radiosensitization involves the inhibition of the repair of DNA damage. Using immunoblot analysis to detect the expression of γH2AX, similar results were obtained for brain tumor cell lines exposed to the combination of VA and radiation (Camphausen *et al.*, 2005). In these studies, whereas γH2AX levels in cells exposed to radiation only and the VA/radiation combination were essentially the same at 1 hr and 6 hr after irradiation, γH2AX expression failed to decrease to control levels in cells exposed to the combined modality. Finally, for the melanoma cell line A375 a radiosensitizing NaB protocol was found to delay the dispersion of radiation-induced γH2AX foci (Munshi *et al.*, 2005).

Thus, for three compounds and in four cell lines generated from three different solid tumor histologies, HDAC inhibition resulted in the prolonged expression of radiation-induced γH2AX, thus suggesting the inhibition of DNA DSBs repair. In these studies, γH2AX was used as a marker of DNA damage. However, rather than simply a consequence of unrepaired DNA DSBs, it is possible that γH2AX may play a causal role in the radiosensitization induced by HDAC inhibitors. H2AX is a variant of H2A histone, and as most other histones, can be acetylated at specific lysine residues (Pantazis and Bonner, 1981). If γH2AX were acetylated by HDAC inhibition, then its function in the DNA damage response may be compromised. Given the varying susceptibilities of histones to acetylation (Strahl and Allis, 2000) and the apparent selectivity of some HDAC inhibitors (Ruijter *et al.*, 2003), it was necessary to determine whether a radiosensitizing HDAC inhibitor treatment protocol affected the acetylation status of γH2AX. Toward this end, U251 cells were exposed to VA, irradiated, and histones collected 30 min later (Fig. 2). As shown in the immunoblot (bottom panel, left side), VA exposure resulted in the acetylation of H2AX as reflected by the bands corresponding to H2AX species of increased molecular weights. γH2AX (lower panel, right side) was only detected in irradiated cells, as expected. However, based on the comparison to H2AX (dotted line), the γH2AX induced by radiation only was acetylated. Irradiation of VA-treated cells induced both this acetylated and a hyperacetylated γH2AX species. Thus, these data indicate that not only is γH2AX acetylated as compared to native

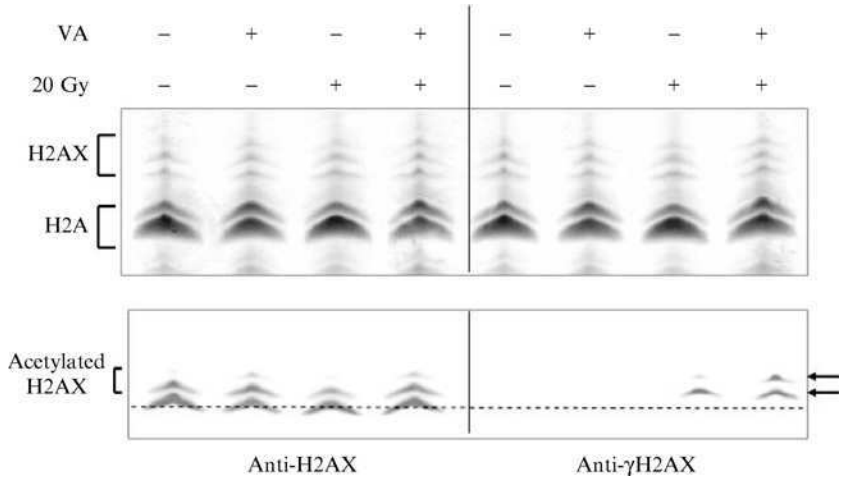


**Figure 2** γH2AX acetylation status after exposure to radiation and/or VA. U251 cells were exposed to VA (1.5 mM) for 24 hr, irradiated (20 Gy), and samples collected 30 min later. Acid soluble extracts were prepared and 100 μg of precipitated histone extracts were loaded on to a 12% AUT gel. The Coomassie blue stained gel (top panel) was photographed to illustrate equal protein loading before transfer for immunoblot analysis. After transfer, the membrane was cut into two pieces with corresponding treatments; the individual membranes were then probed with antibodies to H2AX or γH2AX and developed by ECL (bottom panel). The dotted line indicates H2AX with acetylated H2AX species appearing above in each lane. As shown in the right side of the lower panel, γH2AX is detectable only after irradiation and is acetylated as indicated by its position above that of H2AX (dotted line). However, in cells that were exposed to the VA/radiation combination, an additional γH2AX band is detected corresponding to a hyperacetylated species.

H2AX, but also that VA results in an additional acetylation. The complete time course of hyperacetylated-γH2AX expression after irradiation remains to be determined. Moreover, the half-life of the hyperacetylated γH2AX species after VA removal needs to be determined. If the hyperacetylation of this histone is lost (deacetylated) as rapidly as shown for hyperacetylated histones H3 and H4 after VA removal (Camphausen *et al.*, 2005), then it may account for the requirement of pre- and postirradiation exposure for maximum radiosensitization in this experimental system. Obviously, this is based on the hypothesis that the hyperacetylation of γH2AX compromises its function and results in a reduced capacity to repair DNA DSBs, which remains to be rigorously tested.

In addition to γH2AX results, Munshi *et al.* have presented data that also implicate the inhibition DNA repair in the radiosensitization induced by HDAC inhibitors. In their studies of NaB-induced radiosensitization, DNA repair was evaluated using a host cell reactivation assay, which is based on

reconstitution of an exogenous reporter gene after irradiation. In this assay, NaB treatment was shown to reduce the repair of a transfected reporter plasmid irradiated with 4,000 Gy (Munshi *et al.*, 2005). Whereas these studies suggest that HDAC inhibition inhibits DNA repair, it should be noted that not all repair assays detect this inhibitory activity. We have used the alkaline and neutral comet assays to evaluate the repair of radiation-induced DNA damage in U251 cells exposed to a radiosensitizing VA protocol. According to both assays, VA had no effect on DNA repair (data not shown). However, this lack of an effect using standard DNA repair assays, which detected DNA single and double strand breaks, is consistent with results obtained after irradiation of $G_0$ lymphocytes exposed to NaB (Stoilov *et al.*, 2000). In those studies Stoilov *et al.* showed that whereas NaB had no effect on the initial level of radiation-induced DNA damage or on the repair of single-strand breaks or alkali-labile sites, it did inhibit the repair of chromosome breaks as detected by the premature chromosome condensation technique, suggestive of an inhibition of DNA DSBs (Stoilov *et al.*, 2000). Combining these results with those shown above pertaining to the retention of $\gamma$H2AX, not its induction and the hyperacetylation of $\gamma$H2AX, it may be that HDAC inhibitors affect a late step in DSB repair (i.e., after the ligation of the DNA breaks) involving the remodeling of chromatin structure and the return to preirradiation conditions. Clearly this is speculation and requires considerably more research.

HDAC inhibitors have repeatedly been shown to modify the expression of specific genes, which has been implicated as a mechanism accounting for their antitumor effects. However, as mentioned above, whereas these agents were expected to increase the gene expression, especially tumor suppressor genes, they have also been shown to decrease the expression of selected genes. Along these lines and related to their possible effects on DNA repair, exposure of melanoma cells to a radiosensitizing protocol of NaB has been reported to decrease the expression of the critical repair proteins of *Ku70*, *Ku86*, and *DNA-PKcs* in melanoma cells (Munshi *et al.*, 2005). Moreover, the expression of *SMC* genes, which play a role in the repair of radiation-induced DNA damage, has also been shown to be reduced after 48 hr of VA exposure (Harvey *et al.*, 2004; Marchion *et al.*, 2005). In this study, however, the effects of the 48 hr VA exposure on tumor cell radiosensitivity were not evaluated. Thus, the reduced expression of DNA repair genes may also play a role in HDAC inhibitor-induced radiosensitization. It would be of interest to determine the time course for the "re-expression" of the repair genes, as well as their corresponding proteins after removal of the HDAC inhibitor. A rapid return to untreated levels would be consistent with a maximum enhancement in radiosensitivity with pre- and postirradiation exposure. Hyperacetylation of $\gamma$H2AX and the reduced levels of DNA repair proteins as potential mechanisms of radiosensitization may not be

mutually exclusive. Their relative contributions may depend on the cell type and HDAC inhibitor being used.

Our focus has been on mammalian cells. However, given the information already available on the role of histone acetylation in DNA repair in yeast, it might be expected that the mechanisms of HDAC inhibitor-induced radiosensitization might be better addressed in a yeast system. To our knowledge, the effect of an HDAC inhibitor on yeast radiosensitivity has not been reported. However, whereas this would certainly simplify mechanistic studies, initial work on mammalian cells suggest that HDAC inhibitors do not affect the radiosensitivity of normal cells (Munshi *et al*., 2005) but only those that are transformed. If this tumor-specific effect is validated, then HDAC inhibitors would actually be expected not to influence the radioresponse of a ''normal'' yeast cell.

## VIII. Clinical Application of HDAC Inhibitor/Radiotherapy Combinations

As previously discussed, as a class of drugs HDAC inhibitors enhance the radiosensitivity in preclinical models of numerous cell lines derived from different tumor histologies, including but not limited to colon, glioma, melanoma, and pancreas. This diversity in cell type activity has been shown to be predictive of activity of chemotherapeutic agents in Phase II clinical trials by Voskoglou-Nomikos *et al*. from the National Cancer Institute of Canada (NCIC) (Voskoglou-Nomikos *et al*., 2003). In this study, the authors compared the results of all Phase II clinical trials of chemotherapy agents conducted from 1985–2000 to the results obtained in the preclinical models used to evaluate each drug. In all, 31 compounds were identified and evaluated for this study. When the preclinical models used to develop these drugs were evaluated as predictors of eventual clinical efficacy in Phase II trials, the most significant predictive variable was the drug's activity against a broad range of tumor cell lines with diverse cellular histologies. Therefore, extrapolation of this chemotherapy data suggests that HDAC inhibitors as a class may be successful as radiation sensitizers in the clinic if appropriate strategies are used to test them. However, the work from NCIC also showed that successful preclinical work in one tumor histology was, for the most part, not predictive of a successful Phase II trial in tumors of that same histology. Therefore, the choice of the specific HDAC inhibitor and the tumor histology for the combination Phase II trial must be based on other factors.

As numerous HDAC inhibitors have been shown to possess radiosensitizing properties in preclinical models (Table I), additional characteristics of the agent should be considered to determine the appropriate compound to

test in each clinical scenario. Careful consideration of the pharmacologic properties of the agent, such as the biodistribution and the possible toxicity of administration, allows the determination of an appropriate agent to test in each clinical scenario. For example, several agents including VA and CI-994 readily cross the blood–brain barrier and would, therefore, be logical choices to test in combination with radiation in patients with brain tumors. However, VA would not be a good choice for use in patients with pancreatic carcinomas as one of the major side effects of prolonged VA usage is pancreatitis.

As noted above in the *in vivo* models section, the bioavailability of the HDAC inhibitor in the tissue of interest is critical to the success of radiosensitization with these agents. To aid in determining drug availability and effective HDAC inhibitor levels, several groups have measured histone acetylation in both tumor cells and circulating lymphocytes (Chavez-Blanco *et al.*, 2005; Pauer *et al.*, 2004; Rephaeli *et al.*, 2005; Ryan *et al.*, 2005). Evaluation of histone acetylation in circulating lymphocytes is a particularly attractive method for determining the activity of these agents, as circulating lymphocyte collection is far less invasive than tumor biopsies. Peripheral lymphocyte sampling to evaluate the efficacy of the agent in regards to histone deacetylation may yield similar information and investigation of this marker, prior to radiotherapy, is being included in several ongoing clinical trials (www.cancer.org).

Whereas the availability of numerous HDAC inhibitors can complicate the process of choosing the appropriate agent to test in clinical trials, a more complex issue facing the investigator is the proper group of patients to study the combination with radiotherapy. Two important considerations for patient selection are the role of local control in the tumor type selected and the influence that improved local control has on the patient's progression free survival (PFS), and the use of traditional chemotherapy agents, in combination with radiotherapy, as part of the standard of care for that group of patients. For the purpose of showing enhanced local control through radiosensitization, it would be desirable to have the rate of local failure as the first site of failure to be as high as possible in the tumor type studied. Because the likelihood of local failure in these patients is so high, improvements in local control and PFS are evident after the treatment of fewer patients than would be required in diseases with lower rates of local failure. Additionally, if local recurrence is the primary contributor to progression, the effect of improved local control on PFS can more readily be appreciated with fewer patients.

For example, patients diagnosed with Glioblastoma multiforme will have a local recurrence as the sole site of failure more than 85% of the time. Therefore, if the combination of an HDAC inhibitor and radiotherapy increases the tumor kill locally, then there should be a decrease in local failures and an increase in PFS. Other tumor sites with high local failure

rates, such as unresectable pancreatic carcinomas and unresectable head and neck cancers, which have local failure rates of 30–40% may also be appropriate to study in this regard. However, tumors selected for study with radiosensitizing agents should be chosen based on the likelihood that sensitization will enhance local control and prolong PFS. The importance of additional tumor cell kill on local control and prolongation of PFS should be reflected by improvements in these outcomes after radiation dose escalation, data which is unfortunately not available for most tumors.

An additional consideration in selecting a group of patients for study is the other types of therapy that are part of the current standard of care. The delivery of chemotherapy greatly complicates the preclinical modeling of these agents. Additionally, the combination of these agents with chemotherapy may alter pharmacokinetics and toxicity in unexpected ways. These issues should be considered when contemplating multimodality therapy with these agents. Of note, numerous ongoing clinical studies are currently evaluating HDAC inhibitor with chemotherapy (Table I), and extrapolation from these trials may make triple combination studies safer to conduct and more successful.

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# 7

# Chaperone-Mediated Autophagy in Aging and Disease

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I. Introduction: Different Forms of Autophagy
II. CMA: A Selective Form of Autophagy
  A. CMA: The Beginnings
  B. CMA Substrates and Their Interaction with Cytosolic Chaperones
  C. Lysosomal Receptor for CMA Substrates
  D. At the Other Side of the Lysosomal Membrane
  E. Methods to Measure CMA
III. Physiological Role of CMA
  A. CMA and Starvation
  B. CMA and Oxidation
  C. CMA and Antigen Presentation
IV. CMA and Aging
V. Pathologies Related to CMA
  A. CMA and Nephropathies
  B. CMA and Lysosomal Storage Disorders
  C. CMA and Neurodegeneration
VI. Concluding Remarks
  Acknowledgments
  References

Different mechanisms target intracellular components for their degradation into lysosomes through what is known as autophagy. In mammals, three main forms of autophagy have been described: macroautophagy, microautophagy, and chaperone-mediated autophagy (CMA). CMA is the only autophagic pathway that allows selective degradation of soluble proteins in lysosomes. In contrast to the other mammalian forms of autophagy, CMA does not require vesicle formation or major changes in the lysosomal membrane. Instead, substrate proteins directly cross the lysosomal membrane to reach the lumen, where they are rapidly degraded. The substrate proteins are targeted to the lysosomal membrane by recognition of a targeting motif (a KFERQ-like motif), by a chaperone complex, consisting of hsc70 and its cochaperones, in the cytoplasm. Once at the lysosomal membrane, the protein interacts with a

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lysosomal receptor for this pathway, lysosomal associated membrane protein type 2A (LAMP-2A), and it is translocated across the membrane into the lysosomal lumen assisted by a lysosome resident chaperone. These two characteristics—selectivity and direct substrate translocation—determine the particular role of CMA in different physiological and pathological conditions. In this chapter, we cover current findings on the molecular mechanisms for CMA and the possible pathophysiological relevance of this selective lysosomal degradation. 

## I. Introduction: Different Forms of Autophagy

The degradation of intracellular proteins and other cytoplasmic components plays a very important role in many aspects of a living cell, particularly in the regulation of intracellular homeostasis, removal of misfolded and damaged cellular constituents, and in the cellular response to environmental stressors (Cuervo, 2004a,b; Shintani and Klionsky, 2004). Two major proteolytic systems are responsible for most of the intracellular protein degradation: the ubiquitin-proteasome system and the lysosomes.

The proteasome is the major cytosolic protease, which selectively degrades short-lived proteins (half-life $<12$ hr), including a large percentage of recently synthesized proteins (half-life $<10$ min). This proteolytic complex plays a critical role in the regulation of essential intracellular processes such as, cell cycle progression, cell division, transcription and signaling (reviewed in Ciechanover and Brundin, 2003; Glickman and Ciechanover, 2002; Goldberg, 2003; Myung *et al.*, 2001; Pickart and Cohen, 2004). The lysosomal pathway mainly degrades long-lived proteins with half-lives that can be up to many days (Ahlberg *et al.*, 1985; De Duve and Wattiaux, 1966; reviewed in Cuervo, 2004a,b; Shintani and Klionsky, 2004). In contrast to the proteasome pathway, lysosomes can also degrade complete organelles. This degradation of intracellular components by lysosomes is called autophagy, a process conserved from yeast to mammalian cells. In mammalian cells, three types of autophagy have been described: macroautophagy, microautophagy, and chaperone-mediated autophagy (CMA) (Fig. 1) (reviewed in Cuervo, 2004a,b; Dice, 2000; Shintani and Klionsky, 2004).

Autophagy participates in different cellular functions (reviewed in Levine and Klionsky, 2004). Under normal conditions, it contributes to the continuous turnover of intracellular components, aimed to eliminate both damaged and/or nonfunctional organelles and proteins, and to provide amino acids and essential constituents for the synthesis of new functional intracellular components (Cuervo, 2004a,b; Dice, 2000; Shintani and Klionsky, 2004). Autophagy is also activated in response to certain environmental stressors, such as nutritional deprivation, oxidizing conditions, and host

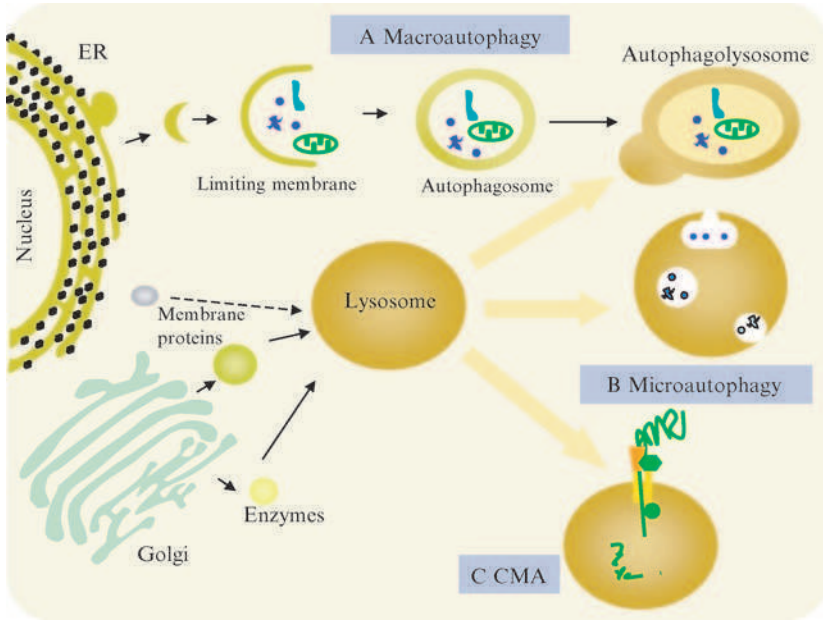


**Figure 1** Types of autophagy in mammalian cells. Three main forms of autophagy are present in most mammalian cells: (A) *macroautophagy* that requires sequestration of substrates in a double membrane vesicle, which then fuses with lysosomes; (B) *microautophagy* that involves direct engulfment of cytosolic components by lysosomes; and (C) *CMA* in which substrates are recognized by chaperones and targeted to lysosomes for degradation after direct translocation into the lysosomal lumen.

invasion by pathogen agents, to remove damaged intracellular components. Autophagy is required throughout an organisms' life, from embryogenesis up to aging, participating in all conditions concerning cell differentiation and tissue remodeling, and often sitting on the junction between cellular survival and cell death (Levine and Klionsky, 2004; Mizushima *et al.*, 2004; Shintani and Klionsky, 2004). Although all forms of autophagy share some common functions, their different mechanisms set the basis for their participation in individually different functions.

In the process of macroautophagy, a *de novo* formed isolation membrane sequesters cytoplasm and organelles destined for degradation (Mizushima *et al.*, 2002). This membrane then seals into a double membrane vesicle—the autophagosome or autophagic vacuole (Mizushima *et al.*, 2002). Through fusion with lysosomes, the autophagic vacuole acquires the enzymes necessary for the degradation of the intracellular components and the inner membrane of the autophagic vacuole. The different steps in macroautophagy are mediated by a group of more than 18 proteins, first described in

yeast and generically known as Atg proteins (Klionsky, 2005; Klionsky *et al.*, 2003). Knockdowns and overexpression of the genes encoding these proteins in different organisms has tremendously advanced our understanding of the role that macroautophagy plays in different physiological and pathological processes (reviewed in Levine and Klionsky, 2004; Mizushima *et al.*, 2004; Shintani and Klionsky, 2004).

In microautophagy, intracellular constituents are directly sequestered by the lysosomal membrane and engulfed for degradation (Ahlberg and Glaumann, 1985; Mortimore *et al.*, 1988). Microautophagy is considered the constitutive form of autophagy that participates in the continuous removal of intracellular proteins and organelles.

The focus of this chapter is on CMA, a selective form of autophagy described only in mammals. Readers are referred to recent reviews to learn more about the molecular components and mechanisms that regulate macro- and microautophagy (Cuervo, 2004b; Klionsky, 2005; Levine and Klionsky, 2004; Mizushima, 2002; Shintani and Klionsky, 2004).

## II. CMA: A Selective Form of Autophagy

In contrast to the nonselective engulfment of soluble cytosolic proteins by macro- and microautophagy, only cytosolic proteins containing a motif biochemically related to the pentapeptide KFERQ in their sequence are degraded in lysosomes through CMA. The substrates first bind to a cytosolic chaperone—the heat shock cognate protein of 70-KDa (hsc70)—and its cochaperones, and then to a lysosomal membrane protein—the lysosomal associated membrane protein type 2A (LAMP-2A) (Fig. 2). After unfolding, the substrate proteins are translocated into the lysosomal lumen for degradation with the help of a lysosomal chaperone (lys-hsc70). Because the substrates are transported across the lysosomal membrane and not engulfed, CMA is a suitable mechanism for degradation of soluble proteins but not organelles. As in the case of macroautophagy, CMA is maximally activated during stress conditions such as starvation, oxidative stress, or exposure to different toxic compounds.

### A. CMA: The Beginnings

CMA was first described in human diploid fibroblasts while following the degradation of radiolabeled exogenous proteins introduced into these cells by erythrocyte-mediated microinjection (Backer *et al.*, 1983). The degradation rates of some long-lived proteins increased by removal of serum, insulin, fibroblast growth factor, or dexamethasone, while these treatments had little

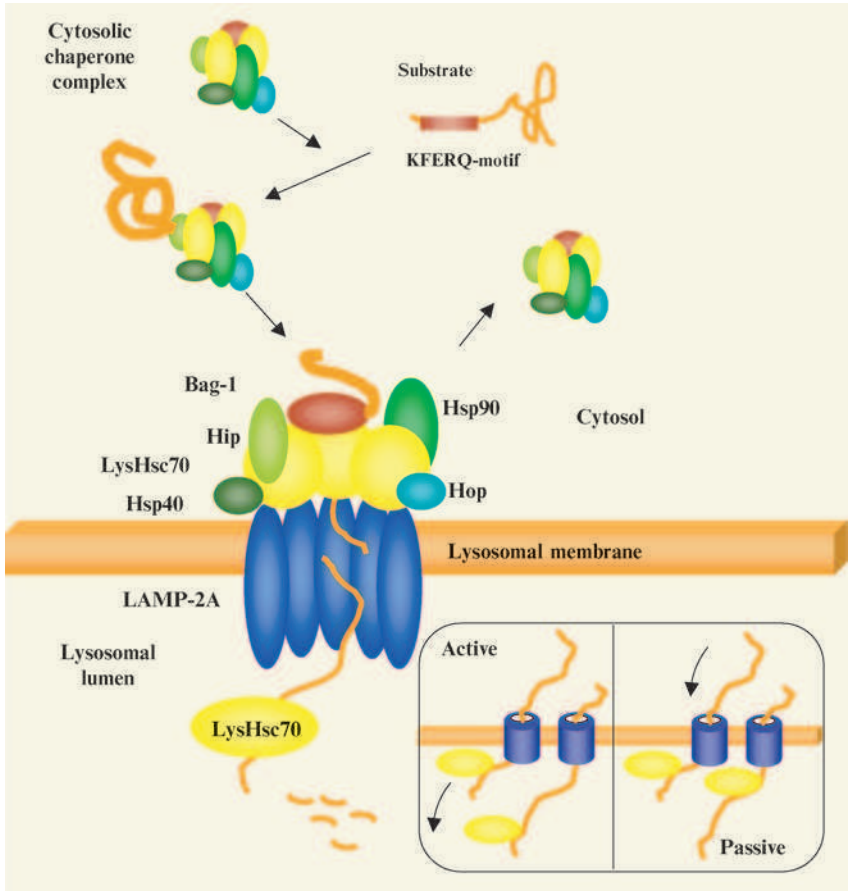


**Figure 2** Schematic model for CMA. The main steps and components of CMA are described throughout the text. Inset shows the two possible mechanisms by which lys-hsc70 may contribute to substrate translocation.

or no effect on the degradation of short-lived proteins or other long-lived proteins. This "selective" degradation of long-lived proteins was shown to occur in lysosomes (McElligott and Dice, 1983).

Independent studies using isolated rat liver lysosomes revealed the existence of direct internalization of different glycolytic enzymes into lysosomes for their degradation (Aniento *et al.*, 1993). Substrate uptake neither did involve morphological changes at the lysosomal membrane, as the ones described for microautophagy, nor did it require formation of intermediate autophagic vacuoles. Subsequent studies revealed that the cytosolic proteins

selectively degraded in lysosomes in the microinjection experiments with culture fibroblasts were also directly taken up by isolated rat liver lysosomes (Cuervo *et al.*, 1994). These studies demonstrated that this selective lysosomal pathway was the same in human fibroblasts and rat liver and was present in many other cell types and tissues (Wing *et al.*, 1991). The observation that chaperones were required for the targeting of substrates toward this selective lysosomal degradation (Chiang *et al.*, 1989) led to the term "chaperone-mediated autophagy" to refer to this lysosomal pathway.

## B. CMA Substrates and Their Interaction with Cytosolic Chaperones

Ribonuclease A (RNase A) was the first CMA substrate identified in the cultured fibroblasts (Dice *et al.*, 1986). A particular pentapeptide in the RNase A sequence, KFERQ, was shown to be necessary for its selective degradation in lysosomes (Dice, 1990). So far, all the other known CMA substrate proteins contain a KFERQ-like motif, which is recognized by hsc70. This motif is composed of a basic (K, R), an acidic (D, E), a hydrophobic (F, I, L, V), and another basic or hydrophobic amino acid and is flanked by a Q on either side. A list of currently identified CMA substrates and their targeting motifs is shown in Table I.

About 30% of cytosolic proteins can be detected by antibodies against the KFERQ-motif, suggesting that this is approximately the number of potential substrates for CMA (Dice, 1990). Interestingly, 80% of the proteins with the KFERQ-like motif can be precipitated by these antibodies, but 20% remain in the cytosolic fraction, suggesting that this motif is not accessible at all times in the substrates. The motif could be hidden in the secondary or tertiary structures of the protein after folding or covered by multimerization or protein–protein interactions, preventing it from being detected by the CMA chaperones.

The KFERQ-like motif in the substrate protein is first recognized by the molecular chaperone hsc70 in the cytosol (Chiang *et al.*, 1989). Binding to the chaperone serves a dual purpose; it facilitates the binding of the substrate protein to LAMP-2A, the CMA lysosomal receptor, and at the same time assists in the unfolding of the substrate protein necessary for its transport into the lysosome. As for most chaperones, the binding of hsc70 to the substrates is modulated by nucleotides (ATP/ADP). The ADP-bound hsc70 binds with high affinity to the substrate protein, while dissociation is promoted when ATP substitutes the ADP. Hydrolysis of the new ATP molecule once again promotes binding to the substrate. A series of cochaperones that form a complex with hsc70 modulate these ATP/ADP binding cycles (Agarraberes and Dice, 2001). For example, hsp40 promotes the

**Table I** Lysosomal Targeting Motifs in Different CMA Substrate Proteins

| Protein Name | Sequence | Properties | Location |
|---|---|---|---|
| Aldolase B | QKKEL | Q++–[ ] | N-terminus |
| | QFREL | Q[ ]+–[ ] | N-terminus |
| | IKLDQ | [ ]+[ ]–Q | N-terminus |
| Annexin I | EFLKQ | –[ ][ ]+Q | N-terminus |
| Annexin II | QKVFD | Q+[ ][ ]– | C-terminus |
| Annexin IV | QELLR | Q–[ ][ ]+ | N-terminus |
| Annexin VI | QEFIK | Q–[ ][ ]+ | C-terminus |
| Aspartate aminotransferase | RKVEQ | ++[ ]–Q | N-terminus |
| c-fos | NLLKE | N[ ][ ]+– | Middle |
| GAPDH | NRVVD | N+[ ][ ]– | C-terminus |
| Glutathione transferase | NKKFE | N++[ ]– | C-terminus |
| Hemoglobin ($\beta$-chain) | QRFFE | Q+[ ][ ]– | N-terminus |
| hsc70 | QRDKV | Q+–+[ ] | Middle |
| | QKILD | Q+[ ][ ]– | C-terminus |
| I$\kappa$B | VKELQ | [ ]+–[ ]Q | C-terminus |
| $\alpha$-2-microglobulin | VDKLN | [ ]–+[ ]N | N-terminus |
| | RIKEN | +[ ]+–N | C-terminus |
| Pax-2 | DVVRQ | +[ ][ ]+Q | N-terminus |
| | QRIVE | Q+[ ][ ]– | N-terminus |
| | QLLRE | Q[ ][ ]+– | Middle |
| 26S proteasome (C8) | IEKLQ | [ ]–+[ ]Q | Middle |
| 19S proteasome (PA28) | QEKVF | Q–+[ ][ ] | Middle |
| Pyruvate kinase | QDLKF | Q–[ ]+[ ] | Middle |
| RNase A | KFERQ | +[ ]–+Q | N-terminus |
| $\alpha$-synuclein | VKKDQ | [ ]++–Q | C-terminus |

[ ] = hydrophobic; + = positively charged; – = negatively charged; c-fos = oncogenic factor GAPDH: glyceraldehyde-3-phosphate-dehydrogenase; hsc70 = heat shock cognate protein of 70kDa; I$\kappa$B = inhibitor of the nuclear factor kappa B; Pax-2 = paired box-related transcription factor-2; RNase A = ribonuclease A.

hydrolysis of ATP into ADP to help the binding of the substrate protein with hsc70; Hip blocks the binding of new ATP molecules, also stabilizing the substrate/hsc70 complex; while Hop and Bag-1 promote substrate release by favoring ATP binding. Some of the cochaperones act directly on the substrate. Thus, hsp90 binds to the unfolded region of a substrate protein to prevent it from aggregation during the cycles of binding/release from hsc70. Other cochaperones act as bridges between hsc70 and the regulatory cochaperones—Hop helps the interaction of hsp90 and hsc70. Blockage of the interaction site of hsc70 with the substrates, depletion of nucleotides or disruption of the chaperone–cochaperone interactions would all block the targeting of substrate proteins into lysosomes (Agarraberes and Dice, 2001).

## C. Lysosomal Receptor for CMA Substrates

The molecular chaperone/substrate complex is then targeted for binding to the lysosomal membrane. Pretreatment of lysosomal membranes with proteases diminished this binding, supporting the existence of a proteinacious docking/receptor component at the lysosomal membrane (Terlecky and Dice, 1993). Known CMA substrate proteins were found to bind to a 96-kDa protein in the membrane of rat liver lysosomes (Cuervo and Dice, 1996). This protein was subsequently identified as the lysosomal associated membrane protein type 2A (LAMP-2A; according to the recent proposed unified nomenclature) (Eskelinen *et al.*, 2005). Overexpression of this protein in culture cells increased CMA activity, while blockage of LAMP-2A with antibodies against its cytosolic tail prevented substrate binding (Cuervo and Dice, 1996). LAMP-2A is one of the protein variants resulting from alternative splicing of the *LAMP*-2 gene (Gough *et al.*, 1995). There are another two protein variants of LAMP-2—LAMP-2B and LAMP-2C. All three isoforms have an identical luminal region, which is highly *N*-glycosylated and believed to protect the lysosomal membrane from luminal ezymes. The last 32 amino acids in their c-terminus, corresponding to their single transmembrane domain and short cytosolic tail, are the differential signature for each of the LAMP-2 isoforms (reviewed in Eskelinen *et al.*, 2005). This region probably determines their different tissue-specific expression and intracellular distribution pattern, as well as their individual functions. CMA substrate proteins bind to the cytosolic tail of LAMP-2A but not LAMP-2B or LAMP-2C (Cuervo and Dice, 1996). Four positively charged amino acids in the LAMP-2A cytosolic tail (KRHH) were proven necessary for the binding of substrate proteins (Cuervo and Dice, 2000b). The specific amino acids in the substrate protein responsible for binding to LAMP-2A remain unknown.

The binding of substrate proteins to LAMP-2A and their subsequent translocation into the lysosomal lumen are the rate-limiting steps in CMA (Cuervo and Dice, 2000a,b). In CHO cells (chinese hamster ovary cells) transfected with LAMP-2A, CMA activity showed a parallel increase to the increase in LAMP-2A (Cuervo and Dice, 2000a). Accordingly, conditions in which levels of LAMP-2A at the lysosomal membrane decrease, such as during aging or in diabetic hypertrophic kidneys, rates of CMA are also reduced (Cuervo and Dice, 2000c; Sooparb *et al.*, 2004). Levels of the LAMP-2A receptor at the lysosomal membrane are regulated through different mechanisms including *de novo* synthesis, blockage of LAMP-2A degradation or dynamic distribution between the lysosomal membrane and matrix, depending on the cellular conditions. The rapid activation of CMA observed during mild oxidative stress is attained through translational upregulation of LAMP-2A (Kiffin *et al.*, 2004). However, in conditions, such as nutritional starvation, when *de novo* synthesis of proteins is limited by the short supply of amino acids, the

increased levels of LAMP-2A at the lysosomal membrane result from decreased degradation of LAMP-2A in this compartment and from the integration of part of the matrix resident LAMP-2A into the lysosomal membrane (Cuervo and Dice, 2000a). Two different proteases participate in LAMP-2A degradation in lysosomes: cathepsin A and a still unidentified metalloprotease (Cuervo *et al*., 2003). Accordingly, in a cathepsin-A knockout mouse in which LAMP-2A degradation is impaired, CMA was found to be upregulated (Cuervo *et al*., 2003).

Different LAMP-2 isoforms may have independent, but also related functions. In complete LAMP-2 knockout mice, which have increased mortality early in life, autophagic vacuoles accumulated in different tissues including liver, pancreas, spleen, kidney, and skeletal muscle. Rates of degradation of long-lived proteins in organs, such as liver, were abnormally low (Tanaka *et al*., 2000). Studies in these animals have revealed that LAMP-2 proteins are involved not only in CMA but also are likely to play a role in macroautophagy and other intracellular functions such as cathepsin targeting and cholesterol trafficking (Eskelinen *et al*., 2002, 2004; Tanaka *et al*., 2000). Further studies focused on each of the LAMP-2 protein variants would help to clarify their individual functions.

## D. At the Other Side of the Lysosomal Membrane

In addition to the cytosolic chaperone/cochaperone complex and the lysosomal membrane receptor, the translocation of substrate proteins into lysosomes by CMA requires the presence of a second chaperone, the lysosomal hsc70, at the luminal side of the lysosomal membrane. Two observations support the role of the luminal hsc70 in CMA substrate translocation: hsc70-blocking antibodies delivered to lysosomes via endocytosis in human fibroblasts in culture reduced rates of CMA (Agarraberes *et al*., 1997); and also, only lysosomes containing hsc70 in their lumen were active for uptake and degradation of CMA substrates (Cuervo *et al*., 1997). No cochaperones have been identified yet for Lys-hsc70.

Lysosomal hsc70 may facilitate substrate entry by binding to the substrate and actively pulling it inside the lysosomal lumen in an energy-dependent manner. However, it is also possible that lys-hsc70 acts passively, locking the protein segment already translocated, so as to prevent any translocation back to the cytosol (Fig. 2).

Levels of lys-hsc70 also change with the activation of CMA. Starvation and oxidative stress increase the amount of lys-hsc70 per lysosome (Cuervo *et al*., 1995a; Kiffin *et al*., 2004). In conditions, such as aging, where CMA activity decreases, an increase in the number of lysosomes containing hsc70 has been described (see later) (Cuervo and Dice, 2000c). Lys-hsc70 may thus

help the activation of CMA during stress, along with the recruitment of lysosomes normally not active for CMA. The mechanisms that regulate the activation of this second group of lysosomes and their physiological relevance remain unknown. Also poorly understood is the pathway followed by hsc70 to enter the lysosomes. So far, no differences have been found between the cytosolic and lysosomal hsc70 other than the prevalence of a more acidic isoform of the protein in the lysosomal lumen (Agarraberes *et al.*, 1997). Since hsc70 has two KFERQ-like motifs, it could be transported into lysosomes by CMA. It is also possible that hsc70 is engulfed by lysosomes through macro- or microautophagy and only a particular isoform (the one with the most acidic isoelectric point) becomes resistant to the lysosomal enzymes and remains in the lysosomal lumen. This could also explain the sequential activation of macroautophagy and CMA during nutritional stress.

### E. Methods to Measure CMA

The activity of CMA can be measured in confluent cells in culture as the increase in the degradation rates of long-lived proteins after removing the serum from the culture medium (Dice, 1990; Majeski and Dice, 2004). Macroautophagy is activated during the first hours of serum removal but formation of autophagic vacuoles does not occur after 4–6 hr of starvation. After this, CMA activity increases progressively, reaching maximal activation at about 10 hr after the removal of serum, and remains at this level for the rest of the starvation period (Dice, 1990; Majeski and Dice, 2004). Because different proteolytic pathways can be simultaneously activated in response to stressors, combinations of different inhibitors have been effectively used to determine the contribution of CMA to the degradation of long-lived proteins under particular conditions. CMA-dependent degradation is defined as that activated by serum removal, sensitive to ammonium chloride (as any other form of autophagy), but insensitive to phosphatidyl kinase 3 inhibitors (which block macroautophagy).

The unequivocal method to measure CMA, however, is by directly tracking the translocation of substrate proteins into lysosomes (Fig. 3). CMA can be reconstituted *in vitro* using the population of lysosomes active for CMA (Aniento *et al.*, 1993; Terlecky and Dice, 1993). After incubation of these lysosomes with the substrate protein in an isotonic medium supplemented with hsc70 and nucleotides, binding of substrate to the lysosomal membrane can be determined as the amount of substrate pulled down with the lysosomes because any protein internalized by the lysosomes will be rapidly degraded (the half-life of substrate proteins in the lysosomal lumen is of

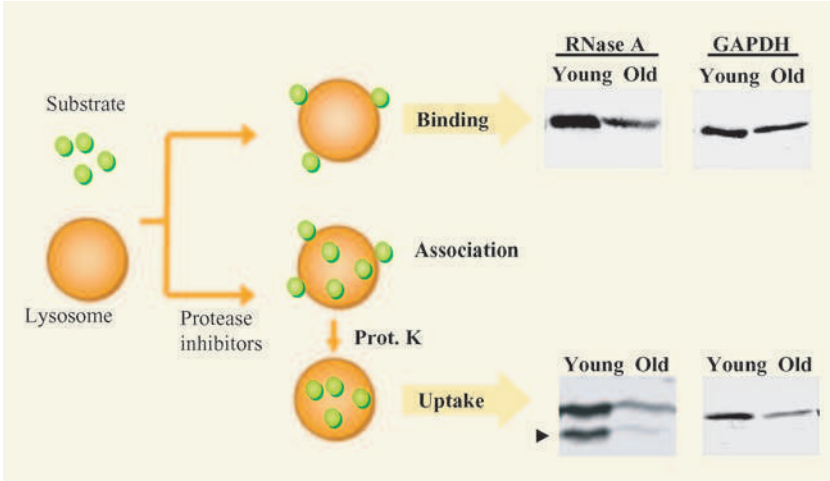


**Figure 3** Measurement of CMA *in vitro*. Binding and translocation of substrate proteins into lysosomes can be reproduced in an *in vitro* system using isolated lysosomes supplemented with the corresponding chaperones and an energy-regenerating system. The basis for this assay is described in the text. Prot. K, proteinase K.

about 5 min). Translocation of substrate proteins can be tracked using lysosomes for which their proteolytic activity has been previously blocked with proteinase inhibitors. The amount of substrate translocated into the lysosomal lumen can be determined as the one resistant to cleavage by an exogenously added protease (Fig. 3).

## III. Physiological Role of CMA

Although degradation of proteins through CMA is likely to occur to some extent under basal conditions (e.g., lysosomes isolated from fed rodents are able to take up and degrade CMA substrates), the lack of selective inhibitors for this autophagic pathway makes it difficult to estimate its contribution to basal protein turnover. Better characterized is the activation of CMA under different conditions primarily related to stress such as nutritional and oxidative stress (Majeski and Dice, 2004; Massey *et al.*, 2004). Recently, exciting new roles have been proposed for CMA, such as a possible participation of CMA in endogenous antigen presentation, as is commented in the last part of this section.

### A. CMA and Starvation

Nutritional stress—serum removal in culture cells or starvation in rodents—sequentially activates both macroautophagy and CMA. The reason and molecular basis for switching from a nonselective to a selective degradation are still unclear but, probably obey to cellular adaptations to a nutrient-limiting environment. Early during starvation, cells respond to the lack of nutrients by accelerating the catabolism of most cytosolic components through macroautophagy. As starvation persists, this adjustment is probably insufficient to help the cell antagonize the adverse conditions. The cell now protects some essential proteins from degradation by shutting down macroautophagy and activating a more selective mechanism, CMA. Activation of CMA allows the removal of nonessential proteins to obtain the amino acids required for the synthesis of essential proteins for cell survival. Increased CMA activity can be detected even after 88 hr of starvation in rodents (Cuervo *et al.*, 1995a). The molecular signaling mechanisms that lead to CMA activation under these conditions still remain unknown. It is possible that some of the products generated by macroautophagic degradation may have a direct stimulatory effect on CMA. However, it is also possible that some endogenous inhibitor of CMA is degraded by macroautophagy, leading to progressive activation of CMA late in starvation. Changes directly in the lysosomal compartment are also possible. It has been reported that activation of CMA always coincides with the relocation of CMA-active lysosomes (those containing higher levels of hsc70 and LAMP-2A) to the perinuclear region of the cells, where they often take the appearance of elongated or even tubular structures (Agarraberes *et al.*, 1997; Cuervo and Dice, 2000b). An attractive possibility could be that these lysosomes relocate to this region of the cell to facilitate homotypic fusion between lysosomes to transfer the luminal chaperone from CMA active lysosomes to those that are not normally active, and consequently, recruit them to this pathway. In fact, we have found that, after prolonged starvation, there is an increase in the total number of lysosomes containing lys-hsc70, supporting the proposed recruitment of a "reservoir" pool of lysosomes for CMA (Cuervo *et al.*, 1995a).

### B. CMA and Oxidation

Many lines of evidence support a role for oxidative stress as a precipitating factor in the pathogenesis of many different disorders (neurodegeneration, inflammatory lung diseases, diabetes, rheumatoid arthritis, artherosclerosis, and motor neuron disorders, among others) and also in the course of physiological aging. Different biochemical and immunological methods

allow the quantification of levels of oxidized proteins in biological systems, as well as the analysis of their intracellular turnover (Brune *et al.*, 2003; Kohen and Nyaska, 2002). A vast amount of literature supports the degradation of oxidized proteins by means of the proteosomal system both *in vivo* and *in vitro* (Friguet *et al.*, 2000; Grune *et al.*, 2003; Keller *et al.*, 2000; Shringarpure *et al.*, 2001; Stolzing and Grune, 2001). Oxidation of some proteins increases their degradation by the 20S proteasome *in vitro*, presumably through exposure of hydrophobic patches, that are recognized directly by the proteasome without the necessity of ubiquitination (Shringarpure *et al.*, 2003). The fact that the treatment of culture cells with proteasome inhibitors or antisense oligonucleotides against essential proteasome subunits results in accumulation of oxidized proteins within cells and decreased cell survival following oxidative stress exposure, supports that the removal of oxidized proteins in a ubiquitin and ATP independent manner by the proteasome also occurs *in vivo* (Shringarpure *et al.*, 2003). However, selective ubiquitinization of oxidized proteins could also be used *in vivo* to target them to the proteasomal system, since a ubiquitin ligase (E3) that selectively ubiquitinizes proteins following oxidation via interaction with iron has recently been identified (Iwai, 2003).

The lysosomal system was never considered to be involved in the removal of oxidized proteins due to the lack of selectivity initially attributed to this system. In fact, most of the work done previously in regards to oxidative stress and the lysosomal system focused on these organelles' contribution to oxidation-induced damage, due to the destabilization of the lysosomal compartment, reported during exposure to severe oxidative conditions (Brunk *et al.*, 1995; Ollinger and Brunk, 1995). However, various evidences pointed toward a possible role of CMA in the removal of oxidized proteins. Added to the selectivity of this pathway, were the fact that (1) CMA activity decreased in aged cells, which have an increased presence of oxidized proteins (Cuervo and Dice, 2000c), (2) the activation of CMA during toxic exposure results in the selective degradation of a protein altered by the toxic compound (Cuervo *et al.*, 1999), and (3) in the presence of antioxidants, degradation of I$\kappa$B via CMA is decreased (Cuervo *et al.*, 1998). Recently, the role of CMA in the removal of oxidized proteins has been confirmed (Finn and Dice, 2005; Kiffin *et al.*, 2004). Oxidized proteins can be detected in the lumen of CMA active lysosomes, but lysosomal levels of oxidized proteins decrease in conditions with declined CMA activity such as aging. In addition, oxidized CMA substrates bind and internalize into isolated CMA active lysosomes at higher rates than their unmodified counterparts. Activation of CMA seems to be part of the cellular response to oxidative stress, since CMA active lysosomes isolated from both rats and cells exposed to prooxidants show higher rates of binding and uptake of CMA substrates, and blockage of CMA in cultured cells decreases the cells' viability following exposure to different prooxidant

compounds (Kiffin *et al.*, 2004; Massey *et al.*, 2006). The mechanisms by which oxidative stress activates CMA-dependent degradation are in fact dual. On one hand, mild oxidative stress alters the conformation of CMA substrates, presumably facilitating their recognition by the chaperone and reducing the time normally required for unfolding. In addition, there is also an upregulation of specific components of the CMA lysosomal translocation complex by means of increased *de novo* synthesis of LAMP-2A and an increase in levels of two of the chaperones associated to the lysosomal membrane (hsc70 and hsp90) (Kiffin *et al.*, 2004). This novel mechanism of CMA activation also suggests that the stress-mediated activation of CMA varies depending on the nature of the stress (Kiffin *et al.*, 2004).

Further evidence for the contribution of CMA to the removal of oxidized proteins has been provided by Finn and coworkers when they showed that the increased presence of ketone bodies, during periods of prolonged starvation, facilitated the removal of CMA substrates because of the mild prooxidant effect of the ketone bodies on the substrates (Finn and Dice, 2005). Ketone bodies, in the form of $\beta$-hydroxybutyrate (BOH), acetoacetate, and acetone, are products of rapid lipid lipolysis and are generated during periods of prolonged starvation as fuels for muscle and the brain (Kalapos, 2003; Sumbilla *et al.*, 1981). The increase in the concentration of ketone bodies during these prolonged periods of nutrient deprivation parallels with the increase in CMA activity (Cuervo *et al.*, 1995a; Dice, 1990; Wing *et al.*, 1991). Even in cells maintained in the presence of serum, addition of ketone bodies induced higher rates of CMA-dependent degradation (Finn and Dice, 2005). BOH-treated substrates were degraded by lysosomes at higher rates than untreated ones because this treatment increased their level of oxidation (Finn and Dice, 2005). As under the mild-oxidative stress conditions reported before (Kiffin *et al.*, 2004), the oxidation mediated by ketone bodies could modify the substrate proteins in such a way that they are targeted and degraded via CMA more efficiently. For example, oxidation could induce conformational changes that may expose hidden KFERQ-like motifs and allow better chaperone interaction. The work of Finn and coworkers not only supports the increase of CMA activity in conditions of oxidative stress but also provides a novel approach for why CMA is activated upon starvation. However, in contrast to the mild-oxidative stress conditions reported before, exposure to ketone bodies alone did not significantly modify the levels of the lysosomal components (receptor and chaperones) involved in CMA (Finn and Dice, 2005). Thus, other, still unidentified, factors may contribute to the activation of CMA during prolonged starvation.

The question still remains as to how the cell determines whether an oxidized protein is degraded by the proteasome or the lysosome. An interesting idea was proposed by Gracy *et al.* (1998) who hypothesized that covalent modifications may convert a non-CMA substrate into a CMA

substrate and target it for degradation by the lysosomal system. These modifications or "terminal markings" could be caused by different biochemical reactions, such as oxidation, deamidation, and/or amino acid conversion, which all occur as the proteins undergo "wear and tear" (Gracy *et al.*, 1998). These altered proteins would be more easily degraded by proteases due to their increased instability and more readily unfolded states. This should also facilitate easier recognition of targeting motifs by chaperones, increasing the degradation via CMA of proteins normally containing the KFERQ-motif. But in addition, Gracy *et al.* (1998) propose that some of the protein modifications could result in development of a KFERQ-like motif in a protein that was not initially a CMA substrate. For example, if a histidine gets oxidized, it would then behave as a negatively charged residue, which could complete a KFERQ-like motif if the other required amino acids were already in place. Although, there is not yet experimental evidence supporting this hypothesis, if proven true, it would greatly increase the pool of cytosolic proteins that are potential candidates for degradation via CMA and consequently would justify the recent efforts by our group and others to restore normal activity of this pathway in aging.

## C. CMA and Antigen Presentation

Antigen presenting cells are involved in presentation of both exogenous and endogenous peptides for recognition by T cells. These peptides are presented on two types of major histocompatibility complex (MHC) molecules, class I and class II. It was classically accepted that MHC-I molecules were involved in the presentation to CD8+ T cells of antigenic endogenous peptides generated by the proteasomal system. Alternatively, MHC-II molecules would be the ones involved in the presentation of antigenic peptides obtained from the extracellular environment or the membrane, by endocytosis or phagocytosis, and present them to CD4+ T cells, after their processing in the endosomal-lysosomal system (reviewed in Pamer and Cresswell, 1998; Princiotta *et al.*, 2003; Rammensee *et al.*, 1993; Villadangos, 2001). Several reports have described endogenous peptides being presented on MHC-II molecules, although, until recently, the mechanisms behind this presentation were unclear (Aichinger *et al.*, 1997; Bonifaz *et al.*, 1999; Brazil *et al.*, 1997; Chicz *et al.*, 1993; Jaraquemada *et al.*, 1990; Lechler *et al.*, 1996; Lich and Blum, 2001; Malnati *et al.*, 1992; Nimmerjahn *et al.*, 2003; Weiss and Bogen, 1991). The participation of autophagy in the presentation of endogenous peptides by MHC-II molecules was proposed in a number of these reports, but only recently, direct evidence has been presented that an endogenous antigen (nuclear antigen 1 of the Epstein Barr virus, i.e., EBNA1) requires macroautophagy for its presentation (Paludan *et al.*, 2005). Dengjel *et al.*

(2005), using a detailed ligand repertoire ("ligandome") of MHC-II ligands, have also reported a significant increase in the presentation of intracellular peptides on MHC-II molecules following the activation of macroautophagy. A decrease in the level of active cathepsins in the endocytic compartment under these conditions seems to also favor the generation of MHC-II peptides, due to less efficient lysosomal protein degradation (Dengjel *et al.*, 2005).

CMA has also recently been implicated as a key player in the presentation of endogenous peptides by MHC-II. Zhou *et al.* (2005) have shown that reduced expression of LAMP-2 by antisense against the entire *LAMP-2* gene, resulted in a reduction in the level of both endogenous and exogenous peptide presentation via MHC-II. Restoration of normal levels of LAMP-2A but not of LAMP-2C, increased cytoplasmic antigen presentation, while the presentation of exogenous peptides was unaltered. A similar effect was observed when intracellular levels of hsc70 were manipulated. Higher levels of hsc70 increased endogenous antigen presentation, while there was a decrease in this presentation when levels of hsc70 were reduced. Even though the participation of the two major players in CMA, LAMP-2A and hsc70 implicates CMA as a key player involved in antigen presentation (Zhou *et al.*, 2005), further analysis is needed to determine how exactly CMA contributes to this process.

## IV. CMA and Aging

Both macroautophagy and CMA activity decrease with age (reviewed in Cuervo *et al.*, 2005; Martinez-Vicente *et al.*, 2005). Induction of macroautophagy, formation of autophagosomes, and their fusion with lysosomes, have all been shown to be impaired in old cells (Donati *et al.*, 2001). Accumulation of undigested products, in the form of lipofuscin, in the lysosomal lumen, seems to be one of the main reasons for defective clearance of autophagic vacuoles with age. In the case of CMA, the pool of lysosomes active for CMA—those containing high levels of the luminal chaperone hsc70—do not undergo major morphological changes with age, neither do they accumulate visible undigested products in their lumen. Binding of substrate proteins to chaperones and their targeting to the lysosomal membrane seems to be preserved until late in life, as is the proteolytic activity of the lysosomal enzymes in CMA-active lysosomes. However, binding and uptake of the substrate proteins into lysosomes are drastically reduced with age, resulting in decreased CMA activity (Cuervo and Dice, 2000c) (Fig. 3). The previously mentioned decrease in the levels of LAMP-2A at the lysosomal membrane with age has been the major defect described so far in the old lysosomes. Levels of LAMP-2A decline before alterations in CMA

activity are manifested. In fact, low levels of the lysosomal receptor are initially compensated through an increase in the number of lysosomes involved in this autophagic pathway. However, at advanced ages, levels of the receptor decrease to such an extent that the CMA defect becomes evident (Cuervo and Dice, 2000c). The reason(s) for the LAMP-2A decrease remains to be elucidated but, altered turnover of the receptor itself and/or changes in its intracellular distribution could be behind the low LAMP-2A levels.

The most direct consequences of reduced CMA during aging, based on its different intracellular functions, would be the accumulation of oxidatively damaged proteins and the deregulation of the response to stress. However, the severity of these consequences would be modulated in each tissue depending on the activity of the other proteolytic pathways. Several lines of evidence support the notion that different proteolytic pathways can compensate for each other to some extent. For example, chronic and acute blockage of the proteasome results in changes in macroautophagy (Ding *et al.*, 2003; Iwata *et al.*, 2005) and cells defective in macroautophagy show abnormally elevated CMA activity (Kaushik *et al.*, unpublished results). Because some subunits of the 20S proteosome contain KFERQ-like motifs and have been shown to be degraded via CMA (Cuervo *et al.*, 1995b), it is likely that impaired CMA activity also has direct consequences on the activity of the ubiquitin/proteasome proteolytic pathway.

## V. Pathologies Related to CMA

The better understanding of the physiological role of CMA, along with the identification of new substrate proteins, has allowed the association of CMA with different pathological conditions. The following sections address some of the pathologies in which CMA activity has been shown to be altered and it may be one of the contributing factors for the development, persistence, and/or defense against those conditions.

### A. CMA and Nephropathies

Although an increase in the levels of the lysosomal luminal resident chaperone hsc70 was observed in a form of acute tubular necrosis induced by inorganic mercury (Hernandez-Pando *et al.*, 1995), the first direct connection of a change in CMA activity associated with any kidney pathology was with the hyaline droplet nephropathy (Cuervo *et al.*, 1999). Chronic exposure to various environmental toxins, such as gasoline derivatives, results in the accumulation of hyaline droplets within the proximal tubule epithelial cells in rat kidneys, which eventually lead to loss of cell function and cell

death (Lehman-Mckeeman *et al.*, 1991). The main component of these hyaline droplets is the lipoprotein $\alpha$-2-microglobulin ($\alpha$-2m), which is the main target of many of these compounds. Even though the type of conformational modification induced by the toxic compounds in $\alpha$-2m is still controversial, it is well accepted that it results in an increased propensity for aggregation and resistance to protease cleavage. Damage occurs preferentially in kidney, because although $\alpha$-2-m is synthesized and secreted from the liver, it is delivered to kidney where it is functionally active (Chaudhuri *et al.*, 1999). As a secretory protein, this lipoprotein functions in the transport of fatty acids into renal epithelial cells. A portion of the intracellular pool of $\alpha$-2m can be detected as a soluble protein in the cytosol in both liver and kidney, although its functional significance remains unknown. This $\alpha$-2m contains in its amino acid sequence a KFERQ-like targeting motif, and it was demonstrated that most of the cytosolic form of the protein is normally degraded in lysosomes via CMA (Cuervo *et al.*, 1999). After exposure to gasoline derivatives, CMA is activated, even under normal nutritional conditions, in liver and kidney. The mechanisms that lead to increased CMA under these conditions still remain unclear. Levels of LAMP-2A at the lysosomal membrane were higher in treated animals when compared to untreated animals (Cuervo *et al.*, 1999). CMA activation could be due to the direct interaction of toxic compounds or their by-products with factors that modulate CMA. However, as in other protein conformational disorders (see later), it is more likely that cells respond to the increased levels of modified $\alpha$-2m in the cytosol by upregulating CMA. CMA may become active upon exposure to such compounds as a means of selectively removing the altered protein from the affected tissues. Although this mechanism seems to facilitate the clearance of the damaged $\alpha$-2m in the early stages of the disease, as the pathology progresses, CMA activation is not enough to remove all of the damaged protein and its accumulation in the kidney results in functional failure of the organ. In contrast to other protein conformational disorders in which late activation of macroautophagy after CMA failure guarantees protein removal, at least for some period of time (see later), compensatory activation of macroautophagy has not been found in these nephropathies.

CMA seems to play an important role in the stress response of the kidney. Although different cytosolic proteolytic systems, such as the calpains and the ubiquitin-proteasome system, are essential in maintaining homeostasis of the kidney under normal growing conditions, CMA has been shown to be a major player in regulating kidney growth (Franch *et al.*, 2002). A decrease in the degradation of KFERQ-containing proteins has been observed upon the treatment of kidney cells with epidermal growth factor (EGF) (Franch *et al.*, 2001). Increasing the levels of different KFERQ-containing proteins, by slowing down their degradation, possibly provides

cellular conditions that favor cell growth. An increase in the levels of a glycolytic enzyme, glyceraldehyde-3-phosphate dehydrogenase, a well-characterized CMA substrate, and of the transcription factor Pax2, which also bears a KFERQ-like motif and has been implicated in renal cell growth, was found upon treatment of kidney epithelial cells with EGF (Franch *et al.*, 2001). The increased levels of these glycolytic enzymes and transcription factors could assist the higher demand for energy as the cells grew (Franch *et al.*, 2001). A decrease in levels of the lysosomal membrane protein LAMP-2A was also seen in these conditions, thereby providing further evidence for the downregulation of CMA during kidney growth. How exactly CMA activity is suppressed in the presence of EGF is unknown but it has been reported that EGF suppresses the degradation of proteins by a mechanism that involves class 1 PI 3-kinase and Ras (Franch *et al.*, 2002).

Decreased CMA activity and increased intracellular levels of KFERQ-containing proteins has also recently been reported during renal hypertrophy associated with acute diabetes mellitus (Sooparb *et al.*, 2004). Growth of the already mature kidney occurs in many disorders, including diabetes and acidosis. Using streptozotocin (STZ)-induced diabetes in rats as a model, Sooparb *et al.* (2004) showed that protein degradation was significantly decreased, while synthesis was increased in the renal cortex. Levels of KFERQ-containing proteins, such as Pax 2, and several glycolytic enzymes were also higher in these induced-diabetic rats (Sooparb *et al.*, 2004), and lysosomal levels of LAMP-2A and hsc-70 were reduced (Sooparb *et al.*, 2004). Overall, these findings provide further evidence for the correlation between kidney growth and chronic suppression of CMA activity (Sooparb *et al.*, 2004).

## B. CMA and Lysosomal Storage Disorders

Lysosomal storage disorder (LSD) is the commonly used term to categorize genetic diseases which result in defects in the degradation of different macromolecules within lysosomes (reviewed in Neufeld, 1991; Wenger *et al.*, 2002). These diseases are often caused by either malfunctioning or lack of a specific enzyme within the lysosome or by defects in delivery of the substrate protein itself to the lysosome. Enzyme malfunctioning can result from direct alterations of its catalytic site, disabling its ability to interact with its substrate protein, or from defects in its intercellular trafficking, resulting in mistargeting of the enzyme out of the lysosome (Mach, 2002). Although for each LSD the substrates accumulated within lysosomes are different, the phenotype that results from this accumulation is often very similar: enlargement of the lysosomal compartment, failure of different lysosomal functions, and eventually, leakage of lysosomal contents into cytosol or extracellular medium

(Neufeld, 1991; Wenger *et al.*, 2002). Most of the classically known LSDs originated from defects in glycases and lipases; however, in recent years, several LSDs have been shown to be the result of decreased presence of specific lysosomal proteases (cathepsins). In fact, the phenotype observed in a long known LSDs, Batten's Disease, has recently been linked to a defect in cathepsin D, by comparison to a knockout mouse for this lysosomal protease (Dawson and Cho, 2000). Among many other lysosomal functions, CMA activity is likely to be affected secondarily to the original defect in many of these disorders.

To date, galactosialodosis is the only LSD in which CMA activity has been shown to be altered. In this LSD, CMA activity does not decrease but is, in fact, increased. This increase occurs due to the presence of higher levels of the CMA receptor, LAMP-2A, on the lysosomal membrane of cells affected by the disorder (Cuervo *et al.*, 2003). The enzyme defective in galactosialidosis, cathepsin A/protective protein, is required to stabilize two glycosidases in the lysosomal compartment (van der Spoel *et al.*, 1998). A defect or lack of this cathepsin results in the malfunctioning of these two enzymes and an intralysosomal accumulation of their substrates. In addition, we found that cathepsin A is also involved in regulating levels of LAMP-2A on the lysosomal membrane (Cuervo *et al.*, 2003) through its previously described serine protease activity (Galjart *et al.*, 1991). The turnover of LAMP-2A, once on the lysosomal membrane, is mediated by the combined action of cathepsin A and a still-unidentified metalloprotease (Cuervo and Dice, 2000a). Association of cathepsin A, a luminal enzyme for the most part, to the lysosomal membrane, is the limiting step in the degradation of LAMP-2A. In cells lacking cathepsin A, LAMP-2A degradation is impaired resulting in continuously higher levels of this receptor on the lysosomal membrane. The constitutive activation of CMA, observed in fibroblasts from both patients with galactosialodosis or cathepsin A knockout mice, is thus a consequence of the abnormally prolonged half-life of LAMP-2A in these cells (Cuervo *et al.*, 2003). It is this constitutive activation, and the subsequent increased degradation caused by it, that may result in the loss of weight and asthenia in patients with this LSD. Worthy of note, is the fact that enzyme replacement resulting in restoration of cathepsin A in the defective fibroblasts reestablishes LAMP-2A degradation and returns CMA activity to normal levels (Cuervo *et al.*, 2003).

Even though for a long time all LSDs were related to a defect in lysosomal enzymes, recently, alterations in different lysosomal membrane proteins have been shown to result in similar phenotypes. A primary defect in the *LAMP-2* gene has been observed in Danon Disease, a lysosomal glycogen storage disorder with normal maltase activity (reviewed in Saftig *et al.*, 2001). However, how CMA is affected in the model system with this disease is difficult to interpret, since in most of these patients mutations occur in the

common region for all LAMP-2 isoforms, so not only is LAMP-2A affected but so are the other two isoforms, namely LAMP-2B and LAMP-2C. As mentioned in the previous sections, Danon disease patients present extensive accumulation of autophagic vacuoles in skeletal and cardiac muscle fibers, which eventually led to muscle weakness, degeneration, and early death by heart failure (Saftig *et al*., 2001; Tanaka *et al*., 2000). Vacuolation has been shown to result from poor clearance of autophagic vacuoles once formed in the cytosol, pointing toward a primary defect in macroautophagy in these patients. The fact that in a particular group of patients the mutation localizes in the LAMP-2B exon and they still present the whole vacuolar phenotype has lead to the hypothesis that LAMP2-B plays a role in macroautophagy. Patients with mutations affecting only LAMP-2A or LAMP-2C have not yet been identified. A better understanding and correlation of what is occurring in Danon Disease in regards to CMA activity would be possible once the function of all three splicing variants is determined.

## C. CMA and Neurodegeneration

Alteration in both lysosomal and proteasomal protein degradation have been described in different neurodegenerative disorders (reviewed in Jellinger and Stadelmann, 2000; Larsen and Sulzer, 2002; Yuan *et al*., 2003). It has become more generally accepted that the defective activity of the proteolytic systems is really related and due to the existence of cross talking among the different proteolytic pathways. Therefore, an alteration in one of the systems will have consequences on the others (Fig. 4).

Neurodegenerative disorders are in fact protein conformational disorders in which the modification (mutation or posttranslational modification) of the structure of a particular protein makes it principally prone to formation of abnormal oligomeric complexes (profibrillar structures, fibrils, and aggregates) that accumulate inside the affected cells and eventually lead to cellular death. This alteration in the way that these proteins organize inside cells also changes the way in which they can be degraded. Most of these proteins, when in a monomeric soluble cytosolic form, can be substrates for the proteasome or lysosomal degradation via CMA (Fig. 4). However, once they organize in these complex structures, only activation of macro- or microautophagy could lead to their degradation. An added problem in these disorders is that the modified proteins and their by-products often have a direct inhibitory effect on the proteolytic systems. Several of these products have been shown to inhibit the ubiquitin/proteasome system in experimental models of Parkinson's and Huntington's disease (Bennett *et al*., 2005; Ciechanover and Brundin, 2003; Grune *et al*., 2004). Also, A$\beta$, the toxic

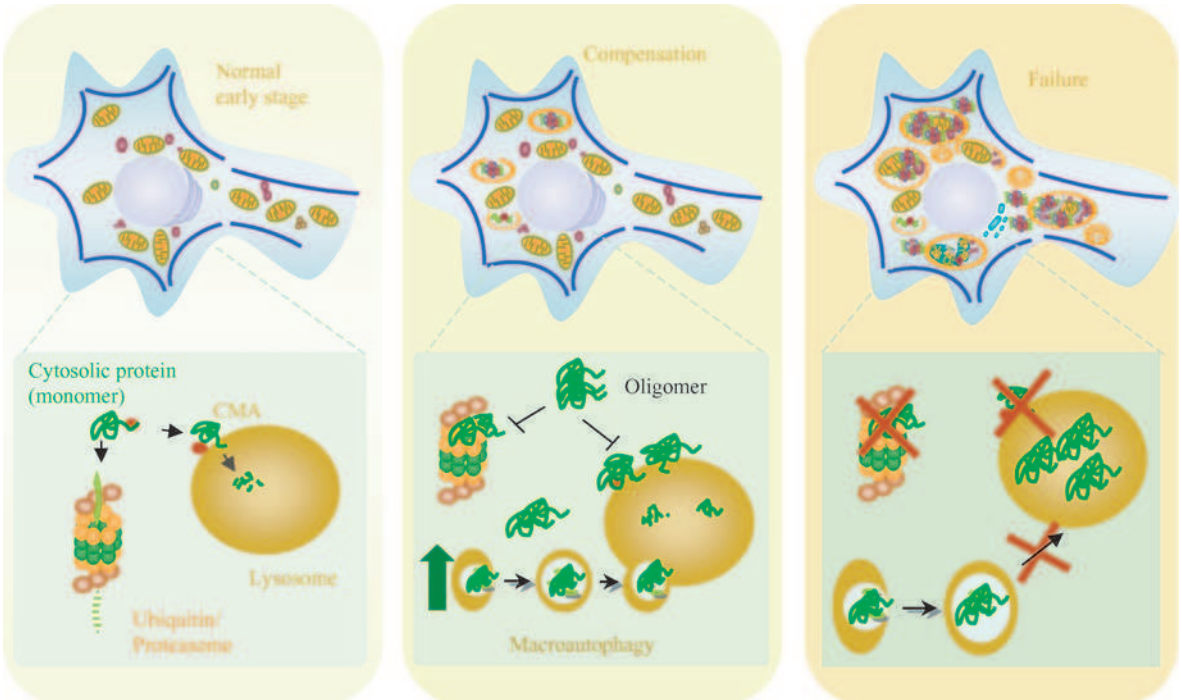


**Figure 4** Role of the different proteolytic systems in protein conformational disorders. The presence of misfolded cytosolic proteins is handled by different proteolytic systems, depending on the level of misfolding/aggregation that the proteins reach. Soluble monomeric misfolded proteins can be degraded by both the ubiquitin proteasome system and CMA. However, as the abnormal proteins organize into oligomeric structures, they can no longer be substrates for these pathways but instead often end up congesting and blocking them. In response to this blockage, macroautophagy is activated to promote the removal of semiaggregate proteins. With time, though, a failure of the macroautophagic pathway occurs, leading to the accumulation of tightly aggregated protein complexes both in the cytosol and inside autophagic/lysosomal compartments. (See Color Insert.)

peptide that accumulates in the brain of patients with Alzheimer's disease, interferes with normal macroautophagic activity in culture cells (Yu *et al.*, 2005). Inhibition of CMA activity by one of these abnormal protein products has recently been shown in some familial forms of Parkinson's disease (Cuervo *et al.*, 2004).

Although, three proteins known to be involved in major neurodegenerative disorders—Amyloid Precursor Protein (for Alzheimer's disease), $\alpha$-synuclein (for Parkinson's disease), and huntingtin (in Huntington's disease)—contain KFERQ-like motifs, potentially making them substrates for CMA, only $\alpha$-synuclein has been shown, so far, to be a substrate for this pathway (Cuervo *et al.*, 2004). Using the *in vitro* system described in previous sections, we have found that wild type $\alpha$-synuclein binds, is taken up and degraded via CMA by intact lysosomes (Cuervo *et al.*, 2004). Furthermore, a portion of intracellular $\alpha$-synuclein interacts with hsc70 and LAMP-2A, and blockage of CMA in culture cells results in decreased rates of $\alpha$-synuclein degradation (Cuervo *et al.*, 2004). In contrast, none of the mutant forms of this protein, which have been implicated in the development of familial forms of Parkinson's disease, can be degraded by CMA (Fig. 4). The mutant proteins bind very strongly to the CMA receptor on lysosomes but do not translocate into the lysosome efficiently (Cuervo *et al.*, 2004). Moreover, the tight binding of these mutant forms of the protein to the lysosomal receptor interferes with the transport of other CMA substrates and eventually results in CMA blockage (Cuervo *et al.*, 2004). We have also recently found that although CMA blockage does not affect cell viability under normal conditions, it makes cells more susceptible to all kinds of stressors, resulting often in enhanced cellular death under those conditions (Massey *et al.*, 2006). Not only mutations but some frequent covalent modifications of $\alpha$-synuclein, such as oxidation, nitration, and formation of dopamine adducts, also modify the ability of this protein to be degraded by CMA (Martinez-Vicente *et al.*, submitted). These results suggest that the mutant forms of $\alpha$-synuclein found in familial PD, and possibly the modified forms of the protein found in the idiopathic forms of the disease, are directly involved in the observed impairment of CMA activity in this disorder.

Since wild type $\alpha$-synuclein can be a substrate for both the ubiquitin/proteasome system and the lysosomes (via CMA) a critical remaining question is what decides the degradation of this protein to occur by one or the other pathway. A recent study by Shin *et al.* (2005) describes the cochaperone CHIP (carboxyl terminus of Hsp70-interacting protein) as being the molecular switch that decides between the two proteolytic pathways. CHIP is composed of three major domains: an amino terminal tetratricopeptide (TPR) domain, a highly charged central domain, and a carboxyl-terminal U-box domain. CHIP has been shown to interact *in vitro* with both hsc70 (the constitutively expressed protein) and hsp70 through its TPR domain

(Ballinger *et al.*, 1999). In addition, CHIP is itself an E3 ubiquitin ligase, involved in ubiquitinylation of particular substrate proteins (Jiang *et al.*, 2001). While the TPR domain is believed to regulate degradation by means of the proteasome, Shin *et al.* (2005) have elegantly shown that the U-box domain is essential and sufficient for targeting of $\alpha$-synuclein for degradation by means of the lysosomes. Although direct degradation via CMA could not be proven, the fact that we and others have found that blockage of macroautophagy does not have any effect on the degradation of wild type $\alpha$-synuclein (Cuervo *et al.*, 2004; Webb *et al.*, 2003), strongly supports CHIP facilitating the degradation of $\alpha$-synuclein via CMA.

Abnormal accumulation of autophagic vacuoles containing neuromelanin and lipofuscin has been described in neurons of Parkinson, Huntington, and Alzheimer disease patients (Cataldo *et al.*, 2000; Kegel *et al.*, 2000; Nixon *et al.*, 2000; Qin *et al.*, 2003; Yu *et al.*, 2004). The increase in macroautophagy seems to be a protective response of the cells, aimed to eliminate these more complex forms of the mutant protein (fibrils and aggregates) that cannot be degraded by the proteasome or by CMA (Fig. 4). It is not clear what triggers the activation of macroautophagy under these conditions, but it could be a direct consequence of the effect of the abnormal protein products on the other proteolytic systems. Thus, acute or chronic inhibition of the proteasome, as well as of CMA, activates macroautophagy (Ding *et al.*, 2003; Iwata *et al.*, 2005; Massey *et al.*, 2006).

In the advanced stages of most of these protein conformational disorders, an increase in autophagic vacuoles is still observed, but it often reflects poor clearance of autophagic vacuoles (failure of macroautophagy) rather than macroautophagy activation (Fig. 4). It is still unclear as to whether or not the increased presence of these autophagic vacuoles occurs due to defects in the fusion of these compartments with the lysosome or due to the inability of the lysosomal enzymes to degrade the aggregate components present inside the autophagic vacuoles.

## VI. Concluding Remarks

Although recent findings have led to better understanding and novel insights into roles of CMA in normal cellular functioning and different pathologies, there are still many aspects about this autophagic pathway that require further investigation. A long-standing question has been which is the transporter or channel for this pathway. Some recent studies point toward LAMP-2A multimerization at the lysosomal membrane being required for translocation (Cuervo and Dice, 2000b). However, it is unlikely that LAMP-2A itself is the transporter for this pathway. Also, the events that take place on both sides of the lysosomal membrane to mediate translocation are still unclear: what is

the role of each of the chaperones and cochaperones on the cytosolic side of the membrane? How does the luminal chaperone recognize the translocated substrate? Where does it find the ATP required for its activity? How is the leakage of lysosomal contents prevented during translocation? Other than the different content of hsc70, very little is known about the differences between CMA active and inactive lysosomes: are there different steps of maturation in the lysosomal biogenic process? What determines their differences in chaperone content? Do both groups of lysosomes participate in other forms of autophagy?

There is still very little information on the changes in CMA in other disorders for which changes in protein degradation, or in other forms of autophagy, have already been reported. A decrease in total protein degradation occurs in many types of cancer (reviewed in Ogier-Denis and Codogno, 2003), and impaired macroautophagy has been found in mammary and ovarian cancer (Liang *et al*., 1999). However, whether CMA activity is altered in oncogenic processes remains unknown. It is not known either how CMA activity is affected in pathologies in which the other autophagic pathways, especially macroautophagy, are primarily impaired, such as in Danon disease or other forms of vacuolar myopathies. Is the decreased CMA activity reported in kidneys of diabetic-rat models a general feature or it is just restricted to particular organs? And in that case, what determines the susceptibility of the affected organs?

Some of these burning questions will be resolved as new CMA substrates are identified, better experimental tools are developed, and factors that may activate or inhibit CMA activity are discovered.

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# 8

# Extracellular Matrix Macroassembly Dynamics in Early Vertebrate Embryos

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I. Introduction
II. Localization and Function of Select ECM Components
  A. Fibronectin
  B. Fibrillin-2
  C. Colocalization
III. ECM Position Fate
IV. ECM Displacement Mapping
  A. Correlated Motion
  B. Displacement Decomposition
  C. Particle Image Velocimetry
V. Tissue Motion Component of ECM Displacement
VI. Local ECM Rearrangements
  A. Magnitude and Diversity
  B. Filament Assembly
VII. Conclusions
  Acknowledgments
  References

This chapter focuses on the *in vivo* macroassembly dynamics of fibronectin and fibrillin-2—two prominent extracellular matrix (ECM) components, present in vertebrate embryos at the earliest stages of development. The ECM is an inherently dynamic structure with a well-defined position fate: ECM filaments are not only anchored to and move with established tissue boundaries, but are repositioned prior to the formation of new anatomical features. We distinguish two ECM filament relocation processes—each operating on different length scales. First, ECM filaments are moved by large-scale tissue motion, which rearranges major organ primordia within the embryo. The second type of motion, on the scale of the individual ECM filaments, is driven by local motility and protrusive activity of nearby cells. The motion decomposition is made practically possible by recent advances

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in microscopy and high-resolution particle image velocimetry algorithms. We demonstrate that both kinds of motion contribute substantially to the establishment of normal ECM structure, and both must be taken into account when attempting to understand ECM macroassembly during embryonic morphogenesis. The tissue-scale motion changes the local amount (density) and the tissue-level structure (e.g., orientation) of ECM fibers. Local reorganization includes filament assembly and the segregation of ECM into specific patterns. Local reorganization takes place most actively at Hensen's node and around the primitive streak. These regions are also sites of active cell migration, where fibrillin-2 and fibronectin are often colocalized in ECM globules, and new fibrillin-2 foci are deposited. During filament assembly, the globular patches of ECM are joined into larger linear structures in a hierarchical process: increasingly larger structures are created by the aggregation of smaller units. A future understanding of ECM assembly thus requires the study of the complex interactions between biochemical assembly steps, local cell action, and tissue motion. 

## I. Introduction

There is a rich history of studies on extracellular matrix (ECM) biology during embryogenesis, starting with the ground-breaking studies of Rudnick, Grobstein, Hay, and Gross (Grobstein, 1954; Gross, 1974; Hay, 1982; Rudnick, 1933). These pioneering studies established the critical role of ECM in development. The discovery and characterization of the integrin family of ECM receptors (Hynes, 1987; Pytela *et al.*, 1985) was another major breakthrough in the field, triggering an avalanche of research studies. The basic molecular intricacies of ECM synthesis, secretion, cytoskeletal interactions, and assembly have now been described. It is established that the ECM provides adhesive substrates and signals for cell migration and differentiation (George *et al.*, 1993; Hay, 1990), modulates the availability of growth factors (Charbonneau *et al.*, 2004; Dallas *et al.*, 2005), and contributes to the mechanical integrity of tissues (Kadler, 2004; Sherratt *et al.*, 2003). To fit these pieces of information into an understanding of how the ECM functions *in vivo* during development and in mature tissues remains a daunting task—requiring further research into the relationships between the various organization levels that span from molecules to organs. Due to the very nature of the problem, the required approaches are multidisciplinary, incorporating anatomical, biochemical, physical, and tissue engineering concepts.

The three-dimensional (3D) organization of the ECM can be as crucial as its molecular composition in determining its developmental functions. Cell—ECM contacts strongly depend on how the ECM is presented to cell

surface receptors, whether it is immobilized, planar, or 3D (Cukierman *et al.*, 2001; Zamir *et al.*, 1999). ECM organization also strongly influences cell shape and motility (Petroll and Ma, 2003; Tomasek *et al.*, 1982): Fibroblasts (Dickinson *et al.*, 1994; Stoplak and Harris, 1982), endothelial cells (Vernon *et al.*, 1995), and neurons (Dubey *et al.*, 2001), are all known to preferentially follow oriented fibers. At the tissue level of organization, mechanical properties, such as anisotropy or load bearing capacity, are determined by 3D ECM structure (Barocas and Tranquillo, 1997; Olsen *et al.*, 1999).

ECM structure, as a physical entity, is molded by mechanical stress (Keller *et al.*, 2003). The most relevant source of mechanical stress is cellular traction force (Oliver *et al.*, 1995; Stoplak and Harris, 1982), which was demonstrated to reorganize collagen filaments *in vitro* (Friedl and Wolf, 2003; Petroll and Ma, 2003). The mechanically-driven ECM reorganization is often not reversible due to modifications in molecular configuration, which in turn enable chemical cross-linking or proteolysis (Wolf and Friedl, 2005). The best-understood example of such reorganization, at the molecular level, is that of fibronectin in which traction forces are needed to change the conformation of individual fibronectin molecules and expose a cryptic binding site (Baneyx *et al.*, 2001; Zhong *et al.*, 1998). Thus, macroassembly, the 3D (and possibly tissue-scale) ECM organization, is not only the sum of all biochemical reactions forming ECM protein complexes, but also involves tissue mechanics and sophisticated cell behavior (Hay, 1982).

While the importance of data obtained from cell culture studies cannot be overestimated, the most commonly used culture methods have inherent limitations in the study of ECM dynamics. Attachment of the ECM to a rigid substrate creates mechanical boundary conditions, which restrict substantial deformation. Thus, the traction stress exerted by a cell affects only the ECM in the immediate vicinity. Embryonic tissues, in contrast, undergo large deformations during organogenesis. These tissue motions are responses to intrinsic mechanical stresses, generated by a concerted action of large cell collectives, many of which are located at distant sites in the embryo (Cowin, 2000; Keller *et al.*, 2003; Trinkaus, 1984). Thus, during morphogenesis, the ECM is expected to exhibit far more substantial rearrangements than those observed in cell cultures. Moreover, *in vivo* ECM macroassembly can be affected by remote morphological events mediated through long-range mechanical stress fields, extending over hundreds of cell diameters. To tackle these challenges, quantitative experimental data on ECM behavior are needed. Such data would facilitate our understanding and allow the formulation and testing of sophisticated mathematical models with quantitative prediction power.

Here we review and report on the macroassembly dynamics of fibronectin and fibrillin-2—two prominent, ubiquitous ECM components, present in vertebrate embryos at the earliest (pregastrulation) stages of development.

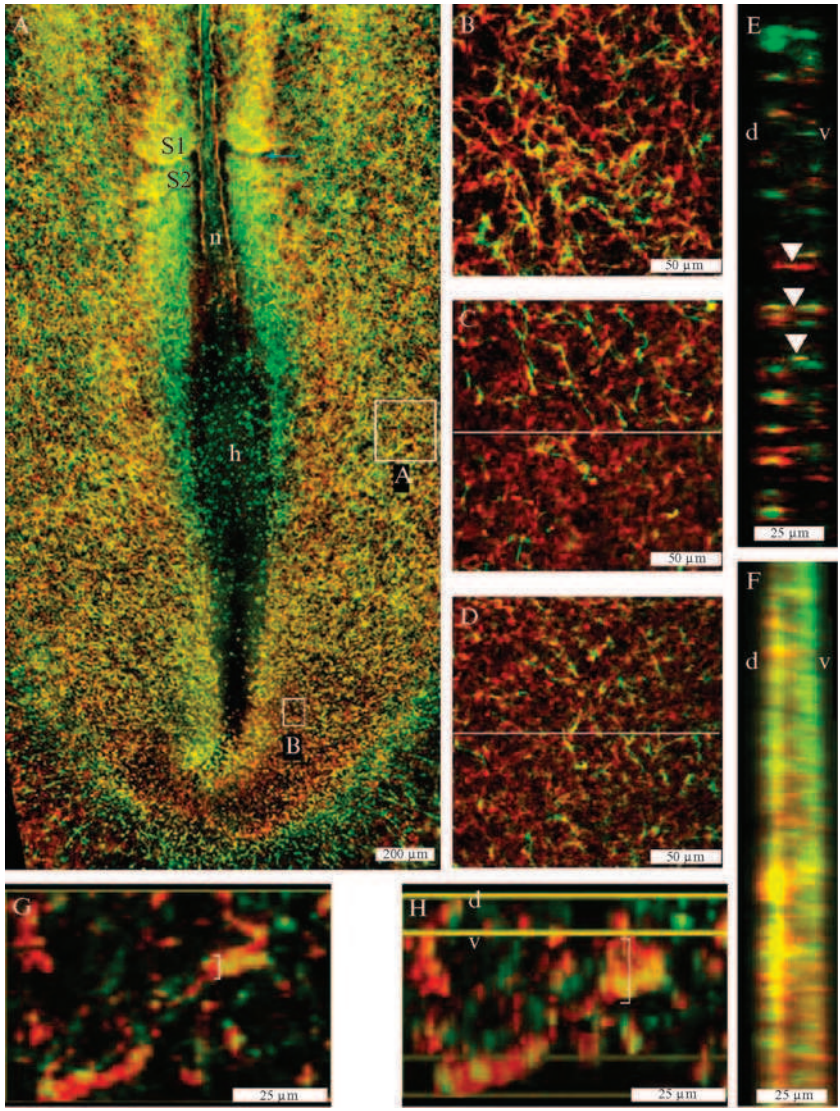


**Figure 1** The localization of fibronectin (red) and fibrillin-2 (green) in a gastrulation/neurulation stage avian embryo. The *en face* views (A–D, G, H) and optical transverse sections (E, F) show the ECM molecules labeled by whole mount JB3 (antifibrillin-2) and B3D6 (antifibronectin) immunostaining in an HH stage 6 (Hamburger and Hamilton, 1951) quail embryo. (A) Wide-field epifluorescence microscopy reveals a similar general pattern of distribution of both fibronectin and fibrillin-2 in the caudal half of the embryo. Fibrillin-2 connects the somites (cyan arrow), and forms long filaments in the presomitic mesoderm, which become more pronounced in time (Fig. 2). Regions analogous to A and B are further analyzed by laser-scanning optical microscopy (B–H). Panels B, C, and D show 2.2 µm thick

We demonstrate that at gastrulation and neurulation stages the ECM is far from a static structure: it moves, rearranges, and its components have well-defined position fates.

## II. Localization and Function of Select ECM Components

### A. Fibronectin

Fibronectin is one of the earliest ECM proteins to be deposited in the embryo. As Fig. 1 demonstrates, fibronectin is present along the basal surface of the ectoderm and endoderm in gastrulating avian embryos, and in filaments around Hensen's node (Krotoski *et al.*, 1986). Studies of amphibian embryos established that fibronectin is a major component of the ECM assembled along the blastocoel roof and other tissue interfaces (Danker *et al.*, 1993; Lee *et al.*, 1984). In fact, every mesodermal cell at late gastrula and early neurula stages appears to be in direct contact with a fibronectin-rich filamentous matrix (Davidson *et al.*, 2004). Recently, Davidson *et al.* (2004) demonstrated that fibronectin is deposited rather rapidly at the mesoderm–endoderm interface during the time when the endoderm involutes over the blastopore lip. The same study also showed that fibronectin-rich ECM is created, removed, and subsequently reestablished during *Xenopus* development: Fibrils are cleared from the dorsal and ventral faces of the notochord, and at the same time appear medially, at the neural/somitic mesoderm boundary. As the neural plate thickens, fibronectin fibrils are reestablished between the dorsal notochord surfaces. The deposition of fibronectin fibers takes place at tissue boundaries, especially at sites of shear motion between tissues.

Fibronectin is mainly known to promote cell adhesion and migration. Fibronectin was shown to be necessary for ingressed mesoblast cell migration away from the primitive streak (Harrisson *et al.*, 1993), and for the extension of the mesendoderm and radial intercalation in *Xenopus* embryos (Davidson *et al.*, 2002; Marsden and DeSimone, 2001). Fibronectin fibrils,

optical sections of boxed area A at the ventral surface, middle, and dorsal surface of the mesoderm, respectively. (E) A digitally reconstructed transverse section along the white lines in (B–D) shows fibronectin and fibrillin filaments spanning the mesoderm (white arrowheads). (F) Anterio-posterior projection of all transverse sections similar to (E) reveal ECM accumulation at the dorsal and ventral surfaces of the mesodermal germ layer. Digitally reconstructed oblique projections of boxed area B (G, H) demonstrate colocalization of filaments (yellow), as well as filaments extending in the dorsal–ventral direction (white brackets). (G) *En face* view, rotated by 10° along the lateral (x) axis. (H) Oblique view, rotated by 50° along the lateral (x) axis. h, Hensen's node; n, notochord; s, somites, d, dorsal, v, ventral.

lining the *Xenopus* animal cap ectoderm (Lee *et al.*, 1984; Nakatsuji *et al.*, 1985), are used as a substrate for mesendoderm migration (Darribere *et al.*, 1988; Winklbauer and Keller, 1996). Fibronectin null mutations in mice are embryonic lethal, with mesodermal defects and failed notochord or somite formation (George *et al.*, 1993). In addition to its role in promoting cell migration, several *in vitro* studies have suggested that fibronectin is required during the assembly of multiple ECM proteins, including collagen types I and III (McDonald *et al.*, 1982; Veiling *et al.*, 2002), fibulin (Godyna *et al.*, 1995; Roman and McDonald, 1993), fibrinogen (Pereira *et al.*, 2002), thrombospondin (Sottile and Hocking, 2002), and LTBP1 (Dallas *et al.*, 2005).

### B. Fibrillin-2

ECM proteins in the fibrillin family are constituents of connective tissue microfibrils (Corson *et al.*, 2004; Sakai *et al.*, 1986; Zhang *et al.*, 1994). Fibrillin-2 is observed in the avian embryo before gastrulation in the form of slender filaments marking the future anterior–posterior axis (Wunsch *et al.*, 1994). During gastrulation, fibrillin-2 continues to display a remarkable pattern widely distributed in association with the mesoderm (Rongish *et al.*, 1998; Visconti *et al.*, 2003) (Fig. 1): a meshwork encases the notochord, and fibrillin-2 cables connect the anterior intestinal portal to the somites. Most notable are the bundles of fibers that run parallel to the embryonic axis. The cranial portion of these bundles encloses the somites, while caudally the cables extend more than 200 μm into the segmental plate mesoderm and approach Hensen's node. Caudal to the node, fibrillin-2 exhibits a different pattern, which consists of punctate, unconnected fluorescent foci. In *Xenopus* embryos, a fibrillin homolog (*xenopus* fibrillin: XF) is expressed from blastula stages onward. At early gastrula stages *XF* expression is restricted to the recently involuted mesoderm and the XF protein is observed as two stripes flanking the presumptive notochord. After neural tube closure, XF demarcates the notochord and the intersomitic clefts (Dr Paul Skoglund, University of Virginia, VA, personal communication).

The importance of the fibrillin family of ECM proteins to the structural and functional properties of connective tissues is demonstrated by the fact that mutations in the human fibrillin genes result in heritable disorders, with patients manifesting ocular, skeletal, and cardiovascular abnormalities (Dietz *et al.*, 1994). In accord, fibrillin-deficient mouse models demonstrated that the absence of normal levels of either fibrillin-1 or 2 proteins is incompatible with the proper function of mature elastic fibers (Arteaga-Solis *et al.*, 2001; Pereira *et al.*, 1997, 1999). Deficiency in both fibrillin-1 and 2 was found to be compatible with gastrulation of mouse embryos, but the embryos died before birth (Dr Luca Carta, Hospital for Special Surgery, NY, personal

communication). In contrast, exogenous expression of a truncated fibrillin in the presumptive dorsal mesoderm of gastrulation stage frog embryos disturbed endogenous fibrillin localization and blocked gastrulation (Skoglund, 1996).

### C. Colocalization

As Fig. 1 demonstrates, the general organization of both fibronectin and fibrillin-2 is surprisingly similar. Both proteins are associated with ECM filaments throughout the lateral embryo, and exhibit a punctate pattern at Hensen's node and in the caudal part of the embryo (boxed region B). Both accumulate at the dorsal and ventral surface of the mesodermal germ layer, in filaments which traverse the mesoderm, and encase the notochord and the somites. While fibronectin and fibrillin-2 distribution is similar, confocal sections show that in general, these molecules are not extensively colocalized. One significant exception is the caudal embryo, where fibrillin-2 immunoreactive filaments often appear decorated with fibronectin foci at the resolution of confocal light microscopy (Fig. 1G and H). Although fibronectin is not an elastic fiber constituent, their association is not uncommon (Goldfisher *et al.*, 1985; Latif *et al.*, 2005). While there is no biochemical evidence for direct binding between fibrillins and fibronectin under physiological conditions, these two ECM components may interact by both binding to versicans (Wu *et al.*, 2005), fibulins (Timpl *et al.*, 2003), matrilin-2 (Piecha *et al.*, 2002), and LTBP1 (Dallas *et al.*, 2000).

## III. ECM Position Fate

Recent advances in optical microscopy and molecular technologies allow the *in situ* visualization of morphogenic processes with micrometer scale resolution (Friedl, 2004; Kulesa, 2004; Lansford *et al.*, 2001). The same technologies also made possible the study of supramolecular, cellular- and tissue-scale ECM assembly, both in cell cultures and *in vivo* (Czirok *et al.*, 2004; Davidson *et al.*, 2004; Kozel *et al.*, 2006; Ohashi *et al.*, 1999; Sivakumar *et al.*, 2006).

Dynamic imaging studies in avian embryos reveal that fibronectin and fibrillin-2 containing ECM filaments are substantially displaced during development (Fig. 2) (Czirok *et al.*, 2004; Filla *et al.*, 2004). During this process, ECM filaments typically retain their characteristic shape and connectivity with adjacent filaments. Position changes, therefore, are interpreted as physical displacements of intact objects rather than as a process involving filament disassembly and reassembly. Based on this approach, one can

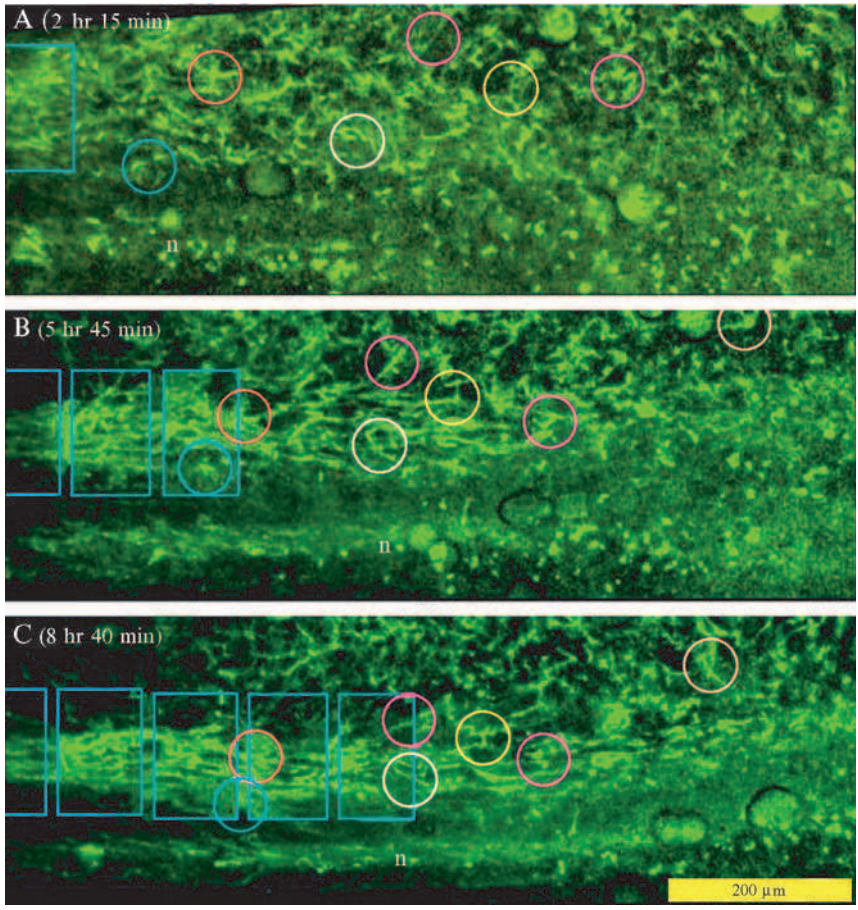


**Figure 2** Fibrillin-2 reorganization coincident with somitogenesis. Selected filaments, marked with the colored circles, visibly alter their relationship with nearby filaments and the axis. Despite this motion, the filaments retain connectedness and shape to some degree. Blue boxes denote formed somites, n, notochord. Reprinted from Czirok *et al.*, 2004.

determine the position fate of ECM filaments—in a manner analogous to cell-fate mapping. This fate is not trivial: ECM filaments are not only anchored to and move with established tissue boundaries, but are rearranged and repositioned prior to the formation of new anatomical features. An example is presented in Fig. 2, where fibrillin-2 filaments associated with the segmental plate mesoderm are assembled into parallel cables at the position of future somites. The demarcation between the axial cables and the less organized lateral meshwork of fibrillin-2 is fuzzy and does not coincide with preexisting anatomical structures. Once somites form, the axial

cables become part of the dense ECM meshwork that encloses and connects all somites. In fact, preexisting ECM filaments with long lifetimes are used in a similar manner during the formation of other early embryonic structures including the foregut, heart, or blood vessels (unpublished data). At present, the relationship between cell and ECM fate maps remains an open problem—in the concluding section we present conjectures on this relationship after further analysis of *in situ* ECM dynamics.

## IV. ECM Displacement Mapping

ECM formation is best studied in caudal regions of early avian embryos, where tissue geometry is relatively uncomplicated. To ensure the simultaneous visualization of all ECM filaments within this area, images are taken in multiple focal planes. To characterize the dynamics of ECM rearrangements, filament segments can be followed through the image sequence, their position registered relative to some stable anatomical reference points (like the intersomitic clefts), and their displacements calculated (Czirok *et al.*, 2004). Although tracking procedures can be performed in 3D, due to the large depth of field of the microscope objective, the resolution along the vertical (z) direction is rather limited. In fact, in the wide-field epifluorescence microscopy setup, the optical depth of field is comparable to the specimen thickness. Therefore, our studies analyze the 2D (x-y) projections of the physical (3D) ECM displacements.

### A. Correlated Motion

The analysis of image sequences reveal that ECM motion is ordered in space, since displacements of adjacent filaments are similar: changes in the relative position of nearby filaments are far smaller than the observed displacement of the ECM composite (Fig. 3A). To visualize temporal changes in ECM motion, fluorescence intensity profiles are calculated along a line parallel to the motion of the filaments. The resulting intensity profiles are shown in Fig. 4C for each frame in the image sequence. The intersection between ECM filaments and the selected line is marked by intensity peaks. As filaments move, the location of the intensity peaks shifts, resulting in continuous curves on the spatio-temporal intensity plot. The smoothness and overall similarity of the curves in Fig. 4C indicate a slowly changing, correlated, and persistent motion where displacements tend to continue over many hours in a similar fashion. Thus, ECM filaments seem to be embedded in a moving and continuous mechanical medium, rather than existing as free-moving or quickly disconnecting and reconnecting polymers.

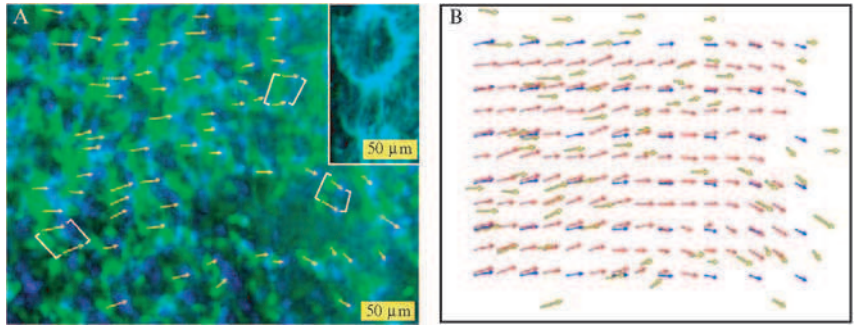


**Figure 3** Detailed view of ECM filament motion. (A) The displacements of fibrillin-2 filaments during a 20 min interval are depicted within a region analogous to boxed area A of Fig. 1, and in a region containing somites (inset). The earlier and latter immunofluorescence pattern is presented in the green and blue channels, respectively. Colocalization of the two channels (cyan) indicates absence of motion (inset). Yellow arrows represent filament displacements, as obtained by manual tracking. The change in filament configuration, as indicated by the similar length of the white brackets, is small compared to the displacements. (B) ECM filament motion, estimated by particle image velocimetry (Zamir *et al.*, 2005). Yellow arrows represent manual tracking results from panel (A). Red arrows represent the results of a two-step predictor–corrector PIV algorithm with 64 and 16 pixel windows, without data smoothing in the corrector step. Blue arrows represent the estimated tissue motion component, determined by the same algorithm with 128 and 64 pixel windows and an additional thin plate spline smoothing.

Images analyzed in Fig. 3 and in Czirok *et al.* (2004) depict every fluorescence-tagged ECM filament, irrespective of their dorsal–ventral location. Because of the absence of ECM filament populations displaying markedly different displacements, we can exclude the possibility of large dorsal–ventral differences in motion. Thus, ECM filaments located either in the mesoderm, at the mesoderm–endoderm or mesoderm–ectoderm boundaries show approximately the same displacements.

## B. Displacement Decomposition

In addition to the above data, we know that although the motion of ECM filaments may be similar, there must be differences, which eventually result in distinct patterns along the dorsal–ventral axis or between different kinds of ECM proteins. It is thus reasonable to distinguish two ECM filament relocation processes—each operating on different length scales. First, ECM filaments are expected to be moved by large-scale tissue motion, which rearranges major organ primordia within the embryo. A second type of motion, on the scale of the individual ECM filaments, could be driven by local motility and protrusive activity of the nearby cells. Although any kind

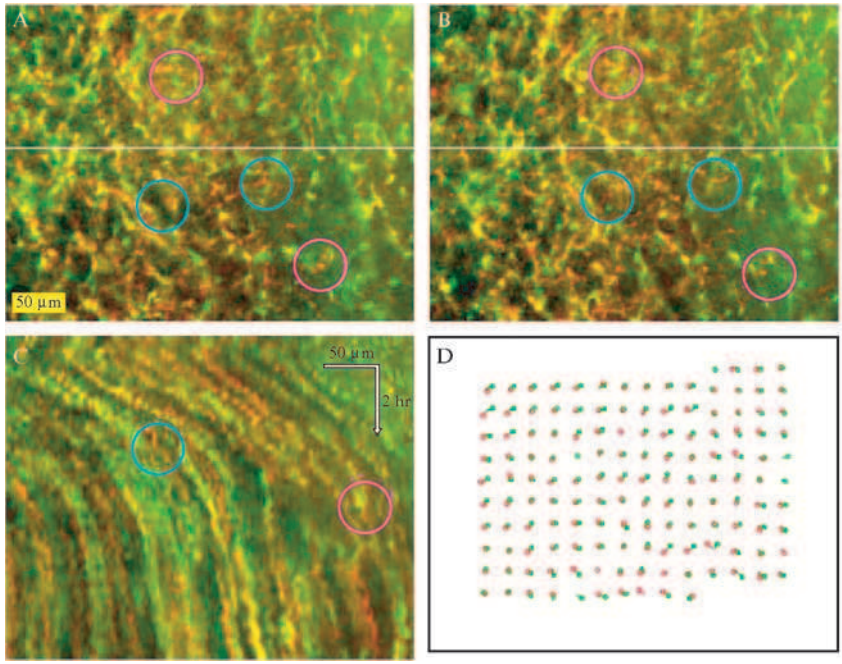


**Figure 4** Comparison of fibronectin (red) and fibrillin-2 (green) rearrangements. Immunolabeled ECM components are depicted at two time points, separated by 20 min (A, B). Despite the general displacement shown in Fig. 3, the relative configurations of fibronectin and fibrillin-2 filaments remained mostly unchanged (magenta circles). However, at certain positions the relative motion of the two ECM components is evident (cyan circles), a difference attributed to local cell activity, (C) Temporal changes in fluorescence intensities along the white line in panels A and B. The continuous curves represent filament trajectories. Within the cyan circle, a fibronectin filament is seen relocating between two fibrillin-2 filaments. In contrast, the two ECM components move as a single composite structure within the magenta circle. (D) The local component of the displacement field, calculated by PIV. The endpoint of the displacement vector is indicated by a circle (fibronectin: red, fibrillin-2: green).

of motion can be arbitrarily divided into two scales, the model posed here predicts that the large-scale motion component is the same for all ECM components, while the local rearrangements must be ECM component specific, to account for the observed differences in the various distribution patterns. Moreover, the local action of the cells is expected to operate on shorter characteristic time scales (minutes) than global tissue movements (hours). As we will learn from the data below, both kinds of motion contribute substantially to normal ECM structure, and both must be taken into account when attempting to understand ECM macroassembly during embryonic morphogenesis.

### C. Particle Image Velocimetry

The decomposition of ECM movements into two processes was made possible by adopting high-resolution (subpixel) particle image velocimetry (PIV) techniques (Zamir *et al.*, 2005) in tracing the filaments. In the PIV analysis of "optical flow," image details are traced through the image sequence without the need for segmentation and recognition of distinct objects. As the red arrows in Fig. 3B demonstrate, a two-step predictor–corrector PIV algorithm approximates the manual tracking very closely. The extraction of the tissue motion component, that is, the motion of a mechanical continuum, can be done by local averaging of the individual ECM filament displacements (Czirok *et al.*, 2004) or by spline smoothing of the results of a coarser PIV calculation (Zamir *et al.*, 2005). Both approaches rely on the presence of local spatial and temporal correlations, that is, on the fact that adjacent ECM filaments continue to move similarly. The second, local, component of the motion is obtained as the difference of the actual displacements (tracked either manually or with high resolution PIV) and the tissue motion component (Fig. 4D).

## V. Tissue Motion Component of ECM Displacement

The overall direction of ECM motion is highly reproducible across embryos (Czirok *et al.*, 2004). Moreover, as Fig. 5 demonstrates, the tissue component of the motion is the same for both fibronectin and fibrillin-2, and is best described as two, counter-rotating vortices on each side of Hensen's node (Fig. 6). This displacement pattern appears to move with the node, as it regresses caudally over time.

The displacement field presented in Fig. 6 has two important implications for morphogenesis: it changes both the local amount (density) and the tissue-level structure of the ECM. Calculation of the divergence of the displacement field reveals how the motion changes the amount of labeled ECM components present within a certain area. As Fig. 7A demonstrates, ECM is condensed around the site of somitogenesis. In contrast, in the caudal embryo, labeled ECM filaments are scattered within an increasing area, so their density decreases. Interestingly, this region of expansion coincides with the deposition and assembly of new, and thus unlabeled, ECM filaments (unpublished data)—a process that may counterbalance the observed local decreased density of labeled filaments. The observed tissue movements can result in ECM density changes of up to 10% per hr, and thus yield a substantial cumulative compression of the ECM over the duration of gastrulation (Zamir *et al.*, 2005). A mathematical decomposition (Abraham *et al.*, 1988) reveals the deformation of the tissue motion component (Fig. 7B). The deformation is maximal between

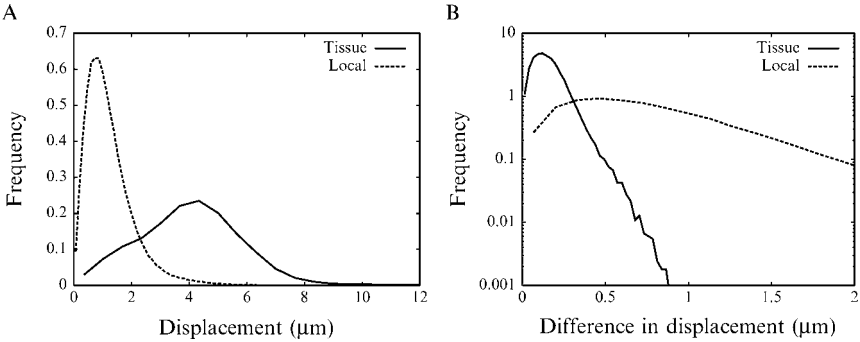


**Figure 5** Quantitative analysis of the tissue and local motion components. (A) Distribution of the magnitude of the tissue (solid line) and local (dashed line) motion components, calculated for fibronectin throughout the caudal embryo. The average magnitudes are markedly different: 4 μm and 1.2 μm for the tissue and the local components, respectively. (B) Distribution of the difference between fibronectin and fibrillin-2 displacements. The magnitude of the difference between the displacement vectors obtained for the two ECM components is calculated throughout the caudal embryo for each motion component (tissue motion: solid line, local rearrangements: dashed line). Compared to the typical magnitude of the tissue displacement, the tissue components are essentially the same for both fibronectin and fibrillin-2 (average difference is 0.15 μm, 3% of the average magnitude). In contrast, the difference in the local component (average is 0.8 μm) is five-fold larger, and is comparable with 1.2 μm, the typical magnitude of the local displacement vectors. Displacements were calculated from 40 images, each taken 5 min apart.

Hensen's node and the somitogenesis front, and appears to play a major role in the transformation of the loose meshwork, characteristic for the segmental-plate associated ECM, into anisotropic, axial cables.

## VI. Local ECM Rearrangements

### A. Magnitude and Diversity

As described in Section IV, local ECM rearrangements are obtained after subtracting the tissue motion component from the displacement data. This local component of ECM displacement, depicted in Fig. 4D, changes quickly in space and time. As Fig. 5 demonstrates, local rearrangements are different for fibronectin and fibrillin-2 and have magnitudes typically five times smaller than that of the tissue motion component. As local ECM rearrangement is presumably correlated with cell motility and protrusive activity, the magnitude of the local component is an approximate measure of the ECM remodeling activity of cells. Figure 8 shows the distribution of the magnitude of the local ECM rearrangement within the caudal embryo. Most active

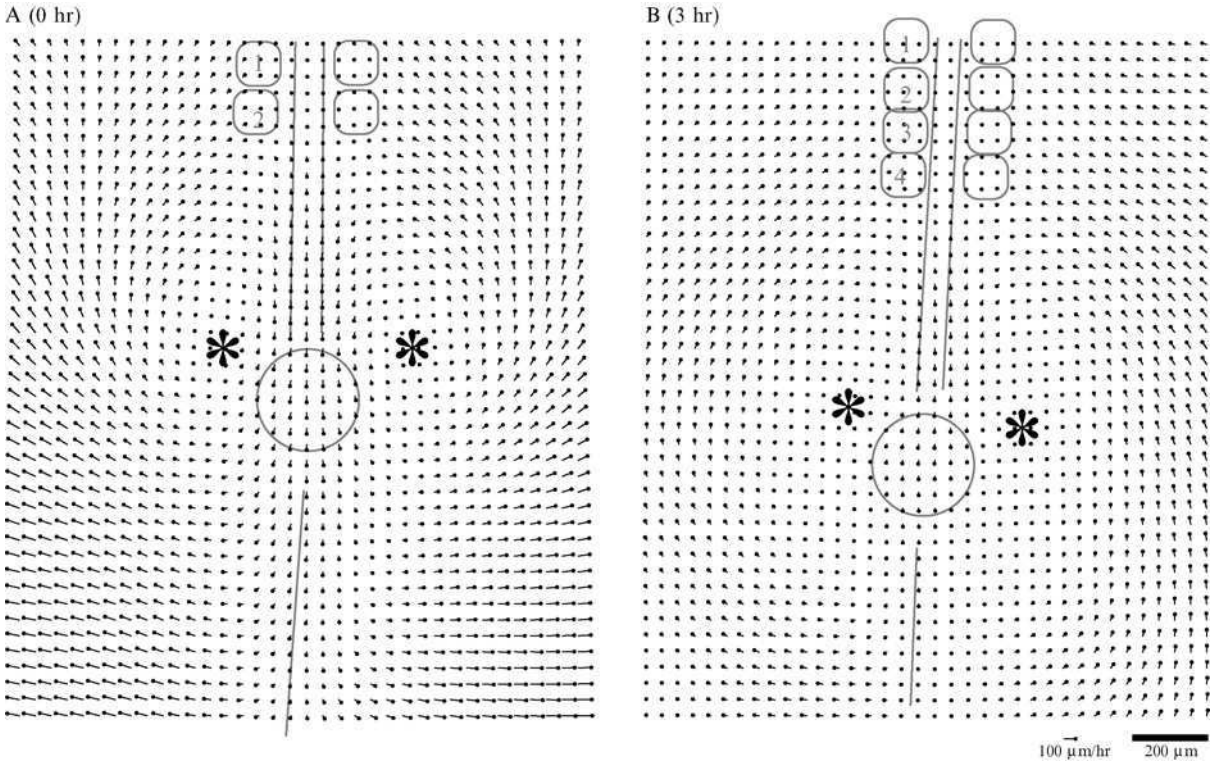


**Figure 6** Tissue component of ECM displacements. Displacement vectors (circles at endpoints) were obtained by PIV and are averaged for each grid point over 10 frames (40 min). The motion pattern obtained from a two-somite embryo (A) and 3 hr later from the same, four-somite embryo (B) are very similar, essentially consisting of two vortices, on each side of the embryonic axis. Centers of rotation are marked by asterisks. The gray lines denote the somites, notochord, Hensen's node, and the primitive streak (from top to bottom).

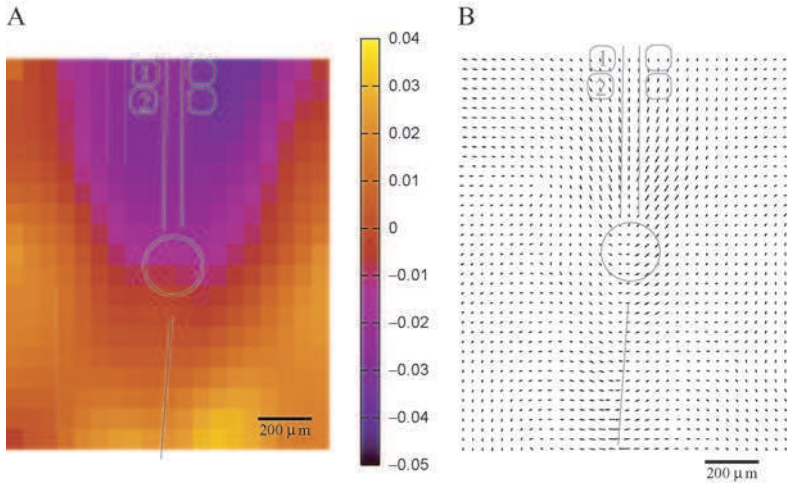


**Figure 7** Analysis of the tissue component of ECM motion. (A) The divergence of the displacement field shows condensation of labeled ECM filaments around the site of somitogenesis (blue) and dilution at the caudal end of the primitive streak (yellow). (B) The motion also deforms the ECM. Tissue deformation is indicated by two-headed arrows: the size of the symbols is proportional to the magnitude of the deformation, and the lines are parallel to the direction of stretch. The gray lines denote the somites, notochord, Hensen's node, and the primitive streak (from top to bottom).

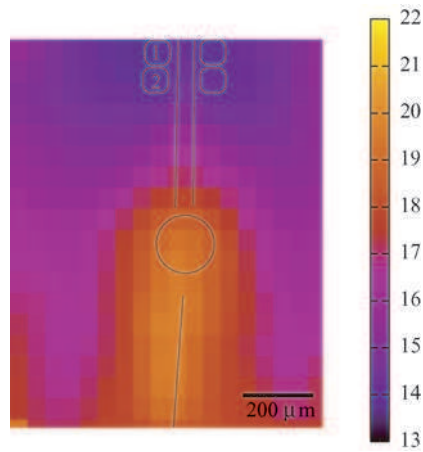


**Figure 8** Magnitude of the local component of ECM rearrangements, averaged over 10 consecutive frames (40 min). Hensen's node and the area surrounding the primitive streak displays especially active local ECM rearrangements (red). The numeric values are given in units of μm/h. The gray lines denote the somites, notochord, Hensen's node, and the primitive streak (from top to bottom).

local ECM reorganization takes place at Hensen's node and around the primitive streak. In this "active" region (red area in Fig. 8) the ECM does not form an interconnected network, fibrillin-2 and fibronectin are often colocalized (Fig. 1) and new fibrillin-2 foci are deposited (unpublished results). The "active" region also coincides with the location where mesodermal cells migrate intensively after ingression (Psychoyos and Stern, 1996; Schoenwolf *et al.*, 1992).

### B. Filament Assembly

Filament assembly, a particularly interesting reorganization process, also takes place in the caudal embryo. Globular patches of immunofluorescence are joined into larger linear structures as shown in Fig. 9. Closer analysis reveals that the process appears to be hierarchical: increasingly larger structures are created by the aggregation of smaller units. Throughout the assembly process, participating ECM filaments appear to consist of both fibronectin and fibrillin-2; thus filaments are "born" as composite structures. While a set of globules and short filaments is often observed to assume a linear pattern, in our pulse-chase experiments some fibers do not appear to be fully connected. Presumably, newly produced or different and thus unlabeled ECM proteins fill in the gaps, but are not visualized.

The dynamics of macroassembly of fibronectin (Ohashi *et al.*, 2002), LTBP (Sivakumar *et al.*, 2006), and elastin (Czirok *et al.*, 2006; Kozel *et al.*, 2006) filaments was recently investigated in various cell culture models. In case of densely populated cell cultures, these studies showed that the ECM is assembled first in distinct globules on the cell surface. Subsequently, through the motion of several adjacent cells, these ECM foci are organized into progressively larger filaments. In the case of elastin, ECM filaments were shown to aggregate in a hierarchical fashion similar to Fig. 9 (Czirok *et al.*, 2006).

## VII. Conclusions

The present data inevitably raise questions about possible relationships between ECM motion and the features of mesodermal cell migration (Psychoyos and Stern, 1996; Schoenwolf *et al.*, 1992). We expect a similar approach to be applicable to cell motion and cell-fate mapping. Using the same arguments as those presented in Section IV, cell displacements can also be decomposed into a tissue and a local motion component–the latter reflecting the autonomous motion of individual cells relative to the tissue (Sepich *et al.*, 2005). We predict that the tissue motion component of the mesodermal

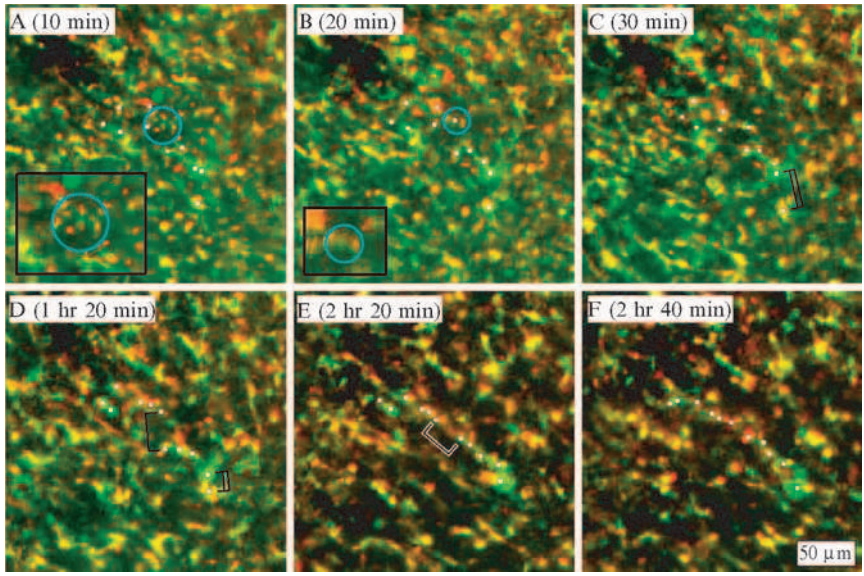


**Figure 9** Hierarchical assembly of ECM filaments. An image sequence (A–F) depicting an area located lateral to the streak (comparable to boxed region B in Fig. 1) as it moves with the tissue. White circles label individual globules of ECM material, which typically contain both fibronectin and fibrillin-2. In the course of 3 hr, the globules assume an ordered, linear shape, which remains stable until the end of observation, 7 hr later (not shown). Brackets indicate areas where previously distinct ECM objects merge (assume a stable linear arrangement). As an example, the filament marked by the cyan ellipse in panel (B) is created from the material enclosed in the cyan ellipse in panel (A). Both areas are shown with greater detail in the insets. During the assembly process, the original punctate immunofluorescence gives rise gradually to a filamentous pattern.

cells and mesoderm-associated ECM are the same. Moreover, as tissue motion can be a response to forces exerted by distant cell collectives, we speculate that the remarkable vortices of DiI-labeled cells reported recently by Yang *et al.* (2002) and Cui *et al.* (2005) could be explained as a tissue mechanical phenomenon, whereby propulsion is generated by forces exerted around the notochord, foregut, and in the area opaca.

This chapter demonstrates that ECM structure *in vivo* is formed through complex interactions including biochemical assembly steps, local cell behavior, and tissue motion. We hope we have conveyed that a future understanding of *in vivo* ECM formation, as well as its function, will require the synthesis of a vast spectrum of studies ranging from determining protein conformations to computer modeling of embryonic tissue movements during organogenesis.

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# Index


**A**

ACB, 126
AcbA, 131
Acerebellar (ace) mutant, in zebrafish, 16
ACG. *See* Adenylyl cyclase G
ACK. *See* Asymmetric cell kinetics
Activating mutations, 94
Adenomatous polyposis coli (APC), 46
*Adenomatous polyposis coli* (APC) gene, 150
Adenylyl Cyclase A (ACA), 120
Adenylyl Cyclase B (ACB), 120
Adenylyl Cyclase G (ACG), 126
Adult stem cells (ASC), 141–142
  cell kinetics symmetry-based strategies for expansion of, 152–157
  comparison of conventional cell lines to SACK-derived, 157–158
  experimental strategies for expansion of, 143–152
  replication mutation risk in expansion of, 160–162
Aggregation, 118, 123
Aging and CMA, 220–221
Agnathans. *See* Jawless animals
Allosteric activators, binding of, 92
Allosteric agonists, 101
2-Amino-8-oxo-9, 10-epoxydecanoic acid, 179
Amyloid precursor protein, 227
Antiapoptotic genes. *See Bcl-2* gene
Antigen presenting cells, 219
Antigen presention, CMA's role in, 219–220
AP2, 14
Aromatic amino acids, 101
ASC. *See* Adult stem cells
ASC expansion
  cell kinetics symmetry-based strategies for, 152–157
  embryonic stem cell-based strategies for, 151–152
  growth factor supplementation approach for, 144, 148–151
  transgenic approach for, 143–148
Asymmetric cell kinetics (ACK), 141, 153–155, 158
Asymmetric self-renewal, 153
Autophagy, 205
  chaperone-mediated. *See* Chaperone-mediated autophagy
  dierent forms of, 206–208
  in mammalian cells, types of, 207
Axenic strains, 117
Axin2/Conductin/Axil, 68

**B**

*Bcl-2* gene, 145
BFR. *See* Bone, formation rate
BMP. *See also* Bone morphogenetic proteins
  activity, level of, 8
  antagonists sclerostin, 58
*Bmp4* expression pattern, 31–32
Bone
  formation rate (BFR), 49, 54
  mass, 51
  remodeling, 44, 50
Bone morphogenetic proteins (BMP), 6, 45, 150–151
Brain, subdivisions of, 17–18
BrdU labeling, 33

**C**

$Ca^{2+}$, physiological role of
  expression cloning of a "CaR" from bovine parathyroid, 87
  inverse relation between serum $Ca^{2+}$ and parathyroid hormone secretion, 86–87
Calcium sensing receptors (CaR), 85
  activation of, 100
  amino acid modulators, 104–105
  in development, 104–105
  domain structure of, 89
  integration, 91–95
  structural domain of, 88–91

cAMP, 134
  binding to specific membrane receptors, 123–124
  as master regulator of *Dictyostelium* development, 123
  removal, 127–128
  signal relay, 126–127
  synthesis, 124–126
cAMP receptors (cARs), 118
Canonical Wnt signaling, 45–46
  Lrp5/Lrp6, 48–58
  pathway, 47
  role of, 70
$Ca^{2+}$ oscillations, distinctive patterns of, 103
CaR. *See also* Calcium sensing receptors
  expression, 105
  signal transduction, 96
cAR1, 122, 127
CaR, translation of extracellular $Ca^{2+}$ by, 97
  $Ca^{2+}$ oscillations, 100–103
  consequences of intracellular $Ca^{2+}$ oscillations, 103–104
  increases in extracellular $Ca^{2+}$, 98–100
  regulation, 89
Carboxyl terminus, 93
  of Hsp70-interacting protein (CHIP), 227–228
*CaR* gene, 105
$\beta$-Catenin, 52–55
Cathepsin A, 224
Caudal embryo, 248
Cell cycle phase distribution, 191–192
Cell-ECM contacts, 238–239
Cell kinetics symmetry-based strategies for ASC expansion, 152–157
Cell mutagenesis in ASC expansion, 160–162
CF. *See* Counting factor
Chaperone-mediated autophagy (CMA), 205, 208–210
  and aging, 220–221
  concluding remarks on, 228–229
  lysosomal membrane, other aspects of, 213–214
  methods to measure, 214–215
  pathologies related to, 221–228
  physiological role of, 215–220
  substrates, lysosomal receptor for, 212–213
  substrates and their interaction with cytosolic chaperones, 210–211
Chemotaxis, 126
CMA. *See* Chaperone-mediated autophagy
CMF. *See* Conditioned media factor
CMFR1, 121
"Coincidence detector", 103
Colocalization, 243
Conditioned media factor (CMF), 121–122
Connective tissue growth factor (CTGF), 58
Countin, 122
Counting factor (CF), 122
Craniofacial morphogenesis, 2
Craniofacial patterning, first milestone, 4–5
  neural plate formation, 5–7
  no-man's land, 7–9
Craniofacial patterning, second step in, 9–10
  ephrins, 23–25
  facial ectoderm and craniofacial patterning, 20–22
  Fgf8, 26–28
  *Hox* gene regulation by AP2, 13–14
  malleable, 12–13
  neural crest cells, *Hox* genes, 10–12
  neuroectoderm and craniofacial patterning, 17–20
  pharyngeal endoderm and craniofacial patterning, 15–17
  species-specific dierences in neural crest cell migration, 13–15
  teeth as a model of species-specific development, 25–26
Craniofacial patterning, third step in
  BMP signaling in fowl, 31
  BMP signaling in teleosts, 31
  species-specific dierences in craniofacial form, 28–30
  three faces of aves, 33
CRD. *See* Cysteinerich domain
CTGF. *See* Connective tissue growth factor
Culmination, 118
Cycling cells, 163
Cysteinerich domain (CRD), 65, 88, 90–92
Cytoplasmic $Ca^{2+}$, 86
Cytosolic chaperones, interaction of CMA with, 210–211

**D**

Danon disease, 224–225
Darkfield microscopy, 123
Dexamethasone, 63
DhkC, 129
Diacylglycerol (DAG), 87
Dickkopfs, 47

*Dictyostelium* cells
life cycle of, 115–116
signal transduction pathways, 117
perspectives, 134
Dictyostelium life cycle regulation, five signal relay
modules of, 117–118
environmental sensing, 130–133
gradient sensing, 123–128
morphogen sensing, 128–130
nutrient sensing, 119
quorum sensing, 119–123
Dierentiation inducing factor-1 (DIF-1), 128
Dimerization, 94
Discadenine, 132
Discoidin-I, 120
Displacement decomposition of ECM filaments, 246–247
Dkk2, 62
Dkk proteins, 64
Dkks. *See* Dickkopfs
*Drosophila* gene armadillo, 52–53

**E**

ECM. *See also* Extracellular matrix
motion, tissue component of, 251
structure, 239
ECM components, localization and function of select, 240
colocalization, 243
fibronectin, 241–242
ECM displacement mapping
correlated motion, 245–246
displacement decomposition, 246–247
particle image velocimetry, 248
ECM displacements
tissue component of, 250
tissue motion component, 248–249
ECM filaments
hierarchical assembly of, 253
relocation, 237–238
ECM rearrangements, local
filament assembly, 252
magnitude and diversity, 249, 251, 252
ECS. *See* Extracellular domain (ECD)
Embryonic development, 5
Embryonic stem cells (ESC)-based strategies for ASC expansion, 144, 151–152
Enamel-specific genes, 25
Endoderm, 15
Endothelial cells, 239
Environmental cues, 118
Environmental sensing, 118
ammonium phosphate and cAMP levels increase, 132
discadenine as an autoinhibitor, 132
germination signals overcoming environmental checkpoint, 132–133
spore encapsulation, 131–132
EPDE, 127
Eph receptors, role of, 23
Ephrins, 23
Extracellular cAMP phosphodiesterase (ePDE), 120
Extracellular domain (ECD), 87
CysRD in, 90–91
cytoplasmic carboxyl terminus, 93
heptahelical domain, 91–93
obligate dimerization of CaR, 94
venus flytrap domain in, 88–90
Extracellular matrix (ECM), 44

**F**

Facial pattern, 1–2
Familial adenomatous polyposis (FAP), 150
FEZ. *See* Frontonasal ectodermal zone
Fgf8, 19
*FGF* genes. *See Fibroblast growth factors* genes
Fibrillin-2
formation, 242
functions, 242–243
*Fibroblast growth factors (FGF)* genes, 148–149
Fibroblasts, 239
Fibronectin
formation, 241
functions, 241–242
Folate, 130
Folic acid, 117
$\beta$ receptors, 119
$\alpha$ receptors, 119
Fowl, BMP signaling in, 31
Frizzled (Fzd) proteins, 61–62
Frontonasal ectodermal zone (FEZ), ectopic transplantation of, 21
Fzd. *See* Frizzled proteins

**G**

Gα4, 130
Galactosialodosis, 224
Galapagos finches, 2–3
Gcn5-related N-acetyltransferase (GNAT) family, 176
GFP, 117
Glucocorticoid, 64
Glycogen synthase kinase (GSK), 46
G protein-coupled receptors (GPCRs), 85, 117
G proteins, 92
Gradient sensing
  cAMP binding, 123–124
  cAMP removal, 127–128
  cAMP signal relay, spatially confined, 126–127
  cAMP signal relay system, 123
  cAMP synthesis, 124–126
Green fluorescent protein (GFP) expression, 50
Growth factor supplementation approach, 144
  using BMP, 150–151
  using *FGF* genes, 148–149
  using *sonic hedgehog (Shh),* 151
  using *Wnt* genes, 149–150

**H**

H2AX acetylation, 192–195
HBM. *See* High bone mass syndrome
HDAC. *See* Histone deacetylase
HDAC inhibitor-induced radiosensitization, mechanisms of, 191–196
HDAC inhibitor(s), 175, 179–180, 186
  effect on tumor cell radiosensitivity, 184–186
  radiotherapy combinations, clinical applications of, 196–198
  *in vitro* radiosensitization by, 180–187
  *in vivo* radiosensitization by, 187–189
Hedgehog (Hh) pathway activity, 151
Hematopoietic stem cells (HSC), 152
Hensen's node, 238
High bone mass (HBM) syndrome, 48
Histone acetylation, 173–174, 176–179
  comparison in tumor and normal cells, 190–191
Histone acetyltransferases (HAT), 176–177
Histone deacetylase (HDAC), 176–178. *See also* HDAC inhibitors
Histone deacetylation, 176–178
Histone hyperacetylation, 177
  *in vitro,* 180–187
  *in vivo,* 187–189
Histone proteins, 176
Homeotic genes. *See Hox* genes
*Hoxa2,* 10
*Hox* genes, 146
  code, 10
  expression, 13
Human bone marrow cells, 151
Huntingtin, 227
Hyaline droplet nephropathy, 221–222
Hyperparathyroidism, 104
Hypocalcemia, 87

**I**

i3 loop, 93
Immortal DNA strand cosegregation, 161
IMPDH. *See* Inosine-5′-monophosphate dehydrogenase
Inactivating mutations, 94
Inosine-5′-monophosphate dehydrogenase (IMPDH), 156–157
Intracellular $Ca^{2+}$ oscillations, pathways for CaR-mediated induction of, 99
Intracellular or transmembrane modulators
  Axin2/conductin/Axil, 68
  kremens, 67–68
  Ror2, 68–69
*In vitro* radiosensitization, by HDAC inhibitors, 180–187
*In vivo* radiosensitization, by HDAC inhibitors, 187–189

**J**

*Jagged-1,* 147
Jawless animals, 12

**K**

Kidney, CMA's role in, 222–223

**L**

LAMP-2B, 225
Lateral neurogenic ectoderm (LNE), 7–8
*Lef1,* 55–57
Lef/Tcf, 55–58

Lef/Tcf transcription factors, 56–57
Lipofuscin, 220
Lipoprotein α-2-microglobulin (α-2m), 222
Lithium, 63
Lithium chloride, 62
LNE. *See* Lateral neurogenic ectoderm
Low density lipoprotein receptor (LDLR), 48
L723Q, 105
Lrp5, 48–51
  osteoporotic mutations in, 49
Lrp6, 51–52
*Lrp6,* 52
Lrp5/6 receptor, 63
LSD. *See* Lysosomal storage disorder
Lymphoid enhancer-binding factor/T cell factor (Lef/Tcf ), 46
Lysosomal associated membrane protein type 2A (LAMP-2A), 212–213
Lysosomal membrane receptors, 212–213
  other aspects of, 213–214
Lysosomal storage disorder (LSD), and CMA, 223–225
Lysosomal targeting motifs in dierent CMA substrate proteins, 211

**M**

α-2m. *See* Lipoprotein α-2-microglobulin
Macroautophagy, 207–208, 214
MAR. *See* Matrix apposition rate
Matrix apposition rate (MAR), 49
Maxillary condensation, 4
Mesenchymal stem cells, 60
Metabotropic glutamate receptors (mGluR), 87
Microautophagy, 207–208
Molecular tête-à- tête, 26
Morphogen sensing, 118
  ammonia controls culmination, 129–130
  cAMP controls morphogenesis, 128
  cell fate influenced by dierentiation inducing factor, 128–129
  folate, 130
Mound, 118
MS-275, 179, 181–182, 187–189
MS-275-induced radiosensitization, 191–193
Multiple cellular mechanisms, 86

**N**

NaB-mediated radiosensitization, 192
Neural crest
  cells, 17
  induction, 8
Neural stem cells (NSC), 147
Neurodegenerative disorders, and CMA, 225–228
Neurons, 239
Neuropathy, and CMA, 221–223
Neurulation, 5
Neutrophils, 134
*Notch* genes, 147–148
Nuclear factor of activated T cells (NFAT), 103
*Nuf* mouse, 105
Nutrient sensing, 119
Nutritional stress eect on CMA. *See* Starvation

**O**

OPPG. *See* Oteoporosispseudoglioma
Oral ectoderm, regionalization of, 26
"Organizer"-like domains, concept of, 28
Osteoblasts, 59
Osteoporosis, 43
Osteoporosispseudoglioma (OPPG), 48
Osteoprotegerin, 54
*Osteoprotegerin (OPG),* 44–45
Oxidized protein, CMA's role in, 216–219

**P**

Pacemaker cells, 123
Parathyroid hormone (PTH), 86–87
Parathyroid hormone related peptide (PTHrP), 96
Particle image velocimetry (PIV) technique, 248
Pathologies related to CMA
  CMA and LSD, 223–225
  CMA and neurodegeneration, 225–228
  CMA and neuropathy, 221–223
PDI, 127
*PdsA* gene, 127
Pharyngeal clefts, 15
Pharyngeal endoderm, 16
Phenylalanine, 85
Phenylalanine-mediated oscillations, 102
Pheromone receptors, 88
Phospholipases, activation of, 96

Physiological role of CMA, 215
in antigen presentation, 219–220
in oxidation, 216–219
in starvation, 216
*Pia* gene, 125
PKC$\alpha$−YFP, 102
PKC-dependent phosphorylation of CaR, 98
Planar cell polarity (PCP), 45
Plasma membrane calcium pump (PMCA), 100
PLC, 123
PldB, 122
Polyamine metabolites, 95
PPE. *See* Preplacodal ectoderm
P53 protein, 155–156
Preplacodal ectoderm (PPE), 8
Prestarvation factor (PSF)
responsive genes, 120
signal transduction pathways, 120
Proteasome, 206
Protein conformational disorders, role of proteolytic systems in, 226. *See also* Neurodegenerative disorders
Protein filamin A, 93
PSF. *See* Prestarvation factor
PufA, 120–121

**Q**

Qucks, 34
Quorum sensing
conditioned media factor (CMF), 121–122
counting factor (CF), 122
prestarvation factor (PSF), 119–121

**R**

Radiosensitization
mechanisms of HDAC inhibitor-induced, 191–196
NaB-mediated, 192, 195
*in vitro,* 180–187
*in vivo,* 187–189
Radiosensitizers. *See* HDAC inhibitor
Receptors, cotransfection of, 94
Restriction enzyme mediated integration (REMI), 117
Rhombomere, transplantation of, 18
Ribonuclease A (RNase A), 210
Rictor, 126
*Ringelschwanz (rs)* mutant mouse, 51
Ror2, 68–69
Runx2, 67

**S**

SACK. *See also* Suppression of ACK agents, 157
SACK-derived ASC strains to conventional cell lines, comparison of, 157–158
Sclereosteosis, 67
Sclerostin, 67
Secreted frizzled related proteins (Sfrps), 65–66
Secreted Wnt inhibitors and agonists
Dkks, 63–65
sclerostin, 67
Sfrps, 65–66
*Wif-1,* 66–67
Serum $Ca^{2+}$, 97
*Sfrp1,* 66
*Sfrp3,* 65
Sfrps. *See* Secreted frizzled related proteins
*Shh* expression, 20
*Sir2,* 177
Slug, 128
*SmlA,* 122
Sodium butyrate, 180
*Sonic hedgehog (Shh),* 19, 151
"Source and sink" model, 129. *See also* Morphogen sensing, ammonia controls culmination
Sox9 positive skeletal elements, 53
Species-specific facial pattern, molecular origins of, 11
Spore dierentiation factors, 130–131
Spore encapsulation of *Dictyostelium,* 131–132
Starvation, CMA's role in, 216
Suppression of ACK (SACK) approach, 144, 152–157
advantages of, 158
future application of regulators and pathways of, 158–160
$\alpha$-Synuclein, 227–228

**T**

TagC, 131
Tartrate-resistant acid phosphatase (TRAP) levels, 60

*Tcf4,* 56
Teleosts, BMP signaling in, 31
Tissue component
  of ECM displacements, 250
  of ECM motion, 251
TORC2, 126
Transforming growth factor beta (TGF$\beta$) family (BMPs), 6
Transgenesis approach for ASC expansion, 144
  limitations in, 143, 145
  using *Bcl-2* gene, 145
  using *HoxB4* gene, 146
  using *Notch* genes, 147–148
Trapoxin, 179
Trichostatin A (TSA), 180
Tumor cell radiosensitization. *See* Radiosensitization

**U**

Unequivocal method, for CMA, 214–215

**V**

Valproic acid (VA), 182
  eects on tumor cell radiosensitivity, 183–184
Venus flytrap domain (VFTD), 89
"Vertebrate archetype," 2
Vertebrate craniofacial complex, 2
VFTD. *See* Venus flytrap domain
Viral infection, 143

**W**

Wnt, 59–60
  signaling, 62, 69, 149–150
  signaling molecules, 58–59
    frizzled, 61–62
    GSK3$\beta$, 62–63
    Wnts, 59–61
*Wnt7b,* 60
*Wnt* genes, 149–150
Wnt inhibitory factor-1 (Wif-1), 67–68

**X**

Xanthosine, 157–158

**Y**

YakA, 120–121
Yak1P, 121

**Z**

Zebrafish, 20

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# Contents of Previous Volumes

## Volume 47


**1 Early Events of Somitogenesis in Higher Vertebrates: Allocation of Precursor Cells during Gastrulation and the Organization of a Moristic Pattern in the Paraxial Mesoderm**
*Patrick P. L. Tam, Devorah Goldman, Anne Camus, and Gary C. Shoenwolf*

**2 Retrospective Tracing of the Developmental Lineage of the Mouse Myotome**
*Sophie Eloy-Trinquet, Luc Mathis, and Jean-François Nicolas*

**3 Segmentation of the Paraxial Mesoderm and Vertebrate Somitogenesis**
*Olivier Pourquié*

**4 Segmentation: A View from the Border**
*Claudio D. Stern and Daniel Vasiliauskas*

**5 Genetic Regulation of Somite Formation**
*Alan Rawls, Jeanne Wilson-Rawls, and Eric N. Olsen*

**6 Hox Genes and the Global Patterning of the Somitic Mesoderm**
*Ann Campbell Burke*

**7 The Origin and Morphogenesis of Amphibian Somites**
*Ray Keller*

**8 Somitogenesis in Zebrafish**
*Scott A. Halley and Christiana Nüsslain-Volhard*

**9 Rostrocaudal Differences within the Somites Confer Segmental Pattern to Trunk Neural Crest Migration**
*Marianne Bronner-Fraser*

## Volume 48

**1 Evolution and Development of Distinct Cell Lineages Derived from Somites**
*Beate Brand-Saberi and Bodo Christ*

**2 Duality of Molecular Signaling Involved in Vertebral Chondrogenesis**
*Anne-Hélène Monsoro-Burq and Nicole Le Douarin*

**3 Sclerotome Induction and Differentiation**
*Jennifer L. Docker*

**4 Genetics of Muscle Determination and Development**
*Hans-Henning Arnold and Thomas Braun*

**5 Multiple Tissue Interactions and Signal Transduction Pathways Control Somite Myogenesis**
*Anne-Gaëlle Borycki and Charles P. Emerson, Jr.*

**6 The Birth of Muscle Progenitor Cells in the Mouse: Spatiotemporal Considerations**
*Shahragim Tajbakhsh and Margaret Buckingham*

**7 Mouse–Chick Chimera: An Experimental System for Study of Somite Development**
*Josiane Fontaine-Pérus*

**8 Transcriptional Regulation during Somitogenesis**
*Dennis Summerbell and Peter W. J. Rigby*

**9 Determination and Morphogenesis in Myogenic Progenitor Cells: An Experimental Embryological Approach**
*Charles P. Ordahl, Brian A. Williams, and Wilfred Denetclaw*

## Volume 49

**1 The Centrosome and Parthenogenesis**
*Thomas Küntziger and Michel Bornens*

**2 γ-Tubulin**
*Berl R. Oakley*

**3 γ-Tubulin Complexes and Their Role in Microtubule Nucleation**
*Ruwanthi N. Gunawardane, Sofia B. Lizarraga, Christiane Wiese, Andrew Wilde, and Yixian Zheng*

**4 γ-Tubulin of Budding Yeast**
*Jackie Vogel and Michael Snyder*

**5 The Spindle Pole Body of *Saccharomyces cerevisiae:* Architecture and Assembly of the Core Components**
*Susan E. Francis and Trisha N. Davis*

**6 The Microtubule Organizing Centers of *Schizosaccharomyces pombe***
*Iain M. Hagan and Janni Petersen*

**7 Comparative Structural, Molecular, and Functional Aspects of the *Dictyostelium discoideum* Centrosome**
*Ralph Gräf, Nicole Brusis, Christine Daunderer, Ursula Euteneuer, Andrea Hestermann, Manfred Schliwa, and Masahiro Ueda*

**8 Are There Nucleic Acids in the Centrosome?**
*Wallace F. Marshall and Joel L. Rosenbaum*

**9 Basal Bodies and Centrioles: Their Function and Structure**
*Andrea M. Preble, Thomas M. Giddings, Jr., and Susan K. Dutcher*

**10 Centriole Duplication and Maturation in Animal Cells**
*B. M. H. Lange, A. J. Faragher, P. March, and K. Gull*

**11 Centrosome Replication in Somatic Cells: The Significance of the $G_1$ Phase**
*Ron Balczon*

**12 The Coordination of Centrosome Reproduction with Nuclear Events during the Cell Cycle**
*Greenfield Sluder and Edward H. Hinchcliffe*

**13 Regulating Centrosomes by Protein Phosphorylation**
*Andrew M. Fry, Thibault Mayor, and Erich A. Nigg*

**14 The Role of the Centrosome in the Development of Malignant Tumors**
*Wilma L. Lingle and Jeffrey L. Salisbury*

**15 The Centrosome-Associated Aurora/Ipl-like Kinase Family**
*T. M. Goepfert and B. R. Brinkley*

**16 Centrosome Reduction during Mammalian Spermiogenesis**
*G. Manandhar, C. Simerly, and G. Schatten*

**17 The Centrosome of the Early *C. elegans* Embryo: Inheritance, Assembly, Replication, and Developmental Roles**
*Kevin F. O'Connell*

**18 The Centrosome in *Drosophila* Oocyte Development**
*Timothy L. Megraw and Thomas C. Kaufman*

**19 The Centrosome in Early *Drosophila* Embryogenesis**
*W. F. Rothwell and W. Sullivan*

**20 Centrosome Maturation**
*Robert E. Palazzo, Jacalyn M. Vogel, Bradley J. Schnackenberg, Dawn R. Hull, and Xingyong Wu*

## Volume 50

**1 Patterning the Early Sea Urchin Embryo**
*Charles A. Ettensohn and Hyla C. Sweet*

**2 Turning Mesoderm into Blood: The Formation of Hematopoietic Stem Cells during Embryogenesis**
*Alan J. Davidson and Leonard I. Zon*

**3 Mechanisms of Plant Embryo Development**
*Shunong Bai, Lingjing Chen, Mary Alice Yund, and Zinmay Rence Sung*

**4 Sperm-Mediated Gene Transfer**
*Anthony W. S. Chan, C. Marc Luetjens, and Gerald P. Schatten*

**5 Gonocyte–Sertoli Cell Interactions during Development of the Neonatal Rodent Testis**
*Joanne M. Orth, William F. Jester, Ling-Hong Li, and Andrew L. Laslett*

**6 Attributes and Dynamics of the Endoplasmic Reticulum in Mammalian Eggs**
*Douglas Kline*

**7 Germ Plasm and Molecular Determinants of Germ Cell Fate**
*Douglas W. Houston and Mary Lou King*

## Volume 51

1 **Patterning and Lineage Specification in the Amphibian Embryo**
*Agnes P. Chan and Laurence D. Etkin*

2 **Transcriptional Programs Regulating Vascular Smooth Muscle Cell Development and Differentiation**
*Michael S. Parmacek*

3 **Myofibroblasts: Molecular Crossdressers**
*Gennyne A. Walker, Ivan A. Guerrero, and Leslie A. Leinwand*

4 **Checkpoint and DNA-Repair Proteins Are Associated with the Cores of Mammalian Meiotic Chromosomes**
*Madalena Tarsounas and Peter B. Moens*

5 **Cytoskeletal and $Ca^{2+}$ Regulation of Hyphal Tip Growth and Initiation**
*Sara Torralba and I. Brent Heath*

6 **Pattern Formation during *C. elegans* Vulval Induction**
*Minqin Wang and Paul W. Sternberg*

7 **A Molecular Clock Involved in Somite Segmentation**
*Miguel Maroto and Olivier Pourquié*

## Volume 52

1 **Mechanism and Control of Meiotic Recombination Initiation**
*Scott Keeney*

2 **Osmoregulation and Cell Volume Regulation in the Preimplantation Embryo**
*Jay M. Baltz*

3 **Cell–Cell Interactions in Vascular Development**
*Diane C. Darland and Patricia A. D'Amore*

4 **Genetic Regulation of Preimplantation Embryo Survival**
*Carol M. Warner and Carol A. Brenner*

## Volume 53

1 **Developmental Roles and Clinical Significance of Hedgehog Signaling**
*Andrew P. McMahon, Philip W. Ingham, and Clifford J. Tabin*

2 **Genomic Imprinting: Could the Chromatin Structure Be the Driving Force?**
*Andras Paldi*

3 **Ontogeny of Hematopoiesis: Examining the Emergence of Hematopoietic Cells in the Vertebrate Embryo**
*Jenna L. Galloway and Leonard I. Zon*

4 **Patterning the Sea Urchin Embryo: Gene Regulatory Networks, Signaling Pathways, and Cellular Interactions**
*Lynne M. Angerer and Robert C. Angerer*

## Volume 54

1 **Membrane Type-Matrix Metalloproteinases (MT-MMP)**
*Stanley Zucker, Duanqing Pei, Jian Cao, and Carlos Lopez-Otin*

2 **Surface Association of Secreted Matrix Metalloproteinases**
*Rafael Fridman*

3 **Biochemical Properties and Functions of Membrane-Anchored Metalloprotease-Disintegrin Proteins (ADAMs)**
*J. David Becherer and Carl P. Blobel*

4 **Shedding of Plasma Membrane Proteins**
*Joaquín Arribas and Anna Merlos-Suárez*

5 **Expression of Meprins in Health and Disease**
*Lourdes P. Norman, Gail L. Matters, Jacqueline M. Crisman, and Judith S. Bond*

6 **Type II Transmembrane Serine Proteases**
*Qingyu Wu*

7 **DPPIV, Seprase, and Related Serine Peptidases in Multiple Cellular Functions**
*Wen-Tien Chen, Thomas Kelly, and Giulio Ghersi*

**8 The Secretases of Alzheimer's Disease**
*Michael S. Wolfe*

**9 Plasminogen Activation at the Cell Surface**
*Vincent Ellis*

**10 Cell-Surface Cathepsin B: Understanding Its Functional Significance**
*Dora Cavallo-Medved and Bonnie F. Sloane*

**11 Protease-Activated Receptors**
*Wadie F. Bahou*

**12 Emmprin (CD147), a Cell Surface Regulator of Matrix Metalloproteinase Production and Function**
*Bryan P. Toole*

**13 The Evolving Roles of Cell Surface Proteases in Health and Disease: Implications for Developmental, Adaptive, Inflammatory, and Neoplastic Processes**
*Joseph A. Madri*

**14 Shed Membrane Vesicles and Clustering of Membrane-Bound Proteolytic Enzymes**
*M. Letizia Vittorelli*

## Volume 55

**1 The Dynamics of Chromosome Replication in Yeast**
*Isabelle A. Lucas and M. K. Raghuraman*

**2 Micromechanical Studies of Mitotic Chromosomes**
*M. G. Poirier and John F. Marko*

**3 Patterning of the Zebrafish Embryo by Nodal Signals**
*Jennifer O. Liang and Amy L. Rubinstein*

**4 Folding Chromosomes in Bacteria: Examining the Role of Csp Proteins and Other Small Nucleic Acid-Binding Proteins**
*Nancy Trun and Danielle Johnston*

## Volume 56

1 **Selfishness in Moderation: Evolutionary Success of the Yeast Plasmid**
*Soundarapandian Velmurugan, Shwetal Mehta, and Makkuni Jayaram*

2 **Nongenomic Actions of Androgen in Sertoli Cells**
*William H. Walker*

3 **Regulation of Chromatin Structure and Gene Activity by Poly(ADP-Ribose) Polymerases**
*Alexei Tulin, Yurli Chinenov, and Allan Spradling*

4 **Centrosomes and Kinetochores, Who needs 'Em? The Role of Noncentromeric Chromatin in Spindle Assembly**
*Priya Prakash Budde and Rebecca Heald*

5 **Modeling Cardiogenesis: The Challenges and Promises of 3D Reconstruction**
*Jeffrey O. Penetcost, Claudio Silva, Maurice Pesticelli, Jr., and Kent L. Thornburg*

6 **Plasmid and Chromosome Traffic Control: How ParA and ParB Drive Partition**
*Jennifer A. Surtees and Barbara E. Funnell*

## Volume 57

1 **Molecular Conservation and Novelties in Vertebrate Ear Development**
*B. Fritzsch and K. W. Beisel*

2 **Use of Mouse Genetics for Studying Inner Ear Development**
*Elizabeth Quint and Karen P. Steel*

3 **Formation of the Outer and Middle Ear, Molecular Mechanisms**
*Moisés Mallo*

4 **Molecular Basis of Inner Ear Induction**
*Stephen T. Brown, Kareen Martin, and Andrew K. Groves*

5 **Molecular Basis of Otic Commitment and Morphogenesis: A Role for Homeodomain-Containing Transcription Factors and Signaling Molecules**
*Eva Bober, Silke Rinkwitz, and Heike Herbrand*

6 **Growth Factors and Early Development of Otic Neurons: Interactions between Intrinsic and Extrinsic Signals**
*Berta Alsina, Fernando Giraldez, and Isabel Varela-Nieto*

7 **Neurotrophic Factors during Inner Ear Development**
*Ulla Pirvola and Jukka Ylikoski*

8 **FGF Signaling in Ear Development and Innervation**
*Tracy J. Wright and Suzanne L. Mansour*

9 **The Roles of Retinoic Acid during Inner Ear Development**
*Raymond Romand*

10 **Hair Cell Development in Higher Vertebrates**
*Wei-Qiang Gao*

11 **Cell Adhesion Molecules during Inner Ear and Hair Cell Development, Including Notch and Its Ligands**
*Matthew W. Kelley*

12 **Genes Controlling the Development of the Zebrafish Inner Ear and Hair Cells**
*Bruce B. Riley*

13 **Functional Development of Hair Cells**
*Ruth Anne Eatock and Karen M. Hurley*

14 **The Cell Cycle and the Development and Regeneration of Hair Cells**
*Allen F. Ryan*

## Volume 58

1 **A Role for Endogenous Electric Fields in Wound Healing**
*Richard Nuccitelli*

2 **The Role of Mitotic Checkpoint in Maintaining Genomic Stability**
*Song-Tao Liu, Jan M. van Deursen, and Tim J. Yen*

3 **The Regulation of Oocyte Maturation**
*Ekaterina Voronina and Gary M. Wessel*

4 **Stem Cells: A Promising Source of Pancreatic Islets for Transplantation in Type 1 Diabetes**
*Cale N. Street, Ray V. Rajotte, and Gregory S. Korbutt*

5 **Differentiation Potential of Adipose Derived Adult Stem (ADAS) Cells**
*Jeffrey M. Gimble and Farshid Guilak*

## Volume 59

1 **The Balbiani Body and Germ Cell Determinants: 150 Years Later**
*Malgorzata Kloc, Szczepan Bilinski, and Laurence D. Etkin*

2 **Fetal–Maternal Interactions: Prenatal Psychobiological Precursors to Adaptive Infant Development**
*Matthew F. S. X. Novak*

3 **Paradoxical Role of Methyl-CpG-Binding Protein 2 in Rett Syndrome**
*Janine M. LaSalle*

4 **Genetic Approaches to Analyzing Mitochondrial Outer Membrane Permeability**
*Brett H. Graham and William J. Craigen*

5 **Mitochondrial Dynamics in Mammals**
*Hsiuchen Chen and David C. Chan*

6 **Histone Modification in Corepressor Functions**
*Judith K. Davie and Sharon Y. R. Dent*

7 **Death by Abl: A Matter of Location**
*Jiangyu Zhu and Jean Y. J. Wang*

## Volume 60

1 **Therapeutic Cloning and Tissue Engineering**
*Chester J. Koh and Anthony Atala*

2 **α-Synuclein: Normal Function and Role in Neurodegenerative Diseases**
*Erin H. Norris, Benoit I. Giasson, and Virginia M.-Y. Lee*

3 **Structure and Function of Eukaryotic DNA Methyltransferases**
*Taiping Chen and En Li*

4 **Mechanical Signals as Regulators of Stem Cell Fate**
*Bradley T. Estes, Jeffrey M. Gimble, and Farshid Guilak*

5 **Origins of Mammalian Hematopoiesis: *In Vivo* Paradigms and *In Vitro* Models**
*M. William Lensch and George Q. Daley*

6 **Regulation of Gene Activity and Repression: A Consideration of Unifying Themes**
*Anne C. Ferguson-Smith, Shau-Ping Lin, and Neil Youngson*

7 **Molecular Basis for the Chloride Channel Activity of Cystic Fibrosis Transmembrane Conductance Regulator and the Consequences of Disease-Causing Mutations**
*Jackie F. Kidd, Ilana Kogan, and Christine E. Bear*

## Volume 61

1 **Hepatic Oval Cells: Helping Redefine a Paradigm in Stem Cell Biology**
*P. N. Newsome, M. A. Hussain, and N. D. Theise*

2 **Meiotic DNA Replication**
*Randy Strich*

3 **Pollen Tube Guidance: The Role of Adhesion and Chemotropic Molecules**
*Sunran Kim, Juan Dong, and Elizabeth M. Lord*

4 **The Biology and Diagnostic Applications of Fetal DNA and RNA in Maternal Plasma**
*Rossa W. K. Chiu and Y. M. Dennis Lo*

5 **Advances in Tissue Engineering**
*Shulamit Levenberg and Robert Langer*

6 **Directions in Cell Migration Along the Rostral Migratory Stream: The Pathway for Migration in the Brain**
*Shin-ichi Murase and Alan F. Horwitz*

7 **Retinoids in Lung Development and Regeneration**
*Malcolm Maden*

8 **Structural Organization and Functions of the Nucleus in Development, Aging, and Disease**
*Leslie Mounkes and Colin L. Stewart*

## Volume 62

1 **Blood Vessel Signals During Development and Beyond**
*Ondine Cleaver*

2 **HIFs, Hypoxia, and Vascular Development**
*Kelly L. Covello and M. Celeste Simon*

3 **Blood Vessel Patterning at the Embryonic Midline**
*Kelly A. Hogan and Victoria L. Bautch*

4 **Wiring the Vascular Circuitry: From Growth Factors to Guidance Cues**
*Lisa D. Urness and Dean Y. Li*

5 **Vascular Endothelial Growth Factor and Its Receptors in Embryonic Zebrafish Blood Vessel Development**
*Katsutoshi Goishi and Michael Klagsbrun*

6 **Vascular Extracellular Matrix and Aortic Development**
*Cassandra M. Kelleher, Sean E. McLean, and Robert P. Mecham*

7 **Genetics in Zebrafish, Mice, and Humans to Dissect Congenital Heart Disease: Insights in the Role of VEGF**
*Diether Lambrechts and Peter Carmeliet*

8 **Development of Coronary Vessels**
*Mark W. Majesky*

9 **Identifying Early Vascular Genes Through Gene Trapping in Mouse Embryonic Stem Cells**
*Frank Kuhnert and Heidi Stuhlmann*

## Volume 63

1 **Early Events in the DNA Damage Response**
*Irene Ward and Junjie Chen*

2 **Afrotherian Origins and Interrelationships: New Views and Future Prospects**
*Terence J. Robinson and Erik R. Seiffert*

3 **The Role of Antisense Transcription in the Regulation of X-Inactivation**
*Claire Rougeulle and Philip Avner*

**4 The Genetics of Hiding the Corpse: Engulfment and Degradation of Apoptotic Cells in *C. elegans* and *D. melanogaster***
*Zheng Zhou, Paolo M. Mangahas, and Xiaomeng Yu*

**5 Beginning and Ending an Actin Filament: Control at the Barbed End**
*Sally H. Zigmond*

**6 Life Extension in the Dwarf Mouse**
*Andrzej Bartke and Holly Brown-Borg*

## Volume 64

**1 Stem/Progenitor Cells in Lung Morphogenesis, Repair, and Regeneration**
*David Warburton, Mary Anne Berberich, and Barbara Driscoll*

**2 Lessons from a Canine Model of Compensatory Lung Growth**
*Connie C. W. Hsia*

**3 Airway Glandular Development and Stem Cells**
*Xiaoming Liu, Ryan R. Driskell, and John F. Engelhardt*

**4 Gene Expression Studies in Lung Development and Lung Stem Cell Biology**
*Thomas J. Mariani and Naftali Kaminski*

**5 Mechanisms and Regulation of Lung Vascular Development**
*Michelle Haynes Pauling and Thiennu H. Vu*

**6 The Engineering of Tissues Using Progenitor Cells**
*Nancy L. Parenteau, Lawrence Rosenberg, and Janet Hardin-Young*

**7 Adult Bone Marrow-Derived Hemangioblasts, Endothelial Cell Progenitors, and EPCs**
*Gina C. Schatteman*

**8 Synthetic Extracellular Matrices for Tissue Engineering and Regeneration**
*Eduardo A. Silva and David J. Mooney*

**9 Integrins and Angiogenesis**
*D. G. Stupack and D. A. Cheresh*

## Volume 65

1 **Tales of Cannibalism, Suicide, and Murder: Programmed Cell Death in *C. elegans***
*Jason M. Kinchen and Michael O. Hengartner*

2 **From Guts to Brains: Using Zebrafish Genetics to Understand the Innards of Organogenesis**
*Carsten Stuckenholz, Paul E. Ulanch, and Nathan Bahary*

3 **Synaptic Vesicle Docking: A Putative Role for the Munc18/Sec1 Protein Family**
*Robby M. Weimer and Janet E. Richmond*

4 **ATP-Dependent Chromatin Remodeling**
*Corey L. Smith and Craig L. Peterson*

5 **Self-Destruct Programs in the Processes of Developing Neurons**
*David Shepherd and V. Hugh Perry*

6 **Multiple Roles of Vascular Endothelial Growth Factor (VEGF) in Skeletal Development, Growth, and Repair**
*Elazar Zelzer and Bjorn R. Olsen*

7 **G-Protein Coupled Receptors and Calcium Signaling in Development**
*Geoffrey E. Woodard and Juan A. Rosado*

8 **Differential Functions of 14-3-3 Isoforms in Vertebrate Development**
*Anthony J. Muslin and Jeffrey M. C. Lau*

9 **Zebrafish Notochordal Basement Membrane: Signaling and Structure**
*Annabelle Scott and Derek L. Stemple*

10 **Sonic Hedgehog Signaling and the Developing Tooth**
*Martyn T. Cobourne and Paul T. Sharpe*

## Volume 66

1 **Stepwise Commitment from Embryonic Stem to Hematopoietic and Endothelial Cells**
*Changwon Park, Jesse J. Lugus, and Kyunghee Choi*

**2 Fibroblast Growth Factor Signaling and the Function and Assembly of Basement Membranes**
*Peter Lonai*

**3 TGF-$\beta$ Superfamily and Mouse Craniofacial Development: Interplay of Morphogenetic Proteins and Receptor Signaling Controls Normal Formation of the Face**
*Marek Dudas and Vesa Kaartinen*

**4 The Colors of Autumn Leaves as Symptoms of Cellular Recycling and Defenses Against Environmental Stresses**
*Helen J. Ougham, Phillip Morris, and Howard Thomas*

**5 Extracellular Proteases: Biological and Behavioral Roles in the Mammalian Central Nervous System**
*Yan Zhang, Kostas Pothakos, and Styliana-Anna (Stella) Tsirka*

**6 The Genetic Architecture of House Fly Mating Behavior**
*Lisa M. Meffert and Kara L. Hagenbuch*

**7 Phototropins, Other Photoreceptors, and Associated Signaling: The Lead and Supporting Cast in the Control of Plant Movement Responses**
*Bethany B. Stone, C. Alex Esmon, and Emmanuel Liscum*

**8 Evolving Concepts in Bone Tissue Engineering**
*Catherine M. Cowan, Chia Soo, Kang Ting, and Benjamin Wu*

**9 Cranial Suture Biology**
*Kelly A Lenton, Randall P. Nacamuli, Derrick C. Wan, Jill A. Helms, and Michael T. Longaker*

## Volume 67

**1 Deer Antlers as a Model of Mammalian Regeneration**
*Joanna Price, Corrine Faucheux, and Steve Allen*

**2 The Molecular and Genetic Control of Leaf Senescence and Longevity in *Arabidopsis***
*Pyung Ok Lim and Hong Gil Nam*

**3 Cripto-1: An Oncofetal Gene with Many Faces**
*Caterina Bianco, Luigi Strizzi, Nicola Normanno, Nadia Khan, and David S. Salomon*

**4 Programmed Cell Death in Plant Embryogenesis**
*Peter V. Bozhkov, Lada H. Filonova, and Maria F. Suarez*

**5 Physiological Roles of Aquaporins in the Choroid Plexus**
*Daniela Boassa and Andrea J. Yool*

**6 Control of Food Intake Through Regulation of cAMP**
*Allan Z. Zhao*

**7 Factors Affecting Male Song Evolution in *Drosophila montana***
*Anneli Hoikkala, Kirsten Klappert, and Dominique Mazzi*

**8 Prostanoids and Phosphodiesterase Inhibitors in Experimental Pulmonary Hypertension**
*Ralph Theo Schermuly, Hossein Ardeschir Ghofrani, and Norbert Weissmann*

**9 14-3-3 Protein Signaling in Development and Growth Factor Responses**
*Daniel Thomas, Mark Guthridge, Jo Woodcock, and Angel Lopez*

**10 Skeletal Stem Cells in Regenerative Medicine**
*Wataru Sonoyama, Carolyn Coppe, Stan Gronthos, and Songtao Shi*

## Volume 68

**1 Prolactin and Growth Hormone Signaling**
*Beverly Chilton and Aveline Hewetson*

**2 Alterations in cAMP-Mediated Signaling and Their Role in the Pathophysiology of Dilated Cardiomyopathy**
*Matthew A. Movsesian and Michael R. Bristow*

**3 Corpus Luteum Development: Lessons from Genetic Models in Mice**
*Anne Bachelot and Nadine Binart*

**4 Comparative Developmental Biology of the Mammalian Uterus**
*Thomas E. Spencer, Kanako Hayashi, Jianbo Hu, and Karen D. Carpenter*

**5 Sarcopenia of Aging and Its Metabolic Impact**
*Helen Karakelides and K. Sreekumaran Nair*

**6 Chemokine Receptor CXCR3: An Unexpected Enigma**
*Liping Liu, Melissa K. Callahan, DeRen Huang, and Richard M. Ransohoff*

**7 Assembly and Signaling of Adhesion Complexes**
*Jorge L. Sepulveda, Vasiliki Gkretsi, and Chuanyue Wu*

**8 Signaling Mechanisms of Higher Plant Photoreceptors: A Structure-Function Perspective**
*Haiyang Wang*

**9 Initial Failure in Myoblast Transplantation Therapy Has Led the Way Toward the Isolation of Muscle Stem Cells: Potential for Tissue Regeneration**
*Kenneth Urish, Yasunari Kanda, and Johnny Huard*

**10 Role of 14-3-3 Proteins in Eukaryotic Signaling and Development**
*Dawn L. Darling, Jessica Yingling, and Anthony Wynshaw-Boris*

## Volume 69

**1 Flipping Coins in the Fly Retina**
*Tamara Mikeladze-Dvali, Claude Desplan, and Daniela Pistillo*

**2 Unraveling the Molecular Pathways That Regulate Early Telencephalon Development**
*Jean M. Hébert*

**3 Glia–Neuron Interactions in Nervous System Function and Development**
*Shai Shaham*

**4 The Novel Roles of Glial Cells Revisited: The Contribution of Radial Glia and Astrocytes to Neurogenesis**
*Tetsuji Mori, Annalisa Buffo, and Magdalena Götz*

**5 Classical Embryological Studies and Modern Genetic Analysis of Midbrain and Cerebellum Development**
*Mark Zervas, Sandra Blaess, and Alexandra L. Joyner*

**6 Brain Development and Susceptibility to Damage; Ion Levels and Movements**
*Maria Erecinska, Shobha Cherian, and Ian A. Silver*

**7 Thinking about Visual Behavior; Learning about Photoreceptor Function**
*Kwang-Min Choe and Thomas R. Clandinin*

**8 Critical Period Mechanisms in Developing Visual Cortex**
*Takao K. Hensch*

**9 Brawn for Brains: The Role of MEF2 Proteins in the Developing Nervous System**
*Aryaman K. Shalizi and Azad Bonni*

**10 Mechanisms of Axon Guidance in the Developing Nervous System**
*Céline Plachez and Linda J. Richards*

## Volume 70

**1 Magnetic Resonance Imaging: Utility as a Molecular Imaging Modality**
*James P. Basilion, Susan Yeon, and René Botnar*

**2 Magnetic Resonance Imaging Contrast Agents in the Study of Development**
*Angelique Louie*

**3 $^{1}H/^{19}F$ Magnetic Resonance Molecular Imaging with Perfluorocarbon Nanoparticles**
*Gregory M. Lanza, Patrick M. Winter, Anne M. Neubauer, Shelton D. Caruthers, Franklin D. Hockett, and Samuel A. Wickline*

**4 Loss of Cell Ion Homeostasis and Cell Viability in the Brain: What Sodium MRI Can Tell Us**
*Fernando E. Boada, George LaVerde, Charles Jungreis, Edwin Nemoto, Costin Tanase, and Ileana Hancu*

**5 Quantum Dot Surfaces for Use *In Vivo* and *In Vitro***
*Byron Ballou*

**6 *In Vivo* Cell Biology of Cancer Cells Visualized with Fluorescent Proteins**
*Robert M. Hoffman*

**7 Modulation of Tracer Accumulation in Malignant Tumors: Gene Expression, Gene Transfer, and Phage Display**
*Uwe Haberkorn*

**8 Amyloid Imaging: From Benchtop to Bedside**
*Chungying Wu, Victor W. Pike, and Yanming Wang*

**9 *In Vivo* Imaging of Autoimmune Disease in Model Systems**
*Eric T. Ahrens and Penelope A. Morel*

## Volume 71

1 **The Choroid Plexus-Cerebrospinal Fluid System: From Development to Aging**
*Zoran B. Redzic, Jane E. Preston, John A. Duncan, Adam Chodobski, and Joanna Szmydynger-Chodobska*

2 **Zebrafish Genetics and Formation of Embryonic Vasculature**
*Tao P. Zhong*

3 **Leaf Senescence: Signals, Execution, and Regulation**
*Yongfeng Guo and Susheng Gan*

4 **Muscle Stem Cells and Regenerative Myogenesis**
*Iain W. McKinnell, Gianni Parise, and Michael A. Rudnicki*

5 **Gene Regulation in Spermatogenesis**
*James A. MacLean II and Miles F. Wilkinson*

6 **Modeling Age-Related Diseases in *Drosophila:* Can this Fly?**
*Kinga Michno, Diana van de Hoef, Hong Wu, and Gabrielle L. Boulianne*

7 **Cell Death and Organ Development in Plants**
*Hilary J. Rogers*

8 **The Blood-Testis Barrier: Its Biology, Regulation, and Physiological Role in Spermatogenesis**
*Ching-Hang Wong and C. Yan Cheng*

9 **Angiogenic Factors in the Pathogenesis of Preeclampsia**
*Hai-Tao Yuan, David Haig, and S. Ananth Karumanchi*

## Volume 72

1 **Defending the Zygote: Search for the Ancestral Animal Block to Polyspermy**
*Julian L. Wong and Gary M. Wessel*

2 **Dishevelled: A Mobile Scaffold Catalyzing Development**
*Craig C. Malbon and Hsien-yu Wang*

3 **Sensory Organs: Making and Breaking the Pre-Placodal Region**
*Andrew P. Bailey and Andrea Streit*

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

*Linda K. Hansen, Joshua Wilhelm, and John T. Fassett*

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

*Cheng-Ming Chuong, Ping Wu, Maksim Plikus, Ting-Xin Jiang, and Randall Bruce Widelitz*

**6 Fur Seal Adaptations to Lactation: Insights into Mammary Gland Function**

*Julie A. Sharp, Kylie N. Cane, Christophe Lefevre, John P. Y. Arnould, and Kevin R. Nicholas*