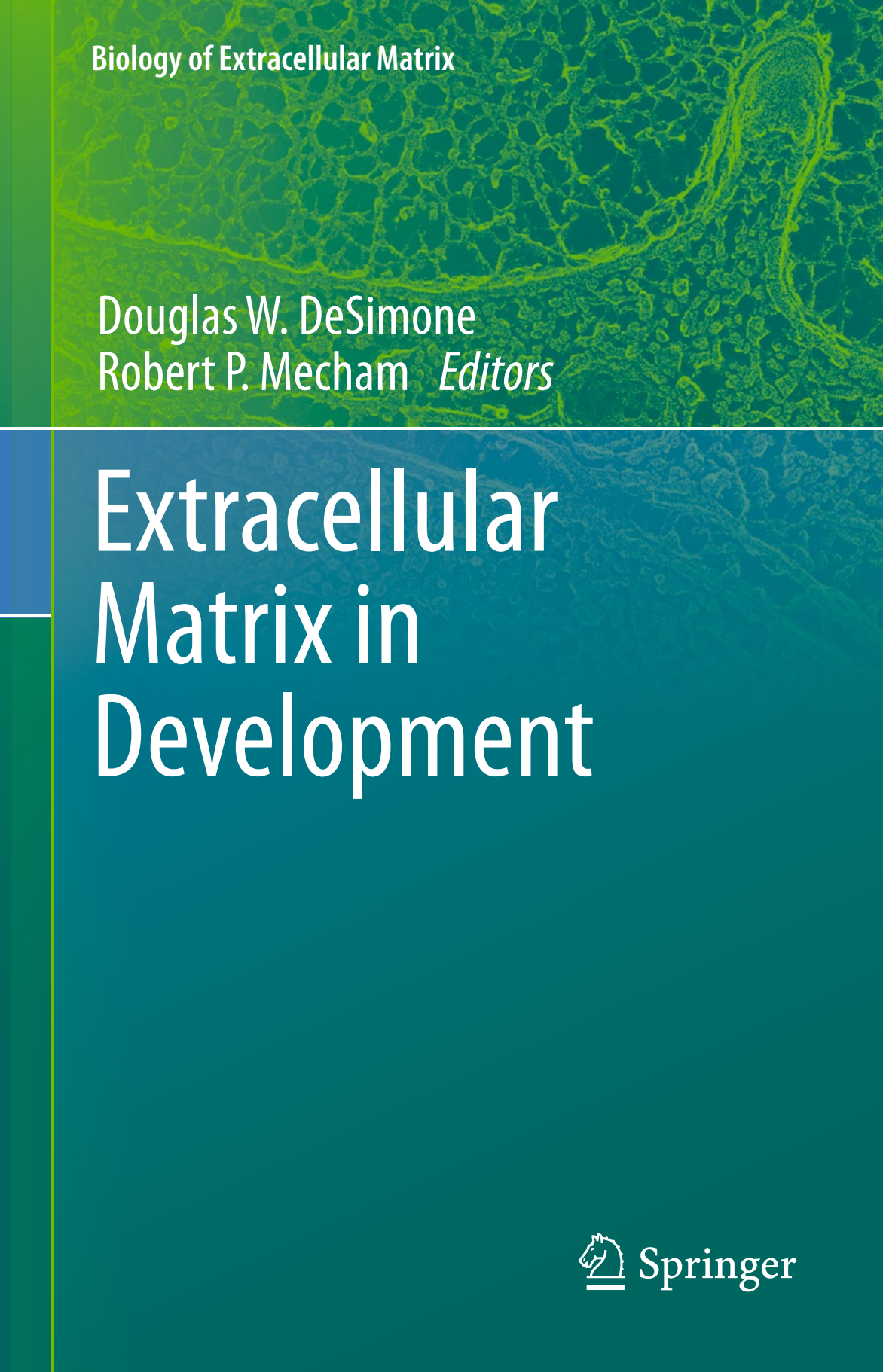
Biology of Extracellular Matrix
Douglas W. DeSimone
Robert P. Mecham Editors
Extracellular Matrix in Development
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Extracellular Matrix in Development

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Preface

The Extracellular Matrix in Development

The biological diversity of various interactive events during development reflects the wide-ranging signals associated with the extracellular matrix (ECM). Matrix components are the source of numerous developmental clocks and, by interacting with cell-surface matrix receptors, provide inductive or permissive signals that tell cells which direction to move, where to stop, and which genes to express when. This volume of the Biology of Extracellular Matrix series explores how the ECM influences development through examples of underlying mechanisms related to developmental processes. The chapters are organized into three broadly defined sections. Part I explores basic questions of how information content in the ECM is organized and how ECM molecules influence cellular and tissue movements in early embryonic events, including gastrulation. Chapters in Part II use the well-studied developmental processes neural crest cell migration and branching morphogenesis to illustrate how ECM provides developmental instructions that initiate and control functional interactions that direct tissue organization and morphogenesis. Also addressed is the importance of ECM in the stem cell niche and how ECM molecules might be used to modulate stem cell differentiation in tissue engineering. One of the most fruitful areas of developmental biology is the use of model organisms to study cell–matrix interactions and the regulatory processes operating during organ and tissue development. The chapters in Part III look at three model systems, moving from the simple *Hydra* to zebra fish and mice, to understand the lexicon of developmental signals associated with the ECM. These three animal systems span a great range of complexity and phylogenetic distance, yet illustrate numerous common principles of cell–matrix interactions in development.

The information code within the ECM is complex. In the first chapter, Cadwallader and Yost illustrate how placement of the sulfate groups on unique sugar side chains of heparan sulfate (HS) proteoglycans influences developmental processes. The collective actions of HS chain biosynthesis result in a mature HS chain with a mix of sulfated and non-sulfated residues. The unique placement and

spacing of sulfate and epimerization modifications create the HS fine structure, which provides specificity for biological regulation. Cadwallader and Yost show how the HS fine structure functions much like a zip code (a "Glycode") to regulate specific growth factor binding. Important players in the sulfation pathway are the *O*-sulfotransferases (OSTs), enzymes that function in the Golgi to add sulfate groups to urinoic acid residues. The authors discuss the importance of each of the OSTs in development based upon studies in model organisms.

One of the surprise findings from the application of live-cell imagining techniques to developing embryos is the extensive cell motion throughout the developing organism. In Chap. 2, Czirok, Rongish, and Little use high-resolution imaging along with sophisticated image analysis to document how tissue movements sweep the ECM to distant positions during early embryogenesis. By studying micro-assembly dynamics of fibronectin and fibrillin-2 in avian embryos, they show that the ECM moves as a composite material, whereby distinct molecular components as well as spatially separated layers exhibit similar displacements. These movement patterns are shared among embryos at equivalent stages of development. The authors discuss the implications of how the large-scale co-movement of cells and the surrounding ECM impacts the establishment and maintenance of ECM-bound morphogen gradients.

Gastrulation, the morphogenetic process that forms and positions the three primary germ layers, is mediated by coordinated tissue movements that are driven by integrated cell behaviors influenced by the ECM. Using the *Xenopus* embryo as a model, Dzamba and DeSimone (Chap. 3) explain the important role of fibronectin (FN) in amphibian gastrulation, where the protein provides a critical substratum for cell adhesion and migration. But beyond these traditional roles, the authors illustrate how FN and other ECM proteins regulate cell polarity and contribute to morphogenetic behaviors through mechanical signaling and by influencing tissue force generation. Although ECM is usually thought of as a static structure, the authors point out that the ECM is very dynamic. Its composition and physical structure is constantly changing during development, which, in turn, is an important way that ECM affects cell behavior.

Chapter 4 addresses how the ECM influences one the most important mechanisms in embryonic development—branching morphogenesis. It has long been known that cell interactions with the interstitial matrix and the basement membrane associated with epithelial cells play a critical role in the branching process. In this chapter, Daley and Yamada highlight several general biological principles by which cell–ECM interactions regulate epithelial morphogenesis, including ECM-mediated or ECM-induced alterations in tissue shape and stimulation of dynamic cell motility, proliferative outgrowth and expansion of the epithelium regulated by growth factors and proteolytic degradation of the ECM, and basement membrane roles in coordinating organization of epithelial tissue architecture. They conclude with a discussion of the implications of this knowledge for the rational design of bioengineered scaffolds in regenerative medicine strategies, as well as their potential relevance to epithelial cancer progression.

Cardiovascular development requires spatial and temporal signals that coordinate cell migration and gene expression in the numerous cell populations that make up the heart and blood vessels. In Chap. 5, Astrof explores how signaling by various growth factors is integrated into precise developmental programs via the extracellular matrix. In the developing vertebrate, connecting the heart to the vascular tree of the embryonic circulation requires asymmetric remodeling that is essential for the separation of arterial and venous circulations. This requires coordinated communication between cells of mesoderm, endoderm, surface ectoderm, and the neural crest. Astrof examines the important interactions between neural crest cells and the surrounding tissues that are critical to development of the cardiac outflow tract and aortic arches. The chapter also contains a detailed discussion of the specific ECM molecules that modulate neural crest development in vivo.

Recent interest in developmental and regenerative biology has been focused on adult and embryonic stem cells that have the ability for self-renewal and, through their pluripotency, possess the ability to mature into tissue cells of many different lineages. Regulating stem cell fate has traditionally relied on presenting growth factors and small molecules in developmentally appropriate ways. In Chap. 6, Choi, Holle, and Engler describe how ECM influences stem cell behavior independent of chemical signals. ECM can impact the immediate cellular microenvironment through changes in stiffness, topography, binding properties, and porosity. These physical properties imparted by the ECM influence cell shape and gene expression. In this context, Choi and colleagues discuss recent advances in nano- or microfabrication techniques, biomechanical and biophysical driven stem cell differentiation, and the mechanisms whereby cells feel their ECM environment.

Because of its ability to regenerate, the *Hydra* is one of the most studied organisms in regenerative biology. Its simple body plan (two layers of epithelial and an intervening ECM) and radial symmetry make it ideal for exploring cell–cell and cell–matrix interactions in development and regenerative processes. In Chap. 7, Zhang and Sarras review the biogenesis of *Hydra* ECM during regeneration and development. They detail the biochemical composition of *Hydra* ECM and the important role of the basement membrane and interstitial collagens in maintaining differentiation of the epithelial cells. *Hydra* ECM is resynthesized and assembled during regeneration, and this process is required before morphogenesis and pattern formation can happen. A major message of this chapter is that ECM biogenesis is essential for *Hydra* morphogenesis, which illustrates the key position ECM plays in regulation of cellular differentiation.

Numerous model organisms have been used to illustrate the importance of ECM in development. Frogs, mice, chicks, and numerous invertebrates have all contributed to our understanding of various steps in the developmental process, but each is limited in how much information it can provide. Although in its infancy as a model to study ECM and interacting proteins, the zebra fish offers numerous advantages that circumvent some problems associated with other animal models. In Chap. 8, Mundell and Jessen review the steadily increasing number of zebra fish studies showing developmental roles for matrix proteins, their receptors, and their modifying enzymes. They provide a framework for the reader to gain an

appreciation for the different ECM proteins, integrins, and metalloproteases present in zebra fish and their potential contributions to embryonic development.

Hyaluronic acid (HA) is present throughout the body where it is present in all tissues. In Chap. 9, Roughley and Moffatt discuss HA's role in skeletal biology, where it plays an important role in chondrogenesis and osteogenesis. HA and its fragments play a major role in endochondral bone formation and possibly intramembranous bone formation through the regulation of chondrocyte, osteoblast, and osteoclast differentiation and action. In the growth plate, its abundance increases from the proliferative to the hypertrophic zone, where it is thought to contribute to cell hypertrophy in addition to its more conventional role in proteoglycan aggregate formation within the ECM. Using mouse models to inactivate hyaluronan synthase (HAS, the enzymes that produce HA in cells), the authors show how loss of HA impairs bone growth and chondrocyte organization. They also discuss how HAS gene mutations lead to other disorders, including malignancies.

Obviously, each of the topics covered in this volume represents significant and distinct areas of matrix biology that merit expanded discussion. To broaden the scope, however, would require a much larger book than is possible for this series. It is hoped that the topics covered in this current volume will provide the reader with a basic understanding of the importance of ECM in influencing fundamental mechanisms of development.

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Contents

Part I
Informational Signals in Extracellular Matrix and Matrix Influences on Cell Movement in the Developing Embryo

Chapter 1
The Glycocode: Translating Heparan Sulfate Fine Structure into Developmental Function

Adam B. Cadwallader and H. Joseph Yost

Abstract Heparan sulfate proteoglycans (HSPGs) are an important component of the cell surface and extracellular matrix. HSPGs function in a wide variety of biological processes, including cell adhesion, signaling, migration, and proliferation. HSPGs are an information-dense family consisting of a core protein to which one or more glycosaminoglycan (GAG) chains are attached. The information contained within the GAG chains allows for great complexity and a specificity to bind and regulate binding of growth factors and morphogens. It is therefore no surprise that HSPGs are involved in many developmental processes, such as neural migration, kidney formation, and placentation. Here we explore how the information-rich GAG chains control distinct aspects of development utilizing a "glycocode" model.

1.1 Fine Structure of Heparan Sulfate Proteoglycans

1.1.1 HSPG Biosynthesis

Heparan sulfate proteoglycan (HSPG) biosynthesis begins with translation of an HSPG core protein. There are three general configurations of HSPG core proteins: (1) a type 1 transmembrane (TM) protein, with a signal peptide and extracellular

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domain, a single-pass TM domain, and a cytoplasmic domain, such as members of the syndecan family; (2) a GPI-linked surface protein, with a signal peptide domain and an extracellular domain, such as members of the glypican family; or (3) extracellular proteins, such as agrin or perlecan. The HSPG core protein transitions toward the cell surface through the Golgi, where GAG chain synthesis is initiated at specific serine residues on the extracellular domain of the core protein. Initially, a four-sugar linker is covalently attached to a serine on the core protein, upon which alternating addition of *N*-acetylglucosamine and glucuronic acid residues creates an immature HS chain. Initial chain modification begins with conversion of select *N*-acetyl groups to *N*-sulfo groups by a family of *N*-deacetyl/*N*-sulfotransferases (NDST). Subsequently, C5-epimerase converts a subset of glucuronic acid residues to iduronic acid residues, and three families of *O*-sulfotransferases add sulfate groups to C2 of the uronic acids, to C6, and more rarely to C3 of the glucosamine residues to create mature HSPG chains (Esko and Selleck 2002).

Unlike the core proteins which are encoded by the genome, HS GAG chain initiation, polymerization, and modification appear to occur in a template-independent fashion in the Golgi. Currently, the mechanism by which mature HS chains are created is poorly understood. Open questions within the field include whether there are specific sub-compartments in the Golgi in which select modifications are made or whether there is an absolute requirement for some modifications to be made before others in vivo. A recent study suggests the biosynthetic pathway is more fluid than previously thought (Raman et al. 2011).

The collective actions of the entire HS chain biosynthetic process result in a mature HS chain with a heterogeneous mix of sulfated and non-sulfated residues (Lindahl et al. 1998; Esko and Lindahl 2001). The unique, sulfated domains created through the placement and spacing of sulfate and epimerization modifications create the HS "fine structure." The specificity of any given HS chain to a particular ligand is thought to be dictated by the HS fine structure and defines the corresponding biological activities (Bernfield et al. 1992; Carey 1997; Rosenberg et al. 1997; Bernfield et al. 1999; Rapraeger 2001; Kramer and Yost 2003; Hacker et al. 2005). Fine structure detail, and ultimately the specificity of any particular HS chain, is defined during the last three steps in the HS chain maturation process, which constitutes the HS *O*-sulfation pathway.

1.1.2 HS O-*Sulfation Pathway*

The *O*-sulfation pathway represents the final three steps in the HS chain biosynthesis pathway—the addition of sulfates to the 2-*O* position of urinoic acid residues, catalyzed by the HS 2-*O*-sulfotransferase family (2-OST); addition of sulfates to the 6-*O* position, catalyzed by the 6-*O*-sulfotransferase (6-OST) family; and the 3-*O* position, catalyzed by the 3-*O*-sulfotransferase (3-OST) family, of glucosamine residues. A single 2-OST family member has been discovered in both invertebrate and vertebrate species (Cadwallader and Yost 2007), but recently a second 2-OST

family member was discovered in zebrafish (Cadwalader et al. 2012). The number of 6-OST genes varies depending upon organismal complexity. Invertebrates, such as *Caenorhabditis elegans* and *Drosophila*, contain one 6-OST gene within their genome. In vertebrate species, the number of isoforms jumps to three in chicken, human, and mouse, with the addition of a splice-variant in the latter two species, and to four in zebrafish (Cadwallader and Yost 2006b). The existence of multiple 6-OST genes in the vertebrate species suggests the possibility of divergent regulation and function, as discussed below.

The 3-*O*-sulfation of glucosamine residues is the rarest HS chain modification, accounting for less than 0.5 % of the total sulfate modification (Colliec-Jouault et al. 1994; Shworak et al. 1994). However, a disproportionate number of 3-OST isozymes are needed to create this modification. The invertebrate model organisms contain one (*C. elegans*) or two (*Drosophila*) 3-OST family members. The number greatly expands in vertebrate species with seven family members in human and mouse and eight family members in zebrafish (Cadwallader and Yost 2006a). The rarity of the 3-*O* modification, as well as the extensive number of 3-OST family members, suggests that the 3-*O* modification acts as functional regulator of HS fine structure formation. A unique understanding of evolution, biochemistry, developmental biology, and gene regulation will be needed to understand the highly complex yet regulated HS fine structure.

1.1.3 Does Diversity Within the O*-Sulfotransferase Families Contribute to the Glycocode?*

There is a wealth of information focused on the biochemical importance of HSPGs and the *O*-sulfation pathway. Studies have looked at how these enzymes modify HS, how the modified HS interacts with various growth factors, and how these interactions are altered through changes in HS biosynthesis in cell culture. However, there is a lack of information about HS fine structure modifications with respect to development or disease. Most studies on HS in vivo function come from work in invertebrates, specifically *C. elegans* and *Drosophila*. While these models helped to define the roles of *O*-sulfation pathway members in an organism, the increased complexity of vertebrate organisms—the increase in growth factor isoforms and concordant increase in HS *O*-sulfation pathway members—provides a better model system for understanding both the complex functions and regulation of HSPGs in an in vivo system.

It is interesting to note that the final steps of the HS biosynthetic pathway represent the stages which contain the most isozymes. Why has there been an increase in the number of 6-OST and 3-OST isozymes within vertebrate species? One intriguing possibility is that the isozymes themselves have specificity. Each member of a given family has the same biochemical function. However, the glycocode hypothesis proposes that each family member selectively performs that

biochemical function in a context-dependent mechanism. For example, every member of the 3-OST subgroup 2 family can transfer a sulfate to its substrate 3-carbon (of glucosamine residues) on a GAG chain, either in vitro or in vivo, but perhaps each member of the family has distinct preferences for 3-*O*-sulfation depending on the sequences of other GAG chain modifications on the substrate HS chain. These other modifications might be quite a distance from the primary modification site and would be challenging to assess biochemically.

Is the expansion of all growth factor families, i.e., FGF and Wnt family members, coordinated with the concordant increase in HS OST family members? *Drosophila* contains 3 FGF genes, while mice and humans contain 22 FGF genes (Bottcher and Niehrs 2005; Thisse and Thisse 2005). The actual number of zebrafish FGF genes has not been accurately determined, but recent evidence suggests approximately 27 (Itoh 2007; Itoh and Konishi 2007). The number of 6-OST and 3-OST isozymes in human is sevenfold greater than in *Drosophila*, comparable to the sevenfold increase in FGF family members from *Drosophila* to human. Has the HS field been led astray by studies focusing on the wrong FGF molecules? The study by Kaminura et al. which concluded a charge density role for HS used *Drosophila* as the model organism (Kamimura et al. 2006). The controversial studies looking at the FGF-FGFR-HS complexes have only examined members of the FGF1 superfamily, namely, FGF1 and FGF2 (Pellegrini et al. 2000; Plotnikov et al. 2000; Schlessinger et al. 2000). The FGF1 superfamily, FGF1 and FGF2, has the most widespread expression in both development and adult of the seven FGF superfamilies (Burgess and Maciag 1989; Baird and Klagsbrun 1991). Does this widespread expression mean a reduced stringency for specific HS fine structures? In contrast, FGF7 has a much more limited expression and correspondingly was shown to require a 3-OST modification (Ye et al. 2001).

The increased number of OST isoforms could act as a sorting mechanism, allowing for the "fine-tuning" necessary to direct growth factor signaling in vertebrate organism development. This rationale has led us to develop a model of HS fine structure function termed the "glycocode" hypothesis. The glycocode hypothesis suggests that the HS fine structure functions much like a zip code to regulate specific growth factor binding. Each growth factor and/or HS-binding ligand recognizes a specific sequence of modifications on HS chains in vivo. Some molecules can recognize more liberal HS fine structures, such as FGF1 and FGF2 not requiring 3-*O*-sulfation, whereas some ligands require more specific sequences, such as FGF7 and antithrombin requiring specific 3-*O*-sulfation. The amount of ligand binding and the location of signaling are controlled entirely by the HS fine structures present on a particular cell. The increase in 6-OST and 3-OST family members, along with diverse expression of each family, creates an advanced sorting mechanism to fine-tune and target growth factor signaling.

1.2 Roles of HSPG Sulfation in Development

While the HS field has primarily focused on the biochemical aspects of HS biosynthesis, recent studies have begun to examine the developmental and disease implications of HS fine structure modification. Early studies focused on the invertebrate species *C. elegans* and *Drosophila*, where the HS biosynthesis pathway is less complex. More recent studies in vertebrates, predominantly mice and zebrafish, have begun to examine how the HS fine structure affects specific organs and tissues.

1.2.1 HS-2-O-Sulfotransferase

The first step of the *O*-sulfation pathway, 2-*O*-sulfation of uronic acid residues, has been studied extensively in many species. Knockout of the 2-OST gene in *C. elegans* (*hst-2*) resulted in multiple phenotypic effects, including aberrant morphology, defects in egg laying, and neuronal defects (Bulow and Hobert 2004; Kinnunen et al. 2005). Surprisingly, knockout of HS2ST in *Drosophila* had only minor defects and showed only partial lethality when both maternal and zygotic HS2ST activity were eliminated (Kamimura et al. 2006). A second gene in *Drosophila*, *pipe*, shows very high homology to the vertebrate HS-2OST genes and was thought to encode a second 2-OST gene. However, work by Zhu et al. showed that while Pipe possesses sulfotransferase activity, the target substrate is not heparan sulfate (Zhu et al. 2005). Work in the chicken showed that HS2ST was expressed throughout the limb bud (Nogami et al. 2004). siRNA knockdown of HS2ST in the developing limb bud resulted in a truncation of the limb and reduced FGF expression throughout the limb (Kobayashi et al. 2007). HS 2-OST−/− mice die neonatally from bilateral renal agenesis (Bullock et al. 1998) and exhibit less penetrant eye and skeletal defects, as well as cleft palate and polydactyl (Bullock et al. 1998; McLaughlin et al. 2003). In contrast to these later-onset phenotypes, morpholino knockdown of maternally encoded HS 2-OST in zebrafish has early embryonic effects. 2-OST-deficient embryos have decreased cell adhesion and fail to initiate epiboly, one of the first steps in gastrulation. It is likely that 2-OST plays multiple roles, in multiple cell signaling pathways, to give this strong and pleiotropic phenotype in early development. Reduced cell adhesion in 2-OST is correlated with decreased b-catenin and E-cadherin at cell membranes, and can be rescued by downstream components of the canonical Wnt signaling intracellular pathway, but not by Wnt8 ligand, suggesting that 2-OST plays an integral role in Wnt signaling during early development (Cadwalader et al. 2012). Additional cell signaling pathways are currently being explored.

1.2.2 HS-6-O-Sulfotransferases

The 6-OST family, with multiple vertebrate family members, has provided insight into both the complexity and the spatial and temporal regulation of HS modification. Invertebrate species, with only one family member, show a range of phenotypes. Knockdown of 6-OST modification in *C. elegans* resulted in neural and cellular guidance defects (Bulow and Hobert 2004) and can suppress overexpression of the axon branching regulator *kal-1*(Bulow et al. 2002). In *Drosophila*, P-element excision of HS6ST resulted in lethality and defects in the tracheal system (Kamimura et al. 2001, 2006). In vertebrate species, in which multiple isozymes exist, targeted knockdown or knockout of individual isozymes showed distinct functions. In zebrafish, two independent phenotypes have been attributed to morpholino knockdown of 6-OST-2. 6-OST-2 is essential during somite specification and differentiation of muscle cells, but acts independently of the somite oscillatory mechanism (Bink et al. 2003). 6-OST-2 knockdown resulted in defects in caudal vein branching morphogenesis, while 6-OST-1a knockdown had no overt phenotype (Chen et al. 2005). To date, only one 6-OST family member has been knocked out in mice, 6-OST-1. Knockout of *Hs6st-1* resulted in growth retardation and other developmental abnormalities, including skeletal and neural growth defects, with most embryos dying between E15.5 and perinatal stages (Pratt et al. 2006; Habuchi et al. 2007; Izvolsky et al. 2008).

1.2.3 HS-6-O-Endosulfatases

While most of the modifications of HS chains occur in the Golgi as the HSPG is transiting to the cell surface, it is important to note that cells have the ability to further regulate the complexity of the HS fine structure after the HSPGs have exited the Golgi. Two related HS-6-*O*-endosulfatases, commonly referred to as the Sulfs, have the unique ability to specifically remove glucosamine-6S residues from HS chains at the cell surface (Dhoot et al. 2001; Morimoto-Tomita et al. 2002). This radically changes the previous view that HS biosynthesis was complete prior to the HS chains moving to the cell surface. To date, the Sulfs have only been identified in vertebrate species. Cranial neural crest cell migration was altered following knockdown of XtSulf1 and XtSulf2 in *Xenopus* (Guiral et al. 2010). Knockout of Sulf1 or Sulf2 in the mouse had little effect on the mouse other than increased mortality (Holst et al. 2007; Lamanna et al. 2007; Lum et al. 2007). However, knockout of both Sulf1 and Sulf2 resulted in high rates of neonatal lethality with skeletal, renal, lung, and growth defects (Holst et al. 2007; Lamanna et al. 2007; Lum et al. 2007; Ratzka et al. 2008).

1.2.4 HS-3-O-Sulfotransferases

The importance of 3-*O*-sulfation in fine structure formation is not well understood. The 3-OST family can be divided into two subgroups based upon biochemical specificity and gene structure. It is thought that all bilaterians have at least one 3-OST from each subgroup (Cadwallader and Yost 2006a; Mizuguchi et al. 2009). One of the defining characteristics for subgroup 1 isozymes is the creation of the fine structure necessary for HS–antithrombin interaction. However, knockout of 3-OST-1 in the mouse failed to provide the supporting in vivo evidence for functions in antithrombin activity (Shworak et al. 2002; HajMohammadi et al. 2003). However, the 3-OST-1 knockout mouse did exhibit strain-specific lethality and intrauterine growth retardation (Shworak et al. 2002; HajMohammadi et al. 2003). Members of the 3-OST subgroup 2 family have been characterized for their ability to generate the HSV-1 binding site in vivo. RNAi-mediated disruption of HS3st-B, the *Drosophila* subgroup 2 homolog, results in a high rate of lethality along with neurological defects and a reduction in Notch signaling (Kamimura et al. 2004). Knockdown of HS3st-A, the subgroup 1 homolog, also exhibited a high rate of lethality (Kamimura et al. 2004). In zebrafish, knockdown of 3-OST-5, a subgroup 1 family member, or 3-OST-6, a subgroup 2 family member, causes left–right laterality defects. Strikingly, each gene functions through a separate mechanism that contributes to asymmetric fluid flow in Kupffer's vesicle, the ciliated organ of asymmetry in zebrafish. 3-OST-5 is required in the FGF pathway, and knockdown diminishes cilia length via decrease in ciliogenic transcription factors FoxJ1a and Rfx2. In contrast, 3-OST-6 knockdown does not alter cilia length, but results in cilia immobility via diminished kinesin motor molecule (Kif3b) expression and loss of dynein arms within the normal-length cilia arm (Neugebauer et al. 2013). The observation that individual 3-OST isozymes that function within the same cells (ciliated cell lineage in Kupffer's vesicle) have distinct functions in distinct cellular pathways suggests that each of these 3-OST family members creates distinct modified domains or "glycocodes" on cell surface proteoglycans. To date, this is some of the strongest functional evidence supporting the glycocode hypothesis.

1.3 Developmental Regulation of the Glycocode

Our working hypothesis is that a unique glycocode regulates specific components of numerous pathways, including the FGF, TGFβ, BMP, Wnt, and hedgehog cell signaling pathways. These codes are embedded in the fine structure or sequences of GAG modifications unique to each cell type. A crucial aspect of the glycocode model is that individual cells regulate the distinct GAG fine structures that they

synthesize. While there is growing evidence that sulfation of HSPGs is important for development, one of the unmet questions in the field is whether specific fine structure sulfation patterns are generated and regulated in a cell-specific fashion. Analysis of HS from different mammalian tissues revealed the tissue-specific composition of samples, pointing to strict regulation of biosynthetic polymer modifications (Maccarana et al. 1996; Ledin et al. 2004). How this regulation occurs in vivo has been a topic of debate in the HS field. Does every cell in an organism express every OST family member? Are the OSTs regulated in a cellular and tissue specific manner? Are complexes of different OST family members selectively forming glycocodes during development?

1.3.1 Developmental Regulation of Distinct OST Family Members

While it was previously assumed that every cell expresses every OST gene, examining the developmental expression patterns of the various gene families from multiple organisms has refined our understanding of the mechanisms governing HS fine structure formation. It was previously thought that 2-OST expression would be ubiquitous given that only one family member exists in vertebrates (with a possible duplication in zebrafish). However, studies in mouse (Bullock et al. 1998), chicken (Nogami et al. 2004), and zebrafish (Cadwallader and Yost 2007) suggest that 2-OST expression is regulated in distinct tissues during development. RNA in situ studies looking at the transcripts encoding multiple isoforms of the 6-OST family in chicken (Nogami et al. 2004), *Xenopus* (Winterbottom and Pownall 2009), mouse (Habuchi et al. 2003; Sedita et al. 2004; Yabe et al. 2005), and zebrafish (Chen et al. 2005; Cadwallader and Yost 2006b) show each isozyme has specific spatial and temporal expression patterns. Not surprisingly, the Sulfs exhibit expression patterns coordinated with 6-OST expression in *Xenopus* (Winterbottom and Pownall 2009; Guiral et al. 2010) and mouse (Lum et al. 2007; Ratzka et al. 2008). Members of the 3-OST family also show a distinct localization patterns when examined in mouse (Yabe et al. 2005; Lawrence et al. 2007) and zebrafish (Cadwallader and Yost 2006a).

Summarizing these expression studies, most of the OST family members are ubiquitously expressed during early stages of embryogenesis. This ubiquitous expression makes sense if a large range of glycocodes are required for relatively pluripotent cells that are poised to receive multiple cell signals. In contrast, temporally and spatially dynamic subsets of OSTs and Sulfs are expressed during later development, suggesting the possibility that each isozyme plays a unique role in building distinct sequences within the glycocode during critical decision points in development. Thus, later in development, perhaps only a restricted set of glycocodes are available in a given cell type. This can be seen firsthand with respect to the 3-OST family and the neural system during development. Every member of

the family is expressed in the neural system (Yabe et al. 2005; Cadwallader and Yost 2006a; Lawrence et al. 2007), but expression is restricted to specific brain areas or specific neurons (Hasegawa and Wang 2008). As development progresses, the glycocode is refined and restricted by the specific OST family members that are expressed in a given cell type during development. Future studies will need to address not only expression in a given organ or tissue, but which cells and cell types are being affected.

1.3.2 GAGosome

Another assumption in the HS field is that the HS polymerization functions independently and occurs prior to HS chain modification. This view may be propagated by the way in which the HS synthesis and modification pathway is presented as a sequential series of biochemical steps (Esko and Lindahl 2001). Recent evidence, including Ext2 regulation of NDST1 and Ext1 (Presto et al. 2008), NDST scaffold formation (Ledin et al. 2006; Raman et al. 2011), as well as HS isolated from various knockout mice (Merry et al. 2001; Li et al. 2003), has begun to question how mature HS chains are produced.

An interesting idea which challenges the stepwise HS chain modification pathway hypothesizes the HS chain modification enzymes expressed by a cell compose a large Golgi resident complex. This complex, most commonly termed the "GAGosome" (Esko and Selleck 2002), could completely synthesize and modify a given section of HS chain at one time. The review by Esko and Selleck provides evidence for the possibility of a complex. Recent findings have extended the evidence for the GAGosome. These findings include (a) the finding that NDST1 competes with Ext1 for binding to Ext2 (Presto et al. 2008), (b) the ability of members of the 3-OST subgroup1 family, which lack a transmembrane domain, to remain in the Golgi (Shworak et al. 1997; Cadwallader and Yost 2006a), (c) the compensation in sulfation when a member of the *O*-sulfation pathway is missing (Merry et al. 2001; Kamimura et al. 2006), and (d) growing evidence that multiple modifications are carried out in a coordinated and specific manner (Victor et al. 2009; Nguyen et al. 2011).

While no direct evidence yet exists for GAGosome formation, how might the GAGosome function within specific cells? We propose the possibility that distinct GAGosomes might contribute to distinct fine structural modifications of GAG chains within a cell. If each member of the 3-OST family has a slightly different specificity (Sect. 1.3), this might be played out in the context of the GAGosome. Furthermore, cells may contain more than one GAGosome type, which would enable cells to produce HS chains with distinct glycocodes, as suggested by distinct roles of different members of the 3-OST family in ciliated cells in zebrafish (Neugebauer et al. 2013).

1.3.3 Charge Density vs. Specificity

The exact nature of the interaction between HS and potential ligands has become a controversial topic within the HS community. The current dogma of the field is that the position and distribution of sulfates on HS chains are necessary for proper ligand binding (Esko and Lindahl 2001; Esko and Selleck 2002). The structural diversity, or specificity, of HS chains is a result of the strictly regulated but variable distribution of sulfate modified residues. This diversity is thought to enable selective interaction with proteins in a sequence and time-specific manner (Esko and Lindahl 2001). There are two major pieces of evidence suggesting that chain specificity is responsible for the HS–protein interactions. The first example is the antithrombin-binding sequence, which is responsible for the anticoagulant activity of heparin (Marcum et al. 1983; Bourin and Lindahl 1993; Princivalle et al. 2001). Each saccharide of the pentasaccharide structure (GlcNAc6S-GlcA-GlcNS3S-IdoA-GlcNS) is essential for high-affinity interaction with antithrombin. The second example often cited is the *N*-unsubstituted GlcN3S residue which mediates the specific binding of herpes simplex gD protein to cell surface HS during viral infection (Shukla et al. 1999).

Opposed to this is the idea that charge density, or the relative amount of negative sulfate charges in a given HS chain length, regulates how factors bind HS chains, and importantly, that the positions of the sulfates are much less critical (Kamimura et al. 2006; Kreuger et al. 2006; Gorsi and Stringer 2007). Therefore, it is thought that many HS–protein interactions may depend more on the overall charge of HS chains than on their specific fine structure. A study from *Drosophila* suggests that the overall charge density of a particular HS chain is more important than fine structure specificity (Kamimura et al. 2006). Mutations in *Hs2st* or *Hs6st* had little effect on FGF-mediated tracheal morphogenesis. Structural analysis of HS chains from these mutants revealed a loss of corresponding sulfation, but a compensatory increase in sulfation at other positions, which maintained the overall level of HS charge density. The *Hs2st*, *Hs6st* double mutant strongly disrupted tracheal morphogenesis. These findings suggest the overall sulfation level, and thus the overall charge level, maybe more important than strictly defined HS fine structures in some developmental contexts.

Several questions need to be addressed before this controversy can be resolved. Knockout of 2-OST in the mouse (Sect. 1.3) (Merry et al. 2001) resulted in major developmental defects, including renal agenesis (Bullock et al. 1998; McLaughlin et al. 2003), and an increase in 6-*O*-sulfation. Thus, a comparable level of charge density still gave a significant developmental phenotype. Knockout of 6-OST-1 in the mouse showed a minimal increase in 2-*O*-sulfation, but this result could be due to multiple isozymes being present. The phenotypes of both knockout mice suggest that fine structure specificity is important for the phenotypes in both mice, and maintaining overall charge density was not sufficient for normal development. Does the difference in phenotypes between *Drosophila* and the mouse knockouts suggest HS specificity differences exist between invertebrate and vertebrate species?

The examples highlighting HS specificity for ligands, namely, the antithrombin-binding site and the HSV-1 gD protein interaction, both contain a 3-O-sulfate modification. Does the 3-OST modification provide the HS fine structure with ligand binding specificity? As stated above, vertebrate species contain at least seven different isozymes to catalyze a modification which accounts for less than 0.5 % of the total HS sulfate modifications. Concomitantly, vertebrate species have seen an increase in growth factors proportional to the increase in 3-OST isozymes. Is the increase in 3-OST isozymes necessary to provide specificity to the increasing numbers of growth factors?

Our glycocode hypothesis proposes that inherent sulfation levels of HS, primarily due to the action of NDSTs, 2-OST, and 6-OSTs, create a low-affinity receptor and that actions of individual members of the large 3-OST family might confer a higher level of specificity. The generation of low-affinity receptors by NDSTs, 2-OST, and 6-OSTs would explain the differences seen in sulfation levels of various organs and tissues. The liver, which contains high inherent levels of HS sulfation, uses HS as a low Kd but abundant receptor for lipoproteins (MacArthur et al. 2007). Cells expressing low-sulfation HS, such as stem cell populations (Pickford et al. 2011), may be protected from hyperexcitation within their niche. High specificity, required by heparan sulfate-binding growth factors, is tightly regulated through the expression of specific isozymes and functions through the GAGosome. We speculate that in most cases, the specificity requires specific modifications, such as a 3-*O*-sulfation or post-synthesis modification provided by the Sulf enzymes. We think the glycocode hypothesis explains the differences in phenotypes seen in specific knockdown of the individual HS *O*-sulfation pathway enzymes.

1.4 Future Directions

The HS biosynthetic pathway is capable of producing 48 unique disaccharide units, yet only 23 units have been characterized in nature (Toyoda et al. 2000; Esko and Selleck 2002). Invertebrate and vertebrate species use the same 23 disaccharide units to make mature HS chains (Toyoda et al. 2000). The only difference between the various species is how these units are put together. Our glycocode hypothesis suggests that the specific placement of sulfates in the fine structure controls the specificity of interaction between HS and individual ligands. Cells have the ability to dynamically regulate expression of each enzyme and perhaps the ability to coordinate how the individual enzymes are packaged into different GAGosomes. While the overall level of sulfation serves as a low Kd binding platform, high specificity requires precise sequence specificity.

Much of the work previously done to identify and characterize specific members of the *O*-sulfation pathway has relied upon in vitro techniques and cell culture. These experiments and their conclusions have made significant contributions to the dogma in the field. Only recently has the field begun to understand the function of HS and specifically the *O*-sulfation pathway in vivo. Several examples are available

where the in vitro and in vivo data simply do not match. Understanding the physiological roles of unique modifications of GAG chains on HSPGs will hopefully lead to a better understanding of complex choices made by cells in the context of their extracellular matrix.

Understanding the roles of the expanded number of 6-OST and 3-OST family members in vertebrates remains a priority for the field. The increased numbers of isozymes suggest that individual family members are highly regulated or essential for specific developmental processes. How does their in vivo expression and function relate with dynamic developmental choices made by cell lineages? Promising new technologies, including computer modeling (Spencer et al. 2010), synthetic organelle systems (Martin et al. 2009), and GAG array technologies (Wakao et al. 2008; Puvirajesinghe et al. 2012), show great potential moving forward. These technologies, along with others, will attempt to define the sequence specificity of numerous heparan sulfate-binding proteins. The key, however, will be in understanding how data derived from in silico applications reflects the fast-paced, dynamic interactions seen in vivo during development.

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Chapter 2
Extracellular Matrix Dynamics in Early Development

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Abstract The extracellular matrix (ECM) is an inherently dynamic structure during development. Large-scale tissue movements sweep the ECM to distant positions during early embryogenesis; furthermore, the previously assembled ECM filaments are extensively reused during later organogenesis. Thus, some organs, such as the heart, inherit previously assembled ECM filaments. This review focuses on the macro-assembly dynamics of fibronectin and fibrillin-2 in avian embryos. Both ECM proteins are ubiquitous in vertebrate embryos at the earliest stages of development. Recent studies compared the movement of these two ECM components and suggested that the ECM moves as a composite material, whereby distinct molecular components as well as spatially separated layers exhibit similar displacements. Utilizing advances in microscopy and high-resolution particle image velocimetry algorithms, two ECM filament relocation processes can be distinguished—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. 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 macro-assembly during embryonic morphogenesis. ECM filaments also fluctuate locally—likely reflecting pulsatile cell contractility in the associated tissues. Suppression of these fluctuations by temporal averaging yields a persistent movement pattern that is shared among embryos at equivalent stages of development.

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Recent studies comparing the motion of embryonic mesenchymal cell populations (newly ingressed mesodermal, pre-somitic, endothelial, and endocardial) all found that the gross displacement of these cells is to a large extent shared by the surrounding ECM. The result being that active motion relative to the local ECM scaffold is more random than previously appreciated. A future understanding of ECM assembly thus requires the study of the complex interactions between biochemical assembly steps, local cell action and the biomechanics of tissue motion.

2.1 Introduction

In addition to providing structure to the interstitial spaces between cells, the ECM acts as a scaffold for cell adhesion. This contact mediates cell migration (movement relative to the ECM) and also allows the cells to exert mechanical forces. Cell binding to the ECM can elicit signal transduction events across the cell plasma membrane (Alberts et al. 2008). The ECM is also believed to play a biochemical and/or biomechanical role in the specification of cell fate. Since ECM constituents bind and sequester soluble growth factors, the ECM can modulate—either restrict or promote—access of ligands to specific cell surface receptors. Thus, the ECM is positioned to regulate the spatial and temporal localization of most growth factors after their secretion. Such ECM-bound growth factors may also stimulate directional cell migration through chemotaxis (Yang et al. 2002; Vasiev et al. 2010). Mechanical cues from the microenvironment are also likely transmitted through ECM–integrin interactions and modulate intracellular signaling and gene expression patterns. For example, the degree of ECM stiffness contributes to cell fate determination of mesenchymal stem cells (Engler et al. 2006). Thus, the local ECM environment can influence cell polarity, survival, proliferation, and differentiation (Rozario and DeSimone 2010).

Although the ECM is a composite structure made up of many different constituents, the three-dimensional (3D) organization of the ECM can be as crucial as its molecular composition in determining its developmental functions. Cellular response to ECM contacts strongly depends on how the ECM is presented to cell surface receptors in vitro: whether it is immobilized, planar, or three-dimensional (Zamir et al. 1999; Cukierman et al. 2001). ECM organization also strongly influences cell shape and motility (Tomasek et al. 1982; Petroll and Ma 2003): Fibroblasts (Stoplak and Harris 1982; Dickinson et al. 1994), 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).

The experimental accessibility of avian embryos makes them especially suitable as model organisms in which to study ECM organization during development (Rongish et al. 1998; Czirok et al. 2004, 2006b). Two ECM components, fibronectin and fibrillin-2, were found to be especially fruitful targets of investigations: Both

can be labeled in vivo by high-affinity antibodies, and both proteins assemble into large, characteristic structures spanning tens of micrometers. During day 1 of gestation, a rich 3D ECM structure is readily apparent. Bundles of fibrillin fibers connect the posterior foregut (anterior intestinal portal, AIP) to the somitic regions, extending well past the first few somite pairs. In addition there are prominent coaxial fibrillin-2 fibers that assemble parallel to the (future) vertebral axis. The cranial portion of these bundles encloses the somites, while caudally the bundles extend more than 200 μm into the segmental plate mesoderm and approach Hensen's node (organizer). Caudal to the node, fibrillin-2 exhibits a strikingly different pattern, which consists of punctate, disconnected foci. The somitic array and the segmental plate mesoderm exhibit an oriented, dense array of paraxial filaments, remarkably different from the loose meshwork-like organization characteristic of the more lateral embryonic regions. In contrast, fibronectin exhibits a finer meshwork-like pattern, with less variability across the lateral plate. Yet, despite these differences, at the resolution of confocal microscopy, most ECM filaments are composite structures containing both proteins (Fig. 2.1).

2.2 ECM Dynamics

2.2.1 Labeling Technique

In order to study ECM dynamics, its constituent proteins can be conveniently labeled in avian embryos by the microinjection of fluorochrome-conjugated monoclonal antibodies, such as JB3, a monoclonal IgG that binds avian fibrillin-2- (Rongish et al. 1998), or B3D6, which recognizes avian fibronectin (Gardner and Fambrough 1983). After antibody microinjection at Hamburger and Hamilton (HH) stages 4–7 (Hamburger and Hamilton 1951), embryos can be recorded for several hours with automated microscopy (Czirok et al. 2002). During the recordings no substantial photo bleaching or other signal deterioration is obvious (Fig. 2.2).

After microinjection, antibodies infiltrate a substantial portion (up to 50–70 %) of the area pellucida in less than an hour. Injection of living avian embryos with ECM antibodies has been in use for over 15 years (Little and Drake 2000). The perturbative effect of microinjected antibodies was assayed by comparing whole-mounted antibody-injected embryos to non-injected controls after 10 h of development. No observable differences were found either in the post-fixation ECM staining pattern or in the anatomy of the embryos. The timing of major morphogenic processes was also the same in antibody-injected embryos and non-injected controls. In particular, the somites form on identical schedules, every 90 min, while the heart loops and commences beating normally (Czirok et al. 2004).

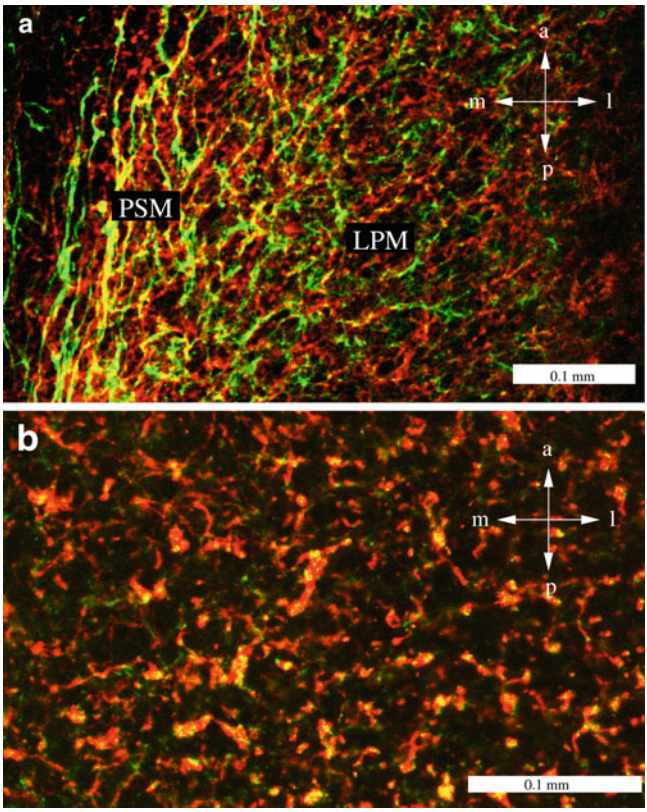

Fig. 2.1 Confocal images of fibronectin (*red*) and fibrillin-2 (*green*) in a 4-somite (Hamburger and Hamilton stage 7) embryo. The images depict a dorsal–ventral (z) projection of the ECM layer located between the mesoderm and the endoderm. Panel **a** shows the ECM associated with the pre-somitic and lateral plate mesoderm. Long fibrillin-2 rich filaments running parallel to the anterior–posterior axis are abundant around the pre-somitic mesoderm. Panel **b** shows an area lateral to the primitive streak. ECM filaments are much shorter and are oriented in random directions. Co-localization of the two ECM components appears as *yellow*. Images were obtained using a 25 × oil immersion objective and a Zeiss LSM510 confocal microscope. *a* anterior, *p* posterior, *m* medial, *l* lateral, *PSM* pre-somitic mesoderm, *LPM* lateral plate mesoderm

2.2.2 Large-Scale ECM Flow Pattern

As reported in Czirok et al. (2004), the fluorescence staining pattern of the ECM changes remarkably during a recording period of 8–12 h (Fig. 2.2). Yet, filament shapes and their relative positions remain fairly similar between consecutive image pairs (separated by 5–10 min intervals)—a feature that makes our tracking procedure possible. In fact, filament motion seems to be as ordered as the slow flow of a viscous fluid: Adjacent filaments move on parallel trajectories.

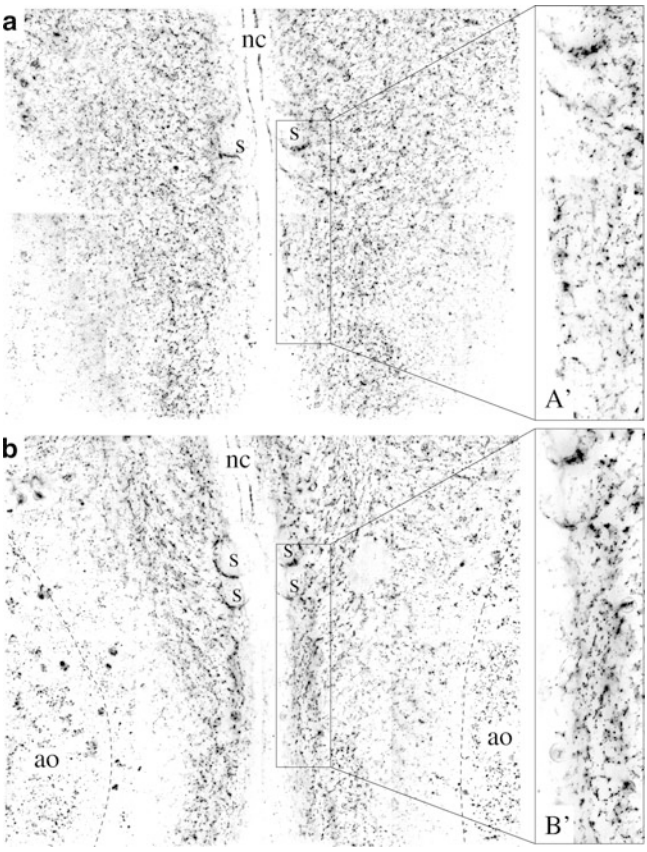

Fig. 2.2 Fibronectin, as visualized by microinjected and fluorophore-conjugated B3D6 antibodies at the beginning of a recording (**a**) and 4 h later (**b**). Images were obtained with a 10 × objective in a widefield setting, subjected to a blind deconvolution to reduce out-of-focus blur; grayscale values were inverted to increase contrast and aid visualization. The pre-somitic mesoderm and the somites are shown at higher magnification in panels **a**′ and **b**′. Notice the increased density of fibronectin filaments surrounding the pre-somitic mesoderm, a consequence of the large-scale ECM motion during the observed time period. The *boxes* are 200 μm wide. *ao* area opaca, *nc* notochord, *s* somite

ECM displacements are extracted either by a manual frame-by-frame tracking procedure (Czirok et al. 2004) or by an automatic pattern-matching algorithm [PIV; Zamir et al. (2005)]. The latter method divides the image into small image tiles. For each tile the algorithm scans the next frame for the image detail of the same size that is most similar to the given tile of the previous frame. The spatial shift between the best matching pattern and the original tile gives an estimate for the displacement of the objects within the tile. During either the manual or the automatic PIV procedures, images are registered in such a way that the somites remain stationary—thus, all motion data are presented in a reference system co-moving with the somites.

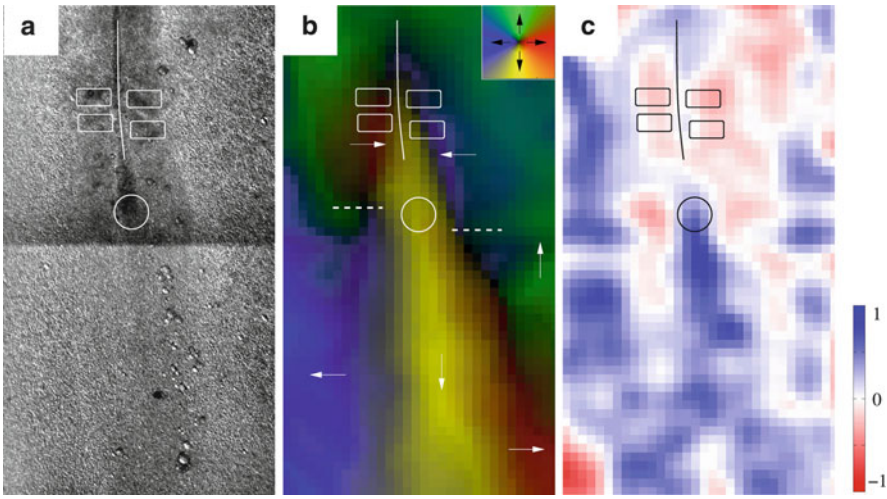

Fig. 2.3 ECM motion pattern in a two-somite (Hamburger and Hamilton stage 6) embryo. (**a**) A ventral view of embryo morphology, visualized by DIC optics. Anatomical features such as somites, the notochord, and Hensen's node are marked with *boxes*, a *vertical line*, and a *circle*, respectively. (**b**) Magnitude and directionality of the persistent ECM movement pattern, represented by intensity and color as shown in the inset. *Arrows* indicate motion direction at selected positions to aid the reader in perceiving motion patterns. *Dashed lines* mark the boundary between the medially and laterally moving regions of the lateral plate mesoderm. (**c**) Accumulation (*red*) or dilution (*blue*) of ECM filaments, calculated from the divergence of the ECM velocity field. The rate of the motion-related increase in ECM density can be substantial; the unit of the scale corresponds to a change of 100 %/h. After Szabo et al. (2011)

The ECM motion pattern can be extracted either by averaging over multiple stage-matched specimens (Czirok et al. 2004) or by averaging over several frame pairs in the case of a single specimen (Szabo et al. 2011). Displacement patterns obtained by either averaging method are highly reproducible. Convergent-extension-like anatomical movements take place lateral to the notochord. Hensen's node and the primitive streak move caudally, and the head region extends cranially. During this period of cranio-caudal axis extension, the ECM surrounding the lateral plate mesoderm (LPM) moves medially. In the caudal embryo, near the primitive streak, the LPM-associated ECM moves laterally. The combination of these displacements results in a vortex-like motion pattern on both the right and left sides of the embryo. The region where the ECM converges medially elongates over progressively later stages of development. The moving boundary separating medial convergence from the more caudal domain, characterized by lateral expansion, is often asymmetric: The domain of medialward movement is larger on the right side of the acquired image frame (the anatomical left side of the embryo). Thus, lateral expansion takes place only at more caudal positions (see dashed lines in Fig. 2.3). This left–right asymmetry in ECM movements around Hensen's node complements recent results, indicating the rotation of cellular components of the node in avians (Cui et al. 2009; Gros et al. 2009).

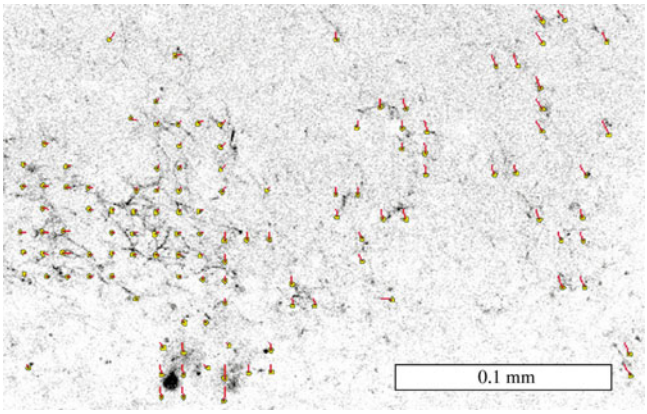

Fig. 2.4 Fluctuating movements of fibrillin-2 filaments, assessed by confocal microscopy. A single, 0.7 μm thick optical plane is shown, with the estimated velocity vectors superimposed. *Red lines* indicate the calculated displacements of the *yellow dots*. As the ECM layer separating the mesoderm from the endoderm is not perfectly flat, only an irregular area within the optical section contains high ECM density. The motion is locally smooth, but varies in direction and magnitude over a scale of 100 μm. After Szabo et al. (2011)

The obtained speeds of ECM movements can exceed 100 μm/h, fast enough to substantially reorganize ECM distribution within a few hours. The observed movements are shared by two distinct ECM components, fibronectin and fibrillin-2. In particular, the cross-correlation coefficient of the simultaneous displacements of fibronectin and fibrillin-2 is 0.91 (Szabo et al. 2011). Similar ECM movements are also present during epithelial tissue morphogenesis in the ancient metazoan, Hydra (Aufschnaiter et al. 2011).

2.2.3 Fluctuations

Surprisingly, velocity field snapshots of ECM movements show considerable variability in the absence of averaging (Szabo et al. 2011). We attribute this phenomenon to intrinsic fluctuations within the tissue. The magnitude and the spatial extent of the fluctuations are somewhat smaller, but comparable to that of the persistent motion pattern. The fluctuations, however, change rapidly: High-frame-rate confocal scans indicated that the correlation time of the fluctuations is, despite their large spatial extent, less than a minute. The fluctuations result in local contractions and expansions of the ECM at apparently random locations (Fig. 2.4), which are similarly short-lived and which lack an obvious correlation with anatomical structures.

The long spatial correlation length (large extent of co-moving area) of the fluctuations is consistent with the idea that the tissue is in mechanical equilibrium; therefore, a local change in cell-generated mechanical forces is expected to

immediately alter tissue deformations elsewhere. Recent studies in fly embryos described a pulsatile, ratchet-like contraction mechanism (Martin et al. 2009). Instead of a uniformly distributed contractile activity spread across the tissue, individual cells were observed to undergo a repeating cycle of asynchronous contractions, with cytoskeletal stiffening followed by relaxation and physical rearrangements. A mechanism similar to that in the fly might explain the ECM fluctuations we observe—however, it is unclear how these pulsatile cycles might be generated in bird embryos or how such fluctuations might affect the large-scale ECM displacements we observe during amniote morphogenesis.

2.3 ECM Restructuring

2.3.1 Remodeling

The observed large-scale ECM movements have a profound impact on tissue scale architecture. As an example, the bundles of ECM filaments that run parallel to the embryonic axis in the vicinity of the pre-somitic mesoderm are formed by these movements. The filaments are assembled from smaller ECM fibrils, units that were previously scattered over an extended area. The medial movement of these nascent ECM filaments, accompanied by stretching along the embryonic axis, both orients and condenses the ECM filaments into larger caliber coaxial cables (Czirok et al. 2004).

Similarly, a substantial component of the future cardiac jelly ECM is drawn from the LPM. A recent study determined that the anatomical origin of ECM filaments that eventually contribute to the developing heart tube can be mapped (Aleksandrova et al. 2012). Extensive tracing of fluorescently labeled endogenous and exogenous fibronectin filaments revealed that (1) ECM flanking the foregut region (AIP) in HH stage 8 embryos contributes to the anterior third of the heart tube, (2) areas located lateral to the caudal segments of the anterior zone, on both sides, are the source of ECM fibrils that are eventually translocated to the middle portion of the cardiac tube, and (3) moving tailward, the region contributing ECM to the caudal heart tube and omphalomesenteric veins maps to the posterior end of the foregut and extends caudally to the level of the second or third somite pair at HH stage 8.

These time-resolved positional mapping data show that preexisting ECM structures are extensively displaced and reused during development. Thus, it is now meaningful to discuss ECM positional fate, in a manner similar to the establishment of fate maps for cell populations. Interestingly, no ECM disassembly was ever observed within our sample of more than 200 recorded embryos.

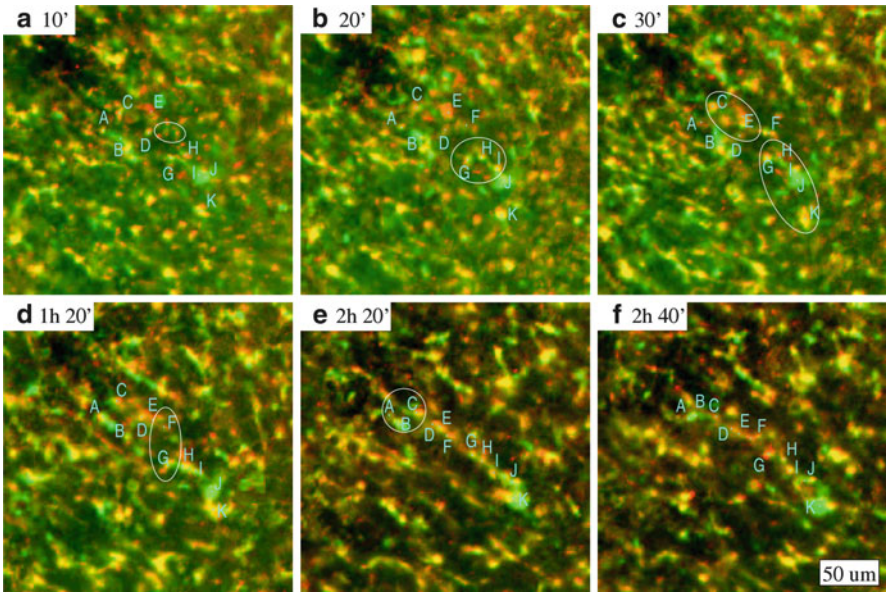

Fig. 2.5 ECM macro-assembly, in vivo. The image sequence depicts a region of interest (ROI) located lateral to the primitive streak. The ROI is moving with the surrounding tissue. Letters denote individual globules of ECM material, which typically contain both fibronectin (*red*) and fibrillin-2 (*green*). In the course of 3 h, the globules assume an ordered, linear shape, which remains stable until the end of observation, 7 h later (not shown). *Ellipses* indicate areas where previously distinct ECM objects merge and are subsumed into a stable linear arrangement. As an example, the filament marked by F in panel (**b**) is created from the material enclosed in the *white ellipse* in panel (**a**). After Czirok et al. (2006b)

2.3.2 *Filament Assembly*

A particularly interesting reorganization process, filament assembly, can be observed to proceed in the caudal avian embryo, near the primitive streak/organizer region (Czirok et al. 2006b). ECM filaments that originally appear as globular patches of fluorescence coalesce into larger linear structures (Fig. 2.5). Closer analysis reveals that the process appears to be hierarchical, whereby elongated larger caliber 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, at the level of resolution in our studies (1 μm), filaments are "born" as composite structures. A similar hierarchical assembly process was observed in densely populated cell cultures, where the ECM is assembled first into distinct globules on the cell surface; this was followed by the appearance of progressively larger filaments which appeared to be organized by the motile activity of several adjacent cells. Thus, when viewed in time-lapse, local cellular behavior appeared to organize ECM assemblies (Czirok et al. 2006a; Kozel et al. 2006; Sivakumar et al. 2006). Similar processes are observed in the caudal

avian embryo with the added complexity that there are long-range tissue displacements occurring concomitant with cell shape change and local motile activity.

2.4 ECM as a Microenvironment for Mesenchymal Cell Migration

2.4.1 Methods to Visualize Cell Motion Relative to the ECM Environment

To compare cellular displacements to that of the local ECM scaffold, simultaneous recordings are required of both the labeled ECM and cell populations. As we discussed in Sect. 2.1, the ECM is conveniently visualized by microinjected, fluorophore-conjugated antibodies. To visualize a specific cell population, one can rely on the same immunolabeling method using antibodies directed against specific cell surface epitopes. Lipophilic dyes that bind to the cell membrane or dyes taken up by the cells can be also administered locally. Transfection and electroporation of fluorescent protein-expressing constructs have also been used to target and label cell populations (Cui et al. 2006). Recently, transgenic quail strains have been developed with promising new possibilities for the visualization of cell and tissue behavior in a live amniote embryo (Sato et al. 2010).

The movement of mesodermal or other mesenchymal cells relative to the local ECM environment is defined using the vector relation of velocity addition:

$$\underbrace{d'_{\text{cell}}}_{\text{total cell displacement}} = \underbrace{d'_{\text{ECM}}}_{\substack{\text{ECM displacement} \\ \text{at the cell's location}}} + \underbrace{d'_{\text{active}}}_{\substack{\text{displacement due to} \\ \text{active cell motility}}} . \tag{2.1}$$

Thus, if both cells and ECM components can be traced at the same location, the difference between the two displacement vectors is attributed to active cell motion. Definition (2.1) is consistent with the various ways cell motility is studied. In most two- and three-dimensional in vitro settings, like invasion into collagen gels, the ECM environment is static ($d_{\text{ECM}} = 0$); hence, all observed cell displacements are due to active cell motility. Furthermore, relation (2.1) ensures that passive objects ($d_{\text{active}} = 0$) move similarly to the ECM—a physical characteristic experimentally verified by tracking injected microbeads into the lateral heart forming fields of quail embryos (Aleksandrova et al. 2012).

It is important to note that relation (2.1) focuses on active cell movements that use the "classical" motility apparatus, which relies upon binding and release of cell–ECM contact points to move the cell body forward (relative to the ECM) (Lauffenburger and Horwitz 1996; Ridley et al. 2003). Other, less understood

cellular activities, such as motility using cell–cell contacts—sometimes referred to as intercellular motility (Gumbiner 2005)—may also result in cell displacements. As reviewed in Montell (2008), germ-band elongation in Drosophila is resulted by a repeating cycle consisting of cellular contraction along the lateral direction and modulation of cell–cell adhesion. In this process contacts between two cells that are located parallel to the anterior–posterior axis are gradually replaced with new contacts between cells that are lined up along the perpendicular (lateral) direction. Thus, intercalation progresses because cell–neighborhood relationships and cell–cell contacts are spatially reorganized in the plane of the epithelium following an ordered pattern of disassembly and reassembly (Bertet et al. 2004). If cells are engaged in a motile activity where they get traction not from the ECM but from the epithelial neighbors, then this activity can be the driving force behind the co-movement of an epithelial sheet and the surrounding ECM.

To employ the above definition (2.1), one needs to establish ECM displacement in the cell's immediate vicinity. This is either readily available from co-tracing adjacent cells and ECM filaments, or must be estimated. As we discussed in Sect. 2.2, ECM displacements are very similar at locations that are sufficiently near one another; thus, a general estimate of ECM displacement is made possible by interpolating from displacement vectors obtained from suitably close locations. In our analysis we interpolate data within a radius of 5 μm.

2.4.2 Mesodermal Cell Movements

An exceedingly important event during amniote embryogenesis is the ingression of epiblastic cells through the primitive streak (Gilbert 2010). During ingression, mesenchymal cells migrate laterally and cranially in an ECM-rich environment. Thus, ingressing mesodermal cells represent, arguably, the premiere example of an embryonic mesenchymal cell population, in amniotes. Zamir et al. (2006) studied the newly ingressed mesodermal cells in HH stage 4 or 5 avian embryos. A portion of the ECM was labeled using microinjected fluorescently conjugated B3D6 antibodies specific for fibronectin. Mesodermal cells were tagged by lipophilic dyes (DiI), applied at the primitive streak. The apparent (total) displacements of labeled cells were manually determined. The ECM displacement field was determined by PIV analysis, and cell-autonomous displacements were obtained using relation (2.1).

In accord with Fig. 2.3, the ECM lateral to the primitive streak was found to move further laterally. Mesodermal cells move in the same direction but faster than the ECM; nevertheless, a substantial portion of the total cellular displacements is also displayed by the ECM. The study found an increasing cranial-to-caudal gradient in cell speed: Cells that emanate from more caudal (posterior) positions in the primitive streak move faster than cells closer to Hensen's node (more anterior). As mesodermal cells move away from the primitive streak, they slow down to speeds that match those of their local ECM scaffold. This change in

relative cell-to-ECM speed is believed to occur concomitantly with the mesenchymal (mesodermal) cells integrating to form a LPM, an epithelium. Note that the formation of the LPM occurs at positions anterior to the sites of cell ingression—again mainly due to tissue movements that result in the continuous regression of Hensen's node. In other words, Hensen's node has moved (caudally) and "passed" the newly forming LPM.

As shown in a recent study by Benazeraf et al. (2010), active motion of the mesenchymal cells, which comprise the pre-somitic mesoderm, is randomly directed. The intensity of mesenchymal cell motion gradually decreases at more anterior positions, at which time/place these cells condense into the pre-somitic mesoderm. Benazeraf and colleagues found, via cells expressing a dominant negative FGF receptor, that FGF signaling is a likely regulator of the intensity of active, randomly directed cellular motility.

These novel data shed new light on the classical cell fate-mapping studies of the mesoderm—it is now clear that for the most part, cells forming either the pre-somitic or the LPM move to their future positions by tissue-level rearrangements (shared by both the cells and the surrounding ECM environment) as opposed to individual cell, directed motile activity of individual cells, relative to the ECM.

2.4.3 Endothelial Progenitor Movements

Endothelial cells of the avian embryo originate as right and left subpopulations of the LPM and then dislocate ventrally into a prominent ECM layer situated between the endoderm and the mesoderm. During the ensuing process, that is, vasculogenesis, primordial endothelial cells assemble a primary vascular plexus within a relatively planar, sheet-like, ECM environment.

Endothelial cells can be tracked by use of microinjected and fluorochrome-conjugated QH1 antibodies, specific for quail vascular endothelium (Pardanaud et al. 1987; Rupp et al. 2004), or using the recently developed transgenic quail line in which endothelial nuclei express yellow fluorescent protein (YFP, Sato et al. 2010). In the Tg(tie1:H2B-eYFP) quail, a fusion between histone H2B and enhanced YFP is driven by the promoter of the TIE1 gene. Thus, transgenic embryos express H2B-eYFP in the nuclei of endothelial and endocardial precursor cells. TIE1+ nuclei are detectable at the earliest stages of vasculogenesis, approximately the same stage when the QH1 epitope appears (Sato et al. 2010).

At stage HH7 there is a substantial medial movement of the forming vascular plexus, a phenomenon predicted by Coffin and Poole (1988). This process, vascular drift, was shown to occur across the entire nascent vascular plexus (Rupp et al. 2004). Early vasculogenesis, however, occurs during a time of vigorous rearrangements in the embryo. In fact, the drift motion of vascular segments is largely coincident with that of the surrounding ECM (Czirok et al. 2008; Sato et al. 2010), an outcome which is to be expected if nascent vessels are embedded in a mechanical continuum. Counterintuitively, active endothelial cell movements at

the onset of vasculogenesis are largely random. Thus, although primordial endothelial cells are actively motile, the majority of their gross medial displacement at these stages is the consequence of medialward drift of the ECM scaffold.

2.4.4 Endocardial Progenitor Movements

Endocardial progenitors are also commonly assumed to migrate from their sites of differentiation in the LPM towards the midline (Gilbert 2010). Yet, as with mesenchymal cells and vascular endothelial progenitors, when endocardial progenitor cell motion is compared to that of the ECM environment, a large degree of similarity is found. In fact, the empirical data show that a distinct medial motion pattern is fully shared between the ECM and the future endocardial cell population. In contrast, the active motion of endocardial cells is randomly directed and of much smaller magnitude. The calculated autonomous speeds of 20 μm/h are in the same range as those reported for endothelial cells moving in culture: 10 μm/h by Szabo et al. (2010) and 50 μm/h by Kouvroukoglou et al. (2000). By way of comparison, the active movement of gastrulating mesoderm cells is somewhat faster, in the range of 60 μm/h (Zamir et al. 2006). Thus, four embryonic mesenchymal cell populations, newly ingressed mesodermal, pre-somitic, endothelial, and endocardial, exhibit similar random motile activity within the context of large-scale tissue (ECM) drift.

2.5 Perspectives

The most likely interpretation for the shared large-scale movement pattern between ECM components (like fibronectin and fibrillin-2) and cells is a model whereby the whole tissue (cells and the associated ECM) moves as a composite material and deforms in response to mechanical forces. The recent finding that microinjected inert particles also translocate to the forming heart, in a manner identical to ECM fibrils (Aleksandrova et al. 2012), is entirely consistent with such a view. On the other hand, cells can actively remodel, pull, and drag the ECM. Therefore, our view that cells can move relative to a conveyor-belt-like ECM that largely contributes to their displacements is an oversimplification. However, it is equally difficult to envision overt, large-scale, relative displacements between tightly interwoven three-dimensional cellular elements and ECM scaffold elements. Furthermore, recent experiments revealed that in the case of an actively moving cell population, for example, vascular sprout cells, local ECM rearrangements accompanying cell invasion are negligible compared to the magnitude of the “rapid” fluctuating ECM motion, described above (Rupp et al. In preparation). Thus, for tissue-scale movements, the ECM indeed acts as an inert conveyor belt. On much smaller scales individual cells can slowly move and remodel the ECM.

The structure of the ECM, as a physical entity, is molded by mechanical stress (Keller et al. 2003). The best understood source of mechanical stress is cellular traction force (Stoplak and Harris 1982; Oliver et al. 1995), which was demonstrated to reorganize collagen filaments in vitro (Petroll and Ma 2003; Friedl and Wolf 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). In the case of fibronectin, for example, traction forces are needed to change the conformation of individual molecules and expose a cryptic binding site required for self-assembly (Zhong et al. 1998; Baneyx et al. 2001).

Lack of information regarding the motors driving the observed ECM (and tissue) movements remains a frustrating problem for developmental biologists and those interested in tissue mechanics (Chuai and Weijer 2009b). Future advances in the field depend on a better understanding of the material properties of the embryonic tissues and how they change over space and time. Knowledge about residual stresses within the tissue is essential to conclusively map the driving forces of tissue motion (Hutson et al. 2003, 2009; Varner et al. 2010).

The large-scale co-movement of cells and the surrounding ECM has implications for the establishment and maintenance of ECM-bound morphogen gradients as well (Chuai and Weijer 2009a). Fibronectin was shown to control the availability of growth factors such as TGFbeta (Fontana et al. 2005; Leiss et al. 2008) and can bind VEGF (Wijelath et al. 2002), in some cases acting in a synergistic manner with heparan sulfate (Stenzel et al. 2011). We predict that the observed substantial ECM movement is likely to deform the concentration fields of the secreted morphogens. Furthermore, as the ECM seems to be extensively reused during morphogenesis, morphogen gradients established at earlier time at some distinct region of the embryo may provide guidance cues at later time stages of development. On the other hand, if the secreted morphogen has a short lifetime, then the ECM displacements can have little bearing on the morphogen signaling. In short, ECM motion likely impacts the bioavailability of morphogen gradients.

Empirical time-lapse data and subsequent computational analysis of multiple prominent morphogenetic events all suggest that earlier studies overestimated the apparent degree of autonomous cell motility in bird embryos. While results regarding relative motion of cells and ECM cannot be simply extrapolated to the morphogenesis of other organs, the findings described in this chapter pose the possibility that what is true during gastrulation, vertebral axis elongation, vasculogenesis, and endocardial morphogenesis may also be true for other organs where mesoderm plays a morphogenetic role. The presented findings also beg the question of whether lessons learned about morphogenetic movements, in amniotes, can be directly translated to understanding morphogenesis in evolutionarily older vertebrates. Several tissue movements, such as the assembly of a heart from bilateral primordia secondary to formation of a foregut, have no direct counterparts in non-amniote vertebrates. Stated differently—due to the striking extent of their millimeter-scale tissue deformations—did morphogenetic strategies evolve in amniotes, which distinguish them from non-amniotes?

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Chapter 3
Extracellular Matrix Functions in Amphibian Gastrulation

Bette J. Dzamba and Douglas W. DeSimone

Abstract The coordinated cell and tissue behaviors that give rise to the movements of gastrulation are influenced by extracellular matrix (ECM). In amphibian embryos fibronectin (FN) is first assembled into fibrils in a spatially and temporally regulated manner at the onset of gastrulation. Studies of the role of FN in amphibian gastrulation have revealed that in addition to providing a substratum for cell adhesion and migration, ECM regulates many other aspects of cell behavior. Cell–ECM interaction modulates cell–cell adhesion. FN contributes to the polarized cell protrusive activities that generate a variety of cell movements. ECM can serve as a repository for growth factors, contributing to the spatial and temporal regulation of growth factor signals. The physical properties of ECM also contribute to morphogenetic behaviors through mechanical signaling and influencing tissue force generation. ECM itself is very dynamic. It is stretched, moved, and remodeled by the cells and tissues whose behaviors it influences.

3.1 Introduction

Gastrulation, the morphogenetic process that forms and positions the three primary germ layers in their proper spatial context, is mediated by coordinated tissue movements that in turn are driven by integrated individual cell behaviors. Extracellular matrix (ECM) influences cell adhesion, migration, and polarity, all of which are critical for gastrulation movements. ECM also maintains boundaries between tissues and contributes to their mechanical properties. In many embryos ECM is synthesized and assembled just prior to or coincident with the initiation of gastrulation movements. Perturbation of various components of ECM and the cellular

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receptors that bind to them confirms the importance of cell–ECM interactions in this process. The current challenge is to understand how complex multifunctional ECMs regulate the dynamic and often "interconnected" cell and tissue behaviors required to resolve the embryonic body plan. Organization of the interacting molecules that comprise ECM is determined by the cells and tissues that synthesize, assemble, and remodel it. Reciprocally, the organization of cells into tissues and the biological properties of those tissues are dependent on the ECM that they produce.

Extracellular matrices are complex macromolecular structures requiring many interacting proteins for their assembly and function. During gastrulation ECM is assembled, disassembled, remodeled, and, in some cases, translocated along with the cells that assemble it. These dynamic features make the elucidation of ECM and ECM receptor functions during gastrulation challenging. Many ECM molecules influence the deposition of other ECM components. For example, laminin is required for the coordinate assembly of basement membrane components such as type IV collagen, nidogen, and perlecan. Thus, lack of laminin leads to the loss of all basement membranes (Miner et al. 2004) making it difficult to ascertain the specific contributions of laminin to cellular behavior apart from those of other basement membrane components. Similarly, inhibition of β1 integrin function can perturb FN fibril formation (Marsden and DeSimone 2001, 2003) affecting not only β1 integrin-dependent processes but also other behaviors that are dependent on FN fibrils. Thus, perturbation of one molecular component is likely to broadly disrupt the ECM/receptor network.

The cells that make up the gastrula often affect each other's behavior as a consequence of their individual activities. For example, some cells assemble matrix that others respond to, while changes in contractility of one cell will alter stresses applied to its neighbors. ECM-derived polarity cues can be transmitted to cells not in direct contact with the matrix (Marsden and DeSimone 2001) although in these instances the mechanisms are unclear.

The tissues undergoing the movements of gastrulation are mechanically linked. When one morphogenetic movement is perturbed, it often leads to the disruption of other movements that are dependent on it. Failure of prospective mesoderm to involute or ingress from a superficial location to the interior of the embryo precludes subsequent migration, illustrating the importance of the proper timing and positioning of cell and tissue rearrangements at gastrulation to later events.

Gastrulation itself ensures that the necessary geometric relationships between germ layers are established to allow organogenesis to proceed. Thus, disruption of organogenesis arising from perturbations in ECM assembly and function may have its roots in abnormal gastrulation.

Many secreted growth factors bind to ECM proteins and the heparan sulfate side chains of proteoglycans. These interactions can modulate growth factor signaling through several mechanisms. ECM binding influences the spatial distribution of signals and potentially contributes to the formation of morphogen gradients. The release of growth factors from ECM "reservoirs" by proteolysis and/or mechanical strain (Wipff and Hinz 2008; Munger et al. 1999; Buscemi et al. 2011) provides a mechanism to activate growth factors at the appropriate time and place to influence

cell behaviors. ECM also contributes to growth factor signaling by "presenting" growth factor ligands to their receptors (reviewed by Hynes 2009; Rozario and DeSimone 2010).

3.2 Models for Studying Gastrulation

The diversity of embryonic architecture associated with different groups of animals has resulted in differing strategies for gastrulation movements (Stern 2004). In contrast to mammals, for example, birds, fish, and amphibians have evolved cellular mechanisms to deal with large yolky eggs and embryos. Nevertheless, many of the same underlying processes involved in cell shape change, migration, and intercalation are used at gastrulation to generate a triploblastic embryo.

Various models offer different experimental strengths for studies of gastrulation. In the case of murine gastrulation, the roles of various ECM proteins and their cellular receptors have been inferred from the phenotypes resulting from their targeted disruption by homologous recombination (Rozario and DeSimone 2010). However, detailed analysis of the ECM and receptor functions during gastrulation in mice has been difficult. Development in utero precludes ready observation of cell and tissue behaviors as they occur. Loss of FN leads to shortening of the anterior–-posterior axis and disruption of mesodermal derivatives such as somites and notochord (George et al. 1993; Georges-Labouesse et al. 1996). Recently ex utero culture and live imaging techniques have been developed that allow early morphogenesis in mouse to be visualized, and these advances will enable analyses of cellular behavior in mutant embryos. (Williams et al. 2012; Yen et al. 2009).

Another factor complicating the analysis of mutant phenotypes in mouse is that integrins and many ECM proteins are derived from multiple gene products. For example, integrin β1 associates with at least 11 α-subunits. Not surprisingly, loss of β1 is peri-implantation lethal (Stephens et al. 1995; Fassler and Meyer 1995), but it has proven difficult to identify precisely the interaction(s) responsible for lethality. Disruption of the α5-subunit leads to the loss of a single integrin heterodimer, α5β1, but the phenotype of embryos lacking this receptor (i.e., defects in posterior mesoderm; Yang et al. 1993) is less severe than the loss of FN, its ligand. It is likely that another FN-binding α-subunit, such as αV, partially compensates for the loss of α5. Interestingly, loss of both αV and α5 leads to a complete deficit of mesoderm, a phenotype more severe than loss of FN, revealing the likelihood that these integrins also function in FN-independent processes (Yang et al. 1999; Yang and Hynes 1996).

Analyses of laminin function in mouse gastrulation are complicated by the fact that the protein is normally present in both embryonic and extraembryonic basement membrane. Laminins 111 and 511 are both expressed during gastrulation. Mice lacking either the β1- or γ1-chains common to both of these laminins have no basement membranes and die at E5.5 before gastrulation begins (Miner et al. 2004; Smyth et al. 1999). Those deficient in the α1-chain still express laminin 511 and can

assemble embryonic basement membrane but die at E6.5 due to the loss of Reichert's membrane, a basement membrane that separates the embryonic and maternal tissues. Expression of extra laminin 511 transgenically allows embryos to begin gastrulation demonstrating that laminin 511 can partially compensate for the loss of laminin 111 when expressed at high enough levels. Laminin 511 is not essential for gastrulation as embryos lacking the α5-chain are able to complete gastrulation but have defects in neural tube closure (Miner et al. 1998, 2004; Miner 2008; Smyth et al. 1999).

Zebrafish (*Danio rerio*) combines the advantages of a genetic system with the ability to image cellular events due to embryo transparency and external development. Roles for ECM have been investigated in post-gastrulation morphogenetic events. Zygotic FN mutants (*natter*) have a cardia bifida phenotype due to a disruption of epithelial organization in the cardiac precursor cells (Trinh and Stainier 2004). Mutants in laminins β1 (*grumpy*) and γ1 (*sleepy*) and fibrillin-2 (*puff daddy*) have defects in notochord formation (Parsons et al. 2002; Gansner et al. 2008). Gastrulation defects are not observed in *natter*, *sleepy*, or *grumpy* mutants, but maternally supplied FN and LN may account for the lack of early embryo phenotypes. Similarly, loss of α5 integrin leads to defects in somitogenesis (Julich et al. 2005; Koshida et al. 2005) and the formation of facial cartilage (Crump et al. 2004) later in embryogenesis. Depletion of syndecan-2 by antisense morpholino injection results in cardia bifida or left–right asymmetry defects depending on whether the yolk syncytial layer or embryo is targeted (Arrington and Yost 2009), but gastrulation appears to be normal. The study of maternal-zygotic mutants is likely to yield additional information on the roles of ECM in gastrulation in the future.

Drosophila is an excellent genetic model of development, but ECM appears to be unimportant for gastrulation in this system. FN and fibrillar collagens are not present in the *Drosophila* genome (Hynes and Zhao 2000). There are only four laminin chains in *Drosophila*. One of two alpha chains pairs with common β- and γ-chains to form two trimers, laminin A and laminin W. Laminin is not detected in the embryo until after gastrulation (Montell and Goodman 1989; Urbano et al. 2009). Mutation of the common β-chain leads to a loss of all basement membranes in the embryo resulting in defects in tissue and organ morphogenesis, but gastrulation is not impaired.

Chick and quail have been used as a source of embryos for observation and experimentation for more than a century. This long history has resulted in the development of multiple experimental techniques for analyzing morphogenesis. Avian embryos are amenable to both experimental manipulation and imaging, particularly of later stage events including somite formation, vasculogenesis, and neural crest migration (Le Douarin 2004; Stern 2005; Davey and Tickle 2007; see also Czirok et al. 2013).

Vertebrate gastrulation has been studied extensively using a variety of amphibians (both anuran and urodele species) although most studies in recent decades have focused largely on *Xenopus laevis*, the South African clawed frog. The early *Xenopus* embryo is well suited to microsurgical manipulations. Explanted

tissues from gastrulating embryos undergo many of the movements of gastrulation when isolated from the embryo and placed in culture. The use of explants to study morphogenetic movements facilitates the imaging and analysis of cellular movements that are obscured in the whole embryo. Explanted tissue is by definition uncoupled mechanically from other tissues in the embryo. This can be advantageous in that it allows region-specific movements to be studied in isolation, but care must be taken to consider how connections to other tissues in vivo might affect normal behaviors. For example, a rectangular explant of dorsal marginal zone tissue will migrate on a FN substrate, but it migrates more slowly than it would in the embryo. However, when it is arranged circumferentially as in the embryo, it migrates at normal velocity (Davidson et al. 2002).

Several techniques have been used to investigate the function of proteins in *Xenopus*. Levels of protein expression can be reduced through the use of antisense morpholinos to block translation. Similarly, mRNA injection can be used to overexpress wild-type, GFP-tagged, dominant-negative, or mutant versions of proteins to look at their structure and function within the context of embryonic tissue. Transcript injection into fertilized eggs ensures expression of the target protein in all cells of the early embryo. Alternatively, because the fate of individual blastomeres from cleavage stage embryos is known and has been mapped (Moody 1987a, b; Dale and Slack 1987), injections can be targeted to affect protein expression in specific lineages and tissues. Many of the proteins involved in early embryogenesis are maternally encoded. *Xenopus* oocytes can be cultured and injected with morpholinos or other antisense constructs to deplete maternal proteins or mRNAs. The oocytes are matured in vitro and then transferred to a host frog to be passed through the oviducts before fertilization (Hulstrand et al. 2010; Schneider et al. 2010; Mir and Heasman 2008). Function-blocking antibodies can also be used to perturb the function of specific molecules. These can be injected into the blastocoel to look at effects on the whole embryo or added to cultured explants to acutely disrupt function. Because the peptide sequence RGD, present in several ECM molecules, is needed for integrin binding (Pierschbacher and Ruoslahti 1984), peptides containing this sequence have been injected into intercellular spaces (e.g., the blastocoel cavity) to perturb ECM–integrin interactions. Due to these experimental advantages, the roles of the ECM and its receptors at gastrulation are arguably best understood in *Xenopus*. We will briefly describe the main tissue movements that occur during *Xenopus* gastrulation and then describe what is known about the role of ECM in each.

3.3 Gastrulation in Xenopus

Gastrulation in Xenopus has been described in detail elsewhere (Davidson 2008; Keller et al. 2003; Stern 2004) but will be summarized briefly here. The major "landmarks" of the gastrulating embryo are depicted in Fig. 3.1. The center of the upper pigmented half of the embryo is referred to as the "animal pole." The "vegetal

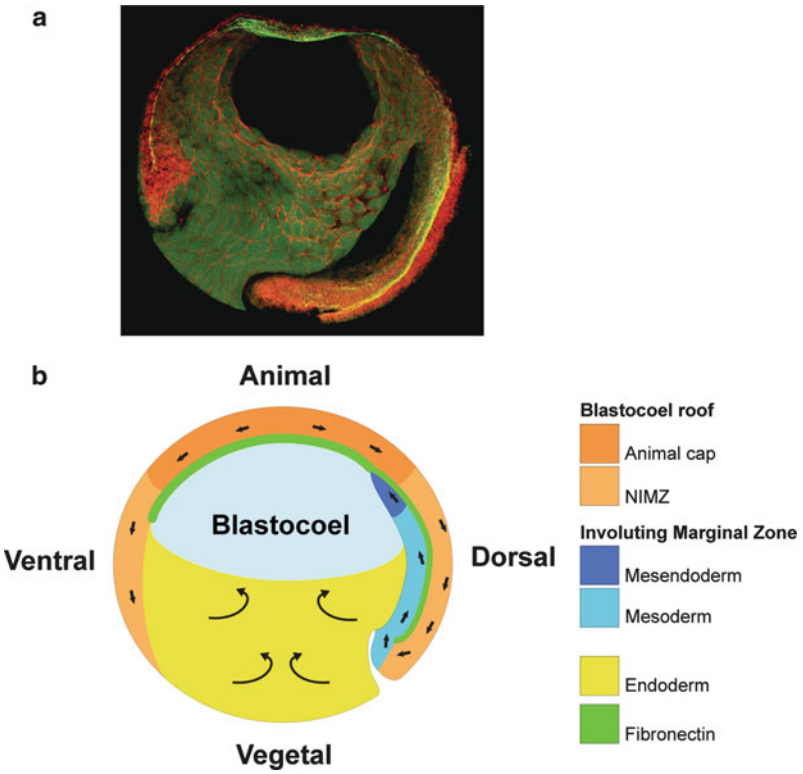

Fig. 3.1 (**a**) Confocal *Z*-stack projection of a mid-gastrula stage *Xenopus* embryo immunostained with antibodies to FN (*green*) and C-cadherin (*red*). (**b**) Diagram of a *Xenopus* early gastrula stage embryo [corresponds generally to embryo shown in (**a**)]. *Arrows* indicate the direction of tissue movements during gastrulation

pole" is in the center of the unpigmented cells on the bottom. The term "marginal zone" is used to describe the band of tissue between the animal and vegetal hemispheres that eventually gives rise to the axial tissues. Part of the marginal zone involutes during gastrulation and is referred to as the involuting marginal zone "IMZ," whereas marginal zone tissue that remains at the surface is termed the non-involuting marginal zone, "NIMZ." The inside of the upper half of the embryo contains the blastocoel, a fluid-filled cavity that forms during cleavage. The bottom of this cavity is referred to as the blastocoel floor. The blastocoel roof is made up of the NIMZ and the "animal cap" tissue above it.

Gastrulation begins internally as the vegetal cells undergo rotation, a movement that positions the IMZ against the BCR. The first visible sign of gastrulation is the apical constriction of some cells in the DMZ to form bottle cells. The formation of bottle cells continues around to the ventral side of embryo to form a ring known as the blastopore. The IMZ cells move from the surface to the inside of the embryo by involuting or rolling around the blastopore lip as a sheet. The sheet, composed of

both mesoderm and endoderm cells and referred to as "mesendoderm," migrates along the inside of the BCR to the animal pole. Cells that involute later are rearranged into a narrower and longer array by convergent extension. The blastocoel roof thins and spreads both before and during gastrulation to cover the embryo in an epithelial sheet through a process termed epiboly (Davidson 2008; Keller et al. 2003).

Early scanning electron microscope studies of *Ambystoma* gastrulae (Nakatsuji et al. 1982) revealed fibrillar structures on the BCR that were aligned along the animal–vegetal axis. Protrusions from the migrating mesendodermal cells appeared to attach and align with the fibrils, suggesting a role for extracellular matrix (ECM) in guiding gastrulation. Subsequent studies demonstrated that the fibrils contained fibronectin (FN) and that injection of antibodies directed against FN, or RGD peptides that block integrin binding, resulted in gastrulation failure in *Pleurodeles*. Involution was blocked and the ectoderm became convoluted, suggesting a failure of epiboly (Boucaut and Darribere 1983; Boucaut et al. 1984a, b, 1985).

The results of similar experiments performed in the anuran *Xenopus* are less clear with phenotypes reported that range from mild to severe. On the mild end of the spectrum are embryos that are pear-shaped at the end of gastrulation and develop into tadpoles with shortened tails but otherwise exhibit apparently normal anterior and axial development (Winklbauer and Keller 1996). On the severe end are embryos that are unable to undergo normal involution (Howard et al. 1992).

The differences in phenotypes noted between urodeles and anurans may reflect differences in the relative importance of FN-mediated cell behaviors in gastrulation in these systems. However, the variations in phenotype severity within the same species may be due to variations between experimental approaches. For example, phenotypes were milder when embryos with punctured BCRs were incubated with RGD peptides than when the peptides were injected directly into the blastocoel (Howard et al. 1992; Winklbauer and Keller 1996). The flanking sequences of the RGD-containing peptides, their effective concentrations, and purity might have varied between studies. Similarly, the polyclonal antisera used to block FN–integrin interaction may have differed in their biological activity. In one study three different polyclonal antisera that immunoprecipitated β1-integrin were compared for their ability to block cell spreading and gastrulation. Two of the three were observed to block cell spreading, and only one of those blocked gastrulation (Howard et al. 1992). Subsequent studies using well-characterized reagents and multiple approaches (Fig. 3.2) have yielded a common set of phenotypes at gastrulation. Monoclonal antibodies to FN (Ramos and DeSimone 1996) or α5β1 (Davidson et al. 2002) that block FN–integrin interaction and FN assembly have been used to probe the roles of FN and integrin at gastrulation (Marsden and DeSimone 2001, 2003; Davidson et al. 2002, 2006). Expression of a chimeric β1 integrin construct consisting of the extracellular and transmembrane domains of hemagglutinin fused to the intracellular domain of β1 (HAβ1) acts as a dominant-negative. It likely interferes with endogenous integrin function by competing for cytoplasmic components critical for integrin function (LaFlamme et al. 1994; Marsden and DeSimone 2001, 2003). An antisense-morpholino was used to deplete

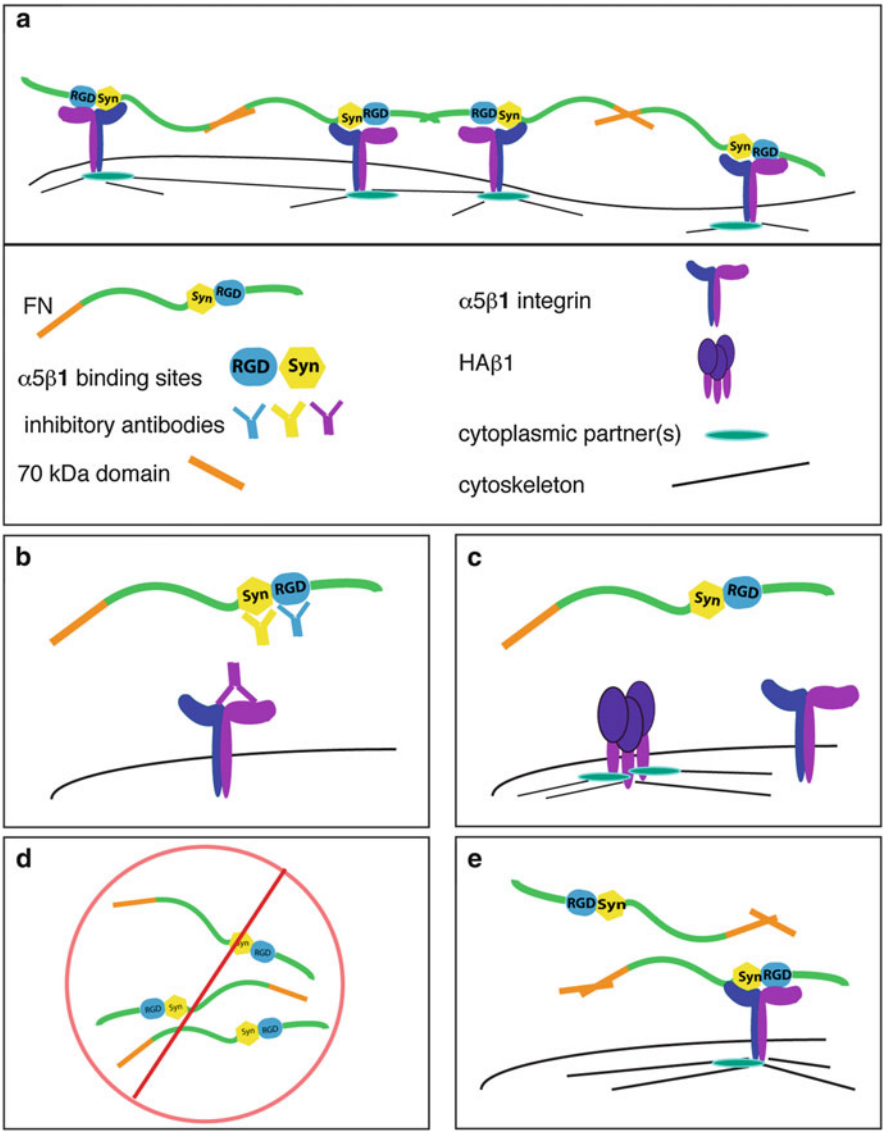

Fig. 3.2 Methods used to disrupt FN–integrin interactions in *Xenopus*. (**a**) Multiple components are involved in FN-dependent cell behaviors. α5β1 integrins bind to FN through the RGD and synergy sites. Cytoplasmic proteins promote bidirectional signaling and the interaction of integrins with the cytoskeleton. FN–FN binding sites mediate fibril assembly. (**b**) Antibodies to FN or α5β1 integrin inhibit FN–α5β1 integrin ligation. (**c**) Dominant-negative HAβ1 competes with endogenous α5β1 integrin for cytoplasmic binding partners that are needed for integrin function. (**d**) Antisense morpholinos block translation of FN. (**e**) Expression of a FN-binding fragment of FN blocks intermolecular FN–FN interactions and inhibits fibril assembly

FN levels by 85–95 % of normal (Davidson et al. 2006). These varied approaches all resulted in a common set of phenotypes demonstrating that both FN and α5β1 integrin are needed for many aspects of gastrulation. Affected embryos are slow to close their blastopores. The BCR ectoderm is thickened, suggesting that epiboly is disrupted. Embryos are shortened along the anterior–posterior axis indicative of defects in convergent extension. The development of anterior–ventral mesodermal derivatives such as heart, blood vessels, and gut is disrupted likely due to abnormal mesendoderm migration (Davidson et al. 2006; Marsden and DeSimone 2001; Ramos and DeSimone 1996; Ramos et al. 1996). Thus, FN and α5β1 integrin mediate many morphogenetic movements during *Xenopus* gastrulation. However, in these embryos, unlike *Pleurodeles*, gastrulation was not completely arrested when FN and/or α5β1 function was perturbed.

The importance of the fibrillar structure of FN was examined using expression of a 70 kDa amino-terminal fragment of FN that blocks the FN–FN interactions critical for FN matrix assembly but does not block α5β1 ligation (Rozario et al. 2009). This approach demonstrated that FN fibrils are needed to promote the thinning of the blastocoel roof but that FN–α5β1 interactions are sufficient to mediate mesendoderm migration and convergence extension (Rozario et al. 2009).

The greater sensitivity of urodeles to FN–integrin perturbations may reflect differences in the mechanisms of migration between urodeles and anurans. Urodele mesendoderm cell migration is less cohesive than it is in anurans (i.e., single cell vs. collective cell migration) consistent with the idea that urodeles may rely more on cell–matrix than cell–cell interaction for migration. The fibrils are also reported to be denser and more oriented in urodeles than anurans (Nakatsuji and Johnson 1983a) again suggesting that cell–matrix interaction plays a more significant role for gastrulation movements in the salamander. Convergent extension, which may contribute by "pushing" the mesendoderm toward the animal pole in the absence of migration, is more robust in anurans than in urodeles (Shi et al. 1987). Thus, urodeles may be more dependent on migration for correct mesoderm positioning.

Thirty years later, our knowledge of how the fibrils first observed by scanning electron microscopy influence gastrulation is greatly expanded. Fibronectin regulates cellular behaviors in many ways to contribute to the coordinated tissue level movements that drive morphogenesis during gastrulation.

3.4 Expression of ECM and Its Receptors During *Xenopus* Gastrulation

The repertoire of ECM molecules expressed during amphibian gastrulation is limited with FN being the most abundant and its roles in morphogenesis best understood. In *Xenopus* FN is translated at the midblastula stage from maternally deposited mRNA (Lee et al. 1984). Zygotically expressed FN transcripts are first detected during gastrulation. Both maternal and zygotic transcripts include the

alternatively spliced EIIIA and EIIIB exons characteristic of cellular FN (DeSimone et al. 1992). FN is initially assembled into fibrils on the inner surface of the animal cap ectoderm and then in the marginal zone where it separates involuting and non-involuting mesoderm; eventually it separates mesoderm from endoderm as well (Lee et al. 1984; Davidson et al. 2004). The only other ECM molecule to receive attention for its role in amphibian gastrulation is fibrillin (Fbn), which is initially expressed at mid-gastrulation along the forming notochord–somite boundary where it contributes to axial extension (Skoglund and Keller 2007; Skoglund et al. 2006). Laminin is reportedly expressed on the BCR of *Pleurodeles* (Darribere et al. 1986; Nakatsuji 1986) but is not detected in *Xenopus* until the end of gastrulation (Fey and Hausen 1990). In other organisms such as chicken and mouse, the ECM at gastrulation includes basement membranes. Collagen VI is expressed during early embryogenesis (Otte et al. 1990), but its role has not been extensively studied.

Integrin α5β1 is expressed on all cells at gastrulation (Gawantka et al. 1992; Joos et al. 1995) and is required for the assembly of FN into fibrils in both *Pleurodeles* and *Xenopus* (Howard et al. 1992; Darribere et al. 1990). The other alpha subunit expressed during gastrulation, αV, is expressed on the surface of cells from all three germ layers in *Xenopus* (Ramos et al. 1996) but is found specifically on the surface of migrating mesodermal cells in *Pleurodeles* (Alfandari et al. 1995). The β-subunit that associates with αV at gastrulation has not been established.

Cells also adhere to FN through cell surface heparin sulfate proteoglycans. Syndecans interact with FN through the heparin-binding domain (HepII) located in the C terminus of the protein (Woods and Couchman 2001). During gastrulation syndecans 1 and 2 are expressed on the BCR (Teel and Yost 1996), whereas syndecan 4 is present on both the BCR ectoderm and involuting mesoderm (Munoz et al. 2006).

Adhesion to ECM is regulated at multiple levels during gastrulation with the timing of ECM molecule and integrin expression being just one factor. Although FN (Lee et al. 1984) and α5β1 (Gawantka et al. 1992; Joos et al. 1995) are both expressed by cells throughout the gastrula, their functional activities are regulated both temporally and spatially. Cell adhesion can be regulated by changes in the activation state of integrins. Cellular mechanisms for integrin "activation" include conformational changes that increase the affinity of integrins for ligand and clustering of integrins leading to changes in avidity (see Margadant et al. 2011, for a recent review). The activation state of integrins changes on some tissues during gastrulation (Ramos et al. 1996). Fibrillar FN is assembled on only a subset of the tissues that express it. The assembled state of FN is essential for some tissue movements, but not others (Rozario et al. 2009), suggesting that FN function is modulated by its macromolecular structure. Cryptic sites that mediate FN–FN interactions and promote FN assembly (Ingham et al. 1997; Hocking et al. 1994) are exposed within FN when it is stretched (Baneyx et al. 2002; Klotzsch et al. 2009). FN binding to α5β1 integrin is mediated by both the RGD sequence in the tenth type III repeat and a "synergy" site located in the ninth type III repeat that acts cooperatively with the RGD site to confer high-affinity binding (Aota et al. 1994).

Models based on a recent crystal structure of the α5β1 ectodomain suggest that the RGD and synergy sites bind to a "trenchlike" structure at the interface of the α- and β-subunits (Nagae et al. 2012). Steered molecular dynamics simulations predict that stretching of FN alters the distance between the RGD and synergy sites and may therefore change the interaction of FN and α5β1 (Krammer et al. 2002). Thus, the movements of gastrulation are likely to modify the activity of FN by exposing or altering functional domains. This posttranslational regulation of FN and integrin allows for the dynamic modulation of adhesive behaviors needed for morphogenesis.

3.4.1 Regulation of Integrin-Dependent Cellular Behavior

Cells from all regions of the blastula and gastrula are able to attach to fibronectin or a recombinant central cell-binding domain of FN containing type III repeats 9–11 which include the RGD and synergy sites known to be important for cell adhesion and spreading on FN (Ramos and DeSimone 1996; Pierschbacher and Ruoslahti 1984; Aota et al. 1994; Bowditch et al. 1994). As prospective mesendoderm and mesodermal cells (i.e., IMZ cells) involute during the course of gastrulation, they gain the ability to spread on FN substrates. This suggests that IMZ cells encounter signals during the course of involution required for changes in their adhesive behavior. In contrast, animal cap ectoderm cells never involute, and while they are capable of attaching to FN, they are unable to spread. However, animal cap cells treated with the mesoderm-inducing factor activin exhibit spreading behavior (Ramos et al. 1996). While recognition of the RGD site alone is sufficient for cell attachment, recognition of both the RGD and synergy sites is required for cell spreading. Cells do not spread on the central cell-binding domain if the synergy site is mutated or blocked by an antibody. Activin does not change surface levels of integrins, further suggesting that the activation state of integrin is being altered to produce the observed changes in adhesive behavior (Ramos et al. 1996). Thus, cell signals received during involution allow cells to recognize the synergy site of FN and change their adhesive behavior. Interestingly, in cultured cells myosin-II-mediated tension can regulate recognition of the synergy site by α5β1 (Friedland et al. 2009) raising the possibility that the signal that allows cells to spread involves mechanical input. Activin can change the mechanoresponsiveness of animal cap cells to forces on cadherin (Weber et al. 2012). As cells involute, they may encounter an activin-like signal either that directly changes integrin activation or that allows them to become more mechanoresponsive to force-induced activation of α5β1. Alternatively, the movements of involution may provide the tension to alter the activation state of α5β1 directly. The hypothesis that the initial movements of gastrulation regulate integrin activity through mechanical signals merits further exploration.

3.4.2 Regulation of Matrix Assembly

FN matrix is first assembled at the onset of gastrulation. Fibrils are localized to the blastocoel roof but not the blastocoel floor. This spatial and temporal regulation of FN is not mediated solely by the expression of FN or its receptor α5β1 integrin as both are expressed by animal and vegetal cells before gastrulation begins (Lee et al. 1984; Gawantka et al. 1992; Joos et al. 1995). As in cultured cells, where the process has been studied extensively (reviewed in Schwarzbauer and DeSimone 2011; Singh et al. 2010), fibrillogenesis in amphibians requires both integrin recognition of the RGD and synergy sites of FN (Darribere et al. 1990; Marsden and DeSimone 2001) and FN–FN interactions. The latter involves the 70 kDa amino-terminal fragment (Winklbauer and Stoltz 1995; Rozario et al. 2009) of FN and the first type III repeat (Darribere et al. 1992). Cell tension, mediated by actin contractility, is critical for FN assembly in both cultured cells (Wu et al. 1995; Pankov et al. 2000; Zhang et al. 1997; Zhong et al. 1997) and *Xenopus* BCR explants (Winklbauer and Stoltz 1995). However, because the cells of the BCR are attached to one another rather than to a rigid substrate, cell–cell adhesion contributes to the generation of tension. In cultured cells integrin activation and focal adhesions are required for FN assembly (Wu et al. 1995; Pankov et al. 2000). As discussed, there is likely no change in integrin activation in the animal cap ectodermal cells that assemble FN during gastrulation (Ramos et al. 1996). Thus, it is unlikely that changes in integrin activation are responsible for triggering FN assembly on the BCR. In fact, activin treatment, which increases integrin activity and decreases cadherin-mediated adhesion, inhibits fibril assembly (Brieher and Gumbiner 1994; Nagel and Winklbauer 1999). As gastrulation begins the shape of BCR cells becomes less rounded and more polygonal indicative of an increase in cell–cell adhesion. Cortical actin assembly also increases as gastrulation proceeds, suggesting that BCR cells are under increased tension. Overexpression of cadherin in early gastrulae leads to a change in the shape of BCR cells from round to polygonal and to precocious FN assembly. Conversely, inhibiting cell–cell adhesion inhibits FN assembly. Thus, the regulation of cell–cell adhesion, in addition to cell–matrix adhesion, modulates FN assembly in the embryo. We have proposed a model in which Wnt-planar cell polarity signaling is a key regulator of FN assembly at gastrulation (Fig. 3.3). Wnt regulates cadherin adhesive activity and the subsequent assembly of cortical actin in the BCR. The downstream Wnt/PCP effectors Rac and Pak are needed for the assembly of cortical actin and the phosphorylation of myosin leading to increased tension in the BCR. This tissue tension is transmitted to FN allowing fibril assembly to occur (Dzamba et al. 2009).

Dominant-negative inhibition of either of the two syndecans expressed on the BCR when FN is assembled abolishes assembly (Kramer and Yost 2002). It will be interesting to further understand how the various players including integrin, cadherin, syndecan, and PCP signaling interact with one another to regulate both FN matrix assembly on the BCR and convergent extension in the mesoderm (Munoz et al. 2006); see Sect. 3.7.3.

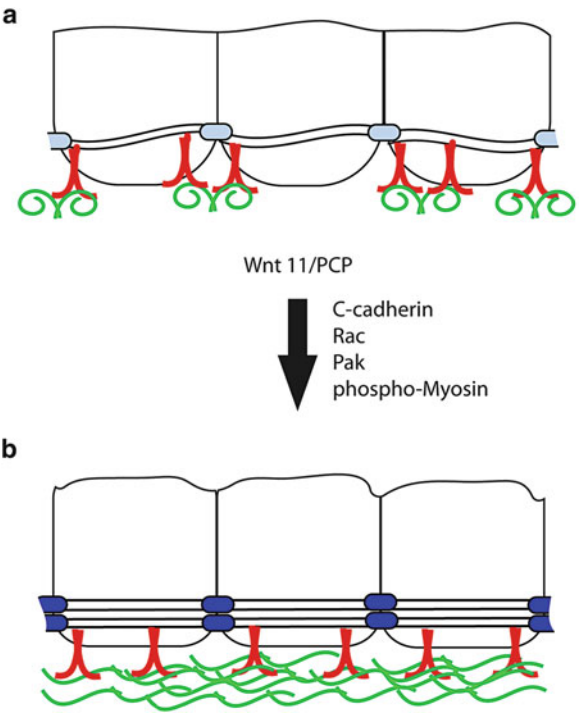

Fig. 3.3 FN assembly on the BCR. (**a**) At the onset of gastrulation, FN dimers (*green*) are bound to α5β1 integrins (*red*) on BCR cell surfaces. Cadherin-mediated cell–cell adhesion (*light blue*) and cytoskeletal tension are low. (**b**) Wnt/PCP signaling increases cadherin-mediated cell–cell adhesion (*dark blue*) and increases cytoskeletal tension through Rac and Pak signaling culminating in the assembly of cortical actin and the phosphorylation of myosin. The tension is transmitted to FN through α5β1 integrins resulting in the exposure of FN–FN interaction sites to mediate fibril assembly

Although FN is initially assembled on the blastocoel roof, ECM is subsequently assembled throughout development at tissue boundaries (Davidson et al. 2004). FN and Fbn are assembled at the notochord–somite boundary where they regulate mediolateral intercalation behavior but are not needed for the formation of the boundary, which forms before Fbn is deposited and is evident even when FN assembly is inhibited (Marsden and DeSimone 2003; Skoglund et al. 2006). Similarly during branching morphogenesis FN is assembled into clefts after their formation has been initiated (Sakai et al. 2003). This suggests that FN is not responsible for creating boundaries but may serve to stabilize them.

The process of somite formation in zebrafish provides a model for how matrix assembly is limited to tissue boundaries. Integrins on adjacent cells within the presomitic mesoderm normally repress one another preventing integrin clustering and FN assembly. Ephrin signaling at the somite border leads to integrin derepression and ligand-independent clustering. The clustered integrins then mediate assembly (Julich et al. 2009). FN assembly in *Xenopus* requires a free cell surface (Winklbauer 1998). The animal cap cells that first assemble FN have a free surface facing the blastocoel, but subsequent assembly often occurs between juxtaposed tissues. Ephrin signaling is involved in the separation of ectoderm and mesoderm in the *Xenopus* gastrula (Park et al. 2011). Perhaps ephrin signaling enables the assembly of FN between those two tissues in a manner analogous to the regulation of assembly between somites in zebrafish. FN assembles around groups of cells

expressing higher levels of cadherin than their neighbors (Dzamba et al. 2009). It is tempting to speculate that differences in cadherin adhesion between tissues also contribute to ECM assembly at tissue borders.

3.5 Mesendoderm Migration

The mesendoderm migrates directionally toward the animal pole of the BCR. The timing of FN fibrillogenesis, coincident with the onset of gastrulation, and the localization of fibrils to the BCR across which mesendoderm cells migrate suggest that FN matrix is important for mesendoderm migration. Indeed experiments in urodeles indicate that it is critical. Perturbing the interaction of migrating cells with FN inhibits mesendoderm migration and gastrulation (Boucaut et al. 1984a, b; Darribere et al. 1988; Darribere and Schwarzbauer 2000). The analysis of the role of FN in mesendoderm migration in *Xenopus* is complicated by the fact that the mesendodermal mantle is able to extend even when FN matrix or FN–integrin interactions are perturbed. Mesendoderm becomes detached from the blastocoel roof but is still able to move toward the animal pole (Davidson et al. 2006; Marsden and DeSimone 2001; Rozario et al. 2009). In fact, the mesendoderm is able to extend even when the BCR is completely removed (Keller and Jansa 1992). This mesendodermal mantle extension is potentially driven through mechanisms other than migration such as vegetal rotation (Ibrahim and Winklbauer 2001; Winklbauer and Schurfeld 1999) or being pushed by the convergent extension of the axial mesoderm "behind it." Even though the mesendoderm can extend in *Xenopus* embryos when FN–integrin interactions are perturbed, there are later defects in mesendoderm-derived tissues such as the gut, heart, and blood, suggesting that FN is needed for "normal" mesendoderm migration (Marsden and DeSimone 2001; Davidson et al. 2006). What remains to be determined is what specific role(s) FN plays in the process.

The mesendoderm cells migrate collectively across the BCR toward the animal pole. Many of the inputs that can guide directional migration (Rorth 2011; Petrie et al. 2009) are influenced by ECM (Fig. 3.4). Cells use haptotaxis to follow cues provided by adhesive gradients (Carter 1967). Such a gradient could be formed by changes in ECM density. Durotaxis (Lo et al. 2000) describes the tendency of cells to migrate from softer to stiffer substrates. ECM can contribute to tissue stiffness. During chemotaxis cells follow gradients of secreted signals (Swaney et al. 2010), many of which bind to ECM. Additionally, the physical alignment of ECM fibrils can provide directional cues through contact guidance (Dunn and Heath 1976). ECM-dependent traction forces contribute to cohesotaxis, a mechanism of directional guidance based on force application to cell–cell junctions (Weber et al. 2012). The relative importance of these mechanisms for guiding mesendoderm migration is not clear.

The question of whether the BCR provides cues promoting the directional migration of mesendoderm has been of interest for nearly 75 years. Spemann

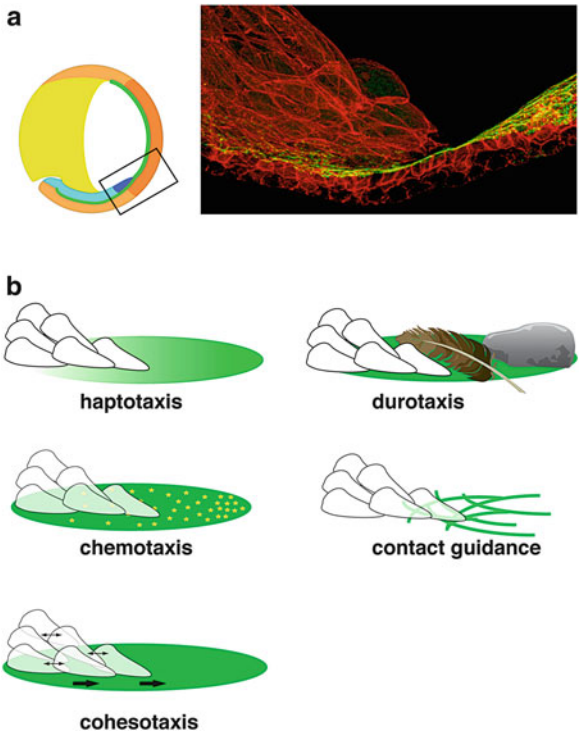

Fig. 3.4 Mesendoderm migration. (**a**) Confocal *Z*-stack projection of mesendoderm migrating on the BCR. The *black rectangle* indicates the region of the embryo that is shown in the micrograph. Immunostaining with antibodies to C-cadherin (*red*) and FN (*green*) reveals the "shingled" arrangement of the migrating cells and the BCR substrate they migrate across. (**b**) Diagrams illustrating multiple mechanisms of directed cell migration that involve ECM

(1938) and later Cooke (1972) found that when a portion of the BCR was removed, rotated 180°, and put back, embryos developed normally. Cooke (1972) concluded that "neurectoderm contains no guiding information for advancing mesoderm cells," a conclusion that is still being tested today. While these experiments suggest that there is no graded information in the BCR, rotating the tissue 180° preserves the animal–vegetal alignment of the fibrils observed by (Nakatsuji et al. 1982) and would still allow for contact guidance. It is not clear how the fibrils become aligned, but it has been suggested that forces generated by the ectodermal cells undergoing epiboly or by migrating mesendoderm cells themselves might be involved (Nakatsuji et al. 1982). A similar example of a sheet of migrating cells arranging matrix and then following its alignment is seen when breast cancer cells, cultured in collagen gels, use Rho-mediated contractility to align the matrix and then preferentially migrate on fibrils that have been arranged perpendicular to the tissue edge (Provenzano et al. 2008). In *Ambystoma* the fibril alignment can serve to bias cell movement in the animal–vegetal rather than mediolateral direction. When animal cap is stretched during substrate conditioning, fibrils are deposited that align with the direction of stretch. Mesendoderm cells follow the "exogenous tension axis" even when it is perpendicular to the original animal pole. This suggests that, at least in *Ambystoma*, fibril alignment can provide contact guidance (Nakatsuji and Johnson 1983b, 1984). In other amphibians, such as *Xenopus*, fibrils show no obvious

alignment (Nakatsuji and Johnson 1983a; Winklbauer and Nagel 1991), suggesting that in these embryos FN influences migration in other ways.

3.5.1 Regulation of Directional Migration by Mechanical Signals

Explanted dorsal tissue that contains both mesendoderm and axial mesoderm (DMZ explant) can migrate directionally on a non-fibrillar FN substrate (Winklbauer 1990; Davidson et al. 2002). Although fibrils assembled by the BCR are not important for the migration of DMZ explants, interaction with FN remains critical. When an actively migrating DMZ explant is treated with inhibitory antibodies either to the RGD site of FN or to integrin α5β1, the explant retracts away from the direction of migration and detaches from the substrate. The direction of retraction suggests that the explant is under anisotropic intercellular tension that is normally balanced by FN–integrin-mediated traction stress. When interaction with the FN synergy site is blocked, the explant remains attached to the substrate, but the leading edge lamellae retract and migration ceases (Davidson et al. 2002). Thus, the synergy site is not needed for the explant to resist intercellular tension but is needed for the extension of lamellae and explant migration. These results agree with the observations made on single mesendoderm cells that RGD–integrin interaction mediates attachment, but protrusive activity additionally requires interaction with the synergy site (Ramos and DeSimone 1996). These experiments also demonstrate that cells can exert traction stress on FN-containing substrates. Traction force is needed to propel migration, but a recent study suggests that it also plays a role in regulating the direction of that migration (Weber et al. 2012).

Directional migration, as seen in the embryo, requires that the orientation of the mesendodermal cell protrusions be coordinated. Both in vivo and in DMZ explants the migrating cells move in a shingled array. The protrusions are polarized toward the direction of migration with cells behind the leading edge extending protrusions underneath their neighbors (Fig. 3.4). Because the group of migrating cells is initially more than one cell layer thick, some of the cells are not in direct contact with FN but are still polarized in the direction of migration (Winklbauer and Nagel 1991; Davidson et al. 2002). This, combined with the observation that single cells do not exhibit polarized protrusive activity or directional migration (Winklbauer and Selchow 1992), suggests that cell–cell contacts play a role in guiding directional migration in *Xenopus*.

Recently it has been shown that mesendoderm polarity is regulated by a mechanoresponsive cadherin complex. Application of localized mechanical force to cell surface C-cadherin, through the use of a magnetic bead coated with the C-cadherin ectodomain, is sufficient to polarize the protrusive and migratory behaviors of single mesendoderm cells plated on isotropic FN substrates. Local ligation of the cadherins is not sufficient to polarize the cells without the application of force. Force on cadherin recruits plakoglobin to the complex and polarizes the keratin intermediate filament network within the cell. Both plakoglobin and

cytokeratin are necessary for polarized protrusive behavior, suggesting that they, together with C-cadherin, form a mechanoresponsive complex (Weber et al. 2012).

Similar behavior is observed in cell pairs plated on FN. Polarized protrusions are directed away from the cell–cell junction, plakoglobin is recruited, and the keratin intermediate filament network becomes polarized. Formation of a junction between the cells is not alone sufficient to generate polarity (Weber et al. 2012). Traction forces are counterbalanced by the force generated by intercellular stresses at the cell–cell junction (Maruthamuthu et al. 2011). Cells plated on poly-L-lysine attach and can form cell–cell adhesions when in contact, but, in the absence of the traction force generated by integrin-mediated adhesion, do not polarize and do not recruit plakoglobin to the junction (Weber et al. 2012). In an intact embryo or DMZ explant, the axial mesoderm likely acts as an anchor generating asymmetric forces across the migrating mesendoderm cells. This asymmetry induces polarized protrusions and is counterbalanced by traction forces generated by integrin–FN interaction (Weber et al. 2012).

3.5.2 ECM as a Repository of Growth Factors

Studies of the behavior of small aggregates of mesendodermal cells reveal that conditioned substrates contain directional cues that are not found in substrates coated with purified FN. Single cells are motile but do not migrate directionally. A minimum aggregate size of 10–20 cells is required for directional migration, suggesting that the cues present in the conditioned substrate require cell–cell adhesion for interpretation (Winklbauer and Nagel 1991; Winklbauer et al. 1992).

ECM regulates the bioavailability of many growth factors (Hynes 2009). It is likely that conditioned substrates differ from planar FN substrates not only because of the fibrillar architecture of the FN but also because other molecules are deposited along with FN.

3.5.2.1 PDGF in Xenopus

PDGFA is expressed by the BCR ectoderm, and its receptor (PDGFRα) is expressed by the migrating mesendoderm cells (Ataliotis et al. 1995). Two forms of PDGFA are generated by alternative splicing (Mercola et al. 1988). The long form (lfPDGFA) contains a positively charged matrix-binding motif that is missing from the short (sfPDGFA) splice variant (Andersson et al. 1994). LfPDGFA binds to FN and its binding is enhanced by heparin (Smith et al. 2009).

Expression of dominant-negative PDGFRα or overexpression of lfPDGFA disrupts gastrulation. The mesendoderm loses adhesion to the ectoderm, and its shingled architecture is perturbed, suggesting that cell–cell interactions may be disrupted. The mesendoderm sends out fewer protrusions and does not extend leading to a loss of anterior structures (Ataliotis et al. 1995; Nagel et al. 2004).

The study of mesendodermal cell aggregates on conditioned substrates suggests that PDGF signaling regulates directionality. Expression of dominant-negative PDGFR in the migrating cells and increasing or decreasing the levels of lfPDGFA in the conditioned substrate lead to a loss of directionality, suggesting that a precise level of PDGF signaling is needed for guidance (Nagel et al. 2004). The interaction of PDGFA with ECM is required for its guidance function in this system as overexpression of sfPDGFA that does not bind to ECM does not disrupt directional migration (Smith et al. 2009). Inhibition of FN fibril formation by treating the animal cap with RGD peptides or cytochalasin during conditioning leads to a loss of directional information (Winklbauer and Nagel 1991) from the conditioned substrate. This might mean that fibrils contain intrinsic directional information or that fibrils are needed for PDGF deposition. It has been suggested that heparin enhances FN–lfPDGFA binding by exposing lfPDGF-A binding sites within FN (Smith et al. 2009). Perhaps such binding sites are also exposed by fibrillogenesis as FN contains many cryptic functional domains that are exposed when the molecule is stretched (Klotzsch et al. 2009).

Unlike mesendodermal aggregates, DMZ explants containing both axial mesoderm and leading edge mesendoderm do not require conditioned substrates to migrate directionally (Winklbauer 1990; Davidson et al. 2002). In these larger explants differences between the two tissues might provide polarity cues. However, directional migration of single tissues or even single cells can occur on purified planar FN substrates. “Donut” explants that contain a 360° ring of mesendoderm without axial tissue also move directionally (Davidson et al. 2002) on planar FN. Mechanical signals through C-cadherin are sufficient to polarize single cells on an isotropic FN substrate (Weber et al., 2012). These observations suggest that more research is needed to understand precisely what role matrix resident PDGFA plays in directional migration. Studies in avian embryos suggest it may act indirectly by regulating cell–cell adhesion.

3.5.2.2 Regulation of Cell–Cell Adhesion by PDGF

Many factors have been proposed to influence the directional migration of mesodermal cells away from the primitive streak in avian embryos. Injection of antibodies to FN does not inhibit ingression but does block subsequent migration. FN immunostaining suggests that the matrix is denser away from the primitive streak (Zamir et al. 2008; Sanders 1984; Harrisson et al. 1993) consistent with a role for FN in haptotaxis (Harrisson et al. 1993). Cells are attracted to FGF4 and repulsed by FGF8, suggesting that FGF-mediated chemotaxis contributes to migration away from the primitive streak (Yang et al. 2002). The directional migration of mesoderm is also strongly inhibited by interfering with PDGF signaling. However, the cells are only weakly attracted to a PDGFA-coated bead, suggesting that PDGF is not influencing directional migration through chemotaxis. Cells expressing dnPDGFRα are unable to respond to FGF4 as control cells do, consistent with an effect of PDGF on the ability of cells to respond to directional cues. PDGF signaling

through PI3K regulates the expression of N-cadherin posttranscriptionally and thus controls directional migration through the regulation of cell–cell adhesion (Yang et al. 2008). It is not known whether N-cadherin functions to signal directional movement through mechanotransduction as C-cadherin does in *Xenopus* or if it is needed for cells to exert tractive forces on each other to mediate intercellular movement. Unlike *Xenopus* mesendoderm, chicken mesodermal cells ingress individually and do not migrate as a cohesive sheet. However, ultrastructural observations suggest that the cells do have contact with one another (Harrisson et al. 1993) making it plausible that forces acting on cell–cell junctions could guide cell migration even when the migrating cells are not arranged in a sheet.

3.5.2.3 Regulation of Cell–Cell Adhesion by Chemokine Signaling

During Zebrafish gastrulation mesoderm and endoderm migration are coupled. Endodermal cells expressing the chemokine receptor Cxcr4a and mesodermal cells, which express the ligand Cxcl12, migrate together. However, in embryos where expression of either molecule has been reduced by MO injection, endodermal cell migration becomes disconnected from mesoderm resulting in the anterior displacement of endoderm and to viscera bifida, a duplication of endodermal derivates such as liver and pancreas (Nair and Schilling 2008). Interfering with FN–integrin interaction using RGD peptides phenocopies the Cxcr4a morphant phenotype, suggesting that FN–integrin interaction may be involved. Integrin β1b expression and adhesion to FN are reduced in Cxcr4a morphant cells. Viscera bifida is rescued by expression of integrin β1b in Cxcr4a morphant embryos, suggesting that chemokine signaling regulates the expression of integrin β1b which in turn regulates the tethering of endoderm to mesoderm (Nair and Schilling 2008). It is unclear whether integrin–FN interactions act directly to tether endoderm and mesoderm. Integrin α5β1 has been shown to mediate cell cohesion in 3-dimensional cell aggregates in the absence of cadherin (Robinson et al. 2003), and FN has been proposed to mediate contact between migrating avian mesodermal cells (Harrisson et al. 1993). Alternatively, integrin–FN interactions may influence tethering indirectly through the regulation of cadherin activity (Marsden and DeSimone 2003); see below.

In summary, FN plays many roles during mesendoderm migration. It acts as a substrate for migration that allows the mesendoderm to adhere to the BCR. Interaction with the synergy site promotes cell protrusion. The direction of those protrusions is regulated by FN through several mechanisms including the establishment of asymmetric force on cadherins that leads to polarized protrusive activity, the storage and presentation of secreted signals such as PDGF, and, in some amphibians, contact guidance provided by fibril alignment.

3.6 Epiboly

The FN on the BCR is not only involved in mesendoderm cell migration. It also involved in the polarized behaviors of the BCR cells themselves. During the course of gastrulation, the ectoderm spreads to cover the surface of the embryo. This process of epiboly is driven by the coordinate radial intercalation of the inner cells of the BCR and spreading of the outer ectodermal cells. The maintenance of thinning in the animal cap region and marginal zone regions of the BCR requires FN (Boucaut et al. 1985; Johnson et al. 1993; Davidson et al. 2006; Marsden and DeSimone 2001; Rozario and DeSimone 2010). The initial thinning of the animal cap occurs before fibronectin assembly and does not require FN–integrin ligation, but the maintenance of the normal two-layered structure requires the presence of FN fibrils. Inhibiting fibril assembly by blocking FN–FN interactions through the expression of the 70 kDa amino-terminal fragment of FN leads to a thicker roof even though FN–integrin interactions are not perturbed (Marsden and DeSimone 2001; Rozario et al. 2009). Interestingly, mechanically stretching the animal cap is sufficient to promote FN assembly (Dzamba et al. 2009). It is possible that the initial FN-independent thinning of the animal cap promotes the initiation of FN assembly. The assembled FN is then needed to maintain the normal thickness of the roof.

3.6.1 ECM Provides Polarity Cues

When antibodies to the RGD or synergy sites are injected into the blastocoel before gastrulation begins, the blastocoel wall in the marginal zone remains close to five cell layers thick throughout gastrulation, demonstrating that FN is required for radial intercalation in that region (Marsden and DeSimone 2001). These regional specific differences in the role of FN in epiboly are not well understood.

During radial intercalation increasing numbers of cells come in contact with FN, suggesting that FN regulates radial intercalation by boundary capture. However, an interesting aspect of the regulation of radial intercalation by FN is that it can act at a distance. Radial intercalation can be observed experimentally by explanting a group of fluorescently labeled deep cells from the BCR marginal zone and overlaying them with the superficial layer of an unlabeled explant. On a nonadhesive substrate, the labeled patch remains coherent as it integrates into the superficial layer; no mixing of cells occurs. In contrast when plated on FN, the labeled cells intercalate between the unlabeled cells consistent with FN regulating cell–cell adhesion (Marsden and DeSimone 2001). The regulation extends to cells not in direct contact with the FN as mixing is seen even several cell layers above the substrate. This suggests that FN is serving a signaling function rather than acting solely by the boundary capture of cells from the superficial layers.

The direction of cell intercalation suggests that FN on the inner surface of the BCR somehow provides polarity cues to the intercalating cells. Normally cell division occurs in the horizontal plane of the BCR. Disruption of FN matrix randomizes the direction of mitotic spindles confirming that FN fibrils are needed for BCR cell polarity (Marsden and DeSimone 2001; Rozario et al. 2009). Like the maintenance of BCR thinning, mitotic spindle orientation requires fibrillar FN (Rozario et al. 2009).

3.6.2 *Integrin as a Regulator of Radial Intercalation in* Drosophila

Although no roles for ECM in *Drosphila* gastrulation have been identified, the β integrin subunit βPS (myospheroid) is required for radial intercalation during mesoderm migration. Mesoderm migration is a multistep process in the fly. The mesoderm invaginates forming a tube inside the embryo. The cells undergo an epithelial-to-mesenchymal transformation, and the tube collapses onto the ectoderm. The cells begin to migrate along the ectoderm toward the dorsal side of the embryo and then radially intercalate to form a monolayer (McMahon et al. 2008, 2010). Although tube collapse and migration appear to occur normally, the monolayer does not form in βPS mutant or morphant embryos consistent with a role for βPS in regulating radial intercalation. Wild-type mesenchymal cells send protrusions into the underlying ectoderm that are absent in βPS morphant embryos, suggesting that integrin is needed for protrusion formation (McMahon et al. 2010). The known *Drosophila* integrin ligands laminin A, laminin W (Montell and Goodman 1989), and tiggrin (Fogerty et al. 1994) are not expressed this early in embryogenesis, indicating that βPS is either acting independent of ligand or that its ligand has not yet been identified.

The possible roles of unligated integrins in regulating cell behaviors have been largely unexplored; however, they are proposed to play a role in somite morphogenesis (Julich et al. 2009). Radial intercalation plays a role in the extension of mesendoderm in *Xenopus* (Davidson et al. 2002; Keller et al. 2003), but radial intercalation during mesendoderm migration has not been studied extensively in amphibians.

3.7 Convergent Extension

The dorsal mesodermal cells that involute after the cells involved in mesendoderm migration contribute to the elongation of the embryo through the process of convergent extension. Convergent extension involves two types of cell behaviors (Fig. 3.5). Radial intercalation gives rise to a thinner longer tissue during early

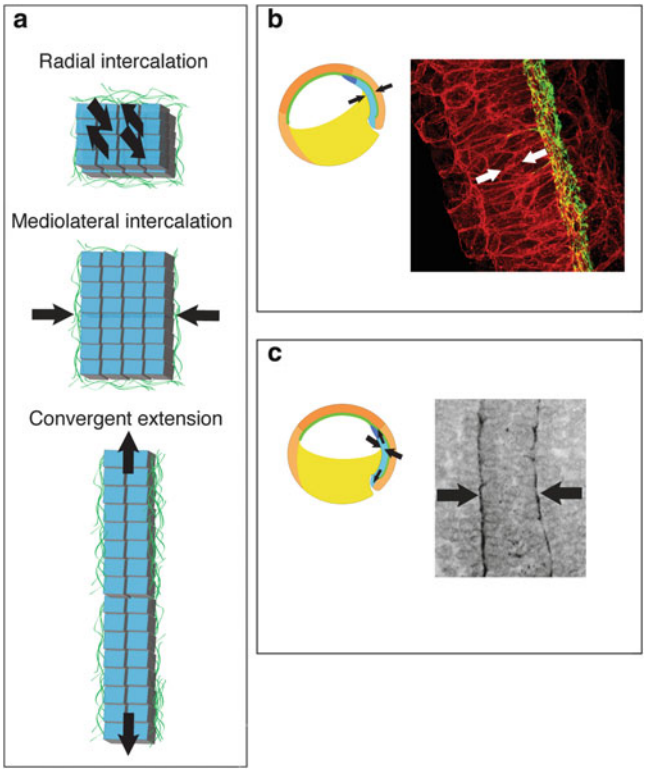

Fig. 3.5 Convergent extension. (**a**) The anterior–posterior elongation of mesoderm through the process of convergent extension involves both radial and mediolateral cell intercalations. (**b**) Confocal *Z*-stack projection of mesoderm undergoing radial intercalation. Immunostaining FN (*green*) is localized to one side of the intercalating mesoderm (*white arrows*). C-cadherin immunostaining (*red*) outlines cell shapes. (**c**) En face view of a mesodermal explant. Immunostaining shows Fbn at the prospective notochord–somite boundary that defines a region of mediolaterally intercalating cells

gastrulation. The cells then move between one another to generate a longer and narrower cellular array (Keller et al. 2003). The cellular movements toward the midline have been termed mediolateral intercalation behavior (MIB). Cells undergoing MIB change their protrusive behavior from multipolar to bipolar and become elongated in the mediolateral direction (Shih and Keller 1992a, b).

Both PCP signaling (Wallingford et al. 2000; Tada and Smith 2000) and integrin α5β1–FN interactions (Davidson et al. 2006; Marsden and DeSimone 2003) are needed for convergent extension. The interactions between PCP and ECM in the regulation of CE are complex (Skoglund and Keller 2010). PCP can act to directly polarize cell behaviors, but can also act indirectly by influencing the assembly of ECM. ECM, in turn, influences cell polarity at least in part through the regulation of PCP signaling.

Both the dorsal and ventral surfaces of the mesoderm undergoing convergent extension are covered with FN (Davidson et al. 2004, 2006). As described above disruption of FN–integrin interaction through the use of antibodies that inhibit the RGD or synergy sites of FN, expression of a dominant-negative β1 construct, or MO-mediated downregulation of FN expression leads to embryos that are shorter and wider than normal, suggesting that convergent extension has been inhibited. Expression of the dominant-negative integrin construct, HAβ1, leads to a loss of FN matrix. Mesodermal cells from embryos expressing HAβ1 do not adopt the normal bipolar phenotype of cells undergoing MIB. Similarly, when DMZ explants from FN morphant embryos are cultured on a nonadhesive BSA substrate, the cells have a smaller length–width ratio than control cells. The elongation of FN morphant cells is rescued when the explants are cultured on a FN substrate, demonstrating that interaction with FN is needed for cell elongation. Further support for the idea that FN regulates cell elongation comes from examining the behavior of cells in Keller sandwiches (Keller et al. 1985). When dorsal marginal zone tissues from two embryos are juxtaposed to one another, the explant converges and extends. When one half of the Keller sandwich is made from an embryo expressing HAβ1, FN is missing from that half of the sandwich. Cells on the side lacking FN are rounded whereas those on the control side extend mediolaterally (Marsden and DeSimone 2003).

There are at least two ways in which FN influences the behavior of cells undergoing convergent extension. Both the cadherin-mediated intercellular movements of cells toward the midline and the regulation of polarized protrusive activity are regulated by FN.

3.7.1 Regulation of Cell–Cell Adhesion by Cell–ECM Adhesion

Precise control of cadherin adhesive activity is necessary for convergent extension (Zhong et al. 1999; Brieher and Gumbiner 1994; Lee and Gumbiner 1995). Aggregates composed of a mixture of cells expressing dnHAβ1 with those expressing a control HA construct sort from one another. Such sorting has been shown to depend on differences in cadherin adhesion strength (Foty and Steinberg 2005; Steinberg and Takeichi 1994). Activation of C-cadherin with an antibody inhibits the HAβ1-mediated sorting, demonstrating that interfering with integrin function is affecting cadherin activity (Marsden and DeSimone 2003) A second cadherin-mediated process, the reintegration of dissociated DMZ cells into intact DMZ explants, is promoted by a fragment of FN containing the RGD and synergy sites. Integrin ligation is not sufficient to promote reintegration when C-cadherin is blocked with an antibody. Conversely, activation of C-cadherin promotes reintegration even when HAβ1 is expressed. Thus, integrin ligation is needed to activate C-cadherin (Marsden and DeSimone 2003).

Another example of integrin ligation influencing cadherin-mediated behavior is during chicken gastrulation when epithelial cells in the epiblast undergo an

epithelial-to-mesenchymal transition (EMT) and "ingress" to form mesoderm. Although the loss of cell–cell adhesion has traditionally been considered a first step in EMT, in this case basement membrane breakdown is seen before loss of cell–cell adhesion. During normal gastrulation laminin and its receptor, integrin α6β1, are removed from the 5–10 cells closest to the midline of the epiblast where ingression occurs. Active RhoA is localized to both the apical and basal surfaces of lateral epiblast cells but is missing basally in the medial cells. Misexpression of RhoA leads to the retention of both laminin and α6β1 at the epiblast midline and inhibits mesoderm ingression. Inhibition of RhoA by C3 exoenzyme, morpholino, or a dominant-negative construct leads to premature basement membrane breakdown. These results suggest a model for the regulation of ingression in which active RhoA is needed for the maintenance of the basement membrane. In the medial cells that undergo EMT, the RhoA GEF, Net1, is downregulated leading to a decrease in RhoA activity and the subsequent loss of basement membrane followed by the loss of adherens junctions (Nakaya et al. 2008). The observation that basement membrane disassembly precedes changes in cell–cell adhesion suggests the possibility that integrin–cadherin cross talk is involved.

3.7.2 FN Regulates Cell Protrusive Activity

In addition to regulating cadherin activity, FN contributes to CE through the regulation of cell protrusive activity. Mesodermal cells in FN morphant *Xenopus* embryos and DMZ explants have more actin-rich protrusions than cells from uninjected embryos. Single cells derived from FN morphant embryos are also more protrusive than control cells. This elevated protrusive activity is attenuated when cells are plated on a FN substrate. Like loss of FN, acute disruption of FN–integrin interaction by treatment with the inhibitory α5β1 antibody P8D4 leads to enhanced protrusive activity, which is rescued by exogenously activating integrin with manganese (Davidson et al. 2006). Thus, FN–integrin ligation can serve to attenuate protrusive activity, a function likely to contribute to the regulation of polarized protrusive activity during MIB. Interestingly, protrusive activity during mesendoderm migration requires interaction with the FN synergy site (Davidson et al. 2002), whereas blocking integrin ligation in converging and extending cells increases protrusive activity (Davidson et al. 2006). Further study of the pathways downstream of integrin ligation in these two cell populations is needed to understand how FN affects them differently.

During mesendoderm migration, as discussed above, the establishment of monopolar protrusive activity is regulated through cadherin (Weber et al. 2012). During convergent extension the orientation of bipolar protrusions and cadherin activity are both dependent on FN. FN is found only under the migrating mesendodermal cells but surrounds the cells undergoing convergence extension; perhaps the difference in the polarity of FN localization is reflected in the different polarities of behavior.

3.7.3 Relationship Between PCP and ECM During CE

The Wnt/PCP signaling pathway is needed for the polarized cell behaviors that drive CE (Wallingford et al. 2000; Tada and Smith 2000; Djiane et al. 2000). One indication of PCP signaling is the recruitment of Dsh to cell membranes (Wallingford et al. 2000; Axelrod et al. 1998; Rothbacher et al. 2000). The interdependence of ECM and PCP signaling is illustrated by the observation that recruitment of Dsh to the membranes of cells is dependent on FN–integrin ligation (Marsden and DeSimone 2001) and also on FN interaction with syndecan-4 (Munoz et al. 2006). Both overexpression and MO-mediated knockdown of syndecan-4 lead to CE defects. Reduction of frizzled-7, a component of the PCP pathway, enhances the phenotype produced by syndecan-4 MO, and syndecan-4 morphant phenotypes can be rescued by a Dsh construct that supports PCP signaling but not canonical Wnt signaling. Syndecan-4 interacts biochemically with both frizzled-7 and Dsh, and these interactions are enhanced by FN. The FN-mediated translocation of Dsh is inhibited by reduction of syndecan-4 expression. Interestingly, although a FN fragment that lacks the heparin-binding domain supports Dsh membrane localization, a substrate comprised of the HepII region of FN that mediates cell adhesion through syndecans does not (Marsden and DeSimone 2001), suggesting that inputs from both syndecan-4 and integrin are needed. Further study is needed to understand how these two adhesion systems interact to influence cell behavior.

Inhibition of Wnt11 not only disrupts CE but also leads to abnormal FN assembly (Dzamba et al. 2009). Small GTPases such as Rac and Rho are downstream effectors of PCP signaling (Habas et al. 2001, 2003). Expression of constitutively active Rho is able to rescue fibril assembly and CE in the presence of dnWnt11 (Dzamba et al. 2009). Convergent extension and FN organization are also disrupted by overexpression or inhibition of the PCP pathway proteins frizzled-7, strabismus, and prickle. Normally FN fibril deposition occurs on tissue surfaces and boundaries (Davidson et al. 2004), but when PCP signaling is disrupted, FN is no longer restricted to surfaces and is seen throughout the mesodermal tissue (Goto et al. 2005). Cells in explants overexpressing strabismus, frizzled-7, or prickle and plated on BSA do not elongate in the mediolateral direction to the same extent as control cells do. The elongation of prickle but not strabismus or frizzled-7 overexpressing cells is rescued by plating on a FN substrate (Goto et al. 2005). This suggests that PCP signaling plays at least two roles in the regulation of MIB. First, PCP regulates the polarized assembly of FN; then it has a second role in regulating cell elongation. Prickle, strabismus, and frizzled-7 all are required for the deposition of FN, but prickle is not necessary for the elongation of cells if a FN substrate is provided. Interestingly, in the tunicate Ciona, prickle (aimless) is required during notochord formation for the maintenance of the polarized distribution of laminin. In this case prickle also regulates the elongation of the cells. Laminin (chongmague) is not needed for the initiation of cell intercalation in Ciona but is required for it is the maintenance of polarized cell shapes (Veeman et al. 2008).

3.7.4 Convergent Extension Is Used in Primitive Streak Formation

Evidence is accumulating that in avian embryos convergent extension is used prior to start of gastrulation in the process of primitive streak formation. This illustrates the ability of the same morphogenetic mechanisms to be used at different times and tissues in various species. The "discovery" of the role of convergent extension also illustrates the opportunities that live imaging provides to illuminate the mechanisms behind long-standing questions in gastrulation. The formation of the primitive streak in avian embryos is accompanied by striking bilateral circular movements of the epiblast cells known as "polonaise movements" (Graeper 1929). Three models have been proposed to account for these movements. The first, oriented cell division (Wei and Mikawa 2000), has been discounted because cells do not divide frequently enough to account for the movements (Voiculescu et al. 2007). Distinguishing between the second two models, chemotactically driven cell migration (Chuai et al. 2006) and mediolateral intercalation (Voiculescu et al. 2007; Lawson and Schoenwolf 2001a, b), has been more difficult. If convergent extension were mediating elongation of the primitive streak, one would predict the involvement of PCP signaling by analogy to frog and fish (Tada et al. 2002; Skoglund and Keller 2010). One group (Chuai et al. 2006) found that perturbation of FGF but not PCP signaling inhibited primitive streak formation. These data support the migration model. On the other hand, Voiculescu et al. (2007) found that PCP signaling is needed for primitive streak formation, and these investigators proposed that the role of FGF signaling is to direct the expression of PCP pathway molecules to the region of the forming primitive streak. The relationship between the movements that lead to primitive streak formation and the ECM that underlies the epiblast has been unclear. When a FN antibody or concanavalin A is used to label the lateral edge of the epiblast ECM, the label is later found at the edge of the primitive streak, suggesting that the matrix moves medially (Sanders 1984). Conversely, autoradiography of radiolabeled transplanted tissue suggested that cells move relative to a stationary ECM (Bortier et al. 2001). Recently, simultaneous time lapse imaging of ECM and the epiblast cells has confirmed that cells and ECM move coordinately during primitive streak formation (Zamir et al. 2008). The coordinate movement of ECM and cells is more compatible with a mechanism of convergent extension driven by intercellular movements than with chemotactic migration across the matrix. Live simultaneous imaging of *Xenopus* gastrula cells and FN demonstrates that FN moves with cells during frog gastrulation as well (Davidson et al. 2008). Understanding the relationship between cell, tissue, and ECM movements will greatly enhance our understanding of gastrulation and other morphogenetic events (Czirok et al. 2013).

3.7.5 *Fibrillin*

Xenopus Fbn, which is most closely related to Fbn-2, is first detected at the prospective notochord–somite border at mid-gastrulation (Skoglund et al. 2006). The assembly of Fbn is dependent on FN both in cultured cells (Sabatier et al. 2009) and in *Xenopus* (Zhou et al. 2009) raising the question of whether some of the effects of FN on convergent extension may be attributable to loss of Fbn. When bipolar cells undergoing intercalation contact the notochord–somite border, protrusions cease at the border, and the cells become monopolar protrusive toward the midline of the embryo (Shih and Keller 1992a). As the formation of the notochord–somite boundary progresses in an anterior–posterior direction, "boundary capture" leads to the zipping up of the mesodermal tissue as cells pull their neighbors toward the border. Fibrillin is the first ECM protein detected specifically at the border and is thus an excellent candidate for mediating boundary capture. Disruption of Fbn at the border using a dominant-negative Fbn construct, MO to Fbn, or a peptide that inhibits Fbn assembly leads to defects in CE (Skoglund and Keller 2007).

Although it is not clear specifically how Fbn contributes to convergent extension, there are several possibilities. Cells transition from bipolar to monopolar protrusive activity when they contact the Fbn containing border. Fbns contain RGD sequences that have been shown to interact with αvβ3 and α5β1 integrins, as well as heparin-binding domains that mediate cell adhesion through an unidentified receptor (Pfaff et al. 1996; Bax et al. 2003, 2007). Perhaps integrin–Fbn, like integrin–FN interaction, regulates protrusion. Similarly, it may act to link cell protrusive activity to traction force. The assembly of ECM might contribute to the stiffening of the dorsal axial tissue that occurs during convergence extension (Moore et al. 1995). A recent study has suggested that the state of the cytoskeleton in the cell cortex may contribute more to tissue rigidity than ECM. However, in those experiments ECM was reduced, but not absent (Zhou et al. 2009), leaving open the possibility that the remaining ECM was sufficient to contribute to tissue stiffness, either directly through the mechanical properties of fibrils or indirectly through modulation of the cytoskeleton by integrin ligation. The observation that FN fibrils are not needed for convergence extension (Rozario et al. 2009) is consistent with the second model.

Fibrillins are also known to modulate TGFβ and BMP signaling (Ramirez and Rifkin 2009). It is not known if this activity of Fbn is involved in regulating convergence extension in *Xenopus*. The regulation of BMP signaling is important for convergence extension in zebrafish, where a gradient of BMP signaling has been shown to modulate cell–cell adhesion. BMP is expressed on the ventral side of the embryo and negatively regulates the stability of cell–cell contacts such that cell movements increase in the dorsal regions of the embryo where BMP signaling is lower and cell–cell adhesion is able to drive intercellular migration (von der Hardt et al. 2007). It is attractive to speculate that Fbn might be involved in setting up adhesive gradients through the regulation of BMP signaling.

3.8 Conclusions

The Merriam-Webster.com definition of matrix "Something within or from which something else originates, develops, or takes form" aptly describes the role of ECM during gastrulation. ECM defines regions within which the movements of morphogenesis occur. In *Xenopus* FN is found under the migrating mesendoderm cells and radially intercalating cells of the BCR. ECM delineates the region that undergoes mediolateral intercalation leading to convergent extension. The development of the polarized movements that drive mesendoderm migration, epiboly, and convergent extension are dependent on interaction with ECM. These movements in turn give rise to embryo form.

The traditional view of ECM as a substrate for cell adhesion and migration needs to be expanded to include its roles in regulating cell polarity, modulating growth factor signaling, and contributing to the mechanical properties of tissues. ECM not only mediates cell–substrate adhesion but also influences cell–cell adhesion. ECM has often been thought of as a static structure, but it is becoming increasingly clear that it is very dynamic. Its composition and physical structure is altered by the tissues it interacts with. These changes, in turn, modulate how ECM affects cell behavior. Future studies focusing on the movements of ECM during morphogenesis are likely to change our understanding of how ECM influences cell behaviors. Recent studies have demonstrated the importance of mechanical signals in influencing cell phenotypes. The role of ECM in mechanotransduction is an important area for future research as ECM both contributes to the mechanical properties of tissues and its own structure is influenced by mechanical forces exerted by those tissues.

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Part II
Extracellular Matrix-Direct Morphogenesis, Growth Factor Signaling, and Maintenance of the Stem Cell Niche

Chapter 4
Cell–ECM Interactions and the Regulation of Epithelial Branching Morphogenesis

William P. Daley and Kenneth M. Yamada

Abstract Branching morphogenesis is a crucial mechanism in embryonic development for establishing the architecture of many mammalian epithelial organs, including the salivary gland, lung, kidney, and mammary gland. It substantially expands the epithelial surface area available for secretion or absorption within many organs. Studies in three-dimensional ex vivo organ culture, including early tissue recombination studies, have revealed that cell interactions with the interstitial extracellular matrix (ECM) and the basement membrane associated with epithelial cells play a critical role in the branching process. Here, we review the dynamic mechanisms by which cell–ECM interactions regulate the morphogenesis and organization of branched epithelial tissue structures. We highlight several general biological principles by which cell interactions with the ECM and basement membrane regulate epithelial branching morphogenesis; these include ECM-mediated or -induced alterations in tissue shape and stimulation of dynamic cell motility, proliferative outgrowth and expansion of the epithelium regulated by growth factors and proteolytic degradation of the ECM, and basement membrane roles in coordinating organization of epithelial tissue architecture. Because an in-depth understanding of cell interactions with the ECM will be important for developing novel approaches to disease therapy, we conclude with a discussion of the implications of these findings for the rational design of bioengineered scaffolds in regenerative medicine strategies, as well as their potential relevance to epithelial cancer progression.

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4.1 Introduction

How complex three-dimensional (3D) structures such as tissues and organs are formed from their precursor epithelial cells is one of the most fundamental questions in developmental biology. Organogenesis requires several distinct but overlapping processes. These include morphogenesis, the physical rearrangement of cells into complex 3D structures, and cytodifferentiation, the process by which cells acquire their own specialized functions. Polarization, or the coordinated orientation of cells within a tissue, also occurs concurrently during organ development and is a central aspect of tissue architecture.

Branching morphogenesis is a conserved developmental mechanism required for the formation of many vertebrate and invertebrate organs, ranging from the *Drosophila* trachea and air sacs, to mammalian lungs, kidneys, salivary glands, and mammary glands. During this process, a primary epithelial bud or tube undergoes dynamic, yet coordinated, cellular rearrangements that give rise to a complex network of branched structures that greatly increase the epithelial surface area for secretion or absorption. While these organs share common structural features in that each consists of a series of highly organized tubules that terminate in a hollow sphere, referred to as an acinus, alveolus, or end bud depending on the tissue type, the specific structure and branching pattern of each of these tissue structures varies significantly (Andrew and Ewald 2010; Lu et al. 2006; Lu and Werb 2008).

Numerous studies over the last several decades have revealed time and again that reciprocal interactions between a cell and its surrounding extracellular matrix (ECM) play a critical role in the acquisition of organ-specific form and function. Known as dynamic reciprocity, such a concept has redefined the traditional view of the ECM as a simple scaffold to support tissue structure; instead, we now view the ECM as both an active player in the acquisition of tissue specificity and as a key determinant of tissue architecture itself (Bornstein et al. 1982; Bissell et al. 1982, 2005).

The ECM is a complex network of macromolecules that is produced by both the connective tissue surrounding an organ and its constituent epithelial cells. Once thought to provide primarily structural support to developing tissues, it is now known that the ECM is extremely dynamic and regulates diverse cellular processes, including cell growth, cell shape change, differentiation, migration, and survival (Daley et al. 2008; Hynes and Yamada 2012; Larsen et al. 2006a; Mecham 2011). The ECM is composed of a fibrous mesh of proteins, including proteins such as collagens, laminins, and fibronectin, proteoglycans, stored growth factors, and the enzymes regulating proteolysis of these components. Cells sense and respond to stimuli in the ECM through cell surface integrins and other receptors, and then with their associated adhesion complexes, which mediate bidirectional signaling between the ECM and cytoplasm (DeMali et al. 2003; Geiger et al. 2001; Harunaga and Yamada 2011). Integrins bind directly to ECM proteins through their extracellular domains and indirectly to the cytoskeleton via adaptor proteins bound to their cytoplasmic tails. The ECM is in a state of constant flux during development,

wound repair, and in disease states, as cells constantly rebuild and reorganize the ECM by degrading and reassembling it (Lu et al. 2011).

The basement membrane is a specialized form of ECM underlying epithelial sheets at their basal surface; it provides structural support, separates epithelial cells from the surrounding stromal compartment, and regulates cell behavior. The basement membrane is composed of a large number of glycoproteins, such as laminins, collagen IV, nidogen, and the heparan sulfate proteoglycan perlecan, which are secreted by epithelial cells and form a scaffold for cell adhesion. The basement membrane is a potent driving force during epithelial morphogenesis and is required not only for the maintenance of tissue structure and organization but is also a key mediator of changes in tissue shape (Yurchenco 2011; Yurchenco et al. 2004).

The goal of this chapter is to review the central role of cell interactions with the ECM and basement membrane in the regulation of epithelial branching morphogenesis. While the *Drosophila* trachea and salivary gland have provided valuable insights into this developmental process, as has endothelial cell tube formation during angiogenesis, these have been reviewed recently elsewhere (Affolter and Caussinus 2008; Herbert and Stainier 2011; Maruyama and Andrew 2012 ; Pirraglia and Myat 2010) and will not be covered here. Instead, we focus mainly on mammalian tissues, namely, the mouse salivary gland, lung, kidney, and mammary gland. We begin with a discussion of some of the fundamental mechanisms of branching morphogenesis with a review of the cell types and cellular behaviors involved. This is followed by a description of the 3D organotypic culture models that have facilitated our understanding of this process. We will then describe some of the classic studies that demonstrated the instructive role of the stromal mesenchyme during epithelial organogenesis, and how these led investigators to identify the ECM as a critical regulator of cell dynamics. Finally, we review our current understanding of several general mechanisms by which cellular interactions with the ECM influence epithelial development and conclude with a brief discussion of the implications of these mechanisms for tissue engineering and our understanding of epithelial cancers.

4.2 Branching Morphogenesis as a Fundamental Developmental Mechanism

A highly organized cell type known as the epithelial cell is the core structural component of most branched epithelial organs. Mature epithelial tissues are characterized by strong cell–cell contacts and adhesion to their neighbors, coordinated cell orientation along the apicobasal axis, and adhesion to a basement membrane at their basal surface (Fig. 4.1a). In the adult differentiated organ, such cells are organized around a centrally located hollow lumen, thus giving rise to a fully polarized epithelial tissue that functions to transport fluids or gases unidirectionally. Epithelial cells are only one of the constituent cell types of an organ; they are also surrounded by diverse supporting cell types, including fibroblasts or mesenchymal cells, neurons, and

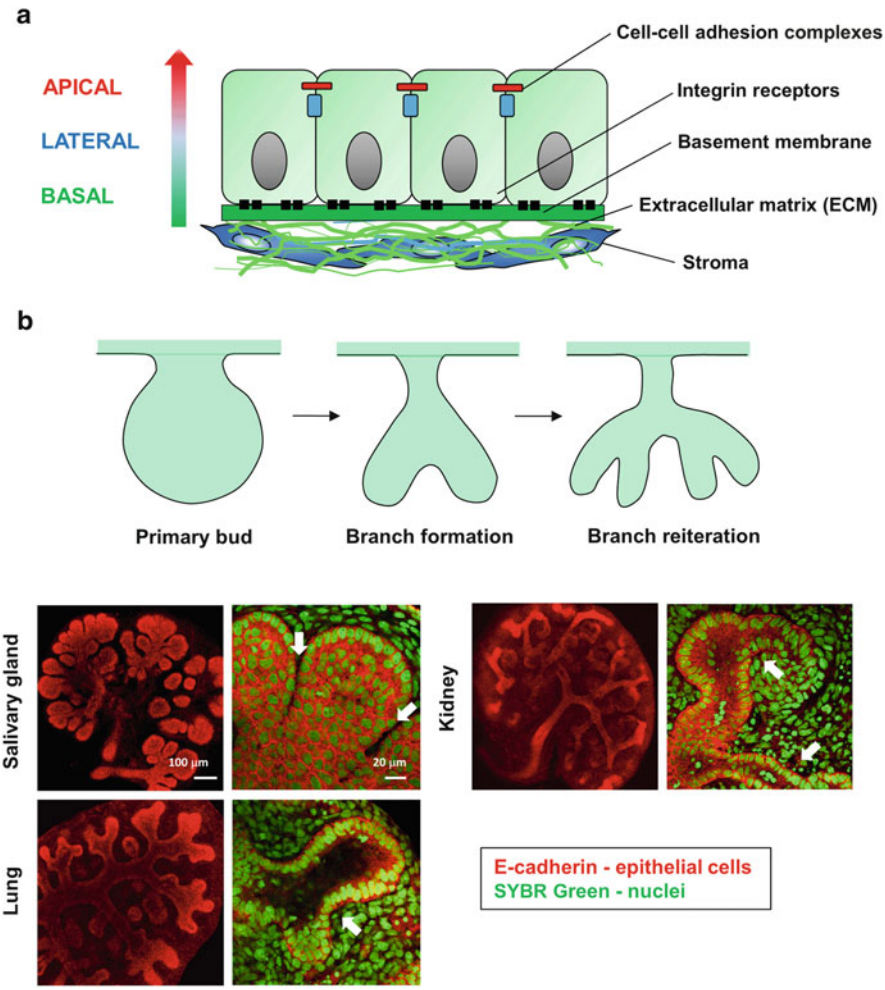

Fig. 4.1 Generalized schematic of epithelial branching morphogenesis. (**a**) Epithelial cells in an epithelial sheet are highly organized along the apicobasal axis. They are characterized by adhesion to their neighbors through junctional complexes in the lateral membrane and adhesion to a basement membrane through integrin receptors at their basal surface. Epithelial cells are separated from the stromal mesenchyme by the basement membrane. The stroma is complex, being composed of fibroblasts and a fibrous extracellular matrix, as well as parasympathetic nerves and blood vessels. (**b**) During branching morphogenesis, a primary epithelial bud or tube is dynamically remodeled to give rise to a complex network of branched structures. Branch formation initiates in the surface of a primary bud and is followed by branch outgrowth to give rise to new epithelial buds. This process is repeated in a reiterative manner to increase epithelial surface area for secretion or absorption. The mode of branch formation can differ between tissues, as illustrated by cleft formation in the salivary gland and lung and bifurcation in the kidney. Staining for E-cadherin delineates the epithelium (*red*) while SYBR Green marks the nuclei (*green*). Scale bars, 100 µm and 20 µm (**b**)

endothelial cells, as well as the ECM that they produce (Fig. 4.1a). Collectively, these are referred to as the stroma. In contrast to epithelial cells, the mesenchyme consists of mostly single cells spread throughout the ECM, with integrin-mediated cell–matrix adhesions predominating over cadherin-based cell–cell adhesions. Dynamic and reciprocal interactions between the epithelium, stromal mesenchyme, other cell types, and ECM are a major driving force during epithelial morphogenesis.

Development of epithelial organs via branching morphogenesis requires the reiterative formation of branches from the surface of a primary epithelial bud and subsequent branch outgrowth or extension (Fig. 4.1b). Multiple modes of branch formation have been described, including bifurcation, side branching, and cleft formation. In the mammary gland and kidney, tubular epithelial structures split in two through a bifurcated elongation with further outgrowth in both directions; new branches can also initiate at sites distant from the distal epithelium, a process known as side branching. In the salivary gland and lung, cleft formation occurs when cells exchange cell–cell contacts for cell–matrix contacts, a process which subdivides an epithelial bud into two new buds. Regardless of the mode of branch formation that occurs, this process is repeated in a reiterative manner to give rise to a highly branched epithelial structure that maximizes epithelial surface area within a minimal packed volume (Andrew and Ewald 2010; Gjorevski and Nelson 2010a).

For each branch that forms during branching morphogenesis, some form of signaling must occur to determine where the branch forms and the direction it extends, the size and particular shape of that branch, as well as where along the existing branch the next branch will initiate. To effectively build a branched epithelial structure, then, such signals must regulate the coordination of a number of basic cellular processes, including cell shape change and rearrangement (Ewald et al. 2008; Villasenor et al. 2010), proliferation (Michael and Davies 2004; Mollard and Dziadek 1998), cell–cell adhesion (Davis and Reynolds 2006; Menko et al. 2002; Walker et al. 2008), cell migration (Ewald et al. 2008; Kadoya and Yamashina 2010; Larsen et al. 2006b), and basement membrane remodeling (Bernfield and Banerjee 1982; Daley et al. 2009; Larsen et al. 2006b). While tremendous progress has been made in the identification of conserved signaling pathways and secreted factors controlling these processes, there are still many unanswered questions concerning how such diverse cellular events are integrated at a systems level (Larsen et al. 2010; Melnick et al. 2009; Monte et al. 2007; Wang et al. 2011).

4.3 3D Cell and Organ Culture Systems for the Study of Branching Morphogenesis

Over the past couple of decades, much of what has been learned concerning the regulation of complex biological systems has come from studies in cell culture. Traditionally, such studies have involved the plating of both primary and

transformed cell lines on artificial, two-dimensional (2D) plastic substrates. While 2D tissue culture has resulted in numerous contributions to our understanding of epithelial cell behavior, many recent studies have demonstrated that traditional 2D cell culture models do not accurately portray cell behavior in vivo (Cukierman et al. 2002; Larsen et al. 2006a; Nelson and Bissell 2006; O'Brien et al. 2002). Cells in vivo are rarely forced to adapt to the flat and rigid surfaces characteristic of 2D cell culture but are instead surrounded by a complex 3D microenvironment consisting of ECM and other neighboring cell types. Because cells seem to signal differently through cell surface integrin receptors when cultured in a 3D matrix than when grown in 2D culture (Cukierman et al. 2001; Doyle et al. 2009), a current trend in cell biology is the development of more physiologically relevant 3D cell culture systems (Griffith and Swartz 2006; Yamada and Cukierman 2007). Such models will enable the prediction of cell behavior in a 3D environment and are of the utmost importance in the field of tissue engineering, which seeks to create artificial tissues by seeding cells on 3D scaffolds that need to mimic the in vivo environment of the ECM.

One common approach that bridges the gap between in vitro studies and the 3D in vivo environment is ex vivo organ culture, which has been used extensively to elucidate the mechanisms regulating branching morphogenesis, especially the role of the ECM in this process. Ex vivo culture protocols have been successfully applied to study the development of embryonic salivary gland (Sakai and Onodera 2008), lung (Carraro et al. 2010; Warburton et al. 2010), kidney (Barak and Boyle 2011), and mammary gland (Debnath and Brugge 2005; Ewald et al. 2008; Lee et al. 2007). Such organs can be readily removed from the embryo, and when placed intact on a filter floating on serum-free media, continue to branch for several days in a manner that closely approximates in vivo development (Sakai and Onodera 2008). Under these conditions, embryonic epithelium dissociated from the surrounding mesenchyme cannot survive alone and must be either recombined with stromal cells or embedded in an ECM gel such as Matrigel and supplemented with mesenchymal growth factors. Ex vivo organ explants are especially suited for studying developmental processes such as branching morphogenesis, because many of the tools of traditional 2D cell culture can be readily applied in organ culture as well; various reagents can be added directly to the culture media, and recent studies have utilized small molecule inhibitors (Daley et al. 2009; Fisher et al. 2001; Larsen et al. 2003), inhibitory antibodies (Kadoya et al. 1995; Walker et al. 2008), antisense oligonucleotides (Hoffman et al. 2002; Shum et al. 1993), viral vectors (Hsu et al. 2012 ; Larsen et al. 2006b), and small inhibitory RNAs (siRNAs) (Rebustini et al. 2007; Sakai et al. 2003) to elucidate the signaling pathways that regulate branching morphogenesis. Furthermore, recent advances in time-lapse microscopy (Ewald et al. 2008; Larsen et al. 2006b; Watanabe and Costantini 2004), combined with the advent of more sophisticated transgenic model animals, have allowed the visualization of cell behaviors during epithelial branching morphogenesis and have shed light on the extremely dynamic nature of this process.

4.4 Epithelial and Mesenchymal Inductive Interactions During Branching Morphogenesis

The importance of cell–ECM interactions during epithelial branching morphogenesis was first hinted at by studies by Borghese and colleagues, who were the first to highlight the instructive role of the stroma in the shaping of epithelial tissues (Borghese 1950a, b). A number of early studies utilized tissue recombination techniques, in which the mesenchyme from one epithelial organ is recombined with the epithelium from another, to demonstrate that the stroma plays a critical role in the determination of epithelial form and function. When mouse pituitary (Kusakabe et al. 1985) and mammary gland (Kratochwil 1969; Sakakura et al. 1976) epithelium were recombined with salivary gland mesenchyme, the epithelium of both of these organs branched in a salivary gland-like manner. In some cases, the inductive effect of the mesenchyme was also shown to affect epithelial cytodifferentiation, since when pituitary epithelium was recombined with salivary gland mesenchyme, it also expressed the salivary gland product α-amylase (Kusakabe et al. 1985). This is not always the case, however, since mammary epithelium, while exhibiting a salivary gland morphology, still synthesized milk proteins following recombination with salivary mesenchyme (Sakakura et al. 1976). It is important to note that the age of the tissue used in such studies was absolutely critical to achieve an inductive response; that is, the mesenchyme loses its ability to induce, and the epithelium its ability to respond, at later stages of development.

While the early investigations described above clearly indicated a critical role for the mesenchyme in the regulation of epithelial branching morphogenesis, they did not address the mechanism by which this occurs. Grobstein originally postulated that epithelial induction by the mesenchyme must be due to a transmissible, diffusible substance (such as an ECM molecule), since the inclusion of a filter between spinal cord cultures (the inducer) and metanephric kidney tubules (the inducee) did not prevent robust branching of the epithelium (Grobstein 1953a, b). This view was later challenged when Saxen and colleagues calculated the diffusion constants of a variety of morphogenetic substances through filters of differing pore sizes and concluded that diffusion alone could hardly account for the prolonged induction time (~72 h) observed in these experiments (Nordling et al. 1971). Subsequently, the presence of small cytoplasmic processes extending from spinal cord mesenchyme through transfilter membranes was documented (Wartiovaara et al. 1974), and when filter pore sizes were reduced so as to prevent the penetration of such processes, tubule induction by both spinal cord cultures and salivary mesenchyme was effectively prevented (Saxen et al. 1976). Such studies have suggested a model in which intercellular contacts, and not diffusible cues, are the chief inductive mechanism by which mesenchymal cells regulate epithelial morphogenesis. While this has never been conclusively demonstrated in vivo, we now know that diffusible substances such as growth factors and other secreted signaling molecules do play an important role in the patterning of branching morphogenesis (see below). Nevertheless, several electron microscopic studies have observed transient contacts between epithelium

and mesenchyme in the salivary gland (Cutler 1977; Cutler and Chaudhry 1973b), lung (Bluemink et al. 1976), kidney (Lehtonen 1975), and tooth bud (Slavkin and Bringas 1976) during the initial stages of branching. In fact, it was demonstrated through salivary recombination experiments that if the epithelium and mesenchyme were separated prior to the stage when these contacts occur and were then recombined after, even though robust branching morphogenesis could still occur, epithelial differentiation into secretory and myoepithelial cells could not (Cutler 1980; Cutler and Chaudhry 1973a). Thus, it is possible that while such direct epithelial-mesenchymal intercellular contacts may not be required for morphogenesis per se, they may instead act as potent inducers of differentiation. Intriguingly, electron micrographs have shown what appears to be fibrillar material similar in appearance to basement membrane at the tips of these contacts (Cutler and Chaudhry 1973b). Taken together with the observation that such contacts appear to protrude through small holes in the basement membrane (Cutler and Chaudhry 1973b), these findings raise the interesting possibility that basement membrane components may be directly involved in epithelial induction.

4.5 ECM Regulation of Epithelial Branching Morphogenesis

The tissue recombination and transfilter studies described above led other researchers to investigate specific stromally derived signaling molecules required for epithelial branching morphogenesis. From such studies, it has quickly become apparent that many ECM components actively regulate or modulate this process; these include collagens (Fukuda et al. 1988; Hayakawa et al. 1992; Nakanishi et al. 1988; Rebustini et al. 2009), laminins (Kadoya et al. 1998, 2003; Rebustini et al. 2007; Yang et al. 2011), fibronectin (De Langhe et al. 2005; Onodera et al. 2010; Sakai et al. 2003), and basement membrane-associated heparan sulfate proteoglycans (Garner et al. 2011; Patel et al. 2007; Shah et al. 2011; Thompson et al. 2010), as well as the cell surface receptors to which such ECM proteins bind (Pozzi and Zent 2011).

Both the ECM and basement membrane are in a state of constant flux during development, as cells reorganize the ECM by degrading and reassembling it (Daley et al. 2008; Lu et al. 2011). The ECM can be remodeled in response to signals transmitted by transmembrane integrin receptors, or by matrix-modifying enzymes such as matrix metalloproteinases (MMPs). MMPs release growth factors and bioactive peptides stored within the matrix, which in turn stimulate a variety of signaling pathways to regulate cell proliferation, survival, and migration. ECM remodeling can also be induced by changes in cytoskeletal tension, which is transmitted by mechanotransducing integrin receptors to effect changes in the microenvironment; conversely, ECM remodeling itself can alter extracellular stiffness, which can in turn be converted into chemical signals that regulate cell behavior.

During branching morphogenesis, the developing epithelium undergoes dynamic changes in tissue shape. Branch formation is followed by proliferative outgrowth of the epithelium to expand bud size for reiterative rounds of branch

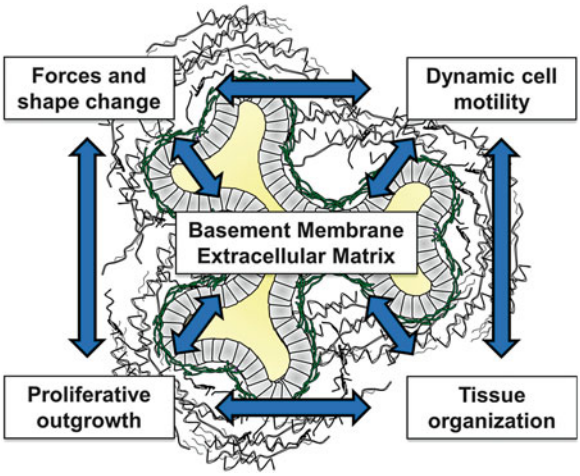

Fig. 4.2 General biological principles by which cell–ECM interactions coordinately regulate branching morphogenesis. Schematic diagram illustrating the dynamic interactions that occur between cells and the ECM/basement membrane during branching morphogenesis. Each of these general principles is discussed in Sect. 4.5 of the chapter text

formation. Remodeling of the basement membrane and ECM plays a critical role in both of these processes and is therefore a key mediator of changes in tissue shape. Cytodifferentiation and tissue polarization are also critical events during epithelial development, and the ECM is intimately involved in the regulation of these processes as well. In the following sections, we review our current understanding of several general biological principles by which dynamic cell–ECM interactions coordinately regulate epithelial branching morphogenesis. These principles are summarized schematically in Fig. 4.2.

4.5.1 Branching Morphogenesis as a Mechanical Process

Morphogenesis is first and foremost a mechanical process. This is due to the fact that a change in the shape of any object requires that forces be applied from within or against the 3D mass that defines that object. Such forces may originate from outside the object and be directed inward, or alternatively, they could be generated from within the object itself and be directed in either an outward or inward direction. In the case of morphogenesis, this solid mass is composed of a closely associated group of cells, and such mechanical forces allow these cells to move great distances and be dynamically rearranged, eventually giving rise to the tissue architecture that is so crucial for tissue function.

Several broad concepts have been proposed to explain branch formation, each of which corresponds to a specific type of force or dynamic internal rearrangement acting on the epithelial surface (Fig. 4.3a). Localized proliferation and directional cell migration,

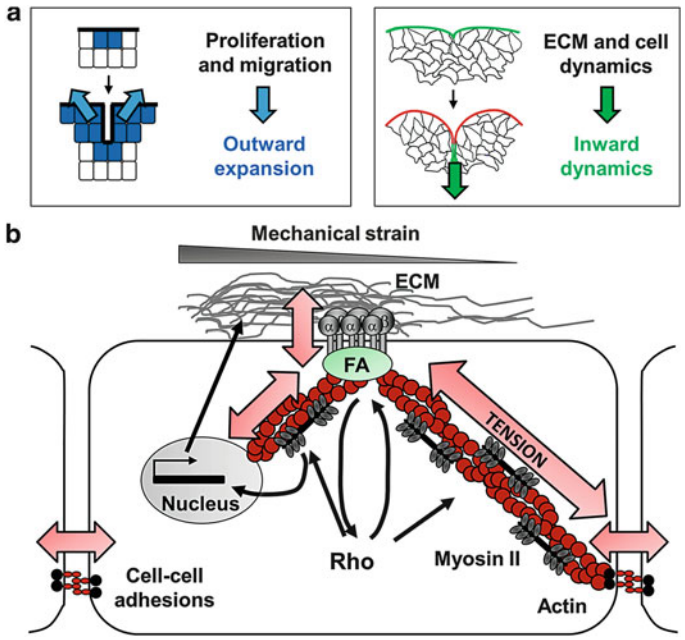

Fig. 4.3 Branching morphogenesis as a mechanical process. (**a**) Several cellular processes have been proposed to contribute to branch formation, each of which involves local tissue dynamics and mechanical forces. Localized proliferation and cell migration occur during branch formation in some epithelial tissues, and if the cells in *blue* were to begin proliferating or migrating while the cells around then do not, this could result in branch formation due to outward-directed epithelial expansion forces (*blue arrows*); an excellent example of this process occurs during the formation of capillary sprouts in angiogenesis (Carmeliet et al. 2009). Localized basement membrane remodeling, in combination with local cell dynamics at nascent branch points, can also facilitate branch formation and inward-directed progression. Newer ECM proteins (*red*), assembled behind older matrix proteins (*green*), might serve as a wedge to help separate dynamically motile epithelial cells (*green arrow*), thereby promoting inward branch progression. The actin cytoskeleton is also required for branch formation and most likely contributes to both of these processes. (**b**) Cytoskeletal forces in mechanotransduction. Cell–cell and cell–matrix adhesions are linked to the actin cytoskeleton through scaffolding complexes at their cytoplasmic domains. Tension generated through the cytoskeleton by actomyosin contraction pulls against these adhesion receptors and is thus transmitted to adjacent cells or the ECM. Such forces can alter the structural and signaling dynamics of adhesion complexes and often result in their strengthening or maturation. Through effects on focal adhesions (FAs), actomyosin-mediated forces can remodel the ECM, which in turn can feed forward to promote further cytoskeletal contraction due to localized matrix stiffening. Mechanical forces can be converted to chemical signals that alter gene expression in the nucleus and promote cell cycle progression as well as epithelial to mesenchymal transition (EMT)

both of which produce outward-directed epithelial expansion forces, have long been known to contribute to branching morphogenesis (Bernfield et al. 1972; Ewald et al. 2008; Larsen et al. 2006b; Michael and Davies 2004; Nogawa et al. 1998). Basement membrane remodeling is also required for branch formation in a number of epithelial tissues (Bernfield et al. 1984; Moore et al. 2005; Sakai et al. 2003), and localized

accumulation of basement membrane components might serve as an inward-directed wedge to stabilize separations between epithelial cells arising from dynamic local tissue rearrangements (see Sect. 4.5.3). Finally, a requirement for the actin cytoskeleton was identified in early studies, in a model in which branch formation is driven by localized contraction involving actin microfilaments, thereby promoting infolding of the epithelial surface (Spooner and Wessells 1972).

Cells and tissues generate mechanical forces through the actomyosin contractile machinery, and the importance of this machinery for a number of morphogenetic processes is now well appreciated. Ingber was the first to propose a model of tissue "tensegrity," in which he hypothesized that all cells and tissues are in a state of constant cytoskeletal "prestress;" that is, all intracellular forces generated from within the cytoskeleton are precisely balanced and resisted by opposing forces generated by the surrounding ECM and adjacent cells. Thus, even subtle changes in the forces experienced by the cells within a tissue can be detected, which then in turn respond by altering their behavior (Ingber 2003a, b). Because both cell–matrix (integrin) and cell–cell (cadherin) adhesion receptors form an interface with the actin cytoskeleton through scaffolding complexes at their cytoplasmic domains, they are uniquely positioned to both sense and transmit cytoskeletal-mediated tension to and from the ECM and neighboring cells (Dzamba et al. 2009; Wang and Ingber 1994; Wang et al. 2001) (Fig. 4.2b). Applied forces can also modulate the structural and signaling dynamics of such adhesion complexes themselves, which often results in their strengthening and maturation, as has been demonstrated for both focal adhesions and adherens junctions in vitro (del Rio et al. 2009; Galbraith et al. 2002; Humphries et al. 2007; le Duc et al. 2010; Pasapera et al. 2010). Through their effects on focal adhesions, actomyosin-mediated forces can induce ECM remodeling, which then can also feed forward to promote increased cytoskeletal contraction through localized matrix stiffening, as has been demonstrated in a number of mammary gland epithelial cell lines (Provenzano et al. 2009) (Fig. 4.3b). Binding of specific ECM molecules to cell surface integrin receptors can also modulate gene expression in the nucleus, which in turn has been shown to promote cell cycle progression. In the next section, we describe mechanisms by which ECM- and cell-mediated forces complement one another to promote dynamic changes in epithelial shape, with a particular focus on cleft formation in the salivary gland, since mechanistic studies of this organ have recently accelerated (Daley et al. 2009, 2011 ; Kadoya and Yamashina 2010; Onodera et al. 2010).

4.5.2 ECM Alters Tissue Shape to Promote Branch Formation

Grobstein and colleagues were the first to demonstrate that collagen is required for salivary gland cleft formation. When embryonic salivary glands were treated with collagenase or with inhibitors of collagen synthesis, branching was inhibited (Grobstein and Cohen 1965; Spooner and Faubion 1980). Conversely, treatment

with inhibitors of collagenase or with exogenous tissue inhibitors of metalloproteinases (TIMPs) promoted cleft formation, with increased accumulation of fibrillar collagen within the cleft region (Hayakawa et al. 1992; Nakanishi et al. 1986a, b). Immunolocalization studies later revealed that both collagen I and III preferentially accumulate at sites of cleft formation, leading to the hypothesis that cleft formation occurs as a result of the contraction or bundling of collagen fibers by mesenchymal cells, which in turn push inward against the epithelium to deform the epithelial surface (Nakanishi et al. 1988). In support of this model, mathematical simulations have recently revealed that external traction forces applied against a solid mass of cells may be capable of producing surface indentations that morphologically approximate salivary epithelial clefts (Wan et al. 2008). However, when glands were cultured in the presence of increasing concentrations of collagen III (Sakai et al. 2003), no stimulation of cleft formation was observed, while collagen I (*Mov-13*) null mice exhibit no defects in salivary gland development (Kratochwil et al. 1986). Thus, these data may be more consistent with a role for collagen accumulation in the stabilization of existing clefts, rather than in the active initiation of cleft formation. Such stabilization could restrict epithelial outgrowth at the base of the cleft, thereby allowing expansion only in adjacent regions.

While collagen fibrils may play an important role in cleft stabilization, the ECM protein fibronectin has been implicated in the active regulation of cleft formation in the salivary gland and other organs (De Langhe et al. 2005; Liu et al. 2010; Sakai et al. 2003). Fibronectin mRNA is preferentially expressed by salivary epithelium in regions adjacent to a forming cleft, and siRNA knockdown of fibronectin in salivary cultures strongly inhibits the first round of cleft formation while the addition of exogenous fibronectin stimulates this process (Sakai et al. 2003). Confocal time-lapse microscopy of mesenchyme-free epithelial rudiments cultured in the presence of fluorescently labeled fibronectin further revealed that fibronectin accumulates at the base of initiated clefts with newer fibronectin progressively being assembled as the cleft deepens (Larsen et al. 2006b). On the basis of such results, a model was proposed in which fibronectin is assembled in a directional manner in progressing clefts, thereby forming a "wedge" between cells that promotes their separation (Larsen et al. 2006b).

Recent work has provided further insight into the mechanisms by which fibronectin is assembled in the basement membrane. Unfolding of dimeric fibronectin molecules bound to cell surface integrin receptors unmasks a cryptic self-association site enabling the assembly of fibronectin molecules to form a fibrillar matrix; this has been shown to require cytoskeletal-mediated forces applied to the cytoplasmic domains of transmembrane integrin receptors (Baneyx et al. 2002; Zhang et al. 1997; Zhong et al. 1998) or cadherins (Dzamba et al. 2009). Consistent with this, ROCK-mediated actomyosin contraction and focal adhesion kinase (FAK) activation promote enhanced cell–matrix interactions in the developing salivary gland and are required for fibronectin accumulation in the basement membrane (Daley et al. 2009, 2011). In the absence of ROCK and myosin II activity, clefts are stalled at the initiation stage and fail to progress, suggesting the existence of a mechanochemical checkpoint required for the transition of

initiated clefts to a progression-competent state (Daley et al. 2009). Interestingly, fibronectin accumulation itself also appears to induce the formation of cell–matrix adhesions and FAK activation in salivary epithelial cells (Daley et al. 2011; Sakai et al. 2003), suggesting the intriguing possibility that a fibronectin-mediated feed-forward loop might promote continued cleft propagation once this process has begun.

Cytoskeletal contraction also promotes branching morphogenesis in the lung (Moore et al. 2002, 2005), kidney (Michael et al. 2005), and mammary gland (Gjorevski and Nelson 2010b), with branch outgrowth preferentially occurring at sites of high cytoskeletal tension and FAK activity in engineered mammary tubules (Gjorevski and Nelson 2010b). While a link between actomyosin contraction and ECM remodeling has not been mechanistically determined in these systems, there is a correlation between basement membrane thinning and branch formation in the developing lung (Moore et al. 2005). Extending the tissue tensegrity model described above, these researchers have proposed a "run-in-a-stocking" mechanism in which localized basement membrane thinning mediated by either actomyosin contraction or localized MMP production allows the invasion of adjacent prestressed epithelium into regions where it is no longer physically restrained by the basement membrane, thus resulting in branch outgrowth (Moore et al. 2005). An exciting area of future research will be to further clarify the mechanistic details of how localized ECM remodeling and cytoskeletal-mediated forces coordinately regulate one another to promote changes in epithelial tissue shape.

4.5.3 ECM Regulation of Epithelial Outgrowth and Expansion via Dynamic Cell Migration

Recent advances in microscopic imaging have enabled the real-time observation of cell dynamics during branching morphogenesis. Confocal time-lapse microscopy using fluorescently labeled cells has revealed a surprising degree of cell motility in the ureteric bud (Chi et al. 2009; Srinivas et al. 1999), mammary gland (Ewald et al. 2008), and salivary gland (Kadoya and Yamashina 2010; Larsen et al. 2006b; Onodera et al. 2010). One common feature observed in all of these studies is that even though branching morphogenesis seems orderly at the tissue level, individual epithelial cells undergo chaotic and dynamic rearrangements. While the significance of these chaotic motions is incompletely understood, it is possible that they facilitate epithelial plasticity to provide an amenable substrate for changes in tissue shape. For example, detailed analyses of local cell dynamics at the base of progressing salivary clefts have revealed that cleft epithelial cells undergo dynamic changes in shape suggestive of a more motile phenotype. Moreover, these cells transiently separate to form intercellular gaps to mediate cleft progression in a relatively stochastic process involving cells located only at the base of the advancing cleft (Onodera et al. 2010).

Cleft progression has also been reported to involve the formation of a small cytoplasmic shelf that protrudes into the cleft (Kadoya and Yamashina 2010). This shelf contains a dense core of actin microfilaments, and the basement membrane appears to invade the groove formed by the shelf and sidewall of the cleft. Taken together with the requirement for actomyosin contraction during cleft formation (Daley et al. 2009, 2011), an intriguing possibility is that the shelf serves as an attachment point for cell–ECM adhesions, with cytoskeletal-mediated forces driving their subsequent maturation (Galbraith et al. 2002). Such cell–ECM interactions might contribute to the shape changes that occur within the cleft epithelium, which in turn would be facilitated by its dynamic nature. Consequently, cooperative interactions between the ECM and local cell dynamics may drive a stepwise progression of the forming cleft.

An important outstanding question, then, is what are the molecular mechanisms that locally promote dynamic epithelial cell motility? A newly emergent theme is that novel regulatory molecules can be induced by the ECM in a highly focal manner. As mentioned in the previous section, the ECM protein fibronectin induces cleft formation in the developing salivary gland, where it might serve as an ECM "wedge" to promote epithelial cell separation. Subsequent experiments with salivary epithelial cells in culture have suggested a dynamic mechanism by which this might occur; when exogenous fibronectin was added to salivary gland cell cultures, it resulted in the downregulation of E-cadherin-mediated cell–cell adhesions and their subsequent replacement by fibronectin-mediated cell–matrix adhesions (Sakai et al. 2003). Recent work has furthered our understanding of the molecular basis of this process. Fibronectin rapidly induces expression of BTB (POZ) domain containing 7 (Btbd7) in a focal region at the base of progressing clefts; Btbd7 in turn upregulates expression of Snail2 and downregulates E-cadherin (Onodera et al. 2010). Because transcription factors such as Snail2 are critical regulators of epithelial-to-mesenchymal transition (EMT) that function to increase epithelial motility and cell scattering (Thiery 2003), it is interesting to speculate that cleft formation may constitute a fibronectin-induced partial EMT occurring at focal locations in the epithelium. This hypothesis is further supported by live imaging of mammary gland cultures, where terminal end buds elongate as a multilayered epithelium exhibiting reduced apicobasal polarity (Ewald et al. 2008), both of which are features of epithelial cells undergoing EMT. Interestingly, EMT scatter factors are transiently upregulated at branch sites in an in vitro model of mammary tissue morphogenesis, where they promote collective cell migration and subsequent branch extension (Lee et al. 2011). Since a role for fibronectin in this latter process has not been investigated, future studies should address the possibility that dynamic signaling from ECM to the nucleus may be a general mechanism to promote labile cell behavior during branching morphogenesis.

4.5.4 ECM Regulation of Epithelial Outgrowth and Expansion via Growth Factor Signaling

Growth factors regulate many critical cellular functions, including but not limited to cell proliferation, migration, survival, and differentiation. Growth factors mediate these effects by stimulating a large range of signal transduction pathways, all of which are initiated upon growth factor binding to cell surface receptor tyrosine kinases. While many growth factors have been implicated in the development of branched epithelial organs (Costantini 2010; Costantini and Kopan 2010; Hogan and Kolodziej 2002; Morrisey and Hogan 2010), particularly well-studied mechanisms involve the fibroblast growth factor (FGF) and epidermal growth factor (EGF) families (Hoffman et al. 2002; Jaskoll et al. 2002; Kashimata and Gresik 1997; Kashimata et al. 2000; Koyama et al. 2003).

Heparan sulfate proteoglycans (HSPGs) are an abundant component of the ECM that exhibit a wide tissue distribution and play an essential role during embryonic development; they are composed of glycosaminoglycan (GAG) chains linked to a core peptide via glycosidic bonds and are key modulators of growth factor signaling in a variety of branching systems (Garner et al. 2011; Meyer et al. 2004; Sarrazin et al. 2011). Early studies documented a correlation between GAG degradation in the basement membrane and the outgrowth of newly formed epithelial buds during salivary gland branching (Bernfield and Banerjee 1982). More recently, the mechanisms by which HSPGs regulate growth factor signaling have begun to be elucidated. When salivary gland organ cultures were treated with heparanase, an enzyme that degrades heparan sulfate from HSPGs, branching morphogenesis and epithelial cell proliferation were increased due to enhanced release of FGF10 from the basement membrane HSPG, perlecan (Patel et al. 2007, 2008). These studies suggest a model in which growth factors are proteolytically cleaved from ECM HSPGs to promote localized proliferation and branching morphogenesis.

Moreover, the affinities of diffusing FGFs for heparan sulfate can control FGF gradients and the resulting morphology of branching morphogenesis (Makarenkova et al. 2009). For example, in the developing lacrimal and submandibular salivary glands, FGF10 binds with high affinity to heparan sulfate, therefore creating a steep gradient with only local diffusion near the source. Thus, only a restricted area of the epithelium experiences this gradient, resulting in directional outgrowth of elongating ducts. In contrast, FGF7 has lower affinity for binding to heparan sulfate and can form a broad gradient that diffuses widely, thereby inducing the formation of multiple buds in multiple directions. This study demonstrates how differences in binding affinity for HSPGs in the ECM can promote differential activities from two structurally similar growth factors that bind to the same growth factor receptor (Makarenkova et al. 2009).

Interestingly, growth factor signaling is in turn capable of modulating the ECM, suggesting the existence of complex feedback loops involving growth factor and ECM signaling. For example, laminin α5 upregulates FGF receptor (FGFR) expression in the salivary gland, which requires downstream signaling through integrin β1.

Conversely, signaling through FGFR was shown to coordinately regulate laminin α5 expression, demonstrating for the first time the existence of a positive feedback loop between the basement membrane and FGFR-mediated growth factor activity (Rebustini et al. 2007). Because all of these growth factors signal through a variety of pathways to stimulate epithelial proliferation and outgrowth, which ultimately function to alter tissue shape, ECM regulation of growth factor activity is yet another example of how the ECM functions as a key mediator of epithelial branching morphogenesis.

4.5.5 Proteolytic Degradation of ECM and the Regulation of Epithelial Branching

Proteolytic degradation of the ECM and basement membrane is another mechanism by which the ECM can be remodeled during development (Lu et al. 2011). This remodeling is mediated by a number of proteases, including matrix metalloproteases (MMPs), serine proteases (serpins), and cysteine proteases (cathepsins). Such proteases influence ECM remodeling and related signaling by cleaving ECM structural molecules into bioactive peptides, releasing growth factors from ECM storage reservoirs (such as HSPGs), and altering ECM structure itself, which can impact epithelial tissue shape (Daley et al. 2008; Page-McCaw et al. 2007).

MMP regulation during branching morphogenesis is complex, and attempts to elucidate individual MMP functions have been hindered by the fact that many MMPs appear to be able to compensate for the absence of another (Page-McCaw et al. 2007). However, salivary glands and lungs isolated from *mmp14* (MT1-MMP, membrane type-MMP1) null mice exhibited decreased branching morphogenesis (Oblander et al. 2005), while overexpression of this MMP in the mammary gland resulted in increased branching (Ha et al. 2001). Interestingly, in embryonic kidney, MT1-MMP expression is enhanced at ureteric bud tips, suggesting that localized basement membrane degradation may be important for epithelial outgrowth (Meyer et al. 2004). In support of this hypothesis, a recent study demonstrated that siRNA depletion of MT2-MMP in salivary gland organ cultures decreased branching morphogenesis, which was attributed to a decrease in the proteolytic release of bioactive NC1 domains from collagen IV in the basement membrane; these authors subsequently showed that collagen IV NC1 stimulates epithelial proliferation and survival through a β1 integrin-dependent mechanism (Rebustini et al. 2009).

MMP activity can also regulate growth factor signaling; for example, HB-EGF, an EGF family growth factor highly expressed in developing salivary glands, must be cleaved by MMPs to yield active growth factor (Powers et al. 2000; Umeda et al. 2001). Interestingly, while siRNA knockdown of MT2-MMP decreased epithelial proliferation and salivary gland branching, this effect could be rescued by exogenous active HB-EGF which increased MT2-MMP expression, suggesting a feedback

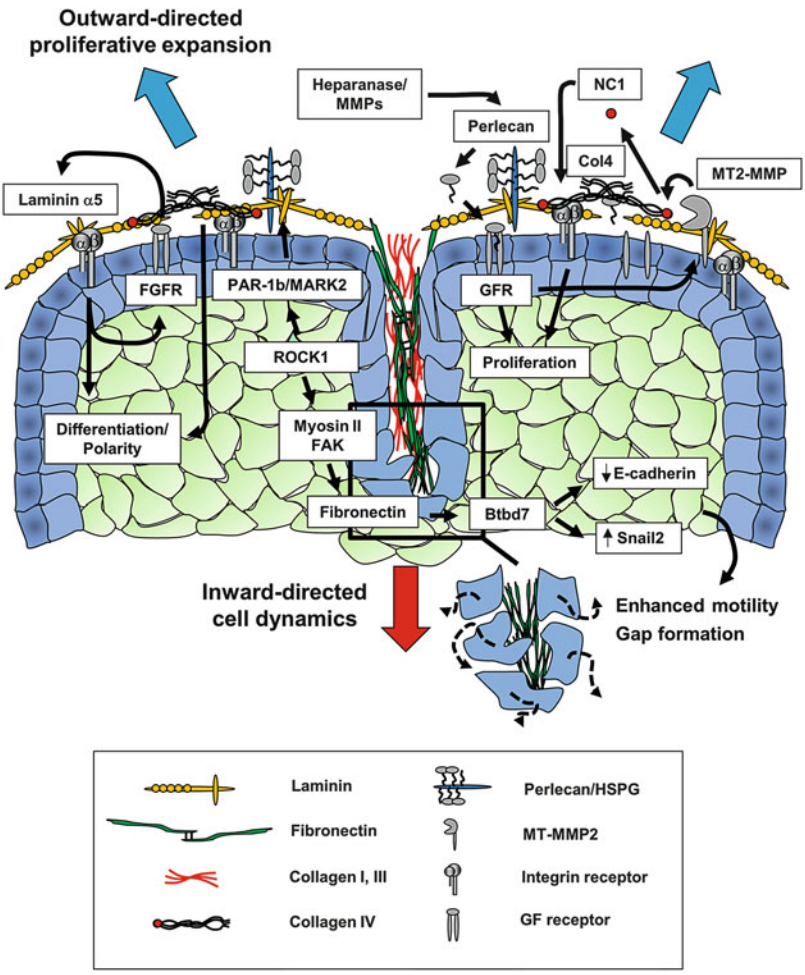

Fig. 4.4 Salivary gland branching morphogenesis is mediated by complex crosstalk involving the epithelial cell-associated basement membrane and stromal ECM. Cartoon schematic illustrating the cellular events by which cell–ECM interactions regulate salivary gland branching morphogenesis. Localized basement membrane remodeling promotes local cell dynamics and is a key mediator of cleft formation and changes in tissue shape. Growth factor-mediated signaling and proteolytic degradation of the ECM converge on various signaling pathways to stimulate epithelial cell proliferation and end bud outgrowth. Basement membrane components such as laminin also regulate cytodifferentiation and tissue polarization. Please see Sect. 4.5 of the chapter text for details

loop involving HB-EGF and MMP expression and activity (Rebustini et al. 2009). Taken together, these studies highlight the complex interplay between MMP activity, growth factor signaling, and basement membrane remodeling, which function coordinately to facilitate branching morphogenesis (Fig. 4.4).

4.5.6 Basement Membrane Regulation of Tissue Polarization and Cytodifferentiation

During epithelial organogenesis, the epithelium not only undergoes overall changes in tissue shape, but its constituent cells are also coordinately organized along the apicobasal axis. In a properly polarized epithelial tissue, cells contact the basement membrane at the basal periphery, while their apical surfaces surround a lumen at the center of the tissue. In fact, the basement membrane itself is a key polarizing factor for epithelial cells in culture and can direct the orientation of apical polarity to the center of developing acini (O'Brien et al. 2001). Thus, it is no surprise that multiple basement membrane components are required for epithelial organization and cytodifferentiation during branching morphogenesis.

Laminins are core components of epithelial basement membranes and are required for the initiation of basement membrane assembly. Consistent with this, laminin α1 is required for salivary gland development, and function-blocking antibodies and inhibitory peptides disrupt basement membrane assembly and reduce branching morphogenesis (Kadoya et al. 1995). Laminin α5 is also an abundant component of basement membranes, and salivary glands from *lama5* null mice lack lumens and exhibit disorganized acinar structures, indicating a role for the α5 chain in cytodifferentiation and polarization (Rebustini et al. 2007). That laminin is required for epithelial polarization is also supported by the finding that salivary glands from α3 integrin-null mice (α3 is part of a major laminin receptor) have thin basement membranes and disorganized tissue structure; they also fail to recruit E-cadherin to cell–cell junctions and showed abnormal cell differentiation (Menko et al. 2001). Laminin receptors in the mammary epithelium, including dystroglycan and integrin α3β1, also directly influence mammary acinar polarization, and they have even been implicated in the expression of the mammary differentiation marker β-casein (Stahl et al. 1997; Weir et al. 2006). Taken together, these studies demonstrate an essential role for a basally localized basement membrane in both the organization and cytodifferentiation of epithelial cells during epithelial development.

While the basement membrane plays important roles in both epithelial morphogenesis and tissue polarization, an interesting question that remained unexplored until recently concerns the molecular mechanisms by which the basement membrane is specifically positioned at the basal periphery of epithelial tissues. Recent studies utilizing salivary gland organ cultures have shed new light on this process. For example, O-glycosylation appears capable of altering basement membrane composition, since salivary glands from mice deficient for the O-glycosyltransferase *Galnt1* exhibit increased intracellular accumulation of laminin α1 and collagen IV, suggesting a role for this posttranslational modification in the secretion of basement membrane proteins. Salivary glands lacking *Galnt1* failed to undergo branching morphogenesis, which was attributed to a decrease in basement membrane-dependent FGF signaling and epithelial cell proliferation (Tian et al. 2012). Another study demonstrated that Rho kinase (ROCK1), independent of myosin II, promotes

basal basement membrane deposition by restricting PAR-1b/MARK2 localization to the basal periphery of epithelial buds (Daley et al. 2012). When ROCK signaling is perturbed, PAR-1b is mislocalized throughout the epithelium, where it drives aberrant deposition of basement membrane within the epithelial compartment. The mislocalized basement membrane accumulations within the epithelium drive the uncoordinated alignment of cells and the formation of ectopic lumens, leading to disorganized tissue structure (Daley et al. 2012). The exact mechanism by which ROCK regulates PAR-1b localization and whether such ROCK-mediated control of basement membrane positioning is a general mechanism used by branched epithelial structures remains to be determined.

4.6 Perspectives and Future Directions

The last 50 years have provided a wealth of knowledge concerning the ways in which the stromal ECM and epithelial cell-associated basement membrane regulate the morphogenesis, organization, and cytodifferentiation of epithelial tissues. Many of the specific functions discussed in the previous sections are summarized schematically in Fig. 4.4, using cleft formation in the salivary gland as a model for branch formation. The mechanisms by which the ECM regulates these processes are not simple and instead involve a complex interplay between ECM assembly and degradation, growth factor- and protease-mediated signaling pathways that regulate cell proliferation, as well as ECM-induced changes in tissue shape and organization. It is important to note that, consistent with the concept of dynamic reciprocity, the epithelium in turn plays a crucial role in the regulation of the ECM. In this final section, we briefly discuss some of the biological implications of these findings and highlight some exciting areas for future study.

4.6.1 Systems Biology Approaches

Tremendous progress has been made in the identification of conserved signaling pathways and secreted factors required for the regulation of epithelial branching morphogenesis. However, how these diverse cellular signaling events interact and coordinate with one another to give rise to a fully functional epithelial architecture remains unknown, and the elucidation of such complex interactions will most certainly require a systems biology approach (Gjorevski and Nelson 2010a, 2011; Larsen et al. 2010; Monte et al. 2007; Wang et al. 2011). Recent progress toward this type of systems level understanding has included the complete description of the 3D branching pattern of the mouse lung (Metzger et al. 2008), in which a genetically hardwired, stereotyped set of three basic branching subroutines was uncovered. Large scale DNA microarrays and serial analysis of gene expression (SAGE) have also been used to elucidate spatiotemporal differences in gene

expression patterns within distinct cell populations of a variety of branching organs (Musselmann et al. 2011; Onodera et al. 2010; Sakai et al. 2002, 2003; Sternlicht et al. 2006; Stuart et al. 2001, 2003). Such approaches should eventually shed light on the multitude of molecular interactions that function together to cooperatively build branched epithelial structures but will require close collaboration with mathematicians for computational modeling of complex data sets. Furthermore, the diverse roles of the ECM in branching morphogenesis have not been considered in many computational studies thus far and will need to be incorporated to generate more complete predictive models.

4.6.2 Tissue Engineering Using Exogenous Extracellular Matrices

The overall goal of tissue engineering is the creation of artificial tissues by seeding cells on 3D scaffolds that attempt to mimic the in vivo microenvironment. One possible approach to this daunting task is to utilize our understanding of normal developmental processes such as branching morphogenesis to engineer functional scaffolds that provide necessary microenvironmental cues. Because recent research has demonstrated the essential role for dynamic cell–ECM interactions in such processes, a recent strategy has been to engineer scaffolds that structurally and functionally resemble native ECM topology. For example, a variety of biocompatible materials have been used to engineer biomimetic nanofiber scaffolds, the diameters of which are in the submicrometer range and approximate the fibrillar structure of the ECM (Ashammakhi et al. 2007a, b; Griffith and Swartz 2006). Salivary epithelial cells have been successfully seeded on such fibers, and can aggregate, self-organize, and undergo branching morphogenesis (Jean-Gilles et al. 2010; Sequeira et al. 2012), as has been observed for salivary epithelial cells embedded in Matrigel (Wei et al. 2007). Because fetal pulmonary cells can also self-organize into alveolar-like structures when embedded in an ECM (Mondrinos et al. 2006, 2007), such a strategy might be a general mechanism by which epithelial cells can be induced to organize on an artificial scaffold. Current studies are ongoing to engineer even more biologically relevant biomimetic scaffolds, and new approaches include the combination of biocompatible polymers with purified matrix proteins and the tethering of bioactive peptides and growth factors to engineered scaffolds (Dvir et al. 2011; Huebsch and Mooney 2009). Such approaches are based on our knowledge that cell interactions with the ECM are dynamic and that the ECM is constantly being remodeled. Because recent work has also highlighted the role of mechanical forces in the regulation of morphogenesis and differentiation (Buxboim et al. 2011; Discher et al. 2005, 2009; Engler et al. 2006), current strategies are also considering the effect of scaffold stiffness on cell organization and cytodifferentation. Thus, close collaborations with cell and

developmental biologists should help bioengineers with the rational design of scaffolds for tissue engineering applications.

4.6.3 *Epithelial Invasion in Branching Morphogenesis and Cancer Progression*

A working hypothesis at the intersection of the fields of developmental biology and cancer research is that tumorigenesis can involve the recapitulation of normal developmental processes at the wrong time, place, and/or extent. In this context, recent studies have highlighted a number of striking similarities between the cellular mechanisms of branching morphogenesis and the invasion strategies used by epithelial tumors during metastasis, most notably in the mammary gland (Ewald et al. 2008; Gray et al. 2010). Confocal time-lapse microscopy of ex vivo mammary cultures has recently delineated the normal procession of cellular events that occurs during mammary gland branching morphogenesis. Such imaging has revealed that ductal branching is preceded by enhanced proliferation and luminal filling to give rise to a multilayered terminal end bud that exhibits reduced apicobasal polarity and migratory behavior (Ewald et al. 2008); this phenotype is remarkably similar to one of the earliest stages in breast cancer progression, a preinvasive lesion known as ductal carcinoma in situ (Burstein et al. 2004; Debnath and Brugge 2005). Furthermore, because ductal carcinoma in situ can be modeled with the MCF-10A epithelial cell line (Debnath and Brugge 2005), many of the molecular changes leading to this preinvasive state have been elucidated; these include the activation of ERK1/2 to promote hyperproliferation and luminal filling (Debnath and Brugge 2005; Pearson and Hunter 2007), which is also required to initiate the same cellular behaviors during terminal end bud branching in the normal mammary gland (Ewald et al. 2008). Thus, it is quite possible that the multilayered, reduced polarity tissue architecture of mammary terminal end buds is a normal developmental mechanism inappropriately recapitulated in ductal carcinoma in situ.

Another hallmark of epithelial malignancy in mammary tissue is a stiffening of the stromal microenvironment caused by the increased deposition of ECM components such as stromal collagen and FN (Paszek et al. 2005; Provenzano et al. 2009; Williams et al. 2008). Such ECM stiffening promotes integrin activation, focal adhesion formation, and downstream activation of FAK and ERK1/2 to drive hyperproliferation and the acquisition of a malignant phenotype. These findings have led to the idea of a constitutively elevated signaling circuit involving Rho/ROCK-mediated adhesion to the ECM and downstream signaling through FAK/MAPK which then feeds forward to promote further ECM remodeling, focal adhesion formation, and enhanced cellular contractility (Paszek et al. 2005; Provenzano et al. 2009; Schedin and Keely 2011). This series of events bears similarities to some of the cytoskeletal-mediated mechanotransduction pathways required for cleft formation in the salivary gland and lung (Daley et al. 2009, 2011;

Moore et al. 2005) and branch outgrowth in the mammary gland (Gjorevski and Nelson 2010a). Interestingly, new studies have also uncovered a role for actomyosin-mediated tension in the promotion of EMT (Gjorevski et al. 2011), a process which may be at least partly recapitulated during both branching morphogenesis and the onset of epithelial malignancy (Thiery et al. 2009). Sophisticated new methods such as atomic force and traction force microscopy will provide further insight into the spatiotemporal distribution of forces in epithelial tissues undergoing branching morphogenesis and allow meaningful comparisons to the tumor microenvironment.

4.7 Conclusions

The dynamic mechanisms of cell–ECM interactions and the ways in which they regulate complex developmental processes such as epithelial branching morphogenesis encompass an exciting area of research with many new exciting discoveries to come. In this chapter, we have considered a number of general biological principles by which cell interactions with the ECM and basement membrane coordinately sculpt mammalian epithelial tissues; these include ECM-mediated forces and alterations in tissue shape, ECM-induced stimulation of dynamic cell motility, proliferative outgrowth and expansion of the epithelium regulated by the ECM, and BM-mediated signaling to coordinate epithelial tissue architecture. Because an in-depth understanding of cell interactions with the ECM will be critical for the development of novel therapeutic approaches to diseases, it is no surprise that the findings considered here have had broad implications for regenerative medicine strategies, as well as for our understanding of epithelial cancer progression. The challenge for the future will be to integrate our new knowledge of the roles of cell–ECM interactions with the vast information on a multitude of other critical regulators of epithelial branching morphogenesis, a daunting task that will require applying a more systems biology viewpoint to understand epithelial organogenesis.

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Chapter 5
Interactions Between Neural Crest-Derived Cells and Extracellular Microenvironment During Cardiovascular Development

Sophie Astrof

Abstract Organ morphogenesis requires coordinated communications between and within different populations of progenitors. Development of the mammalian asymmetric aortic arch artery tree is an excellent model system to study genes and mechanisms orchestrating complex inter-tissue interactions during organ morphogenesis. In this system, the initially symmetric vascular tree connecting the heart to the embryonic circulation undergoes asymmetric remodeling in a highly stereotyped manner. This morphogenetic process is essential for the separation of arterial and venous circulations and requires coordinated communication between cells of mesoderm, endoderm, surface ectoderm, and the neural crest. While a number of key signals have been identified, the means by which these signals are integrated to drive this morphogenetic process is not well understood. One possible means by which signaling by various growth factors can be integrated into precise developmental programs is via the extracellular matrix. This review will examine roles of extracellular matrix proteins in mediating growth factor signaling between neural crest cells and the surrounding tissues during development of the cardiac outflow tract and aortic arch arteries.

5.1 Introduction

Neural crest (NC) derivatives play a major role in cardiovascular development by modulating morphogenesis of the cardiac outflow tract (OFT) and aortic arch arteries (AAAs) (Fig. 5.1). Proper development of this vascular system is essential for delivering deoxygenated blood to the lungs and oxygenated blood to the rest of the body. Congenital malformations of this system lead to the most common and

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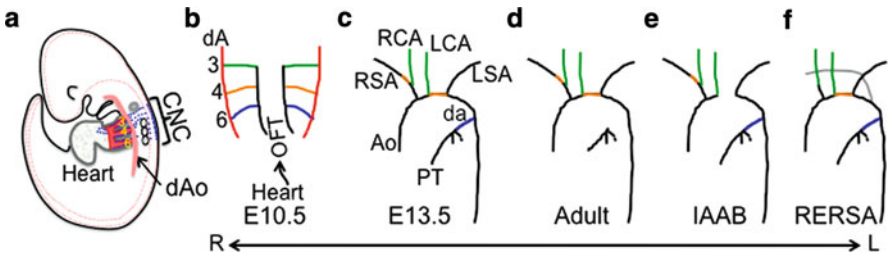

Fig. 5.1 Cardiac neural crest and morphogenesis of cardiac outflow tract and aortic arch arteries. (**a**) Left-side view of E9.5-10 mouse embryo. NC-derived cells are shown as *blue dots*. *Bracket* marks the position of the CNC in the neural tube. Pharyngeal arch arteries are numbered. *dAo* dorsal aorta. (**b**) Ventral view of the symmetric arch arteries at E10.5. (**c**–**d**) Ventral views of the remodeled arch arteries in normal configuration and (**e**–**f**) in examples of disease configurations. *L* left; *R* right; *RCA*, *LCA* right and left carotid arteries; *RSA*, *LSA* right, left subclavian arteries; *PT* pulmonary trunk; *Ao* aorta; *da* ductus arteriosus. (**d**) da closes after birth and degenerates, leading to separate aortic and pulmonary circulations. (**e**) Premature degeneration or defective formation of the left fourth arch artery (*orange*) leads to interrupted aortic arch type B (IAAB). (**f**) Degeneration or aberrant formation of the right fourth pharyngeal arch results in the absent RSA; instead, the persistent right segment of the dorsal aorta (*grey*) forms vascular ring around the trachea, termed *RERSA*, retroesophageal right subclavian artery

severe forms of human birth defects, which are often lethal if not corrected by surgery in the first few days of life (Roger et al. 2011). A high proportion of AAA remodeling defects are observed in patients with DiGeorge syndrome, the most common chromosome microdeletion syndrome in humans, in 50 % of patients with Down syndrome and in up to 90 % of patients with heterotaxy (defective left–right patterning) (Moon 2006). Therefore, understanding genes and mechanisms orchestrating morphogenetic processes leading to formation and remodeling of the cardiac OFT and AAAs is essential for gaining insights into etiology, diagnosis, prevention, and treatment of a large proportion of human congenital heart defects.

NC is a population of progenitor cells located in the dorsal neural tube of vertebrate embryos. Cardiac neural crest (CNC) resides within a particular region of the neural tube located between the otic pit and the fourth somite (Chan et al. 2004; Leatherbury et al. 1990). Descendants of these cells play pivotal roles in OFT septation resulting in formation of two separate vessels, the aorta and the pulmonary trunk, development of cardiac conduction system, OFT valves, as well as in asymmetric morphogenesis of the AAAs. Mutations in genes compromising CNC development (directly or indirectly) give rise to lethal cardiovascular defects in model vertebrate organisms and in humans (Wurdak et al. 2006) (Fig. 5.1a, b). Development of the asymmetric cardiac outflow vasculature requires coordinated communication between and within different populations of progenitor cells. This review will examine the role of cell–extracellular matrix (ECM) interactions in mediating inter-tissue communications between NC-derived cells and the surrounding tissues during development of the cardiac OFT and AAAs.

Similar to other NC-derived populations, CNC-derived cells encounter changing extracellular microenvironment as they transit from the dorsal neural tube to their final destinations, and their interactions with various tissues are fundamental, not only to the development of the CNC-derived lineages but also to the development of tissues in close proximity to the CNC (Graham et al. 2005; Wurdak et al. 2006). For example, signaling between CNC-derived cells and precardiac mesoderm is essential for addition of the cardiac precursors to the OFT of the heart. Defects in these interactions result in shortened OFT, leading to malrotation and malalignment of major arteries with respect to the left and right ventricles of the heart (Waldo et al. 2005). Similarly, interactions between blood vessel endothelial cells (derived from the mesoderm) within the pharyngeal arches 3, 4, and 6 and the CNC-derived cells are essential for recruitment of the CNC, their differentiation toward arterial vascular smooth muscle cell (VSMC) fate, and remodeling of the initially symmetric pharyngeal arch artery blood vessels into their final asymmetric arrangement (High et al. 2008) (Fig. 5.1b). Signaling between the pharyngeal endoderm, mesoderm, surface ectoderm, and the CNC is essential for navigation, proliferation, survival, and differentiation of the CNC-derived cells, as well as for the downstream morphogenetic events such as OFT rotation, septation, and asymmetric remodeling of the pharyngeal arch arteries (Fig. 5.3).

5.2 Extracellular Microenvironment Regulates Migration and Navigation of CNC-Derived Cells

How interactions between transiting NC-derived cells and surrounding tissue microenvironment mediate cardiovascular morphogenesis is not well understood. There is evidence that short- and long-range signaling interactions between all embryonic germ layers are important for NC migration, navigation, cell fate specification, and cardiovascular morphogenesis (Calmont et al. 2009; Goddeeris et al. 2007; Graham et al. 2005; Kirby and Hutson 2010; Liu et al. 2004; Macatee et al. 2003; Park et al. 2008; Rentschler et al. 2010; Stuhlmiller and Garcia-Castro 2012; Urness et al. 2011) (Fig. 5.2). The extracellular milieu, which includes large ECM glycoproteins, proteoglycans, and their cellular receptors, plays essential roles in modulating interactions between the CNC and components of the pharyngeal microenvironment.

5.2.1 Migration of NC Cells Toward Their Target Destinations

Genes and mechanisms regulating routing of NC-derived cells toward their eventual destinations are not well understood. A process termed contact inhibition of locomotion (CIL) has been hypothesized to facilitate directional migration of cell

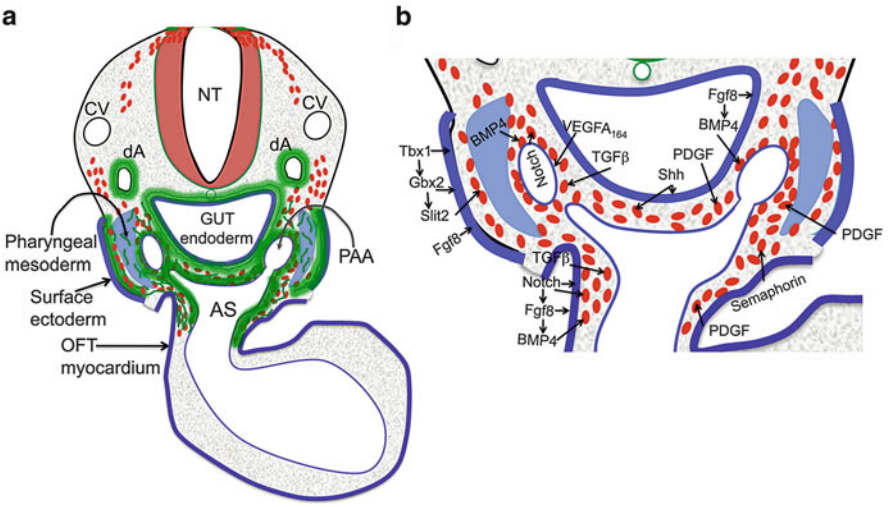

Fig. 5.2 Signaling within pharyngeal microenvironment. (**a**) Tissue-specific enrichment of FN protein in the pharyngeal region. A schematic cross section through E9.5 embryo. *NT* neural tube, *CV* cardinal vein, *dA* dorsal aorta, *PAA* pharyngeal arch artery, *AS* aortic sac, *OFT* outflow tract. *Green* marks areas enriched in FN protein. Neural crest-derived cells are *red ovals*. Dorsal is at the *top*; ventral is at the *bottom*. (**b**) Enlarged pharyngeal region shown in (**a**) depicting examples of signaling pathways known to orchestrate signaling between endoderm, mesoderm, surface ectoderm, and the CNC during cardiovascular morphogenesis. FN protein is enriched at strategic positions and is predicted to modulate some of the depicted intercellular signaling pathways

cohorts during embryogenesis (Rovasio et al. 1983). In the case of the NC, CIL involves transient homotypic interactions between NC-derived cells, preventing cells at the front from moving back, while heterotypic interactions between NC-derived cells and cells of other tissues do not have the same effect and allow NC cell invasion. Noncanonical Wnt signaling and activation of Rho GTPases play essential roles in this process by limiting polarized lamellipodial protrusions to the leading edge of NC cells traveling in front of the group (Carmona-Fontaine et al. 2008). In addition to CIL, NC cell co-attraction mediates migration of NC cells in cohorts. In fish and frog, this process is mediated by the complement fragment C3a and the C3a receptor, both of which are produced by NC-derived cells and are required for cohesive migration of NC streams (Carmona-Fontaine et al. 2011). Thus, NC cell homotypic CIL and co-attraction cooperate to promote directional movement of swarms of NC-derived cells toward their target destinations.

Recent experiments in chick and genetic manipulations in mouse indicate the existence of cues playing fine-tuning roles in migration and navigation of NC-derived cells (Erickson et al. 1980; Gammill and Roffers-Agarwal 2010; Kulesa and Gammill 2010). In vitro studies suggest that locomotion of NC cells depends on composition of the ECM and the complement of ECM receptors expressed by NC-derived cells. For example, compared with CNC, cephalic NC cells isolated from the neural tube regions anterior to the otic pit exhibit different migratory

properties when presented with increasing levels of FN. Migration speed and persistence of cephalic NC cells do not change when these cells are plated on a wide range of FN concentrations. However, increasing concentrations of FN lead to decreased speed and increased migrational persistence of CNC cells (Xu et al. 2006). Migrational persistence is defined as a length of time traveled in a straight line.

One reason for different migratory responses of NC cells originating at different rostrocaudal levels of the neural tube could be due to a varied complement of integrins, a major class of cell surface ECM receptors (Delannet et al. 1994; Haack and Hynes 2001; Hynes 2002; Testaz et al. 1999). Cranial NC cells migrate faster on laminin1 [LM-111 according to new nomenclature (Aumailley et al. 2005)] relative to trunk NC cells. This has been attributed to a fast recycling rate of integrin α6 in these cells (Strachan and Condic 2004). Trunk NC cells migrate 2–3 times faster on FN than on LM-111 or collagen I, on which they exhibit a more persistent migration (Rovasio et al. 1983). Trunk NC cells preferentially adhere and migrate on FN when given a choice between FN, collagen I, LM-111, or glass. Although when these cells are cultured on LM-111 for more than 24 h, their adhesion to LM-111 improves, suggesting that NC-derived cells can adjust their properties depending on the type of ECM substrate they encounter (Rovasio et al. 1983).

Navigation of NC-derived cells in the embryo may be modulated by a combination of attractive and repulsive cues. For example, attractive properties of fibronectin (FN) can be modulated by versican and type 6 semaphorins, Sema6A and Sema6B, all of which act as NC cell repellents (Dutt et al. 2006; Testaz et al. 2001; Toyofuku et al. 2008). Different classes of semaphorins expressed along the dorsoventral axis further modulate the routing of NC cells toward their destinations (Gammill and Roffers-Agarwal 2010; Kulesa and Gammill 2010; Toyofuku et al. 2008). For example, NC cells transiting through somites migrate through the anterior of somite's sclerotome. This pattern is determined by neuropilin (Nrp) 2 expressed both by NC cells in the anterior part of each somite and by semaphorin (Sema) 3F expressed in somites' posterior, repelling NC-derived cells from this region. Global genetic ablation of either Nrp2 or Sema3F allows NC cells to traverse through the entire width of a somite, without disturbing somite's anterior–posterior polarity itself. Interestingly, wild-type NC cells avoid Sema3F + territories, while Nrp2-deficient NC cells are not repelled by Sema3F (Gammill et al. 2006).

5.2.2 Extracellular Microenvironment and Differentiation of NC-Derived Cells

NC progenitor cell fate can be changed by surgical transplantation from one position to another along the neural tube (Trainor et al. 2002). Such transplantation experiments indicated that NC progenitor cell fate is plastic and can be determined by short- and long-range signals emanating from tissue microenvironment

(Le Douarin and Kalcheim 1999). In addition to modifying NC migration, various components of ECM play distinct roles in modulating differentiation of NC-derived cells. For example, plating NC cells from either cephalic or trunk regions on FN promotes their differentiation toward smooth muscle cell lineage, while plating on LM-111 or collagen IV does not (Costa-Silva et al. 2009). These experiments suggest that varying levels and distributions of ECM molecules in vivo modulate migration and differentiation of NC-derived cells.

5.3 Examples of ECM Macromolecules Modulating Neural Crest Development In Vivo

ECM proteins modulate a multitude of signaling pathways involved in cell survival, proliferation, migration, and differentiation and are prominently expressed within embryonic tissues (Giancotti and Tarone 2003). Intriguingly, there is a large body of evidence showing that ECM proteins are synthesized and distributed in nonuniform patterns within embryos (e.g., Fig. 5.3). For example, immunofluorescence staining experiments showed that vitronectin, collagen, laminin, fibulin1, perlecan, and FN proteins are highly abundant in some and greatly underrepresented in other sites of the embryo and genetic ablation of fibulin1, α5 chain-containing laminins, perlecan, or FN indicated that ECM plays essential and distinct roles in NC (and CNC) development in vivo (Coles et al. 2006; Cooley et al. 2008; Copp et al. 2011; Costell et al. 2002; Delannet et al. 1994; Duband and Thiery 1987; Gammill et al. 2007; Mittal et al. 2010; Peters and Hynes 1996). Intriguingly, expression studies of genes encoding ECM macromolecules indicate that ECM mRNAs and proteins are dynamically regulated during embryogenesis and are expressed and/or accumulate as "hot spots" at specific embryonic sites implying that ECM proteins play tissue-specific roles during embryonic development (Coles et al. 2006; Cooley et al. 2008; Copp et al. 2011; Mittal et al. 2010) (Fig. 5.3).

5.3.1 Laminin α5

Laminins are extracellular protein heterotrimers composed of α, β, and γ chains. The mRNA and protein of laminin α5 chain, a component of laminins 511, 521, and 523 (Aumailley et al. 2005) are distributed nonuniformly in the mouse embryo (Copp et al. 2011). At the early times of NC development, at embryonic day (E) E8.5, *LAMA5* mRNA is expressed by cells in the upper third of the dorsal neural tube, in endoderm, surface ectoderm including that of pharyngeal arches, and, weakly, in the pharyngeal arch mesenchyme (Coles et al. 2006). By E9.5, *LAMA5* mRNA becomes downregulated in the neural tube anterior to the forelimb bud, while it remains expressed in the posterior neural tube, pharyngeal arches, and the endoderm

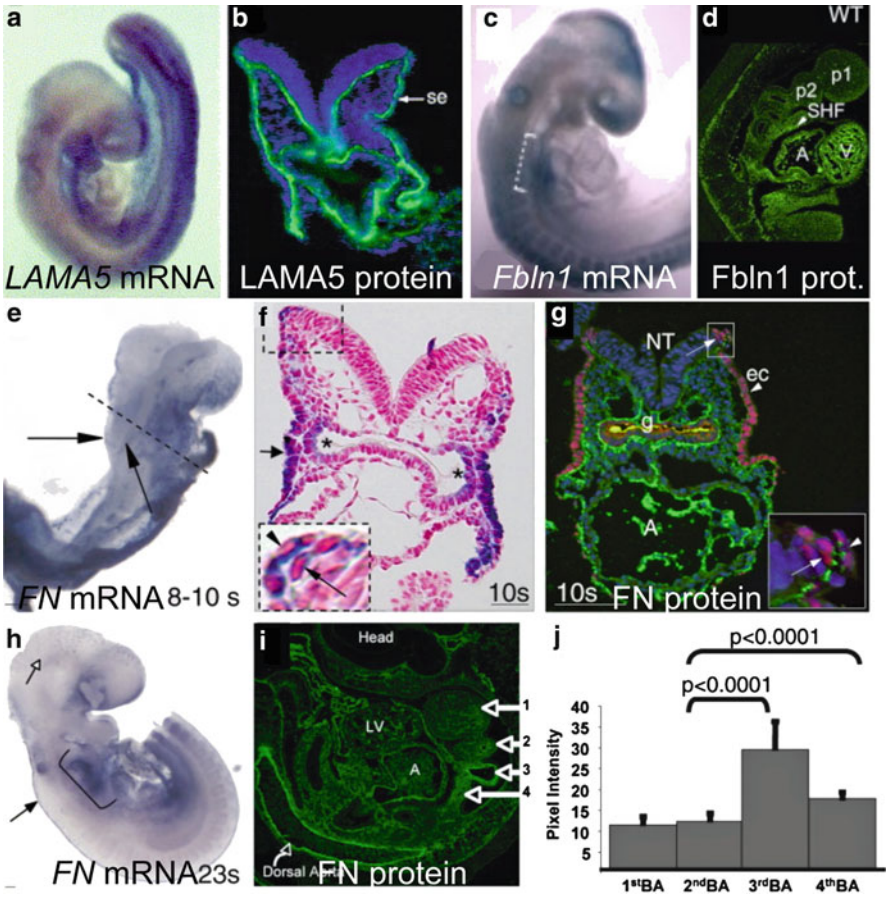

Fig. 5.3 Extracellular matrix proteins are expressed in nonuniform patterns during embryogenesis. (**a**, **c**, **d**, **h**) E9.5 right side views. (**a**) *Laminin alpha 5* (*LAMA5*) mRNA. (**b**) E8.5. Transverse section. LAMA5 protein (*green*) (Coles et al. 2006). (**c**) *Fibulin1* mRNA. (**d**) Fibulin1 protein. Sagittal section (Cooley et al. 2008). (**e**–**j**) Expression of *FN* mRNA and protein. (**e**) *FN1* mRNA is expressed at the edges of the dorsal neural tube in 8–10 somite embryos (*black arrows*). *Dotted line* is an approximate plane of section shown in (**f**). (**f**) *FN* mRNA is expressed by the dorsal neural ectoderm (*long arrow* in the inset) and the surface, nonneural ectoderm (*arrowhead* in the inset). *Asterisk* marks *FN1* mRNA in pharyngeal pocket endoderm and *short arrow* points at pharyngeal ectoderm. (**g**) Transverse section through a region containing CNC cells in a 10-somite wild-type embryo. FN protein (*green*) is present between TFAP2α+ neural (*arrow*, inset) and surface (*arrowhead*, inset) ectoderm. (**h**) *FN1* mRNA expression is maintained at E9.5 in the dorsal neural tube (*black arrow*) and within the region of branchial arches 3, 4, and 6 (*bracket*). *FN1* mRNA is also expressed by microvascular endothelial cells in the head (*open arrow*). (**i**) Sagittal section through E9.5 embryo showing expression of FN protein. (**j**) FN protein is enriched in branchial arches 3 and 4, compared with arches 1 and 2 (Mittal et al. 2010). (Printed with permission from *Developmental Biology*)

(Copp et al. 2011). However, laminin α5 protein is enriched at the basal sides of foregut endoderm, surface ectoderm, and the neural tube. This pattern is distinct from the pattern of *LAMA5* mRNA (Coles et al. 2006; Copp et al. 2011) (Fig. 5.3a, b). The presence of laminin α5 protein in the basement membrane surrounding the neural tube suggests that laminin α5 protein diffuses away from the sites of its synthesis and that its enrichment at certain embryonic sites could be determined by expression patterns of cognate laminin receptors. Genetic ablation of *LAMA5* leads to widening and fusion of NC cell cohorts, reduction in the size, and a delay in the formation of initial ganglionic condensations during early neurogenesis between E8.5 and E10.5, but remarkably gangliogenesis largely recovers by E11. These observations (supported by in vitro data) suggest that α5 chain-containing laminins play restrictive role(s) in NC migration by facilitating proper routing of NC-derived cells to their destinations (Coles et al. 2006). The authors of this paper also suggest an intriguing idea that the initial delay followed by recovery of gangliogenesis at a later developmental time in *LAMA5* mutants implies a role for *LAMA5* in cessation of NC cell migration upon arrival at their destinations (Coles et al. 2006).

5.3.2 *Fibulin1*

The mRNA of the ECM protein fibulin1 is highly expressed at the dorsal neural tube, somitic and pre-somitic mesoderm, and in mesoderm containing presumptive cardiac progenitors at E8.5 (Cooley et al. 2008). At E9.5, *fibulin1* mRNA is downregulated from the trunk neural tube anterior to the forelimb bud, while it remains expressed in all pharyngeal arches. Interestingly, the localization of fibulin1 protein does not match that of its mRNA at this time: fibulin1 protein is highly enriched in the embryo mesenchyme lateral to the neural tube and dorsal to the foregut, while it is virtually excluded from the pharyngeal arch mesenchyme, containing Pax3+ cells (NC cells); in addition, fibulin1 is highly enriched within the cardiac OFT (Cooley et al. 2008) (Fig. 5.3c, d). Global deletion of fibulin1 leads to defects in cranial NC-derived structures such as facial skeleton, CNC-related defects in thymus, and aortic arch artery development and in the development of cranial nerves. Elevated apoptosis in NC-rich areas suggests that fibulin1 is important for NC cell survival. Fusion of cranial NC streams in fibulin1 mutants suggests that fibulin1 is important for the establishment and/or maintenance of proper routes or boundaries of NC paths and implies that fibulin1 acts as a repellent for NC cells contributing to cranial nerves IX and X (Cooley et al. 2008).

5.3.3 *Fibronectin*

FN gene, multipotent neural crest, and a closed, endothelial-lined vasculature are vertebrate innovations (Hynes 2012). Investigation into the fate of the CNC in global FN-null mouse mutants demonstrated that FN is not required for

specification of the CNC precursors or for their exit from the dorsal neural tube. In global FN-null mutants, CNC cells reach their distal destinations in the pharyngeal arches and the cardiac OFT at appropriate developmental times and in grossly normal patterns, indicating that FN is not required for CNC migration. Instead, FN is required to maintain normal CNC cell numbers, by promoting CNC survival and proliferation (Mittal et al. 2010).

FN is alternatively spliced at EIIIA, EIIIB, and V regions, and there is evidence that alternative splice variants of FN containing EIIIA and/or EIIIB exons are also involved in modulating CNC development. (Astrof et al. 2007; Astrof and Hynes 2009; Peters and Hynes 1996). The sequences of EIIIA and EIIIB are virtually 100 % conserved among vertebrates, while the sequence similarity between EIIIA and EIIIB is low. Alternative splicing of these exons is dynamically regulated such that they are nearly always included into FN protein around newly developing blood vessels during embryogenesis or in pathologies involving new blood vessel growth, while their expression is undetectable around mature, quiescent vasculature in an adult (Astrof and Hynes 2009; Ffrench-Constant and Hynes 1989; Ffrench-Constant et al. 1989; Peters et al. 1996). Concurrent deletion of EIIIA and EIIIB exons from the *FN* gene leads to embryonic lethality depending on the genetic background (Astrof et al. 2007). 80 % of EIIIA/EIIIB-double nulls die by E10.5 and exhibit severe cardiovascular defects on mixed 129/C57BL/6J genetic background. Of note are the small size of pharyngeal arches, dilated arch arteries, and paucity of mesenchymal cells in the cardiac OFT, suggesting that EIIIA and/or EIIIB isoforms of FN play important role(s) in the development of the CNC (Astrof et al. 2007). Further studies are needed to explore potential roles of these splice variants in CNC development and in morphogenesis of the cardiac OFT and AAAs.

5.3.4 *FN mRNA Is Dynamically Regulated During Embryogenesis*

Detailed knowledge of cellular source(s) of ECM proteins and the identity of their target cell populations is important for understanding the role of ECM during organ morphogenesis. Examination of *FN* mRNA and protein expression at different stages of embryogenesis indicated that the expression of this gene is dynamically regulated in time and space (Mittal et al. 2010; Pulina et al. 2011). For example, by the late headfold stage, FN protein surrounds the ventral node, and absence of FN protein in global FN-null embryos leads to defective intercalation of cells within the node leading to piling of nodal cells on top of each other and defective morphogenesis of the node (Pulina et al. 2011). This leads to aberrant specification of the left–right asymmetric body plan, which in turn is required for the proper asymmetric development of the cardiovascular system (Ramsdell 2005). At E8.0, *FN* mRNA is highly expressed by the lateral plate mesoderm, by anterior definitive endoderm, and by endothelial cells of the nascent heart tube. At a slightly later time point, in

embryos containing 8–10 somites, *FN* mRNA is upregulated in the NC as well as in the pharyngeal pocket endoderm, in pharyngeal surface ectoderm, and in splanchnic mesoderm, containing presumptive myocardial progenitors (Mittal et al. 2010).

At E9.5, *FN* mRNA is maintained in the dorsal neural tube and is present in the newly emigrating NC cells (Mittal et al. 2010). At this time, *FN* is also expressed at distinct locations within pharyngeal epithelia: pharyngeal pocket endoderm and surface ectoderm (Figs. 5.2f and 5.3a) and in the pharyngeal mesoderm. Accordingly, we found that FN protein is enriched in specific embryonic locations, which correlate with the "hot spots" of *FN* mRNA (Figs. 5.3g, i, j, and 5.2a). Pharyngeal endoderm, ectoderm, and mesoderm comprise pharyngeal microenvironment regulating CNC cell proliferation, survival, differentiation, and remodeling of the AAAs and the cardiac OFT. The presence of FN in this area suggests that this protein could play an important role in intercellular and inter-tissue interactions regulating development of organs containing NC-derived cells.

Our mRNA expression studies indicated that *FN* is expressed by populations of progenitors known to give rise to all components of the heart and the cardiac OFT vasculature: CNC, precardiac mesoderm, endothelium, and epicardium. Being highly conserved among vertebrates, the distinct spatiotemporal distribution of *FN* mRNA and protein implies that tissue-specific and timed synthesis of FN is biologically important. What is the significance of tissue-specific expression of FN (or other ECM macromolecules)? And is there a difference in intracellular signaling responses to paracrine and autocrine sources of FN? Recent studies indicated that tissue-specific synthesis of FN by breast epithelial cells or by retinal astrocytes plays important roles in morphogenesis of these organs. FN produced by breast epithelial cells is important for their proliferation and lobuloalveolar development (Liu et al. 2010). FN produced by retinal astrocytes is required for directional, VEGF-dependent migration of blood vessels during retinal angiogenesis (Stenzel et al. 2011b). We also have evidence that tissue-specific synthesis of FN by pharyngeal endoderm, mesoderm, or neural ectoderm in the embryo plays essential roles in morphogenesis of the cardiovascular system (S. Astrof, unpublished). Because FN is essential for cardiovascular development and because cell adhesion to FN is known to modulate a multitude of intracellular signaling pathways in vitro, investigation of tissue-specific autocrine and paracrine functions of FN would provide invaluable insights into mechanisms of intercellular communications essential for cardiovascular morphogenesis.

In summary, nonuniform expression patterns of ECM proteins suggest that NC-derived cells traversing the embryo encounter nonuniform and dynamic tissue microenvironment, containing different gradients and assortments of ECM proteins in time and space. Cell adhesion to different ECM proteins elicits diverse intracellular signaling responses based on the presence of other signaling molecules and on mechanical properties of tissues (Engler et al. 2006; Giancotti and Tarone 2003; Hynes 2009). Studies cited above support the notion that interactions between the NC-derived cells and the ECM modulate NC cell fate and morphogenesis of tissues containing, derived from, or influenced by the transiting NC cells.

5.4 Integrins Are Important for the Development of the Cardiac Neural Crest

Integrins are a major class of cell surface receptors that bind ECM and activate ECM-dependent intracellular signaling cascades regulating cell adhesion, migration, survival, proliferation, self-renewal, and differentiation in a context-dependent manner. There are 18 alpha and 8 beta subunits in mammals; they are known to comprise 24 distinct integrin heterodimers. Integrin beta 1 chain can combine with 12 distinct alpha chains forming 12 different integrin heterodimers with diverse ligand specificities (Hynes 2002). Thus genetic deletion of integrin beta 1 leads to the ablation of 12 distinct integrins, depleting cells from receptors with binding specificities for a wide range of ECM proteins, including laminins, collagens, FN, and other RGD-containing constituents of ECM.

Genetic experiments in mice indicate that integrins play important roles in NC development (Breau et al. 2006) and the CNC in particular. Ablation of integrin beta 1 using Wnt1-Cre transgenic mice leads to embryonic lethality by E12.5 (Turlo et al. 2012). Unexpectedly, CNC migration does not depend on β1-containing integrins. Instead, beta 1-deficient CNC cells cannot support proper development of the pharyngeal arch arteries, and these mutants die due to severe dilation and hemorrhage of these vessels (Turlo et al. 2012). Consistent with this finding, prior experiments in the mouse and chick showed that integrin heterodimers containing alpha 5 or integrin alpha 4 subunits (forming α5β1, α4β1, and α4β7 integrins) were also not required for NC migration (Haack and Hynes 2001). Global ablation of integrin alpha 5 indicated that this integrin was important for proliferation and survival of the CNC (Mittal et al. 2010). Taken together these studies indicate that individual integrin heterodimers or a combination of 12 β1-containing integrins are not required to mediate NC migration.

Cell–ECM interactions mediated by integrins engage a number of downstream signaling cascades, and NC-specific mutations in genes encoding downstream effectors of integrin activation, such as Rac1, Cdc42, and FAK, give rise to defective development of the cardiac OFT and reduced differentiation of CNC-derived cells around pharyngeal arch arteries toward VSMC cell fate leading to aberrant remodeling of the pharyngeal vascular tree (Fuchs et al. 2009; Thomas et al. 2010; Vallejo-Illarramendi et al. 2009).

However, mechanisms by which integrins and their downstream effectors mediate CNC-dependent cardiovascular development are not well understood.

ECM proteins are thought to function as platforms for integrating signaling by diverse components of extracellular milieu and orchestrating intercellular interactions during tissue morphogenesis (Hynes 2009). Analogously, we propose that integrin-mediated cell–ECM interactions in the pharynx integrate combinatorial inputs from a variety of growth factor signaling pathways to orchestrate the complex process of AAA morphogenesis (Fig. 5.2). In the section below, we provide examples of how this integration could take place.

5.5 Roles of Cell–ECM Interactions in Modulating Signaling Pathways During CNC Development and Cardiovascular Morphogenesis

5.5.1 Fibroblast Growth Factor Family

Signaling by fibroblast growth factors (Fgf) Fgf3, Fgf8, Fgf10, and Fgf15 individually and/or in combination is intimately involved in morphogenesis of the cardiovascular outflow system. In vitro studies demonstrated that cell adhesion to FN facilitates Fgf signaling, and it is possible that cell–ECM interactions modulate tissue-specific responses to Fgf in vivo as well (Miyamoto et al. 1996). Fgfs including Fgf8 and other growth factors bind FN (and probably other ECM components), and Fgf8 was shown to function as a morphogen in vivo (Martino and Hubbell 2010; Toyoda et al. 2010). Therefore, FN and probably other ECM macromolecules could facilitate cellular responses to Fgf8 and other growth factors by modulating their spatial distribution and bioavailability. Understanding how and which components of the ECM are important for Fgf8 signaling in vivo is important because Fgf8 plays a requisite (albeit an indirect) role in CNC development and regulates morphogenesis of the cardiac outflow system. Deregulated signaling by Fgf8 is implicated in the pathogenesis of human DiGeorge syndrome, the most common chromosomal microdeletion syndrome in humans (Moon 2006).

While Fgfs signal to CNC cells, Fgf signaling to the CNC is not required for OFT septation and pharyngeal arch artery remodeling (Park et al. 2006, 2008; Zhang et al. 2008). Instead, Fgf signaling to the pharyngeal surface ectoderm, to pharyngeal endoderm, and to cardiac progenitors in the splanchnic mesoderm is required indirectly, in part by regulating BMP signaling, to modulate CNC cell fate, number, proliferation, survival, and activation of MAPK signaling pathway. Consequently, tissue-specific autocrine Fgf8 signaling to surface ectoderm is required for the formation of the fourth pharyngeal arch arteries, and autocrine signaling to precardiac mesoderm and pharyngeal endoderm mediates rotation and septation of the cardiac OFT. Interestingly, in addition to regulating gene expression of transforming growth factor family members, Fgf8 signaling regulates expression of semaphorins and plexins. Thus defective septation of the cardiac OFT in the conditional Fgf8;Isl1$^{Cre/+}$ mutants may be due in part to defective navigation of the CNC cells (Cai et al. 2008; Macatee et al. 2003; Moon 2006; Park et al. 2006, 2008; Urness et al. 2011; Vincentz et al. 2005).

The precise complement of intracellular components mediating Fgf signaling is not fully understood. Similar to other RTKs, cell adhesion to FN leads to Fgf receptor (FGFR) phosphorylation in a ligand-independent manner (Zou et al. 2012). Downstream signaling mediated by FN via integrins and by Fgfs via FGFRs may involve common intracellular mediators activated by both pathways, including FAK, src family kinases, src substrate, p130^{Cas}, and Crkl (Li et al. 2003; Zou et al. 2012). In vivo signaling by Fgf8 is mediated in part by phosphorylation of

FRS2α and Crkl (Moon et al. 2006; Zhang et al. 2008). Fgf-mediated phosphorylation of Crkl and Erk1/2 in vivo is dependent on FAK, a well-known downstream effector of integrin signaling (Vallejo-Illarramendi et al. 2009). Global deletion of Crkl leads to similar (albeit milder) phenotypes seen in hypomorphic Fgf8 mutants (Moon et al. 2006). Upon integrin-mediated cell–ECM adhesion, Crkl associates with $p130^{Cas}$ and becomes recruited to focal adhesions in integrin-, src-, and $p130^{Cas}$-dependent manner (Li et al. 2003). In this light, it would be interesting to investigate the detailed cardiovascular abnormalities in global $p130^{Cas}$ mutants, which are reported to die at about E11.5–E12.5 from severe cardiac and vascular defects (Honda et al. 1998). Crkl can also facilitate Fgf8 signaling in adhesion-independent manner, by binding directly to the phosphorylated Tyr463 within the YELP motif of FGFR1 upon cell stimulation with Fgf8. This binding leads to activation of Rac1, Cdc42, and PAK in DOCK180-dependent manner and allows full activation of Erk1/2, Raf1, and MEK1 (Seo et al. 2009). The importance of these studies is underscored by the finding that Crkl is required for NC survival in vivo (Moon et al. 2006). Significantly, Crkl is located within the 22q11 region commonly deleted in human DiGeorge patients (Moon et al. 2006). Therefore, mutations in Fgf8, Crkl, and $p130^{Cas}$ and in other mediators of Fgf8 signaling may contribute to the severity and variability of symptoms observed in these patients. As mentioned above, ECM may facilitate Fgf signaling by binding to and localizing Fgf ligands at specific embryonic locations. In addition, syndecans serve as common co-receptors for FN-mediated integrin signaling and Fgf-mediated FGFR signaling (Arrington and Yost 2009; Horowitz et al. 2002; Saoncella et al. 1999), suggesting the existence of common FN–integrin–syndecan–Fgf–FgfR signaling complexes in cells. Taken together, there exists compelling evidence for the crosstalk and possible synergy between FN-, integrin-, and Fgf-mediated signaling pathways. Future studies investigating details of these interactions would provide important insights into mechanisms underlying defects in cardiovascular morphogenesis in human patients with congenital heart defects.

5.5.2 Platelet-Derived Growth Factor Family

Signaling by platelet-derived growth factor (PDGF) ligands is mediated by homo- or heterodimers of PDGF receptors α and β. Concurrent deletion of both PDGFRs from the NC is early neonatal lethal due to cardiovascular insufficiency and results in 100 % penetrant defects in thymic development, ventricular septation, cardiac OFT septation, and premature regression of the right fourth pharyngeal arch artery, leading to the presence of vascular rings around trachea (Richarte et al. 2007). These abnormalities are classically attributed to defective development of the CNC. Formation, delamination, migration, proliferation, and survival of CNC cells were not significantly affected in NC-specific PDGFRα/β-double null mutants. Interestingly, there were fewer CNC cells present within the cardiac OFT implying a role for CNC-specific PDGF signaling in the navigation of CNC cells into the heart

(Richarte et al. 2007). Usually, defective persistence and remodeling of pharyngeal arch arteries is associated with low numbers or defective differentiation of CNC cells into αSMA+ VSMCs. However, normal numbers of αSMA+ cells were present around the AAAs between E11.5–E13.5, the window of time during which the symmetric pharyngeal arch artery vascular tree attains its mature asymmetric configuration (Fig. 5.1b). Expression of PDGFR ligands by the pharyngeal epithelia (PDGF-AA) and endothelium (PDGF-BB) suggests that signaling interactions between pharyngeal microenvironment and CNC play requisite roles in asymmetric remodeling of pharyngeal arch arteries even in the presence of normal numbers and differentiation of CNC-derived VSMCs (Richarte et al. 2007).

Seminal studies in the 1990s demonstrated that cell adhesion, and in particular, cell adhesion to FN mediated by integrin α5β1, is required for RTK activation and signaling downstream of EGF, PDGF, and FGF receptors (Miyamoto et al. 1996). Recent studies indicate that cell adhesion leads to pre-activation of RTKs in a ligand-independent manner and potentiates growth factor signaling (Giancotti and Tarone 2003). In case of PDGF signaling, cell adhesion to FN (but not to collagen I, IV, or LM-111) leads to a ligand-independent activation of PDGFRs, mediated by α5β1 and/or by αv-containing integrins (Veevers-Lowe et al. 2011). Extracellular domains of PDGFRs and integrin α5β1 interact by virtue of their binding to heparin sulfate (FN is heparinated) and co-localize in lamellipodia of migrating cells. FN and PDGF-BB additively activate PDGFRβ signaling, and PDGF-BB can directly bind to the extracellular domain of integrin α5β1 (Veevers-Lowe et al. 2011). Cell adhesion to FN enhances PDGF-BB-mediated cell migration in integrin α5β1-dependent manner. FAK is required for FN- and integrin α5β1-mediated phosphorylation of PDGFRβ and for the subsequent phosphorylation and activation of Akt. Taken together, these experiments suggest that cell–FN adhesion regulates PDGF signaling and directional migration by a combination of at least two modes: (1) by facilitating assembly of integrin–FN–growth factor–RTK signaling complexes and (2) by activating integrin signaling (Veevers-Lowe et al. 2011).

5.5.3 Vascular Endothelial Growth Factor Family

Vascular endothelial growth factor (VEGF) family consists of VEGF-A, VEGF-B, VEGF-C, and VEGF-D members, which have different roles in vascular and lymphatic development in normal and pathological conditions (Lohela et al. 2009). VEGF-A growth factors exist in several isoforms arising from alternative splicing of a single gene. VEGF-A isoforms of increasing lengths have increased capacity for binding ECM macromolecules, including FN, and these interactions potentiate VEGF-A signaling to cells (Wijelath et al. 2002). The shortest VEGF-A isoform, VEGF-A_{120}, does not bind ECM. Individual VEGFs play integral roles in vascular development, and global VEGF-A haploinsufficiency in $VEGF^{+/-}$ mutants leads to early embryonic lethality due to severe vascular insufficiency (Carmeliet et al. 1996). Soluble and matrix-bound VEGF-A isoforms play distinct roles in

cardiovascular development (Stalmans et al. 2003; van den Akker et al. 2007). VEGF-A_{164} isoform has intermediate ECM-binding capacity compared with VEGF-A_{120} and VEGF-A_{188} and is the only VEGF-A isoform that can bind Nrp1 (Lohela et al. 2009). Mice lacking VEGF-A_{164} isoform (e.g., mutants expressing either VEGF-A_{120} or VEGF-A_{188} isoforms) develop a range of facial, thymic, and cardiovascular abnormalities similar to those observed in patients with DiGeorge syndrome, and mutations in the promoter of human VEGF-A are associated with increased severity in cardiovascular defects of DiGeorge patients (Stalmans et al. 2003). Cardiovascular abnormalities in mice lacking VEGF-A_{164} include defective septation of the cardiac OFT and aberrant/random remodeling of AAAs. Interestingly, *VEGF-A* mRNA is highly expressed in the pharyngeal pocket endoderm, while its co-receptor Nrp1 is expressed in the pharyngeal endoderm, in arch core mesoderm, and in NC-derived cells; Nrp1 mutants also exhibit defective septation of the cardiac OFT and aberrant remodeling of the AAAs (Kawasaki et al. 1999). These observations suggest that signaling by VEGF-A within the pharyngeal microenvironment and/or to the CNC is important for CNC development and vascular morphogenesis. Signaling by VEGF-A_{164} regulates transcription of *Tbx1*, a gene located within human 22q11 interval and often mutated in DiGeorge patients; *VEGF-A* and *Tbx1* genetically interact to mediate the proper development of pharyngeal arch arteries (Stalmans et al. 2003). Taken together, these studies support the notion that VEGF-A_{164}-Nrp1-Tbx1 axis indirectly regulates CNC development, vascular patterning, and penetrance of cardiovascular congenital phenotypes in human patients (Calmont et al. 2009; Guris et al. 2006).

Recent studies indicate that cell adhesion to ECM via β1-containing integrins is required for differential signaling activities elicited by soluble and matrix-bound VEGF-A_{164} isoforms. Matrix-bound but not soluble VEGF promotes integrin β1–VEGFR2 association and trafficking to focal adhesions. This association is required for the prolonged (~30 min) phosphorylation of specific VEGFR2 residues induced by the matrix-bound (but not soluble) VEGF and activation of P38 downstream of VEGFR2. In contrast, VEGFR2 phosphorylation by soluble VEGF is downregulated within 5 min of stimulation and does not lead to activation of P38 (Chen et al. 2010). Taken together, these studies suggest that cooperation between matrix-bound VEGF and signaling pathways engaged by cell–ECM interactions facilitate VEGF-mediated septation of the cardiac OFT and remodeling of the pharyngeal arch arteries.

5.5.4 Sonic Hedgehog

Sonic hedgehog (Shh) signaling to the CNC is tightly regulated. Either increased or reduced Shh signaling to the CNC-derived cells leads to defective cardiovascular development (Goddeeris et al. 2007). Shh produced by the pharyngeal endoderm is required for CNC survival and consequently for the aorticopulmonary septation and arch artery remodeling (Goddeeris et al. 2007). Autocrine Shh signaling to the

endoderm is also important for these processes, while paracrine Shh signaling to mesodermal precursors of the cardiac OFT is important for the OFT septation and arch artery remodeling. These studies indicate that autocrine signaling by Shh to the pharyngeal endoderm and paracrine signaling to early cardiac precursors lead to production of a secreted factor(s), modulating CNC cell fate and vascular remodeling; however, mechanisms of this regulation are not well understood (Goddeeris et al. 2007).

How cell–ECM interactions regulate Shh-mediated signaling and cardiovascular development within the pharyngeal microenvironment is not well understood; however, they are probably important since cell–ECM communications are known to modulate Shh activity (and vice versa) in other biological contexts. For example, conditional ablation of β1-containing integrins in intestinal epithelial cells leads to downregulation of Shh, Ihh, and FoxA2. Conversely, ectopic expression of integrin β1 leads to increased expression of Shh and FoxA2 in a manner dependent on activation of Akt, one of the downstream integrin effectors (Jones et al. 2006).

Extracellular ligands, mechanical forces, and intracellular factors can activate integrin signaling by modulating integrin's three-dimensional conformation (Astrof et al. 2006; Hynes 2002). Shh negatively modulates NC cell adhesion and migration by inhibiting conformational changes that lead to integrin β1 activation but not its cell surface expression (Fournier-Thibault et al. 2009; Testaz et al. 2001). This inhibition is mediated by solid phase rather than soluble Shh, and remarkably, it is independent of Shh-mediated gene transcription, translation, cell fate specification, or signaling involving the Patched-Smoothened-Gli pathway (Fournier-Thibault et al. 2009; Jarov et al. 2003). These experiments show that in addition to and independent of its role in patterning the neural tube (NT), Shh modulates the balance between cell–cell and cell–ECM adhesion by modulating integrin activation in NC progenitors in vivo (Fournier-Thibault et al. 2009). Ectopic expression of Shh in the NT abrogates activation of integrin β1, attenuating cell–ECM interactions necessary for the detachment of the NC cells from the NT, and promotes cell–cell adhesion by activating Cad6B, Daam1, and RhoB. These changes in cell–cell and cell–ECM interactions occur much earlier than the expected alterations in dorsal–ventral NT cell fates induced by ectopic Shh. The inhibitory role of Shh in integrin activation and NC cell adhesion and migration can be counteracted by agents that artificially activate integrins, such as Mn^{2+} (Fournier-Thibault et al. 2009) but not by cyclopamine or forskolin, known inhibitors of canonic Shh signaling. Reducing the dose of Shh in vivo by sequestration of Shh leads to excessive delamination of NT cells (Testaz et al. 2001). Interestingly, the inability of CNC to regenerate following ablation of the CNC progenitor territory in the dorsal NT in the chick has been linked to excessive Shh signaling (Hutson et al. 2009). Therefore, it would be interesting to determine whether CNC regeneration in this model could be induced by integrin-activating agents such as Mn^{2+}. The role of a potential cross talk between Shh and cell–ECM signaling pathways within the pharyngeal microenvironment in CNC development, OFT septation, and arch artery remodeling remains to be investigated.

5.5.5 *Wnt*

Wnt signaling to the NC is important for the maintenance/abundance of NC progenitors as well as specification/balance of NC progenitor cell fates (Hari et al. 2002, 2012; Ikeya et al. 1997). Wnt signaling regulates and is regulated by cell–ECM interactions (De Langhe et al. 2005; Rallis et al. 2010); however, the role(s) of cell–ECM interactions and Wnt signaling during cardiovascular morphogenesis are not well understood.

5.5.6 *Notch*

Notch signaling to the CNC and to cardiac progenitors of the OFT myocardium is important for regulating OFT septation and vascular remodeling in the pharynx and has been the subject of several recent reviews (Jain et al. 2010; Rentschler et al. 2010). Recent in vivo studies indicate that cell–ECM interactions modulate Notch signaling during embryogenesis. For example, α4 chain-containing laminins facilitate blood vessel morphogenesis by regulating Notch signaling through integrin β1-mediated upregulation of *VEGFR2* and *Dll4* mRNA (Stenzel et al. 2011a). Signaling by β1-containing integrins during somitogenesis is important for activation of Notch signaling in a cell-autonomous manner by activation of integrin-linked kinase (ILK) (Rallis et al. 2010). It will be interesting to investigate whether cell–ECM interactions modulate Notch signaling during OFT septation and arch artery remodeling.

5.5.7 *Transforming Growth Factor Beta Family*

Signaling by transforming growth factor beta (TGFβ) family members to the CNC is required for the OFT septation and pharyngeal arch artery remodeling, in part by regulating differentiation of CNC cells into VSMCs and expression of αSMA (Kaartinen et al. 2004; Stottmann et al. 2004; Stottmann and Klingensmith 2011; Wurdak et al. 2006). Numerous BMP and TGFβ ligands and their antagonists orchestrate development of the CNC and CNC-dependent cardiovascular morphogenesis (Kirby and Hutson 2010). TGFβ ligands regulate synthesis of the ECM proteins (Massague 1998); conversely cell–ECM interactions play an integral role in regulating signaling by TGFβ family members (Shi et al. 2011). Most members of this superfamily are synthesized in the inactive, latent form. At least two modes of regulation are known to activate TGFβ ligands: (1) by proteolysis, to release TGFβ homodimers from their pro-domains; this could be facilitated by association between αv-containing integrins with cellular or secreted metalloproteinases or (2) by mechanical forces, via integrin αv-mediated pulling and stretching of the inactive, TGFβ-containing latent complexes (Wipff and Hinz 2008).

Members of TGFβ family of proteins are synthesized as pro-peptides; their pro-domains are cleaved off during exocytosis but remain non-covalently associated with the mature TGFβ homodimers. These complexes (termed small latent complexes, SLCs) render TGFβ inactive. TGFβ–SLCs are covalently bound to latent TGFβ-binding proteins (LTBPs 1, 3, or 4), forming large SLC–LTBP latent complexes via disulfide bonding of the pro-domain to the LTBPs (Todorovic et al. 2005). In the extracellular milieu, LTBP1 is covalently cross-linked to ECM proteins including FN, VN, and/or fibrillin via transglutamination (Wipff and Hinz 2008). TGFβ-containing SLC–LTBP1 complexes are inactive but can be activated in vivo by proteolytic cleavage severing the covalent association of the pro-domain with LTBP and releasing active TGFβ homodimers free of their pro-domains or by a pulling force exerted by cells on SLC–LTBP1 complexes (Munger et al. 1999). The latter has been best illustrated for the activation of TGFβ1–SLC–LTBP1 complexes. The pro-domains of TGFβ(1–3) have an RGD motif that can bind to and activate αv-containing integrin heterodimers. The tensile force generated by the interactions between integrin cytoplasmic tails and the actin cytoskeleton exerts a pulling force on the SLC and is counteracted by the covalently bound LTBP1 anchored to the ECM, leading to stretching of the SLC–LTBP1 complex (McMahon et al. 1996; Sivakumar et al. 2006). Upon stretching of the SLC–LTBP1, the embedded TGFβ homodimers pop out, and free from their pro-domains, they are competent to bind TGFβ receptors and activate downstream signaling; mutations that interfere with stretching of the SLC–LTBP1 complex inhibit release of the active TGFβ (Shi et al. 2011).

Consistent with these studies, TGFβ signaling is downregulated in global LTBP1 mutant mouse embryos, which develop lethal cardiovascular defects characterized by aberrant septation of the cardiac OFT and AAA remodeling defects (Todorovic et al. 2007). In addition, elegant recent studies demonstrated the role of αvβ8-mediated activation of TGFβ signaling during remodeling of retinal vasculature (Hirota et al. 2011). In these studies, αvβ8 integrin expressed by retinal astrocytes is required to activate TGFβ signaling in the closely apposed endothelial cells of retinal blood vessels, and the absence of αv or β8 on the astrocytes leads to reduced TGFβ response in the endothelial cells, blunted endothelial tip cell morphology, and aberrant development of the retinal vascular plexus (Hirota et al. 2011). FN expressed by retinal astrocytes is also required for retinal vascularization by modulating directionality, alignment, and numbers of endothelial filopodia, in part by binding to and facilitating signaling by VEGF-A (Stenzel et al. 2011a).

The importance of ECM proteins in modulating TGFβ signaling and human pathology is underscored by the discovery that integral ECM proteins fibrillins modulate activity of TGFβ and BMPs (Neptune et al. 2003; Nistala et al. 2010). Fibrillin-1 binds and modulates activity of TGFβs, and deficiency of fibrillin-1 causes human Marfan syndrome characterized in part by dilation and rupture of the ascending aorta (Neptune et al. 2003). Decrease or absence of fibrillins causes increased availability of active TGFβ, leading to increased canonical and noncanonical TGFβ signaling (Holm et al. 2011). Interestingly, the increase in noncanonical TGFβ signaling pathway mediated by activation of Erk1/2 appears to be the

main cause of abnormal aortic pathology in the fibrillin-1 mutant mouse model of Marfan syndrome (Habashi et al. 2011; Holm et al. 2011). In conjunction with integrins, abundance, composition, and mechanical compliance of the ECM modulate activity of TGFβ family members by regulating mechanical properties of the microenvironment (Wipff and Hinz 2008). For example, while integrin α5 does not appear to bind TGFβ-containing SLC complexes, FN fibrillogenesis mediated by integrin α5 is important for αvβ6-mediated TGFβ signaling, and activation of latent TGFβ is compromised in FN-null or integrin α5-null cells (Fontana et al. 2005). Because FN binds LTBP1 and facilitates activation of latent TGFβ1-containing complexes (Fontana et al. 2005) and because both LTBP1 and FN are synthesized by the endoderm within the pharyngeal microenvironment, it would be interesting to examine whether FN regulates TGFβ signaling and AAA remodeling.

5.5.8 Semaphorins, Plexins, and Neuropilins

Semaphorin signaling is essential for navigation of NC-derived cells through the embryo, for the proper development of the CNC and, subsequently, for septation of the cardiac OFT and AAA remodeling (Feiner et al. 2001; Schwarz et al. 2008; Toyofuku et al. 2008). CNC cells traversing the embryo encounter repellent and attractant types of semaphorins. Recent experiments in the chick and mouse suggest that cues provided by the action of several diffusible types of semaphorins expressed at the time of CNC migration and their receptors expressed by the CNC cells could facilitate (a) exit of the CNC from the dorsal neural tube, (b) navigation of the CNC to their destinations, and (c) CNC entry into the cardiac OFT (Feiner et al. 2001; Schwarz et al. 2008).

Semaphorin and neuropilin signaling regulate (and could be probably regulated by) cell–ECM interactions during vascular remodeling. Class 3 semaphorins, Sema3A and 3F, provide repulsive cues for NC migration by signaling through NC-expressed Nrp1 and Nrp2, to pattern cranial neurons and ganglia (Schwarz et al. 2008). This mode of action is independent of the requirement for Nrp signaling to the endothelium, which plays an important role in vascular remodeling (Kawasaki et al. 1999; Schwarz et al. 2008). Signaling by Sema3A and 3F inhibits integrin activation in vitro and in vivo, negatively regulates directional persistence of migrating vascular endothelial cells, and is required for remodeling of the primitive vascular plexus in chick and mouse embryos into the stereotypic, yet nonuniform plexus of large and small blood vessels (Serini et al. 2003). Nrp1 regulates integrin-mediated endothelial cell adhesion in a manner that is separable from its roles in VEGF-mediated activation or Sema3-mediated inhibition of cell adhesion (Valdembri et al. 2009). Interestingly, Nrp1 potentiates cell adhesion to FN but not to other large ECM glycoproteins vitronectin, collagen I, or LM-111, suggesting that composition of extracellular microenvironment imparts specificity to Nrp1-mediated vascular remodeling. Nrp1 potentiates integrin α5β1-dependent cell spreading and FN matrix assembly on cell surfaces by facilitating integrin

endocytosis and recycling. The extracellular portion of Nrp1 binds activated integrin α5β1, while the intracellular SEA motif present within the short, 50-amino acid cytoplasmic tail of Nrp1 facilitates binding of Nrp1 to endocytic adaptor protein GIPC1 and a minus-directed motor protein Myo6 and targets activated α5β1 integrins into Rab5/Rab21-containing endocytic vesicles, which rapidly recycle back to the plasma membrane. These experiments demonstrated that endothelial Nrp1, GIPC1, and Myo6 are required to stimulate α5β1-mediated cell adhesion to FN (Valdembri et al. 2009).

Cardiovascular defects in Nrp1-null embryos are similar to those observed in Sema3C mutants, suggesting that Sema3C signaling mediated by Nrp1 participates in AAA remodeling and OFT septation (Feiner et al. 2001; Kawasaki et al. 1999). Interestingly, Sema3C signaling to endothelial cells mediated by endothelial PlexinD1 and its Nrp co-receptors is required for cardiac OFT septation and AAA remodeling (Gitler et al. 2004; Zhang et al. 2009). Thus a possible mechanism underlying aberrant remodeling of pharyngeal arch arteries in Nrp1, PlexinD1, and Sema3C mutants could be due to defects in integrin-mediated cell–ECM interactions and signaling. In this regard, NC-specific mutations in genes encoding downstream effectors of integrin activation, such as Rac1, Cdc42, and FAK, give rise to defective development of the cardiac OFT and reduced differentiation of CNC-derived cells around pharyngeal arch arteries toward smooth muscle cell fate and aberrant remodeling of the pharyngeal arch arteries (Fuchs et al. 2009; Thomas et al. 2010; Vallejo-Illarramendi et al. 2009). Intriguingly, FAK-deficient CNC cells within the OFT do not express Sema3C (Vallejo-Illarramendi et al. 2009), implicating integrin-mediated activation of FAK in regulation of semaphorin expression and signaling. Future studies will determine whether cell–ECM interactions modulate attractive and/or repulsive properties imparted by semaphorin signaling to endothelial, NC, or other cell types during cardiovascular development.

Mutations leading to decreased expression of Sema3C and PlexinA2 have been observed in human patients with defective septation of the cardiac OFT (Kodo et al. 2009). These mutations disrupted the function of the transcription factor Gata6, which directly binds to and activates transcription from Sema3C and PlexinA2 promoters (Kodo et al. 2009; Lepore et al. 2006). Function of Gata6 in the CNC cells is required for the septation of the cardiac OFT and remodeling of AAAs (Lepore et al. 2006). Therefore, understanding genes, pathways, and interactions regulating expression and signaling by semaphorins and plexins would provide further insight into human congenital cardiovascular disorders.

5.6 Conclusions

ECM components such as FN, laminins, collagens, and others have long been known to modulate growth factor signaling in vitro; however, signaling pathways engaged by the ECM macromolecules in vivo and especially during embryogenesis

are not well understood. Examples given above illustrate how cell–ECM interactions could facilitate signaling by a variety of growth factors important for the proper morphogenesis of the cardiac outflow vasculature. We hypothesize that the dynamic and nonuniform distributions of ECM macromolecules within embryos impact combinatorial signaling outcomes and orchestrate inter-tissue communications, which affect cellular positions, shapes, fates, and functions during OFT septation and AAA morphogenesis.

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Chapter 6
Engineered ECM Microenvironments and Their Regulation of Stem Cells

Yu Suk Choi, Andrew W. Holle, and Adam J. Engler

Abstract Stem cells possess numerous therapeutic benefits since it is possible to reproducibly control their ability to mature into different cell types even after prolonged culture in vitro; this ability makes stem cells well suited for tissue engineering and regenerative applications. Consequently, understanding stem cell differentiation is a crucial step for those applications. Regulating stem cell fate has traditionally relied on presenting small molecules such as growth factors and cytokines in developmentally appropriate ways, but such a view overlooks other important niche characteristics. Recently, extracellular matrix (ECM) properties have been shown to influence cellular behavior independent of chemical signals, and this has shifted the differentiation paradigm to include ECM properties, e.g. topography, stiffness, composition, porosity, and cell shape/size. Recent advances in bioengineering have enabled versatility in patterning cell types with controlled chemistries, geometries, and sizes. In this chapter, we detail the recent advances in nano- or microfabrication techniques, the biomechanical- and biophysical-driven stem cell differentiation, and the mechanism of how cells "feel" their ECM environment.

6.1 Introduction

Tissue engineering and regenerative medicine aim to repair and/or regenerate injured or diseased tissues by implanting a combination of cells, biomaterials, and/or soluble factors (Atala 2008; Vacanti and Langer 1999); tissue-engineering strategies generally focus on fabrication using those three building blocks

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ex vivo, whereas regenerative medicine generally relies on guiding the host's healing capabilities. In both approaches, there is a need for cell sources that can function as mature cells, support endogenous cells, synthesize a three-dimensional extracellular matrix (ECM), or release paracrine factors. Adult and embryonic stem cells possess promising characteristics that make them ideal candidate cell sources for these applications. Stem cells have the ability to self-renew, i.e. replicate into identical daughter cells, and to be at least multipotent, i.e., be able to mature into tissue cells of many different lineages (or as with embryonic stem cells—ESCs—they must be pluripotent and able to mature into all cell types). When stem cells are isolated from their in vivo niche, differentiation is often less controlled, easily misdirected, and results in high heterogeneity (Ding and Schultz 2004). For these reasons, it is important to understand the underlying mechanisms in stem cell self-renewal and differentiation and to find the right switches for differentiation into specific lineages.

Many studies have used biochemical approaches by culturing cells in a media cocktail with growth factors, sera, etc. adopted from culture media for those mature cell types to induce stem cells to become those lineages. More recently, the interaction between cells and their three-dimensional, fibrillar extracellular matrix (ECM) has been shown to be regulated by properties of the matrix that arise from its composition (Flaim et al. 2005) and organization (Reilly and Engler 2010). As the matrix assembles, forming either a denser network or additional cross-links between fibrils, its biophysical properties change: it becomes less porous, rougher, stiffer, etc. These characteristics make it more than a simple scaffold to which cells adhere. Significant efforts have shown that matrix topography (Dalby et al. 2007), stiffness (Engler et al. 2006), and structure/cell shape (McBeath et al. 2004) can dictate stem cell fate. In this chapter, we will highlight fascinating results indicating both the extent to which ECM factors control stem cell fate and the mechanisms that regulate this process. More specifically, we will focus the discussion on the effects that biophysical properties have on the stem cell fate decision process and the potential mechanisms underlying mechanotransduction in stem cells. Given the variety of stem cells and their differing potency, we will largely restrict our examples to those using adult stem cells including bone marrow-derived stem cells (BMSCs) and adipose-derived stem cells (ASCs), both of which are mesenchymal in origin.

6.2 Topography

Cell adhesion to ECM is a necessary feature in many tissues for fundamental cellular behavior such as proliferation, migration, differentiation, development, and death (Gumbiner 1996). Through adhesions, cells can sense the important ECM property of surface topography, which ranges from nano- to microscale features such as matrix fibrils (Geblinger et al. 2010; Stevens and George 2005;

Vogel and Sheetz 2006). Adhesion-mediated sensing of ECM topography plays a key role in various cellular processes including cell adhesion (Karuri et al. 2004; Ranucci and Moghe 2001), migration (Kaiser et al. 2006; Kim et al. 2009), morphological change (Teixeira et al. 2006), cell growth (Liliensiek et al. 2006; Rowland et al. 2010), and differentiation (Chang et al. 2002). In tissues in vivo, cells reside in natural ECM, which is a complex 3D fibrous structure with a wide distribution of fiber density and spacing. These complicated 3D ECMs provide biochemical, biomechanical, and biophysical cues, all of which are very different with flat 2D tissue culture plates. As such, cell responses to 3D matrices are different from their responses to 2D substrates, even with the same basic material components, e.g. ESCs recognize 2D protein coatings of fibronectin in a fundamentally different way than on 3D matrix (Reilly and Engler 2010).

Different adhesion structures can be observed on 3D matrices compared to 2D protein coatings that arise from differences in which proteins localize to the adhesion. For example, localization of $\alpha_5\beta_1$ and $\alpha_v\beta_3$ integrins, paxillin, selected cytoskeletal components, and tyrosine phosphorylation of focal adhesion kinase (FAK) all change when moving from a surface to a matrix. Moreover, enhanced biological activity within the fibrillar, 3D adhesions can be observed (Cukierman et al. 2001). Even roughened 2D substrates have implicated topographical cues in the regulation of cell fate (see discussion below).

The widespread use of 2D cell culture methods has limited the ability to study cell behavior in 3D. However, new techniques have recently been developed to fabricate 3D materials with topographical diversities at the nano- and microscale. Therefore, it is now possible to learn more about the interaction between stem cells and ECM using reductionist approaches in synthetic models as these methods more closely mimic natural 3D ECM. However, mimicking natural 3D ECM is very complicated and has limited the variety of materials, resulting in many studies utilizing simpler 3D or even pseudo-3D matrices to answer similar questions as we outline next.

6.2.1 Nano- and Microtopography Fabrication Techniques

Compared to flat substrates, 3D matrices better mimic the complexity and surface morphology of the physiological ECM environment. Thus, there is an emerging interest in 3D matrix fabrication with nano- and microtopography tools developed over the past few decades. Conventional methods such as salt-leaching (Levenberg et al. 2003), vacuum-drying (Mikos et al. 1993), and gas-foaming (Montjovent et al. 2005) methods suffered drawbacks due to a lack of interconnectivity between pores as well as a lack of spatial control over the pores' arrangement and location. The use of organic solvents also limited the choice of materials. New fabrication methods permit one to fabricate structures at the length scale of a cell and below, which now allows one to directly investigate a cell's interactions with its material. Initially,

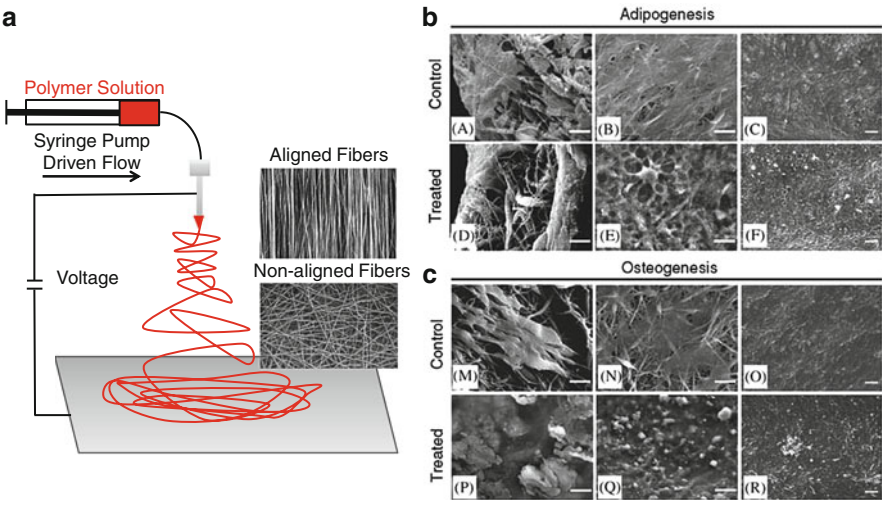

Fig. 6.1 Electrospun ECM mimetic materials. (**a**) Schematic of an electrospinning apparatus. Insets are images of fibers captured from rotating cylindrical substrates (aligned fibers, *top*) and stationary substrates (random fibers, *bottom*). (**b**) Adipogenic and (**c**) osteogenic differentiation of human BMSCs on electrospun poly(ε-caprolactone) nanofibers with or without biochemical factors. Scale bar: 5, 15, 50 μm. Reprinted from Li et al. (2005a)

these lithographic tools were developed to make microchips for small electric devices, but they have been widely adopted by bioengineers and introduced to cell biologists. There are many reviews which cover these techniques in depth (Curtis and Wilkinson 1997; Flemming et al. 1999; Martinez et al. 2009; Seidlits et al. 2008), and we will summarize them here briefly.

6.2.2 *Electrospinning*

Many materials lack the fibrillar structure of matrix, but electrospinning, which involves applying a high-voltage electric field to a jet of polymer solution coming out of a syringe, overcomes this limitation (Li et al. 2002, 2005a; Matthews et al. 2002; Stitzel et al. 2001). Briefly, a syringe or pipette emits a jet of polymer solution, which is collected on a grounded substrate in the presence of an electric field that controls jet dynamics (Fig. 6.1a); if the substrate is rotated during collection, aligned fibers can be produced, otherwise orientation is random (Fig. 6.1a, inset). Various polymer types can be spun into fibers with diameters ranging from several nanometers to tens of microns in diameters. Even though electrospinning requires relatively simple instruments, conditions necessitate optimization to fabricate the desired fiber size, orientation, and uniformity prior to cell culture. Other variables including solvent evaporation rate, polymer solution conductivity, needle to collector distance, and solution viscosity further confound experimental setup, requiring one to pay particular attention to the type and quality

of fibers produced to ensure that it accurately represents a fibrillar and 3D network. Despite these difficulties in controlling polymer properties, electrospinning produces materials that are extremely versatile and may contain mixed fibers (either within a fiber or using fibers of different composition) with tunable orientation, mechanics (based on cross-linking and fiber weaving), and dimensionality. Most commonly, BMSCs have been plated on electrospun materials in differing culture media conditions, but despite growth factor signaling, between each set of material conditions, a varied propensity to differentiate has been observed for adipogenic and osteogenic differentiation (Fig. 6.1b, c) (Li et al. 2005a).

6.2.3 Lithography

One parameter that is difficult to modulate reliably with electrospinning is porosity, which is often affected by fiber density, alignment, etc. Nano- and microfabrication using lithographic techniques have also been used to build 3D structures similar to matrix, but these methods have direct control over the important parameters that are not addressed with electrospinning. The most popular lithographic methods use light-sensitive polymers called photoresist and photomasks that allow one to selectively expose the polymer to light to make simple micron-scale features. To briefly outline the process illustrated in Fig. 6.2a (steps i–v indicated below), photoresist polymer is spin coated onto a wafer (e.g., silicone or glass, i), photomask is placed on top of the film to selectively protect regions of the wafer (ii), and the wafer is exposed to UV light (iii). The exposed region will remain as a positive or negative feature depending on the type of photoresist material after it is developed in a buffer (iv). With the template wafer generated by photolithography, the features can be transferred to elastomers or polymers such as polydimethylsiloxane (PDMS) or polyacrylamide (PA) gel easily using soft lithography (v).

Unfortunately, the number of polymers that can be used in this technique is limited as many react poorly with the photoresist. Feature size is also limited by diffraction limitations, i.e., the maximum resolution is dependent on the wavelength of the light source used during photoresist exposure (>200 nm) (Flemming et al. 1999). More sophisticated versions of lithography such as electron beam lithography (EBL) (Dalby et al. 2007) and nano-imprint lithography can achieve higher resolution down to 3 nm. The latter tool especially can generate complex hierarchical 3D structures. Despite the capability to generate complicated and precise features, the fabrication process can be both expensive and time consuming and also requires special equipment.

3D features can also be fabricated with other microscale lithographic methods such as stacked polymer films with micro-features. This method is utilized to stack 5–35 layers of polymer sheets containing network features of vascular channels that are connected to adjacent networks vertically by machined-through holes (Bettinger et al. 2005) (Fig. 6.2b, c). A temperature-sensitive cell sheet technique can also generate 3D tissue-like structures by stacking cell sheets (Takahashi et al.

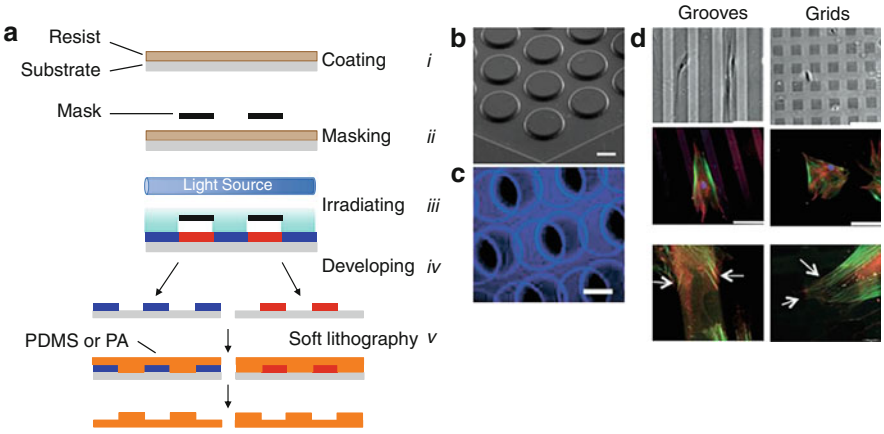

Fig. 6.2 Lithographically produced ECM. (**a**) Schematic diagram of the photolithographic process. (**b**) Poly(glycerol sebacate) (PGS) microfluidic devices casted from silicon mold. (**c**) Composite fluorescent micrographs of PGS after flowing DAPI solutions to demonstrate the patency of multilayer cell culture system. Scale bar: 200 μm. Reprinted from Bettinger et al. (2005). (**d**) Representative images of human BMSCs on hydrogenated amorphous carbon (a-C:H) groove and grid nanopatterns at day 7. Human BMSCs aligned along the grooves and adopted the shape of the grids. *Arrows* indicate vinculin expression at focal adhesions. Scale bar: 100 μm. Reprinted from Martino et al. (2009)

2011). The three-dimensional printing technique is another method of fabricating biomaterials with very defined topography using computer-aided design models and an advanced inkjet printer (Mironov et al. 2003). 3D printing allows precise control of the architecture design and the interconnectivity of pores. Despite its advantages, 3D printing uses organic solvents that can limit the feasibility of incorporated soluble factors, and an optimization of material-solvent ratios is required to attain desired features.

6.3 3D Topography of ECM as a Regulator of Stem Cell Fate

As outlined above, tissue engineering has undergone a biomimetic renaissance; in a field originally driven by novel polymer chemistry, the paradigm has been shifting towards incorporating a better understanding of matrix biology into scaffold design, making scaffolds more biocompatible, increasing cell viability, decreasing cytotoxicity, and increasing adhesion. This has resulted in a series of discoveries on how stem cell fate is controlled using topographical cues. For example, using the fact that matrix is fibrillar, Hashi and coworkers found that human BMSCs were viable and aligned on fibronectin-coated microspun poly(L-lactic acid) (PLLA); the fibril size mimicked the size of collagen fibrils (0.4–1 μm in diameter). They also showed that human BMSCs attached significantly faster to collagen-coated

electrospun PLLA nanofibers (~400 nm in diameter) than to flat collagen film (Hashi et al. 2007). Thermo-responsive hydroxybutyl chitosan nanofibers have also been used to differentiate BMSCs. Unlike previous efforts where growth factors were added (Li et al. 2005a), here the material alone was shown to differentiate BMSCs into a myogenic lineage without any biochemical induction, as assessed by nuclear elongation and expression of myogenin, desmin, pax7, and collagen IV (Dang and Leong 2007). This material also displayed myogenic biomechanical cues with a stiffness of 10–15 kPa (i.e., myogenic stiffness, discussed in Sect. 6.4) which may explain why growth factors were not required; in combination, matrix-based cues may be more potent.

To better appreciate how matrix cues interact with each other and with growth factors, recent efforts have focused on creating combinatorial experiments. For neurogenesis, BMSC differentiation has demonstrated the effects of both topographical cues and biochemical cues (Prabhakaran et al. 2009). A PLLA-co-poly-(3-caprolactone)/collagen (PLCL/Coll) material was electrospun, creating 230 nm nanofibers, and BMSCs were differentiated into a neurogenic lineage using neurogenic induction factors such as brain-derived neurotropic factor (BDNF), epidermal growth factor (EGF), and β-mercaptoethanol. The same group has also shown that human BMSCs can be induced into an epidermal lineage on the same material when cultured alongside epidermal factors EGF and 1,25-dihydroxyvitamin D_3 (Jin et al. 2011). Electrospun polycaprolactone (PCL) scaffolds with randomly oriented nanofibers (~700 nm in diameter) induced a chondrogenic fate in human BMSCs in the presence of transforming growth factor-beta 1 (TGF-β1) (Li et al. 2005b) comparable to cell aggregates or pellets, a widely used protocol for chondrogenesis of MSCs in vitro. Silk fibrin nanofiber scaffolds containing bone morphogenetic protein 2 (BMP2) and in the presence of osteogenic media can differentiate human BMSCs towards an osteogenic lineage with higher calcium deposition (Li et al. 2006). While each group does observe differentiation, different growth factors and polymers form the cell environment, and thus, comparisons of efficacy are difficult at best. Comprehensive analysis of matrix-mimetic properties is vital to developing a further appreciation of how matrix cues can be combined in electrospun scaffolds for stem cell differentiation.

3D lithography has also been employed to regulate stem cell fate. For example, PDMS with 350 nm-wide gratings causes BMSCs to elongate and upregulate neuronal differentiation markers such as microtubule-associated protein 2 (MAP2) compared to unpatterned controls (Yim et al. 2007). Other simple nano- or microstructured patterns, such as micropillars, have been shown to promote differentiation in mouse ESCs (Lovmand et al. 2009; Markert et al. 2009). In human BMSCs, two studies have shown that 200 nm feature sizes are optimal for cell adhesion and self-renewal (Dulgar-Tulloch et al. 2009; Khor et al. 2007). In these examples, most but not all substrates referred to as 3D provide large topographical cues on an otherwise flat substrate. Contrary to electrospinning, with materials produced by lithographic methods, it would appear that their combination with growth factors provides less synergy, i.e., the cell response is dominated by the topographical cue. For instance, the combination of nanotopography and

biochemical cues such as retinoic acid further enhanced the upregulation of neuronal marker expressions, but nanotopographical patterns of grooves and grids showed a stronger effect compared to retinoic acid alone on unpatterned surface (Martino et al. 2009) (Fig. 6.2d). As with electrospinning, most studies of nano- or micro-3D topography have shown that BMSCs differentiate into specific lineages not only by 3D topography but also by biochemical factors, e.g. differentiation media as used in traditional protocol. It is clear that much more work is required to reproducibly control multiple intrinsic matrix properties in these 3D materials before we can fully appreciate how their properties combine to direct stem cell fate.

6.4 Modulating Cell Shape and Size: A Corollary to Matrix Porosity

Although 3D substrates can mimick natural ECM structure and/or topography more than 2D, 2D patterns can more easily regulate cell shape and size by controlling ligand placement; thus, it enables one to study important matrix-based questions using reductionist approaches. Cell morphology is one of the earliest ways to distinguish the status of cell, though it cannot be a definite marker by itself. Rather than allowing cells to spread on non-patterned plastic dishes, controlling spatial organization of ECM can be used to culture cells in a specific shape or size to direct stem cell fate. Adhesive ECM ligands or proteins with a specific pattern can be immobilized onto nonadhesive materials, which can restrict cell spreading. The size and shape of the pattern can be varied for the purpose of the experiments. Usually there are two purposes: restricting the size and shape of cell adhesion sites for single cell culture and aligning cells with a specific design.

6.4.1 2D Matrix Pattern Techniques: Soft Lithography

Soft lithography is the most commonly used technique to create chemically adhesive patterns on substrates to restrict cell shape and size (Kane et al. 1999; Qin et al. 2010). It typically involves a “soft” elastomeric material (the “stamp”), cast from a master mold, which is usually made from a silicon wafer with a patterned set of features made in relief of the desired stamp pattern. Prior to stamping or “microcontact printing” (μCP), it is important to ensure that the nonadhesive area is non-fouling, i.e., does not readily adsorb matrix proteins from any source, serum or otherwise, so that nonspecific cell binding to the substrate is limited. This will ensure that cell adhesion remains unbiased. Several methods in surface chemistry can produce a relatively non-fouling surface. Precoating surfaces with albumin is the most common method used to prevent deposition of other serum proteins (Nelson et al. 2003), but its efficacy is limited. Synthetic materials including poly(ethylene glycol) (PEG),

also known as poly(ethylene oxide) (PEO), have also been widely used and are often attached to other materials to render them inert. Covalently attaching PEG to muscle adhesive proteins has been shown to render the proteins inert when attached to gold and titanium surfaces for up to 2 weeks in vitro (Dalsin et al. 2003). Although PEG is effective in preventing nonspecific protein adsorption, its non-fouling capability is dependent on surface chain density and can be damaged by oxidants. As an alternative, tetraethyleneglycol dimethylether (tetraglyme), which forms a highly non-fouling cross-linked structure (Lopez et al. 1992), has been shown to significantly reduce blood platelet (Tsai et al. 2002) and monocyte (Shen and Horbett 2001; Shen et al. 2001) adhesion in vitro. Triblock copolymers, which spontaneously assemble on hydrophobic surfaces and are commercially known as poloxamers or Pluronics®, have been extensively studied and are also extremely non-fouling (Amiji and Park 1992; Liu et al. 2002; Neff et al. 1999). Alkane thiols have also been extensively used but are typically printed only on metal substrates such as gold. Despite this, they have the advantage that their electrical activity allows one to dynamically modulate adhesion (Mrksich 2000).

Prior to treating the substrate to make it non-fouling, microcontact printing (μCP) must be employed. μCP's popularity originates from its simplicity, cost-effective means of production, and flexible platform from which one can design any 2D shape. Moreover, one can choose to print on a wide array of surfaces using many different stamp materials. The μCP process as a cell-patterning tool is illustrated in Fig. 6.3a. First, an elastomeric stamp (e.g., PDMS) is fabricated by casting a liquid-phase polymer over a master mold (usually made by photolithography). After curing of the elastomeric stamp, an "ink" protein (e.g., fibronectin) is adsorbed onto the stamp. The substrate is then chemically functionalized so that covalent binding occurs between the protein and substrate when the stamp is in contact with the substrate. Finally, it is common practice to backfill the non-stamped area with a non-fouling chemical (e.g., Pluronics®). What results is a matrix protein pattern in a variety of shapes, as illustrated in Fig. 6.3b. Cells conform to the pattern when placed onto the substrate, though it should be noted that for patterns that are not regular shapes, such as the letters "UCSD," cells can bind to the bottom and side of the "U" and span the region where there is no protein coated on the substrate. On uncoated regions, cells do not adhere, and no focal adhesions can be observed (Fig. 6.3c).

In practice, the μCP technique can print patterns as small as 1 μm with normal PDMS stamps provided that a photomask of suitable resolution is used when creating the stamp. This tool is able to make patterns with two different proteins by stamping with one and subsequent backfilling with the other. Creating more complex structures is challenging but can be accomplished by several consecutive stampings (Lauer et al. 2001; Wheeler et al. 1999) or via a multi-step stamp (Tien et al. 2002). Nonetheless, the transfer efficacy can be variable and may not be very consistent or reproducible.

Soft lithographic patterns can also be generated by stencil-based methods in which a membrane containing holes of the desired shape and size is placed over the substrate prior to stamping. Thus, instead of stamping the desired feature onto the

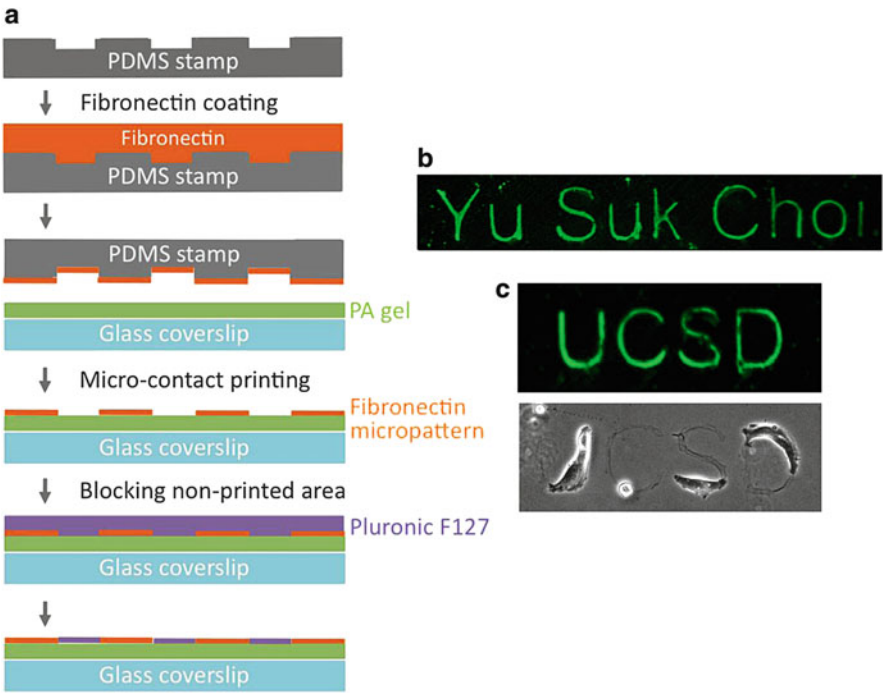

Fig. 6.3 Microcontact printing provides 2D matrix-based spatial control of cells. (**a**) Schematic of the μCP method. (**b**) Fibronectin was successfully μCP onto PA gel with various shapes. (**c**) Human BMSCs recognized fibronectin patterns and did not adhere to the non-fouling areas

substrate, this technique selectively allows for protein coating in the holes of the stencil, while the areas outside the holes remain protected by the stencil. Stencil materials can include those used in the μCP technique, but many also include very stiff grids (e.g., metal). Cells may be seeded prior to or after removal of the micropatterned holes, and the substrate can be backfilled with either matrix proteins or a non-fouling chemical (Folch et al. 2000).

6.4.2 Microfluidic Patterning

Another useful method to pattern cells employs microfluidic patterning (μFLP) (Fiorini and Chiu 2005). μFLP shares the same basic chemistry as microcontact printing methods, but it uses a microfluidic channel to deliver protein solutions to selected areas of the substrate. Backfilling of the nonadhesive areas is achieved either by using capillary action or applying suction at one of the ports of the device. As with the μCP technique, μFLP can usually be performed in parallel due to the reusability of the microfluidic channels (Delamarche et al. 1997; Rhee et al. 2005).

However, one advantage of this method over μCP is that it is possible to produce features without drying the surface. μFLP is also more capable of controlling ligand concentration and generating gradients or evenly distributed mixtures of multiple proteins (Chiu et al. 2000). On the other hand, μFLP requires substantially more proteins compared to the small volume required to coat a stamp in μCP.

6.4.3 Other Patterning Methods

While both of these methods are extremely common means of patterning ECM proteins, several other tools have been developed to pattern adhesive islands for cells. For example, inkjet printing has been adapted for patterning by utilizing an "ECM protein ink" and printing on glass (Khan et al. 2010; Roth et al. 2004; Sanjana and Fuller 2004; Turcu et al. 2003). While the inkjet tool is cost efficient, spot resolution is limited, with the smallest size spots created being around 100 μm. These techniques involve patterning ECM proteins, but an alternative approach has been to pattern the placement of cells with an optical tweezers technique. Objects with a higher refractive index, such as cells or particles, can be captured in an optical trap with focused laser light and positioned as desired (Ashkin et al. 1987), just as ECM proteins can be stamped where desired. While it has a resolution similar to the μCP technique and allows for versatile patterning, this method is the most expensive.

The techniques reviewed above can be divided into two categories: passive cell attachment on an adhesive pattern and active cell deposition. Many problems relating to long-term, single cell culture can be avoided by combining these techniques. Ongoing efforts to fabricate chemical patterns with a specific shape and size by soft lithography and then deposit cells one by one on those patterns by optical or electrical methods may improve this.

6.4.4 Regulation of Stem Cells by Cell Shape and Size

Though cell size and shape may not initially appear to be matrix related, ECM porosity can directly affect cell size and shape in 3D culture as well as in vivo and alter cell behavior (Reilly and Engler 2010). This was first observed in fibroblasts and endothelial cells, in which cell survival was found to be dependent on cell spreading (Chen et al. 1997; Roskelley et al. 1994; Watt et al. 1988). These observations were extended to BMSCs, in which an adipogenic–osteogenic differentiation switch depends on cell size; unspread cells favored adipogenic commitment, whereas well-spread cells preferentially became osteogenic despite the presence of growth factors for both lineages (McBeath et al. 2004) (Fig. 6.4a, b). Cell spreading affects Rho GTPase activity (Ren et al. 1999), and McBeath and coworkers found that size-dependent fate regulation was mediated by RhoA activity

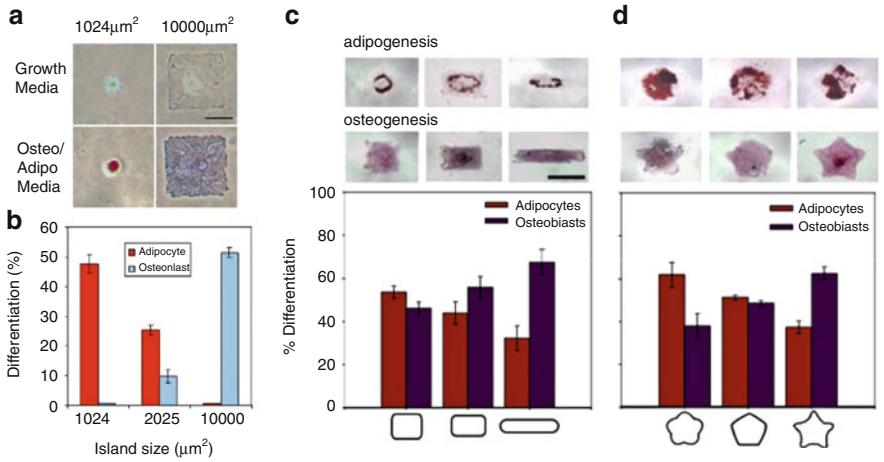

Fig. 6.4 The response of stem cells to various matrix micropatterns. (**a**, **b**) BMSCs plated onto micropatterned ECM proteins of an indicated size in growth media or mixed differentiation media. Cells were stained for lipid retention (*red dye*) or alkaline phosphatase production (*purple*), and the percentages were quantified versus the overall cell population. Reprinted from McBeath et al. (2004). Scale bar: 50 μm. (**c**) Percentage of cells captured on *rectangles* of varying aspect ratio differentiating to adipogenic or osteogenic lineage. (**d**) Percentage of cells differentiating to either lineage when captured on fivefold *symmetric* shapes. Scale bar: 50 μm. Reprinted from Kilian et al. (2010)

and cell tension. A similar size-dependent switch between chondrogenic and smooth muscle cell fates has also been observed in mixed media conditions, again dependent on Rho GTPases (Gao et al. 2010). In addition to size, Kilian and coworkers demonstrated the importance of shape in regulating cell tension and ultimately BMSC fate. Cells patterned in rectangles with increasing aspect ratio (Fig. 6.4c) and in pentagonal shapes of different subcellular curvature (with the same area; Fig. 6.4d) displayed adipogenic or osteogenic differentiation depending on whether they exhibited decreased or increased actomyosin contractility, respectively. More contractile cells had enhanced c-Jun N-terminal kinase (JNK) and extracellular related kinase (ERK1/2) activation in conjunction with elevated wingless-type (Wnt) signaling (Kilian et al. 2010). What underlies these findings is the significant change in cytoskeletal structure during a switch between lineages (Yourek et al. 2007). The signaling that accompanies this informs the cell nucleus of the lineage to adopt.

One important aspect of controlling cell shape and size that we have not yet discussed is that while it provides an excellent, reductionist approach to understanding cell and matrix biology, it eliminates the natural variation in cell size that is dictated by a matrix with a range of porosity. While some of the techniques we have previously covered, such as electron beam lithography (EBL), can be used to make random topographies (Dalby et al. 2007), similar methods are not available with cell size and shape unless matrix patterns are intentionally made slightly larger

than the typical cell size. However, that approach is likely complicated by problems with multiple cells binding within a given feature.

6.5 Matrix Stiffness

Although matrix composition has been shown to influence stem cell differentiation (Flaim et al. 2005), especially in the context of how it combines with other properties such as stiffness (Rowlands et al. 2008), we will focus discussion of our final intrinsic matrix property on the ECM's Young's modulus, which is commonly referred to in biology as stiffness, rigidity, or elasticity. As far back as the early 1980s, the scientific community was beginning to develop an appreciation for the forces that a cell could impart on its microenvironment (Harris et al. 1980). More recently, it has become clear that the forces generated by cells are transduced through the matrix and provide important signals to promote focal adhesion assembly (Pelham and Wang 1997), direct migration (Chen et al. 2000; Guo et al. 2006), and regulate proliferation (Hadjipanayi et al. 2009) and apoptosis (Wang and Thampatty 2008). Matrix stiffness sensing occurs via a similar Rho GTPase machinery as cell shape and size sensing (Peyton and Putnam 2005) by sensing the strains the cell imposes on the matrix (Fu et al. 2010). While the sensing mechanisms will be further explored in Sect. 6.6, these findings have led to a new approach in stem cell differentiation in which it is hypothesized that ECM stiffness might also direct stem cell fate (in addition to the intrinsic properties mentioned above).

6.5.1 Young's Modulus

Before continuing, it is important to thoroughly define Young's modulus (E), which is the response of a material to an applied stress (σ, the force per area measured in Pascals, Pa) and the resulting strain (ε, unitless present elongation). Young's modulus can most simply be calculated by the linear relationship $E = \sigma/\varepsilon$, especially at low strain and in an elastic material, such as a hydrogel or rubber. Young's modulus is measured in Pascal (Pa) or Newton per square meter (N/m^2). In soft biological tissues, stiffness varies from the soft brain, ~1 kPa (Gefen and Margulies 2004), to the stiff but not calcified bone where cells reside, >30 kPa (Discher et al. 2005). The Young's modulus of calcified bone is on the order of ~5 GPa (Choi et al. 1990).

6.5.2 Fabricating Substrates with Variable Stiffness

Most materials used to mimic native ECM stiffness are either reconstituted natural matrices or synthetic hydrogels. The former provides better mimicry of biological

activity, but suffers from difficulty in separating chemical and mechanical changes in matrix properties, i.e., increased ECM density stiffens the matrix but also increases ligand density in collagen gels (Raub et al. 2010) and fibronectin matrices (Mao and Schwarzbauer 2005). The latter allows one to decouple biophysical and biochemical changes but requires matrix ligands to be covalently attached to the synthetic polymer. Mechanical properties including stiffness can be modified depending on (1) porosity and pore size, (2) polymer concentration and its molecular weight, and (3) the degree of cross-linking (Vincent and Engler 2011). We will next examine two of the most widely used synthetic hydrogels below.

Polyacrylamide (PA) is the most commonly used material for the protein separation step in gel electrophoresis, but thin film preparations when functionalized with protein can support cell adhesion (Pelham and Wang 1997). PA gels have advantages that make them a popular choice to study the effect of matrix stiffness on stem cell differentiation, including cost efficiency, widely tunable mechanical properties, and easy functionalization with cell-adhesive proteins or ligands. PA polymerization occurs via a free-radical reaction when a radical donor (most commonly ammonium persulfate) reacts with an acrylamide and bis-acrylamide mixture (Wang and Pelham 1998). The stiffness of the PA gels varies from ~0.1 to 100 kPa (Tse and Engler 2010; Wang and Pelham 1998), based on the ratio of acrylamide to bis-acrylamide. This highly tunable material can be used to make a gradient gel by utilizing different methods such as mixing two droplets of different stiffness (Lo et al. 2000), by using photoinitiator and a gradient photomask (Tse and Engler 2011), or by preparing a gradient of cross-linker concentration in a microchannel device (Zaari et al. 2004). Gradient techniques allow one to study the cell behavior "durotaxis," which is the characteristic migration of a cell along a stiffness gradient. As stated earlier, PA gels are normally non-fouling, which enables electrophoresis, but to ensure protein attachment, PA gels used for cell culture have proteins covalently grafted onto their surface, often with the heterobifunctional cross-linker *N*-sulfosuccinimidyl-6-(4′-azido-2′-mitrophenylamino) hexanoate (sulfo-SANPAH) (Beningo et al. 2002).

PDMS is an elastomer most often used to make microfluidic channels or stamps for μCP as discussed previously. PDMS is polymerized by mixing elastomer with curing agent at a desired ratio and baking for a few hours at 90 °C. By varying the ratio of elastomer and curing agent, reaction time, and reaction temperature, the stiffness of PDMS can be varied between ~12 and 2,500 kPa (Gray et al. 2003; Tzvetkova-Chevolleau et al. 2008). The polymerization process provides good optical clarity, making PDMS an ideal substrate for microscopic applications. The surface can also be easily modified to adsorb proteins for cell adhesion. PDMS is also a very good material for generating a matrix with refined (~10 μm) topography when it is combined with photolithography, as previously discussed. High-density pillar arrays can be fabricated by pouring PDMS solution over a photolithography-micropatterned mold (Tan et al. 2003). By using a defined pillar stiffness, one can detect cell pulling force by calculating the angle changes of the pillar based on the physical dimensions of pillars (Fu et al. 2010). Deformable pillars allow for the quantification of substrate deformation and traction forces generated by cells.

6.5.3 *Stiffness Measuring Techniques*

Measuring stiffness for small matrix samples is not as simple as reconstructing a stress–strain curve as previously described. Microindenters, atomic force microscopes (AFM), and rheology have all been used to accomplish this task as traditional compression and extension tests for such small samples are not feasible. Microindenters were originally developed to measure Young's modulus for a high range of stiffnesses (Tai et al. 2005) and have been adapted to measure softer ranges of stiffness, which are more relevant to biological stiffnesses in the range of kPa to MPa (Ebenstein and Pruitt 2004). Microindentation for soft materials usually uses a spherical tip (of several microns in diameter; Fig. 6.5a, top) attached to the end of a flexible microneedle to probe the material (Fig. 6.5a, bottom). Deflection of the tip, which provides the force to indent the material (obeying Hooke's law for springs; F = spring constant $\times$ tip deflection), is detected by optical microscopy and the modulus is obtained by comparing the displacement to a standard curve using the Hertz model (Hertz 1881) (Fig. 6.5b). Microindentation measurements of soft hydrogels such as polyacrylamide substrates have been shown to correlate well with atomic force microscopy (AFM; will be discussed below) in the range from 2 to 40 kPa (Jacot et al. 2006). Since this technique relies on visual displacement of tip, it is relatively easy and inexpensive to use, but these benefits are offset by measurement resolution, which dramatically affect the modulus.

AFM is a type of scanning probe microscope (SPM), which is similar to microindentation but which differs in several specific ways to improve resolution. First, it uses piezo motors to finely control the XYZ motion of the indenter and sample stage such that only the AFM probe's dimensions limit resolution; probes for cell-based applications typically have a spherical or pyramidal tip with a radius of contact with the material as small as 10 nm. AFM tips are typically made out of silicon nitride with a gold coating to make them reflective. Secondly, probe interactions with the substrate, i.e., deflection, are measured by a photodetector capturing light from a laser or intensified light-emitting diode that has been reflected off of the gold-coated AFM probe (Fig. 6.5c) (Drake et al. 1989). Hooke's law is then used to convert probe deflection to force, as with microindentation. AFM is also capable of converting deflection into nanoscopic changes in sample height so that it can be used to create nanoscopic topographical images as well as perform force measurements.

AFM had been used extensively to measure the elasticity of hydrated tissues and substrates (Binnig et al. 1986; Drake et al. 1989; Engler et al. 2007; Richert et al. 2004). Young's modulus can be calculated using indentation models of a pyramid/cone developed by Sneddon (1965) or a sphere by Hertz (1881), assuming that the material or matrix is thick enough to mask contributions from the underlying rigid support. An estimate of the thickness required for this assumption is ~20 μm (Engler et al. 2004) using a modified Hertz model which can correct for material thickness (Dimitriadis et al. 2002). Highly technical and expensive, AFM may not be the best choice for labs, but the spatiotemporal control that comes with AFM

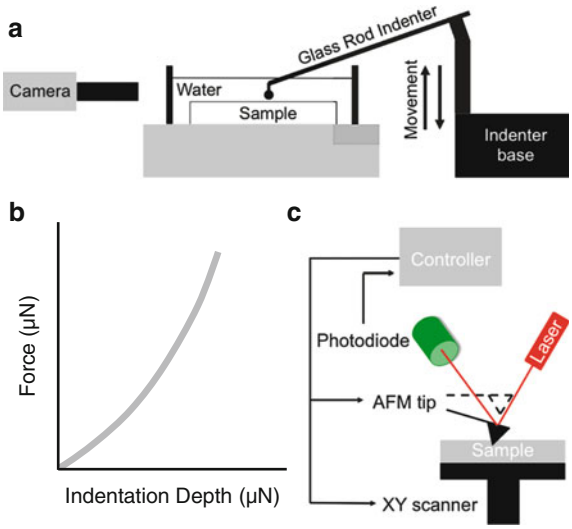

Fig. 6.5 Stiffness measuring techniques. (**a**) Schematic of microindenter. The microindenter tip is a sphere of low melting point glass 170 μm in diameter. Scale bar is 100 μm. The sample is held stationary while the indenter base is moved using a micromanipulator, increasing or decreasing the force the spherical tip exerts on the sample. Images of the indenter tip are captured for each loading or unloading step. (**b**) This plot graphically details the algorithm used to compute the elastic modulus values from force versus indentation depth data. (**c**) Schematic of AFM. The photodiode detects the deflection of the cantilever by sensing the position of the reflected beam. After calibration, the deflection can be translated to force. The cantilever is pushed into the sample during which the cantilever deflects until a defined trigger force is reached

enables one to measure spatial changes in matrix properties with the resolution of 20 nm (Flores-Merino et al. 2010) and over weeks in culture for dynamic materials (Young and Engler 2011).

Both techniques introduced above may accurately measure the stiffness of materials with a linear elasticity; however, many natural ECMs are often not linearly elastic, may undergo strain stiffening (increasing deformation from the cell results in a stiffer matrix), and are anisotropic (material properties are not the same in each direction). Thus for a 3D fibrillar fibronectin matrix, Young's modulus from multiple locations may not be identical, which leads to larger standard deviations within a data set (Engler et al. 2009a). Thus, it is very important to choose the right tools depending on your sample materials and to employ the other measurement techniques that probe different mechanical modes, for instance, rheology. Briefly, the rheometer measures stiffness as it applies a shear stress or a shear strain on top of the surface of a material using a rotating cylinder (Macosko 1994). Direct comparison of multiple stiffness measurements can be accomplished by converting shear to Young's modulus (Fung 1977). Especially for PA hydrogels, Young's moduli measured by AFM and rheometer have shown good agreement (Yeung et al. 2005).

6.5.4 Controlling Stem Cell Fate by ECM Stiffness

In addition to biochemical signals from the ECM, cells must feel or sense mechanical or physical aspects of their environment and respond to their extracellular stimuli appropriately over time. Recent studies have been able to test the hypothesis that stem cell fate can be directed by a single ECM mechanical property, stiffness, decoupled from ligand density (Engler et al. 2006). Human BMSCs cultured on various stiffness PA gels (collagen I coated) showed that the stiffness of the matrix defined the specific lineage differentiation. Cells on brain-mimicked soft (~1 kPa) gels differentiated into a neurogenic lineage, muscle-mimicked firm (~10 kPa) gels induced myogenesis, and matrices with bone-like stiffness (>30 kPa) were found to differentiate cells to an osteogenic lineage (Fig. 6.6a). The mechanism of stiffness-mediated lineage specification appeared to be related to cell contractility and tension by non-muscle myosin II, as shown by the loss of specific lineage differentiation after blebbistatin treatment; this result is qualitatively similar to those seen with micropatterned cells (McBeath et al. 2004). More recently, human ASCs were shown to exhibit the same behavior in response to changes in matrix stiffness (Fig. 6.6b), but it was also found that 2% of these cells fused together to form multinucleated cells on matrices of myogenic stiffness (Choi et al. 2012), an observation never seen in BMSCs and an order of magnitude better than ASCs chemically induced to become muscle (Di Rocco et al. 2006). Fusion rate was decreased by blebbistatin and increased by lysophosphatidic acid (Choi et al. 2012), again suggesting that cell contractility and tension govern this process.

Rarely, however, is stiffness uniform within a tissue; the spatial gradients created by this variability can induce stiffness-directed migration, i.e., durotaxis (Lo et al. 2000). While matrix-directed durotaxis has been observed in fibroblasts and smooth muscle cells with supraphysiological gradients (Isenberg et al. 2009; Lo et al. 2000; Zaari et al. 2004), only recently have physiological gradients, ~1 kPa/mm, been fabricated. Human BMSCs were found to migrate prior to differentiating into the lineage supported on the stiffer portion of these matrices. Since residual expression of the previous lineage was detected (Tse and Engler 2011), it is not clear how "plastic" or terminally differentiated stem cells are when differentiated by matrix stiffness. Further evidence of this is that stiffness-induced differentiation can be confounded by growth factor-mediated differentiation: a cell on a soft substrate becoming a neuron can be confused with myogenic induction media, leading to the expression of multiple fates within a cell (Engler et al. 2006). Similarly, single ASCs undergoing stiffness-induced myogenesis replated onto stiffer matrix begin to express Runx2, an osteogenic marker, implying their plasticity. Only when ASCs fuse together to become a myotube is plasticity lost (Choi et al. 2012) (Fig. 6.6c).

It should be noted that all of the observations described thus far include cells cultured in 2D or pseudo-3D substrates. To study stiffness effects on stem cell fate in 3D, human BMSCs have been cultured in thixotropic gels of varying rheological

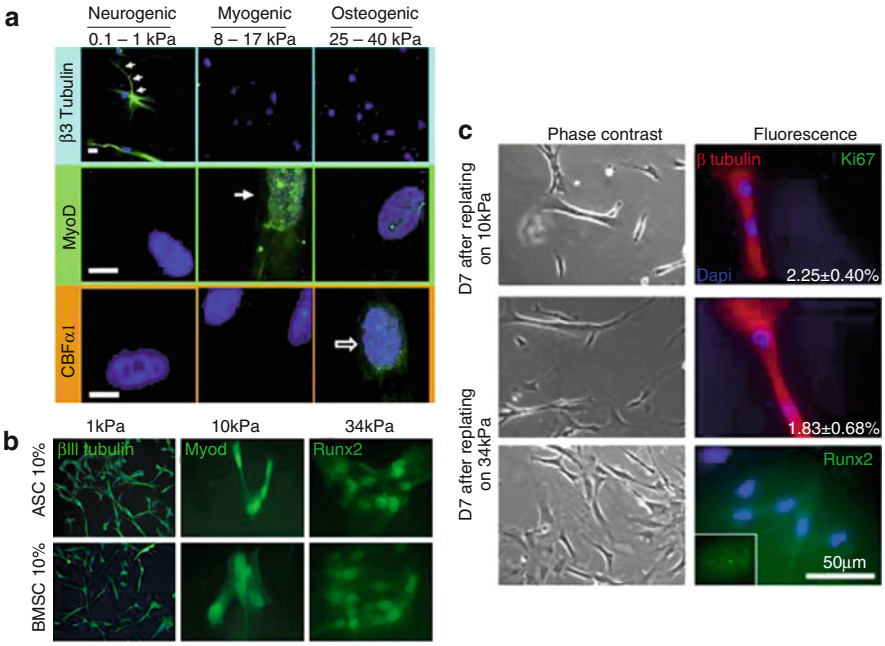

Fig. 6.6 Controlling stem cell fate by ECM stiffness. (**a**) Lineage specification of human BMSCs into neuro-, myo-, and osteogenic cells on brain, muscle, and bone-mimicked hydrogel. Reprinted from Engler et al. (2006). (**b**) ASCs are shown to simply reflect the qualitative stiffness sensitivity of BMSCs. (**c**) ASC-derived myotubes, when replated onto permissive (10 kPa) or nonpermissive (34 kPa) stiff matrix, maintain their fused state. Singly nucleated ASC felt osteogenic stiffness on 34 kPa gels and expressed punctuated osteogenic marker, Runx2. Reprinted from Choi et al. (2012)

properties. Similar to previous findings in 2D culture (Engler et al. 2006), cells in 3D expressed neurogenic, myogenic, and osteogenic markers as an increasing function of matrix stress, respectively (Pek et al. 2010).

6.6 Mechanotransduction: Signaling via the Matrix

While the discussion thus far has focused on extracellular cues, it is important to at least briefly describe the mechanisms by which cells can sense their extracellular environment. The term "mechanotransduction," which describes this sensing, is a relatively new term to the cell biology field. More specifically, it refers to the mechanism through which cells convert mechanical stimuli into biochemical signals. Here we will review how stem cells feel their mechanical environment, e.g., topographical cues, porosity, and matrix stiffness, transduce that mechanical stimuli into biochemical signals, and ultimately control their cellular fate. While we will focus specifically on focal adhesion-based mechanisms as they relate specifically to the extracellular matrix, many other reports of potential mechanosensing mechanisms are nicely reviewed elsewhere (Discher et al. 2009; Engler et al.

2009b; Guilak et al. 2009; Jaalouk and Lammerding 2009; Janmey et al. 2009; Vogel and Sheetz 2006). In order for a focal adhesion-based mechanotransducing system to operate, three events are required: (1) cell attachment to the ECM via cell adhesion molecules (CAMs) such as integrins, (2) cytoskeletal contractility, and (3) adaptation to contractility by FA-associated sensors.

6.6.1 Integrins: Making Connections

Cell adhesion is a result of the binding of a protein on the cell's surface to the ECM surrounding the cell. While there are a number of different proteins comprising the cell adhesion molecule (CAM) family, those most responsible for cell–ECM linkage, integrins, are part of large macromolecular complexes known as focal adhesions (FAs). Cell–ECM attachment is mediated by pairs of heterodimeric transmembrane integrins, which are often specific for a particular matrix protein, e.g., fibronectin and $\alpha_V\beta_3$ (Giancotti and Ruoslahti 1999; Howe et al. 1998). These integrin pairs are formed by alpha and beta subunits, with extracellular domains that recognize and bind to amino acid sequences, most notably the Arg–Gly–Asp (RGD) attachment site (Ruoslahti 1996). Upon initial cell attachment to the ECM, increasing numbers of integrins will arrive to reinforce the nascent focal contact, followed by cytoskeletal proteins, such as F-actin stress fibers, and cytoplasmic linker proteins that connect the integrins to the cytoskeleton, resulting in the formation of mature focal adhesions (Lagunas et al. 2011). At this point, the cell is fully attached to the ECM, and the cytoskeleton can begin to actively sample the cellular microenvironment for mechanical information.

6.6.2 The Cytoskeleton: Generating Forces

Recent work has demonstrated the importance of the cytoskeleton, most notably actomyosin structures, in cell differentiation (Engler et al. 2006; McBeath et al. 2004). The process of cytoskeletal contractile force generation begins once integrins have completed binding to the ECM. Guanine exchange factors (GEFs), recruited by focal adhesion proteins like p130Cas (Cote and Vuori 2007) or focal adhesion kinase (FAK) (Premont et al. 2004), catalyze the activation of Rho GTPases, including RhoA, Rac, and CDC42 (Parsons et al. 2010). The activation of Rho GTPases allows for the phosphorylation of ROCK, a key step in the Rho/ROCK contractility pathway. Once ROCK has been phosphorylated, it activates myosin light chain (MLC), which promotes actin–myosin binding and contraction (Chen et al. 2010). This pathway functions as a positive feedback loop: an increase in contractility results in further reinforcement of focal adhesions via stress fiber recruitment, resulting in further contractility (Novak et al. 2004; Vicente-Manzanares et al. 2009). However, contractility alone does not provide

an explanation for the cellular mechanotransduction observed in response to engineered ECM microenvironments, and thus, the role of focal adhesion proteins must be further explored, as they play an important role in linking the actin cytoskeleton to integrins.

6.6.3 *Focal Adhesion-Based Mechanosensors: Bridging the Gap*

Cellular responses to porosity, topographical, or stiffness cues are not passive but are a result of active contractility of the cell on its environment. As evidence, it has been shown that while matrix stiffness regulates BMSC differentiation into different lineages, the use of the non-muscle myosin inhibitor blebbistatin blocks stiffness-induced lineage specification (Engler et al. 2006). Cellular contractility against substrates of different stiffnesses results in different levels of force exposure in the linker proteins, connecting integrins to the cytoskeleton. This has led to the suggestion that cellular forces may change the conformation of linker proteins, exposing binding sites critical for downstream signaling. It seems that desired matrix stiffness is critical for a mechanosensing protein function (Reilly and Engler 2010).

To date, over 100 proteins have been categorized as being involved in focal adhesion (Geiger et al. 2001). Many of these proteins have been implicated as mechanosensors, though often little is known about how each specifically signals and/or transduces force. Though it could vary based on the protein, either a biphasic or monotonically increasing response is likely required for binding. In the former, force is required to open a cryptic site, but overextension can completely unfold the site, rendering it nonfunctional (Fig. 6.7). With the latter, additional force could continue to expose cryptic regions, as with talin (del Rio et al. 2009). Proteins that undergo functional unfolding in response to physiological force are known as "molecular strain gauges" (Holle and Engler 2011).

One such molecular strain gauge, talin, has been studied in detail and is known to link integrin dimers and actin filaments (Gingras et al. 2008). To accomplish sensing, another key mechanosensing protein, vinculin, is recruited to talin when the cryptic vinculin binding site in the talin rod is exposed as a result of unfolding (del Rio et al. 2009; Gingras et al. 2006; Margadant et al. 2011; Vicente-Manzanares et al. 2009). Another force-sensitive focal adhesion protein, p130Cas, has been shown to unfold under force in response to changes in the physical microenvironment (Sawada et al. 2006). More molecular strain gauges are in the process of being discovered, but linking focal adhesion protein unfolding to altered cell behavior requires a direct link to signaling pathways know to affect gene expression, of which there exists little evidence. However, many signaling proteins or regimes have been implicated in the mechanotransduction response, including MAPK (Uchida et al. 2010), PI3K (Hamamura et al. 2012), MKL1 (Huang et al. 2012), and X-ROS and calcium ion signaling (Prosser et al. 2011).

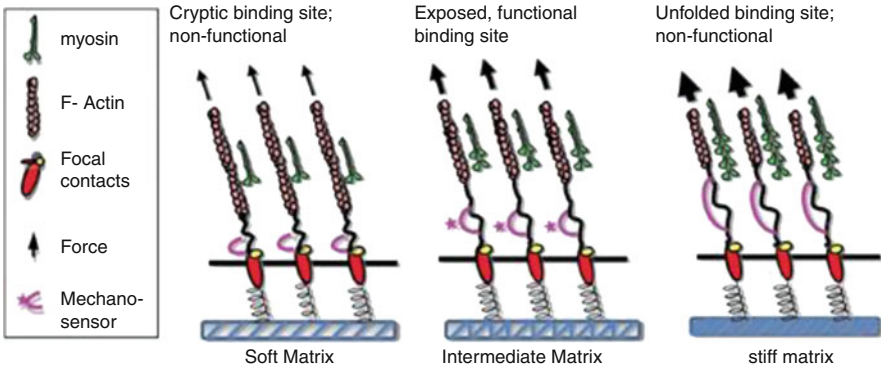

Fig. 6.7 Proposed force-sensing mechanism within stem cells. Changes in protein folding as forces are exerted to expose binding sites. Cells on soft matrix with weak intracellular forces cannot sufficiently change the conformation of a mechanically sensitive protein of interest to expose a cryptic binding site, making it nonfunctional. By comparison, cells on stiff matrix generate high tension, which causes the protein to unfold to such a degree that the binding site is rendered nonfunctional. However, cells on matrix with optimal elastic properties may apply the appropriate amount of forces such that the cell may change the conformation of the protein, making the cryptic binding site accessible. Reprinted from Reilly and Engler (2010)

The challenge, then, lies in the pursuit of a fully characterized mechanotransducing system, starting with integrin binding to the ECM and cytoskeletal contraction, followed by processing of the cellular contractile stress via a focal adhesion-based sensor that in turn activates a signaling cascade which results in changes in gene expression in the nucleus. By engineering-controlled ECM microenvironments of a given cell, these systems can be analyzed more easily. The synergy in this field lies in the fact that as more is learned about mechanotransduction, knowledge is gained that can be applied to designing more effective engineered ECM microenvironments, which in turn become more effective at eliciting cellular behaviors that elucidate the mechanotransduction process itself. Once these complete systems have been discovered, they can be utilized in the design of clinical therapies aimed at combating disease resulting from faulty mechanotransduction.

6.7 Discussion and Conclusion

In this chapter, we reviewed how the ECM environment had an influence on the fate of stem cells. Based on the findings and evidence available now, more study is required regarding underlying mechanisms to address these gaps in stem cell differentiation and also to develop more natural ECM-like matrices to induce stem cells into desirable lineages. As the role of topography and stiffness of ECM continues to be uncovered, the data collected will facilitate the fabrication of ECM

that more accurately mimic the complex biomechanical and biophysical properties of tissues. Stem cell studies in 2D have provided useful knowledge to understand mechanical stimuli on stem cell differentiation; however, stem cells reside in vivo in 3D. Thus, engineering more accurately mimicked 3D ECM may be the next step forward to improve stem cell differentiation.

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Part III
Model Organisms and the Lexicon of Developmental Signals Associated with the Extracellular Matrix

Chapter 7
ECM in *Hydra* Development and Regeneration

Xiaoming Zhang and Michael P. Sarras Jr

Abstract The body wall of *Hydra* is organized by two layers of epithelia (ectoderm and endoderm) with an intervening extracellular matrix (ECM), termed mesoglea by early biologists. Morphological studies have determined that *Hydra* ECM is composed of two basal lamina layers located underneath each epithelial cell layer and a centrally located intervening interstitial matrix. Molecular and biochemical analyses of *Hydra* ECM have established that it contains components similar to those seen in more complicated vertebrate species. These components include laminin, type IV collagen, and various fibrillar collagens. *Hydra* ECM components are synthesized in a complicated manner involving cross talk between the epithelial layers. Any perturbation to ECM biogenesis leads to a blockage in *Hydra* morphogenesis. Blockage in ECM–cell interactions in the adult polyp also leads to problems in epithelial transdifferentiation processes. In terms of biophysical features, *Hydra* ECM is highly flexible, a property that facilitates continuous movements along the organism's longitudinal and radial axis. This is in contrast to the more rigid matrices found in vertebrates. The flexible nature of *Hydra* ECM can in part be explained by the unique structure of the organism's type IV collagen and fibrillar collagens. This chapter focuses on (1) the general structure of *Hydra* ECM, (2) its molecular composition, (3) the biogenesis of *Hydra* ECM during regeneration, (4) *Hydra* ECM in development, (5) cell–ECM interaction in *Hydra*, and (6) *Hydra* ECM remodeling during development.

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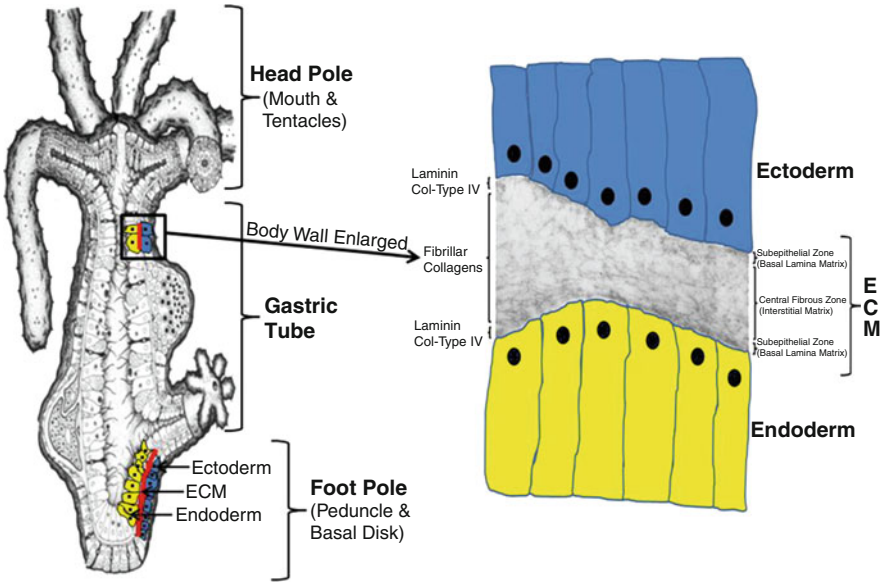

Fig. 7.1 Hydra body plan formed of an epithelial bilayer with an intervening extracellular matrix (ECM). *Hydra* exists as a gastric tube with a mouth and several tentacles at the head pole and a peduncle and a basal disk at the foot pole (*left* diagram). The entire body wall of *Hydra* (from the tip of the tentacles to the basal disk) is organized as an epithelial bilayer with an intervening ECM along the entire longitudinal axis of the organism. *Hydra*'s ECM is structured as two subepithelial zones (i.e., basal lamina matrix) with an intervening central fibrous zone (i.e., interstitial matrix). As shown in the composite diagram to the right that utilizes a transmission electron micrograph of *Hydra* ECM interposed between a drawing of two cell layers (ectoderm and endoderm), *Hydra* laminin and type IV collagen are localized to the two subepithelial zones of the matrix, while *Hydra* fibrillar collagens (e.g., Hcol-I) are localized to the central fibrous zone or interstitial matrix

7.1 Introduction

Hydra, as a member of Cnidarian, arose early during metazoan evolution in approximately 580 million years ago before the divergence of protosomes and deuterostomes. *Hydra* is the first of existing Cnidarian groups containing defined epithelial cell layers with junctional complexes present at the apical pole of the cells. Therefore, *Hydra* has true epithelial tissues. *Hydra* polyps' body plan is organized as a hollowed tube with a gastric (body) region in the middle, a mouth (also known as the hypostome) and a tentacle ring (which is usually composed of 6–8 tentacles) at one end known as the head pole, and a peduncle and basal disk at the other known as the foot pole (Fig. 7.1). Because of this cylindrical body shape, a *hydra* polyp has a radial symmetry at any horizontal body plane. Therefore, the study of development and regeneration on *Hydra* is mainly focused on the longitudinal axis. *Hydra* polyps use their basal disks to adhere to a surface, while their tentacles can catch preys and deliver them to the mouth opening. Nutrients are digested inside the gastric tube, and wastes are expelled from either the mouth or

the basal disk opening. The wall that surrounds the entire polyp from the tip of the tentacles to the bottom of the basal disk is structured as an epithelial bilayer with an intervening extracellular matrix (ECM) (Fig. 7.1). The ectodermal epithelial cells contain muscle fibers arranged longitudinally, whereas the endodermal epithelial cells have muscle fibers arranged circularly. At normal stage, these two groups of muscle fibers contract alternatively making hydra polyps exercise periodic elongation and retraction. In the gastric region, in between the epithelial cells are the interstitial cells (I-cells) that include phenotypes such as nerve cells, nematocytes, secretory cells, gametes, and differentiating I-cell precursors (Bode 1996).

Hydra polyps reproduce most of the time through budding at the "budding zone," which is located at the junction between body and peduncle (Fig. 7.1), although sexual reproduction via gametes is possible under special environmental conditions. The development of a bud starts with a tissue evagination followed by tentacle formation, body elongation, and base region constriction (Otto and Campbell 1977). A detached bud is proportionally the same as an adult polyp. Therefore, the budding process of a *Hydra* polyp has drawn great interests from developmental biologists because it involves many aspects of development from cell–cell interaction, molecular signaling, to pattern formation.

When the head or the foot is cut off, a *Hydra* polyp can regenerate the missing structure in 30–36 h (reviewed by Holstein et al. 2003). A complete normal *Hydra* polyp can be regenerated from a gastric region tissue as small as 5 % of the entire polyp (reviewed by Bode 2003). In 1972, Gierer et al. achieved regenerating *Hydra* polyps from aggregated cell pellets that were formed by non-enzymatically dissociated *Hydra* cells. This robust regenerating capacity granted *Hydra* a powerful model to study regeneration. However, studies have indicated that the high regenerative capacity of *Hydra* is solely due to the epithelial cells because polyps lacking any I-cells are fully capable of complete body regeneration (Bode 2011), although they are unable to feed themselves because of the lack of nerve cells. *Hydra* has also been used as a model for analysis of cell–ECM interactions during morphogenesis and cell differentiation.

Over the last three decades, biochemical and molecular studies have established that the ECM of *Hydra* is composed of a broad spectrum of matrix components reflective of that seen in more complicated vertebrate systems. These matrix molecules have been shown to have an important role in morphogenetic and cell differentiation processes in *Hydra*. These studies have also established the structural basis for the biophysical properties of *Hydra* ECM. Combining the early ultrastructural analyses with recent molecular and biochemical studies, the current understanding of *Hydra* ECM is a highly flexible matrix with elastic properties. This characteristic of *Hydra* ECM is mainly based on the macromolecular structure of its collagens including type IV and other fibrillar collagens.

This chapter includes discussions of *Hydra* ECM in regard to (1) its general structure, (2) its molecular composition, (3) the biogenesis of *Hydra* ECM during regeneration, (4) *Hydra* ECM in development, (5) cell–ECM interaction in *Hydra*, and (6) *Hydra* ECM remodeling during development.

7.2 The General Structure of *Hydra* ECM

7.2.1 *Early Studies on the Morphological Structure and Biochemical Composition of* Hydra *ECM*

Ultrastructural studies of *Hydra* ECM using electron microscopy technique identified a broad spectrum of structural components. *Hydra* ECM, also termed mesoglea, was described as an amorphous matrix of low density containing fine fibrils ranging from 5 to 50 nm in diameter (Hess 1957; Wood 1961; Gauthier 1963). From both sides, ectodermal and endodermal cells send cellular processes from their basal surfaces into the ECM and form cell–cell contacts across the mesoglea (Hess 1957; Wood 1961; Haynes et al. 1968).

In 1968, Davis and Haynes provided a detailed analysis of *Hydra* ECM with polyps being either relaxed or contracted. In agreement with previous studies, they reported that *Hydra* ECM was approximately 0.5–2.0 μm in diameter and noted that it was thickest in the body region and thinnest in the tentacles. Using TEM analysis, they indicated that the *Hydra* ECM had three structural components, namely, an amorphous ground substance, particulate materials, and fibrils. The fibrils consisted of three types. The smallest fibrils were 5–9 nm in diameter, and their density in the ECM varied depending on whether the polyps were in a relaxed or contracted state. When a polyp is contracted, these fibrils were randomly arranged and more densely packed, while in a relaxed state, the fibrils were in a more orderly arrangement and less densely packed. A second type of fibril that was less abundant was a thicker banded fibril that ranged from 36 to 45 nm in diameter and had a periodicity of approximately 30 nm. The third class of fibrils consisted of short thin fibrils that formed bundles oriented perpendicular to the longitudinal axis of the polyp. These fibrils extend from the basal surface of the ectodermal cells into the ECM suggesting some type of linker function.

Studies by Shostak et al. (1965) showed that isolated *Hydra* ECM was very elastic and could be stretched to twice its original length. It would retract to its original length when released from tension. In this regard, it was noted in the ultrastructural studies of Davis and Haynes (1968) that in a contracted state, the fibrils of the central fibrous zone become irregular and fold upon themselves.

Biochemical studies targeting at the macromolecular composition of *Hydra* ECM by Barzansky and Lenhoff (1974) and Barzansky et al. (1975) provided additional insight to its structure. Utilizing a combination of techniques including gel chromatography, amino acid analysis, and thin layer chromatography for sugar moiety analysis in conjunction with radiotracer precursor labeling, they reported that *Hydra* ECM had biochemical characteristics similar to vertebrate basal lamina. They also concluded that the amino acid profiles suggested the presence of collagens in *Hydra* ECM. Experiments using lathyritic agents known to cause reduced cross-linking of collagens indicated a role for these macromolecules in *Hydra* morphogenesis (Barzansky and Lenhoff 1974).

The combination of morphological and biochemical studies provided such a view that *Hydra* ECM exists as a highly flexible and elastic structure as compared to normal ECM of vertebrates. Also in contrast to vertebrate ECM, the collagens of *Hydra* ECM are more easily extracted due to a reduced amount of cross-linking as will be discussed later in the *Hydra* collagen sections (Deutzmann et al. 2000). The unique properties of *Hydra* ECM provide the necessary rigidity for maintenance of body shape while at the same time permitting sufficient plasticity to allow extensive but reversible shape changes along the longitudinal and radial axis.

Since 1991, with a purpose to further clarify the macromolecular structure of *Hydra* ECM and to elucidate the role of cell–ECM interactions during cell differentiation and morphogenesis, Sarras and his colleagues published a series of papers that utilized a combination of biochemical, molecular, and cell biological approaches. Below is an overview of what is currently known about the overall structure of *Hydra* ECM.

7.2.2 Hydra *ECM Is a Trilaminar Structure Composed of Two Subepithelial Basal Lamina Matrices and a Central Interstitial Matrix*

In order to investigate the structural details of *Hydra* ECM, it is essential to have a more in-depth understanding of the molecular composition of it. Once ECM molecules are in hand, antibodies and probes can be developed as tools for structural studies. Therefore, a series of studies toward this goal was started in late 1980s. The work involved (1) isolation of *Hydra* ECM and use of biochemical and immunological approaches to analyze the purified matrix preparation, (2) use of the purified *Hydra* ECM preparation to generate a battery of *Hydra*-specific polyclonal and monoclonal antibodies, and (3) use of these *Hydra*-specific antibodies as reagents to screen expression cDNA libraries and as probes to characterize the distribution of matrix components in *Hydra* ECM using morphological techniques. In addition, *Hydra*-specific antibodies and isolated matrix component domains were also used as blocking reagents to study the role of cell–ECM interactions in *Hydra* using a number of regeneration bioassays. These functional studies were later complemented with antisense RNA studies to selectively knockdown *Hydra* ECM components during regenerative processes.

Initial biochemical and immunological studies using antibodies generated to vertebrate ECM components indicated that *Hydra* ECM had a spectrum of matrix components similar to those observed in more complicated invertebrates and in vertebrates. Specifically, evidence for the presence of collagen type IV, laminin, heparan sulfate proteoglycan, and fibronectin-like molecules was present (Sarras et al. 1991a). Pulse-chase autoradiographic studies in conjunction with translational and posttranslational processing inhibitor studies supported the presence of collagen and proteoglycan components (Sarras et al. 1991a). Use of *Hydra*-specific

monoclonal antibodies (Sarras et al. 1993) in combination with special ultrastructural staining techniques (Sarras et al. 1994) revealed that *Hydra* ECM is composed of distinct structural regions. A central fibrous zone that appeared similar to interstitial matrix occupies the majority of H*ydra* ECM. Flanking the central interstitial matrix, adjacent to the basal plasma membrane of each epithelial cell layer, there are two much thinner basal lamina-like regions named the subepithelial zones (Fig. 7.1). Subsequent studies showed that laminin chains were confined to the subepithelial zones (i.e., basal lamina) (Sarras et al. 1994) and type I-like collagen was confined to the central fibrous zone (i.e., interstitial matrix) (Deutzmann et al. 2000). A recent study using vertebrate monoclonal antibody to the NC1 domain of collagen type IV has localized this macromolecule in the basal lamina layers, co-localizing with laminin (Shimizu et al. 2008).

Using confocal microscopy, scanning electron microscopy, and immunohistochemistry techniques, a study using *Hydra*-specific antibodies to laminin, collagen type IV, and collagen type I has demonstrated that *Hydra* ECM is a porous structure with multiple trans-ECM pores ranging from 0.5 to 1 μm in diameter and about six pores per 100 mm^2 in density. Cellular processes from the ectoderm and endoderm utilize these pores to form cell–cell connections within the ECM. These cellular processes are still in contact with the basal lamina which forms a cylinder around the cellular processes as they pass through the ECM (Fig. 7.2) (Shimizu et al. 2008). The most up-to-date understanding of the overall structure of *Hydra* ECM is shown in Figs. 7.1 and 7.2. A list of the major components of *Hydra* ECM with their general properties is shown in Table 7.1.

7.3 Molecular Components of *Hydra* ECM

7.3.1 Laminins

7.3.1.1 The Laminin Family of Matrix Proteins

Laminins represent a family of glycoproteins that are a major component of basement membranes (basal lamina). To date, at least sixteen different laminin heterotrimers have been identified in mammals, and a number of laminins have been identified in invertebrates such as *Drosophila*, *Caenorhabditis elegans*, and sea urchin (Burgeson et al. 1994; Richards et al. 1994; Iivanainen et al. 1995; Martin et al. 1995; Miner et al. 1995; Champliaud et al. 1996; Benson et al. 1999; Hutter et al. 2000). Laminins are involved in basement membrane assembly and are an important component in the supramolecular architecture of matrix (Yurchenco and O'Rear 1994; Yurchenco and Cheng 1994; Timpl and Brown 1996; Yurchenco 2011). The heterotrimer isoforms are generated from multiple subunits to include the α, β, and γ chains. In vertebrates, there are many different α, β, and γ chains found. Humans have at least 16 heterotrimeric laminin species (Aumailley et al. 2005). At least some of these chains

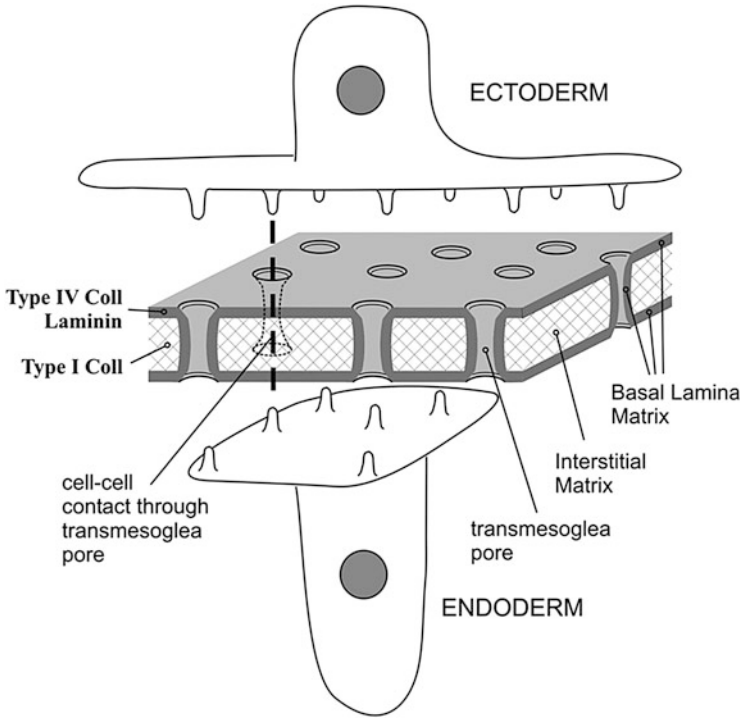

Fig. 7.2 Model of *Hydra* ECM structure showing cellular processes sheathed by a basal lamina and extending through the ECM from both the ectoderm and endoderm. These processes allow for communication between the two cell layers through a cellular extension system that is sheathed by a basal lamina and thereby separated from direct contact with interstitial matrix components [Scheme taken from Shimizu et al. (2008) with permission]

may exist as alternatively spliced forms (Kallunki et al. 1992; Galliano et al. 1995; Talts et al. 1999). ECM superstructure is based on assembly of a self-binding laminin mesh-like polymer, formation of a self-binding type IV collagen network polymer, and subsequent binding of various other ECM components to these networks (Colognato and Yurchenco 2000). Interaction between laminin domains of this network and cell surface ECM-binding proteins such as integrins and dystroglycan is involved in matrix assembly processes (Colognato et al. 1999) and cell signaling events (Darribere et al. 2000; Wickstrom et al. 2011; Yurchenco 2011; Alexi et al. 2011). In turn, matrix assembly influences cell differentiation and morphogenesis.

7.3.1.2 *Hydra* Laminin

Hydra laminin chains have been cloned and functionally analyzed (Sarras et al. 1993, 1994; Zhang et al. 2002). As stated above, *Hydra* laminin is localized to the two subepithelial zones (basal lamina) of *Hydra* ECM. A partial sequence for a *Hydra*

Table 7.1 Major components of *Hydra* ECM

ECM component	General properties
Basal lamina ECM components	
Laminin	Contains α, β, and γ subunits in a trimeric cruciate (HLM) structure as viewed by rotary EM. Cloning studies indicate that the alpha subunit is a vertebrate α5-like or *Drosophila*-like chain, while the beta subunit is a vertebrate β1-like chain containing at least one defined cell binding domain (FTGTQ)
Collagen type IV (Hcol-IV)	A homotrimeric glycoprotein formed from three collagen IV α1-like chains. Each subunit contains a collagen domain at its N terminus and a smaller non-collagenous domain (NC1) at its C terminus. The collagen domain contains RGD cell binding motifs. TEM rotary shadow studies indicate that polymerization of Hcol-IV molecules within the ECM involves interaction of the NC1 domains. A typical 7S domain at the N terminus appears not to be present in the mature ECM
Interstitial ECM components (fibrillar collagens)	
Fibrillar collagens type I (Hcol1)	A homotrimeric glycoprotein formed from three collagen type I α1-like chains. Each chain contains an N-terminal propeptide and C-terminal propeptide. During processing the C-terminal propeptide is removed, but the N-terminal propeptide is retained in the mature molecule. This results in the formation of flexible fibrils but not thickened banded fibrils as typically seen in vertebrate type I collagens. Hcol-1's flexibility is enhanced by a reduction in its proline content and a loss of critical lysines involved in lysyl cross bridging
Hydra collagen types 2, 3, 5, and 6 (Hcol2, Hcol3, Hcol5, and Hcol6)	*Hydra* fibrillar collagens Hcol 2, 3, 5, and 6 were characterized only from cDNA sequence analysis and subsequent computer modeling, and so unlike Hcol1 where the protein was also purified and biochemically analyzed, there is some degree of speculation about the in vivo nature of these proteins

See the proposed structures of the fibrillar collagens in Fig. 7.3

β1-like chain was initially reported (Sarras et al. 1994). Studies have since been completed for description of the entire ORF of the β1 chain and partial description of an α-5-like chain (Zhang et al. 2002). No γ chain has thus far been identified, although the typical trimeric form for isolated *Hydra* laminin has been observed by rotary shadow TEM analysis (Zhang et al. 2002). While *Hydra* laminin is localized to the two subepithelial zones (basal lamina) of *Hydra* ECM, it is synthesized exclusively by the endoderm, which means that the molecules have to diffuse through the mesoglea to reach the ectodermal layer (Sarras et al. 1993, 1994). The location at the

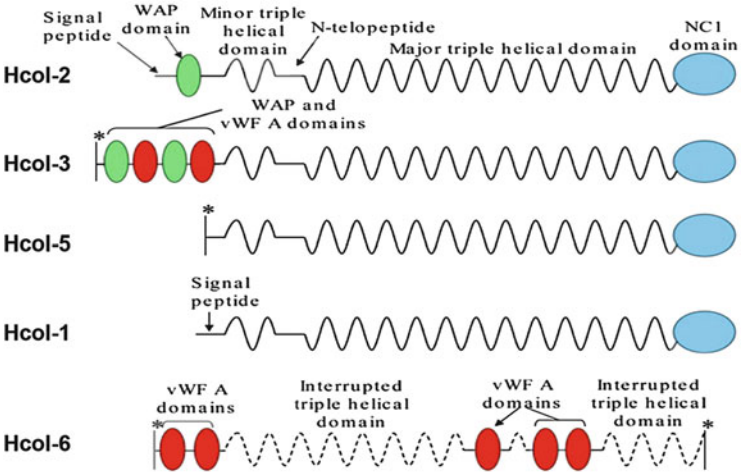

Fig. 7.3 Summary chart of the proposed structure of each of the *Hydra* fibrillar collagens. The complete cDNA sequences are known for fibrillar collagens Hcol-1, 2, 3 and 5, and the deduced structures are based on rigorous biochemical structural analysis (Zhang et al. 2007; modified with permission of the author)

basal region of both cell layers suggests that laminin is required for proper cell function and differentiation. During *Hydra* ECM regeneration, laminin secretion from the endoderm precedes the secretion of *Hydra* collagen-I that arises from the ectoderm (Deutzmann et al. 2000), and inhibition of laminin secretion through an RNA antisense technique will block collagen secretion (Shimizu et al. 2002). Earlier studies have already established that antibodies to *Hydra* laminin will block *Hydra* morphogenesis (Sarras et al. 1993) and other ECM-related processes such as cell migration (Zhang and Sarras 1994).

While the mechanism(s) of signal transduction in *Hydra* is not fully known, some published data (Agbas and Sarras 1994) and unpublished studies (Sarras laboratory) suggest the involvement of integrins, the primary class of ECM receptors in higher animals (Darribere et al. 2000; Wickstrom et al. 2011). The *Hydra* ECM-receptor data mainly pertain to a region in the short arm of *Hydra* laminin β1-like chain. In this regard, sequence analysis of the β1-like chain indicates the substitution of a FTGTQ sequence for the YIGSR receptor-binding sequence observed in vertebrates. Although the role of the YIGSR sequence in signal transduction-mediated processes has been questioned, published studies do indicate (1) its potential use as an inhibitor of human pre-B leukemic cell growth and metastasis using SCID mice models (Yoshida et al. 1999) and (2) its role in the guidance of axon growth cones (Hopker et al. 1999). Such studies and others support its involvement in cell signaling processes. More recent studies also validate the existence of the YIGSR ECM-receptor sequence (Saleh et al. 2008).

The substituted FTGTQ sequence in *Hydra* laminin β1-like chain has also been shown to interact with the cell surface under both in vitro and in vivo conditions (Sarras et al. 1994). Affinity purification studies indicated that the FTGTQ sequence can interact with a *Hydra* integrin-like protein (Agbas and Sarras 1994). To fully understand the relationship between pattern formation and ECM structural assembly in *Hydra*, further analysis of (1) laminin-mediated cell signaling processes and (2) the role of laminin in the biogenesis and assembly of *Hydra* ECM is required. As an update, it is important to note that based on publication of the *Hydra* genome in 2010, it is now known that true integrin molecules are present in the genome of this organism (Chapman et al. 2010). Based on this sequence data, more extensive structural and functional studies regarding cell–ECM interaction can now be conducted.

7.3.2 Collagens

Collagens are found in all animals and are the most abundant protein of the extracellular matrix. The basic structure of this large protein family consists of multiple Gly-X-Y repeats. Extensive structural and functional diversity among collagens is accomplished by introducing interruptions in the triple helical domains and inclusion of various globular domains (Prockop and Kivirikko 1995; Olsen and Ninomiya 1999; Ricard-Blum 2011). Some collagens, like the basement membrane collagen type IV, are found in all animals, whereas others are limited to particular groups. The fibrillar collagens for instance have previously only been found in vertebrates and have not been identified in invertebrates. In contrast, specialized collagens such as the cuticle collagens *of C. elegans* (Kramer 1994) and the mini-collagens of *Hydra* nematocyst capsules (Kurz et al. 1991) have only been found in invertebrates. Surprisingly, Kramer (1994) has reported that the cuticle collagens of *C. elegans* are encoded by more than a 100 genes. These observations indicate how diversity in collagen structure is utilized to meet the needs of a broad spectrum of specialized extracellular matrices. Accordingly, analysis of the structure of specialized collagens provides us with insight into the organization and function of ECM in both vertebrates and invertebrates.

While indirect evidence had suggested the existence of collagens in the ECM of *Hydra* (Hausman and Burnett 1971; Barzansky and Lenhoff 1974), more recent structural and functional analysis has provided a clear understanding of the types of collagens that exist in this invertebrate (Deutzmann et al. 2000; Fowler et al. 2000). These collagens include a basement membrane-type (*Hydra* collagen type IV, Hcol-IV) and an interstitial-type (*Hydra* fibrillar collagen, Hcol-1). Other *Hydra* fibrillar collagens have been more recently identified (Zhang et al. 2007). As will be discussed, Hcol-IV and Hcol-1 collagens have been characterized at both the cDNA and protein level.

7.3.2.1 *Hydra* Collagen Type IV

Collagen type IV is the second most prominent constituent of basement membranes after laminin. These collagens are glycoproteins composed of three subunits that form a polymerized network in conjunction with laminin (Hudson et al. 1994; Borza et al. 2000; Colognato and Yurchenco 2000). Currently, six different types of collagen type IV subunits are known to exist in vertebrates (Yurchenco and O'Rear 1994; Hopker et al. 1999; Borza et al. 2000; Vanacore et al. 2011). Invertebrate collagen type IV molecules have previously been identified in organisms such as *Drosophila* (Blumberg et al. 1988), *C. elegans* (Guo et al. 1991), and a number of other species (Kuehn 1994).

As reported by Fowler et al. (2000), *Hydra* ECM also contains a collagen type IV. Analysis of the cDNA clone revealed a protein of 1,723 amino acids, including an interrupted 1,455 residue collagenous domain and a 228 residue carboxyl-terminal non-collagenous domain. Hcol-IV is similar to all known α(IV) chains but, again, most closely resembles vertebrate and invertebrate α1(IV) chains. Like *Hydra* fibrillar collagen, Hcol-IV also forms homotrimeric molecules. Electron microscopy reveals an irregular network of rodlike structures interrupted by globular domains. This network can be depolymerized by reducing agents to dimeric collagen molecules, joined via their C-terminal non-collagenous domains. Under extensive denaturing conditions, depolymerization can only be taken to the dimeric but not monomeric stage. This suggests that the individual polypeptide chains are quantitatively held together by non-reducible cross-links in addition to disulfide bonds. This behavior is quite different from the vertebrate collagen type IV that needs pepsin digestion for solubilization. For vertebrate collagen type IV, a model has been proposed in which four molecules aggregate via their N-terminal domains to form a spider-like structure. The interactions are stabilized via disulfide bonds and lysine-derived cross-links, resulting in a highly protease-resistant 7S domain. In addition, the C-terminal globular domain, NC1 domain, binds to itself, mainly via disulfide bridges, to form a linear dimer.

Both interactions at the N-terminal and C-terminal ends lead to the proposal of an open network structure that can further polymerize via lateral aggregation of the triple helical domains (Yurchenco and O'Rear 1994; Kuehn 1994; Reinhart-King 2011). In contrast, in *Hydra*, while C-terminal interactions and lateral aggregation occur, a stable 7S domain is not formed. A similar collagen type IV has also been reported in the worm *Ascaris suum* (Noelken et al. 1986). This form of collagen type IV favors a more flexible ECM than that seen in typical vertebrate matrices.

7.3.2.2 *Hydra* Fibrillar Collagens

Hydra Fibrillar Collagen 1

Fibrillar collagens make up the majority of matrix components within the interstitial matrix of the animal kingdom. Likewise, *Hydra* fibrillar collagens are the major

components of *Hydra* ECM (Deutzmann et al. 2000). The cDNA for the *Hydra* fibrillar collagen, Hcol-I, encodes a protein of 1,412 amino acids. Hcol-I was the first fibrillar collagen to be identified in *Hydra*. The polypeptide isolated from *Hydra* ECM has an apparent molecular weight of 155 kDa. The subunit chains of Hcol-I form homotrimeric molecules that constitute the majority of the fibrils within the central fibrous zone (interstitial matrix). Sequence comparisons clearly define Hcol-I as a fibrillar collagen. The highest similarity is to the α chains of vertebrate collagens type I and II. A similar degree of similarity is found between Hcol-I and invertebrate sea urchin collagen (Exposito and Garrone 1990) and a sponge fragment (Exposito et al. 1992). Corresponding to the similarity at the sequence level, Hcol-I also exhibits the characteristic domain structure of fibrillar collagens, consisting of a central triple helical domain flanked by an N-terminal propeptide-like domain and a C-terminal propeptide (Fig. 7.3). It is noteworthy that the triple helical domain with 340 uninterrupted GLY-X-Y repeats has exactly the same length as the fibrillar collagens of vertebrates, suggesting similar fibril-forming possibilities.

Despite marked similarities in the primary structure, there are distinct differences in the supramolecular organization of vertebrate fibrillar collagen networks as compared to that seen in *Hydra* ECM. Hcol-I forms a network of fine fibrils rather than thicker banded fibrils as seen by electron microscopy of vertebrate interstitial matrices. In contrast to vertebrate collagens that require pepsin digestion for solubilization, large polymeric structures of Hcol-I can be isolated from the ECM of *Hydra* under native conditions.

Several factors are responsible for the special structure of *Hydra* Hcol-I. These factors include (1) a low content of proline in the triple helical domain that is only about 40 % that of vertebrate collagens, (2) a reduced degree of interchain cross-linking due to the lack of classical consensus sequences for lysine/lysine-aldehyde derived covalent bonds, and (3) most importantly, altered posttranslational processing that results in retention of the N-terminal propeptide-like domain in the mature molecule. Combined, these factors result in a more flexible fibrillar collagen that can bend on itself as suggested by the early ultrastructural studies of Davis and Haynes (1968).

Other *Hydra* Fibrillar Collagens: Hcol2, Hcol3, Hcol5, and Hcol6

Next to be identified through molecular cloning techniques were four fibrillar collagens named Hcol2, Hcol3, Hcol5, and Hcol6 (Zhang et al. 2007). Hcol6 was only a partial sequence of a collagen gene with a unique structural organization consisting of multiple von Willebrand factor-A domains interspersed with interrupted collagenous triple helices. Hcol2 and Hcol5 have major collagenous domains of classical length (1,020 amino acid residues), whereas the equivalent domain in Hcol3 is shorter (969 residues). The N-propeptide of Hcol2 contains a whey-acid-protein four-cysteine repeat (WAP) domain, and the equivalent domain of Hcol3 contains two WAP and two von Willebrand factor-A domains (Fig. 7.3).

Phylogenetic analyses revealed that the *Hydra* fibrillar collagen genes form a distinct clade that appeared related to the protostome/deuterostome A-clade of fibrillar collagens. Database searches revealed that Hcol2, Hcol5, and Hcol6 are highly conserved, which also provided preliminary evidence for the expression of a B-clade fibrillar collagen. In situ hybridization indicated an ectoderm expression pattern along the entire longitudinal axis of *Hydra* for the Hcol2 and Hcol3 fibrillar collagens as was previously reported for Hcol1 (Deutzmann et al. 2000). *Hydra* Hcol2 and Hcol3 also have high expression in the tentacles and forming buds. Fibrillar collagen Hcol5 has high expression in the tentacles, foot process, and forming buds of adult polyps, while Hcol6 expression is high at the base of the tentacles and in forming buds. During head regeneration, it is expressed in the ectoderm as observed with Hcol1. Hcol6 is expressed to a much lower degree than the other fibrillar collagens, and its expression pattern is restricted to the base of the tentacles and to forming buds (Zhang et al. 2007).

Non-ECM Collagens of *Hydra*

To be complete, it should be pointed out that *Hydra* has a variety of what are called "mini collagens." These collagens are localized within the capsule of nematocysts and are not associated with *Hydra*'s ECM. They have a unique and interesting structure due to their role in maintaining high hydrostatic pressures within the cavity of the nematocyst capsule. Because these collagens are not associated with *Hydra*'s ECM, they will not be further discussed in this chapter. Readers are referred to articles pertinent to their unique structure and function (Kurz et al. 1991; Holstein et al. 1994; Beckmann and Ozbek 2012).

7.3.3 *Evidence for Other Types of Matrix Components in* Hydra *ECM*

During functional studies using a pharmacological approach, evidence was obtained by Sarras et al. (1991a, 1993) and Zhang and Sarras (1994) indicating that proteoglycans also exist in the *Hydra* ECM. These experiments involved analysis of the effect of molecules that block proteoglycan biosynthesis during head regeneration (Sarras et al. 1991a), *Hydra* cell aggregate formation, and during cell migration as studied in *Hydra* grafting experiments (Zhang and Sarras 1994). The validity of these pharmacological studies was confirmed using pulse-chase autoradiographic techniques to demonstrate that proteoglycan-associated molecules such as SO4 were in fact blocked from appearing in the ECM following treatment with agents such as β-xyloside as compared to its inactive isomer, α-xyloside. These studies indicated that any blockage in proteoglycan biosynthesis resulted in a blockage in *Hydra* morphogenesis (head regeneration and cell

aggregate morphogenesis) and a retardation in normal I-cell migration, suggesting that proteoglycans are components of the *Hydra* ECM. Genbank search on NCBI website for *Hydra* proteoglycan EST sequences and proteoglycan proteins returned with several results of predicted *Hydra* sequences similar to vertebral proteoglycans. As of this date, however, proteoglycans have not been isolated from *Hydra* ECM, and therefore these evidences are indirect in nature.

7.4 Biogenesis of *Hydra* ECM Is Intimately Tied to Regeneration

Hydra ECM synthesis and normal assembly are required for general morphogenesis, pattern formation, and cell differentiation to occur in the adult polyp. This has been determined through head and foot regeneration studies and through experiments utilizing *Hydra* cell aggregates. Development of *Hydra* cell aggregates involves complete morphogenesis of several *Hydra* polyps within 48–72 h from a cell pellet formed from non-enzymatically dissociated cells obtained from the adult polyps (Gierer et al. 1972; Bode 1974, Sarras et al. 1993). In all processes studied, including head regeneration, foot regeneration, and development of *Hydra* cell aggregates, formation of a new ECM must occur before morphogenesis can proceed. If ECM biogenesis is blocked or perturbed in any way, morphogenesis is stopped. This has been shown through a broad array of approaches to include (1) use of pharmacological reagents (Sarras et al. 1991a), (2) blocking antibodies to *Hydra* ECM components (Sarras et al. 1993), (3) fragments of ECM components that are used to compete in the normal polymerization of the matrix components (Zhang et al. 1994), and (4) antisense RNA to ECM components introduced through electrophoresis techniques (Shimizu et al. 2002).

The sequence of events associated with *Hydra* ECM biogenesis indicates that cross-talk occurs between the ectoderm and endoderm and through signals emanating from the ECM to both epithelial layers. A general description of *Hydra* ECM biogenesis is depicted in Fig. 7.4. Removal of the head or foot or a simple incision along the gastric tube wall of *Hydra* results in a retraction of the ECM from the cut edge. This is explained by the unique elastic properties of *Hydra* ECM. Together with epithelial cell migration to cover the wound, a region with sealed epithelial bilayer that has no ECM between the two cell layers is created (Shimizu et al. 2002). This ECM-deficient bilayer changes its morphology so that cells become flattened compared to the more cuboidal to columnar cellular phenotype normally seen along *Hydra*'s body wall (Fig. 7.4). Soon after sealing (within 1 h post-decapitation), this bilayer immediately begins to synthesize a new ECM. The process involves initial upregulation of ECM component genes within 3 h of decapitation (in the case of head regeneration experiments as shown in Fig. 7.4). In this process, basal lamina mRNA expression (e.g., laminin and Hcol-IV) is specific to endodermal cells, while interstitial matrix components' expression (e.g., Hcol1,

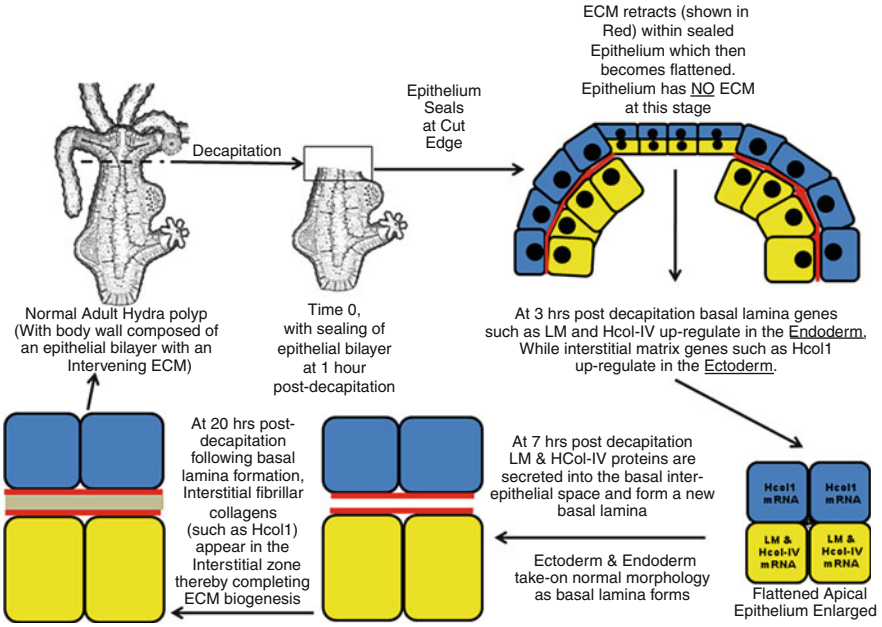

Fig. 7.4 ECM biogenesis following decapitation of *Hydra*. Experimentally induced regeneration in *Hydra* is typified by surgical removal of the head pole in the adult *Hydra* polyp. This is a simple procedure involving excision of the head pole with a small scalpel. When decapitation is initiated, the open head pole (the gastric cavity is now open to the aqueous environment) seals within 1 h by fusion of the two cut ends of the apical epithelial bilayer (Shimizu et al. 2002). The elastic properties of *Hydra* ECM cause the entire matrix to retract at the cut ends resulting in the sealed epithelial bilayer to now lack an intervening ECM. Because of the lack of an ECM, the apically sealed epithelial bilayer alters its morphology and becomes flattened as compared to the normal cuboidal-columnar phenotype that body wall cells display. Within 3 h, an upregulation of ECM genes is observed in the ectoderm and endoderm (Shimizu et al. 2002). This upregulation could be due to the abrupt lack of an ECM and subsequent ectoderm/endoderm cell to cell signaling. By 7 h, basal lamina components such as laminin and collagen type IV (Hcol-IV) are translated and secreted into the basal inter-epithelial compartment where the ECM is normally located. The basal lamina (BL) polymerizes at the basal border of both the endoderm and ectoderm. Once this occurs, the epithelial bilayer begins to resume its normal phenotype. Following the appearance of the BL and 20 h after decapitation, interstitial fibrillar collagens such as Hcol1 begin to be translated and appear between the two previously formed basal lamina layers adjacent to the basal extracellular border of the ectoderm and endoderm (Shimizu et al. 2002). This likely involves signaling events between the basal lamina components and the ectodermal cell layer from which Hcol1 is produced. These signals between the basal lamina components and basally located ECM-receptor plasma membrane complexes (e.g., integrins) trigger initiation of ECM-specific cellular pathways that are required for biogenesis of the ECM. Once the interstitial matrix polymerizes, the ECM is now completely polymerized and the normal adult Hydra body wall structure is reformed. This permits pattern formation processes to complete head regeneration morphogenesis

2, and 3) is specific to the ectoderm layer of cells. Within 7 h of decapitation, basal lamina (BL) proteins are secreted from the endoderm, and a newly formed BL is seen associated with the basal extracellular border of both the ectoderm and

endoderm. Through ECM-receptor systems (Agbas and Sarras 1994), BL components trigger synthesis of fibrillar collagens, such as Hcol1, that are secreted from the ectoderm and polymerize in the interstitial matrix zone between the two BL layers (Shimizu et al. 2002). The linkage between BL formation and interstitial matrix component translation and secretion is supported by RNA antisense experiments in which laminin translation is blocked and subsequent Hcol1 translation and secretion from the ectoderm is prevented (Shimizu et al. 2002), thereby blocking ECM formation and perturbing morphogenesis of a head or foot structure. The regeneration of *Hydra* cell aggregates formed from dissociated cells goes through a cystic stage which has an epithelial bilayer that lacks an ECM. If one uses pharmacological agents, blocking antibodies to *Hydra* ECM components, or fragments of ECM components to perturb ECM polymerization in *Hydra* cell aggregate, morphogenesis of the aggregates beyond the cystic stage will not occur (Zhang et al. 1994).

Taken in total, these studies indicate that (1) ECM biogenesis is essential for *Hydra* morphogenesis and (2) ECM biogenesis is a complicated process involving cross talk between the endoderm and ectoderm as well as signaling from the ECM to the cells of the bilayer. This cross talk involves sequential synthesis of basal lamina (endoderm) and interstitial matrix (ectoderm) components that will in turn signal appropriately timed translation and secretion of ECM proteins resulting in the polymerization of a well-ordered matrix. As with higher invertebrates, this also involves ECM-receptor complexes that trigger appropriate signaling pathways associated with ECM biogenesis processes.

7.5 *Hydra* ECM in Development

Since embryogenesis in *Hydra* does not occur regularly, development in *Hydra* can be studied through the processes of bud formation and tentacle growth. In addition, *Hydra* cell aggregate regeneration involves the entire process of polyp development from de novo and, therefore, is also a good model to study *Hydra* development.

When a bud forms, the evagination of the epithelial bilayer brings the ECM with it. As the epithelial bilayer protrudes quickly in budding (Otto and Campbell 1977), the ECM is stretched to a very thin layer (Aufschnaiter et al. 2011). This provides more evidence to support the elastic property of *Hydra* ECM. At this time, the thickness of the ECM is only about 50–75 % of that of the body region. Nevertheless, the ECM is still associated with the bud and the trilaminar structure is likely maintained. While the ECM is stretched thin within the bud, the epithelial cells of the bud start to upregulate the synthesis of ECM components, namely, laminin and collagens type I and IV. This is shown by in situ hybridization experiments using molecular probes to these ECM components (Zhang et al. 2002, 2007). During this process, the sequence of events is proposed to involve the following steps: (1) epithelial cell protrusion stretches the ECM thin; (2) the thinning of the ECM sends signal(s) to epithelial cells on both sides to make more ECM; and (3) as a result, the ECM increases in thickness

within the bud which affects bud morphogenesis and tentacle formation. The nature and pathway(s) of this signaling process needs further study.

The ECM within tentacles is much thinner than that of the body region and remains thin during the life of the organism. In situ hybridization experiments (Deutzmann et al. 2000; Fowler et al. 2000; Zhang et al. 2002, 2007) indicated that ECM synthesis is concentrated in the proximal region of the tentacles with the highest level of synthesis at the base. ECM synthesis of the tentacle gradually disappears at its distal region. Aufschnaiter et al. (2011) have recently published a set of in vivo studies indicating that the ECM of tentacles moves from a proximal to distal position and finally disappears. Combining the two sets of data (in situ hybridization and in vivo ECM labeling), it is reasonable to propose that tentacle ECM is brought over from the body region when tentacles start to emerge, continue to be synthesized in the proximal region during tentacle extension, and are displaced toward the tentacle distal tips. There is no data indicating that tentacle emergence also involves ECM stretching as observed in budding; however, both biological events start with epithelial cell evagination and protrusion. It is possible that tentacle emergence and budding both employ the same signaling mechanism as a result of ECM stretching and thinning.

7.6 Cell–ECM Interactions in *Hydra*

Cell–ECM interactions are essential during normal tissue homeostasis, in a broad spectrum of disease states, as well as in developmental processes (Matejas et al. 2010; Kim et al. 2011; Watt and Fujiwara 2011; Wickstrom et al. 2011; Yurchenco 2011). Here, we discuss cell–ECM interactions by including the following processes: (1) cell proliferation, (2) cell migration, (3) cell differentiation, and (4) morphogenesis. These cell–ECM interactions in *Hydra* are listed in Table 7.2 which includes references of the studies. A role for the ECM in the modulation of cell proliferation in *Hydra* was first reported by Zhang et al. (1994). These studies showed that introduction of exogenous matrix protein domains during ECM formation in *Hydra* resulted in alterations in cell proliferation rates. Likewise, alteration of ECM structure or exogenous introduction of blocking antibodies to matrix components or matrix component domains would retard I-cell migration in *Hydra* grafting experiments (Zhang and Sarras 1994). In vitro studies with isolated nematocytes have also indicated dependence of cell–ECM interactions for cell migratory processes in *Hydra* (Gonzalez-Agosti and Stidwill 1991; Ziegler and Stidwill 1992; Stidwill and Christen 1998). As discussed above, a number of studies involving head regeneration (Sarras et al. 1991b) and morphogenesis of *Hydra* cell aggregates (Sarras et al. 1993) have clearly shown that any perturbation of ECM formation will result in blockage of *Hydra* morphogenesis. In addition, studies involving *Hydra* cell aggregates (Zhang et al. 1994) or *Hydra* foot regeneration (Leontovich et al. 2000) have shown that perturbation of ECM structure or ECM turnover can affect cell differentiation in the adult *Hydra* polyp. To understand the

Table 7.2 Developmental processes in *Hydra* that have been reported to involve cell–ECM interactions

Developmental process	References
1. Porosity of *Hydra* ECM as it facilitates cell–cell communication between the ectoderm and endoderm	Shimizu et al. (2008)
2. *Hydra* ECM as it relates to cell turnover and the budding process	Münder et al. (2010) and Aufschnaiter et al. (2011)
3. Cell proliferation during morphogenesis as monitored in *Hydra* cell aggregates	Zhang et al. (1994)
4. Cell migration under in vivo conditions in grafting experiment and under in vitro conditions with isolated *Hydra* nematocytes using ECM-coated culture plates	Gonzalez-Agosti and Stidwill (1991), Ziegler and Stidwill (1992), Zhang and Sarras (1994), and Stidwill and Christen (1998)
5. Cell differentiation/transdifferentiation of basal disk cells and battery cells and cell differentiation in Hydra cell aggregates	Zhang et al. (1994), Yan et al. (1995, 2000), and Leontovich et al. (2000)
6. Epithelial morphogenesis of the head or foot as monitored in regeneration experiments and in the adult polyp as monitored in Hydra cell aggregate experiments	Barzansky and Lenhoff (1974), Sarras et al. (1991a, 1993, 1994), Yan et al. (1995, 2000), Deutzmann et al. (2000), and Fowler et al. (2000)

signaling process of the cell–ECM interactions in *Hydra*, the signaling motifs on the cell surface or in the ECM are considered. These signaling motifs can be either exposed or cryptic.

The action of *Hydra* matrix metalloproteinases has been shown to be involved in exposing potentially cryptic signaling sites within ECM components (Leontovich et al. 2000). *Hydra* matrix metalloproteinase (HMMP) has been shown to specifically affect the maintenance of the phenotype of basal disk cells through mechanisms that remain unclear (Leontovich et al. 2000). A similar observation has been reported for *Hydra* metalloproteinase-1 that is localized to the tentacle ECM and has been shown to be involved in the maintenance of tentacle battery cell phenotypic markers (Yan et al. 1995, 2000). As reviewed by Schmid et al. (1999), the importance of cell–ECM interactions during morphogenesis and cell differentiation extends beyond *Hydra* to a number of classes within Cnidaria. Most recently, a new in vivo labeling technique for *Hydra* collagen 1 and laminin was used to track the fate of ECM in all body regions of the animal and to track the relationship between epithelial cell movement and that of the underlying ECM (Aufschnaiter et al. 2011). It has been known that the epithelial cell layers in *Hydra* are constantly displaced toward the tentacle tips, the basal disk, and into the buds (Shostak and Globus 1966; Campbell 1967, 1973). Therefore, all cells in a *Hydra* polyp (except cells in the hypostome region) are constantly moving. What happens to the ECM during epithelial cell movement was not clearly understood before. These recent ECM studies revealed that *Hydra* epithelial cells moved together with the associated ECM, although the pace of movement of the ECM varies at different regions of a polyp (Aufschnaiter et al. 2011). Sometimes, epithelial cells and ECM

move together, whereas other times cells move faster than that of the ECM. During the evagination of buds and tentacles, extensive movement of epithelial cells relative to the matrix was observed together with local ECM remodeling (Aufschnaiter et al. 2011). From this study, we now confirm that the *Hydra* ECM is not a stationary skeleton that supports cells to move on it, but rather, it moves dynamically along with the epithelial cells.

7.7 ECM Remodeling During Development in *Hydra*

While the extracellular metalloproteinases work to expose the ligands for cell–ECM signaling, they are also involved in remodeling the ECM constantly to meet the needs for maintaining the polyp's shape and structures. Several cDNA fragments of *Hydra* matrix metalloproteinases have been cloned, and their expression patterns were observed at different regions of the polyp such as base of the tentacles, upper body column, lower body region, peduncles, and base of the bud (Zhang lab unpublished data). One matrix metalloproteinase, MMP-A3, was specifically expressed at the junction between the base of the bud and the body of the mother polyp. It appeared when the base of the bud started to constrict, which is a necessary step to make the bud detach, and lasted until shortly after the bud fell off from the mother polyp (Münder et al. 2010). The constriction at the base of the bud is the beginning of a boundary setup between the bud tissue and that of the mother polyp. Several signaling pathways are involved in this boundary formation (Münder et al. 2010). In addition, collagen biosynthesis and cross-linking is needed to carry out the constriction process (Aufschnaiter et al. 2011). When using 2,2-dipyridyl, a chemical inhibitor of prolyl hydroxylase, on a budding *Hydra* polyp, the constriction stopped and the bud failed to detach (Aufschnaiter et al. 2011). These studies suggest that ECM synthesis, assembly, and remodeling occur at the same time and at the same position for a common biological process that takes place in *Hydra*. This dynamic and intimate association between ECM synthesis and remodeling may happen throughout the body axis of *Hydra*.

7.8 Conclusion

Hydra ECM is a flexible and elastic matrix positioned between two epithelial layers. Hydra ECM has multiple pores that house epithelial cell processes that arise from both sides of the bilayer and that contact each other within the matrix. It is organized as a trilaminar structure with two thinner subepithelial zones and one thicker central interstitial matrix. Laminin and type IV collagen occupy the subepithelial zones, whereas fibrillar type I collagen forms the majority of the interstitial matrix. Both epithelial cell layers contribute to ECM synthesis with laminin from the endoderm and collagens from the ectoderm. The trilaminar

structure is maintained throughout a *Hydra* polyp and moves with epithelial cells, but each at different rates. *Hydra* ECM is resynthesized and assembled during regeneration, and this process is absolutely needed before other developmental processes, such as morphogenesis and pattern formation, can happen. During development and regeneration, *Hydra* ECM interacts with epithelial cells via signaling processes between the two. Remodeling of *Hydra* ECM happens constantly throughout the polyp by using a family of matrix metalloproteinases which are specifically located at different regions of the polyp.

The functional role of *Hydra* ECM in a variety of its cellular and developmental processes is seen replicated in more complicated vertebrate systems (Kim et al. 2011; Kruegel and Miosge 2010; Watt and Fujiwara 2011; Wickstrom et al. 2011; Yurchenco 2011). Given the early divergence of *Hydra* during metazoan evolution, this serves to reinforce the fundamental nature of ECM to the cell biology of all metazoans. This also reminds us that although *Hydra* is considered a relatively "simple" organism, it does not mean that its cellular and molecular biology is anything but simple to understand. In this regard, future studies with *Hydra* should be directed at (1) understanding how cell signaling pathways coordinate the formation of ECM by the epithelial bilayers of *Hydra* and (2) the precise mechanisms underlying the role of ECM in *Hydra* morphogenesis and cell differentiation.

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Chapter 8
Extracellular Matrix Remodeling in Zebrafish Development

Nathan A. Mundell and Jason R. Jessen

Abstract The importance of extracellular matrix proteins in the development of chick, frog, and mouse embryos has been recognized for decades. Accordingly, with the emergence of zebrafish as a genetic and developmental model system, there has been a steady increase in the number of studies showing developmental roles for matrix proteins, their receptors, and their modifying enzymes. The goal of this chapter is to highlight some of the extracellular matrix and interacting proteins present in the developing zebrafish embryo and discuss examples of morphogenetic processes requiring extracellular matrix protein expression, assembly, and turnover.

8.1 Introduction

Extracellular matrix proteins influence multiple cell behaviors including adhesion, polarity, migration, differentiation, growth, and survival. It is therefore not surprising that functional roles for matrix proteins have been identified in processes as diverse as zebrafish gastrulation, somitogenesis, neuronal migration, organogenesis, and jaw development. However, the study of extracellular matrix and interacting proteins in zebrafish is in its infancy. A cursory examination of the literature indicated that 90 % of zebrafish publications mentioning collagen, fibronectin, laminin, or matrix metalloproteinases were published in the last decade. The

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goal of this chapter is to highlight the types of zebrafish developmental processes where extracellular matrix and interacting proteins have been identified as playing important roles. We also provide a framework for the reader to gain an appreciation for the different extracellular matrix proteins, integrins, and metalloproteases present in zebrafish and their potential contributions to embryonic development.

8.2 Zebrafish Extracellular Matrix and Interacting Proteins

8.2.1 Fibronectin

Fibronectin is a large multidomain dimeric glycoprotein (dimer size is ~440 kDa) expressed in both embryonic and adult tissues. Vertebrates contain two types of fibronectin; soluble/globular fibronectin is a major component of blood plasma, while insoluble cellular fibronectin is a major extracellular matrix protein that is assembled from a dimer into a fibrillar meshwork (Schwarzbauer and Sechler 1999). It has become clear from work on frog embryos that the state of the fibrillar fibronectin matrix can have different effects on morphogenetic cell movements (Rozario et al. 2009) (see Chap. 3). Integrins are the major cell surface receptors for fibronectin, functioning to link cells to the extracellular matrix (Mao and Schwarzbauer 2005). Generation of a fibrillar fibronectin matrix is a cell-mediated process requiring integrin receptors, cell adhesion, and mechanical forces (reviewed by Schwarzbauer and DeSimone 2011) (see also Chap. 3). Fibronectin also associates with other extracellular matrix proteins such as collagen and is responsible for fibrillogenesis of both fibronectin and collagen networks (Dallas et al. 2006). Loss of fibronectin in the mouse is embryonic lethal producing numerous problems associated with mesoderm morphogenesis including somite and heart defects (George et al. 1993; Georges-Labouesse et al. 1996). The zebrafish genome encodes two fibronectin isoforms, Fibronectin1 and Fibronectin1b (Zhao et al. 2001; Sun et al. 2005). *Fibronectin1* mRNA exhibits a restricted expression pattern in the early embryo (prior to 24 h post-fertilization) localizing to the mesoderm, lateral plate mesoderm, posterior notochord, tail bud, and yolk syncytial layer (Thisse et al. 2004; Trinh and Stainier 2004). At later stages (after 24 h post-fertilization) *fibronectin1* is detected in the tail bud, otic vesicles, and pharyngeal arches (Rauch et al. 2003). The *fibronectin1* gene is disrupted in *natter* mutants characterized by a flattened hindbrain and cardia bifida (Chen et al. 1996; Jiang et al. 1996; Trinh and Stainier 2004). Homozygous *natter* mutant embryos exhibit defects in epithelial organization and the timing of myocardial precursor cell migration (Trinh and Stainier 2004) (discussed in Sect. 8.3.6). *Fibronectin1b* expression is also restricted localizing to somitic and paraxial mesoderm at early embryonic stages and to epidermis, posterior somites, and perhaps the liver at later stages (Thisse et al. 2004). There is currently no *fibronectin1b* mutant, but studies conducted using antisense morpholino

oligonucleotides have shown a requirement for *fibronectin1b* during somitogenesis and muscle development (Julich et al. 2005; Koshida et al. 2005; Snow et al. 2008) (discussed in Sects. 8.3.2 and 8.3.3). Similar to the mouse and frog, knockdown of zebrafish fibronectin also disrupts convergence and extension cell movements associated with gastrulation (Latimer and Jessen 2010) (discussed in Sect. 8.3.1).

8.2.2 Laminin

Laminins form heterotrimeric protein complexes that act as key components of all basement membranes as well as other types of extracellular matrices. Laminins consist of different combinations of alpha, beta, and gamma subunits, and mammals possess at least 16 distinct complexes that are encoded by 12 *laminin* genes. Mutations in laminin subunits contribute to a variety of human diseases including cardiomyopathy and muscular dystrophy (Mostacciuolo et al. 1996; Knoll et al. 2007). The zebrafish genome encodes at least 10 laminin orthologs with distinct and overlapping mRNA expression patterns. Notable expression domains include the eye, brain and spinal cord, pharyngeal arches, notochord, and trunk muscle (reviewed by Sztal et al. 2011). Mutations in Laminin1 and Laminin2 subunits produce strong developmental defects. For example, loss of either *lamininβ1* (*grumpy*) or *lamininγ1* (*sleepy*) prevents proper formation of both the intersegmental blood vessels and the basement membrane adjacent to the notochord resulting in a failure in notochord differentiation (Parsons et al. 2002). Mutation of the *lamininα1* gene (*bashful*) causes a milder notochord phenotype that can be enhanced by simultaneous disruption of *laminina4* (Pollard et al. 2006). Mutation of *lamininα1* also disrupts the directed migration of hindbrain motor neurons but not their ability to undergo proper axonal guidance (Paulus and Halloran 2006) (discussed in Sect. 8.3.4). By contrast, mutations in *laminina2* (Hall et al. 2007) and *lamininβ2* (Jacoby et al. 2009) genes produce dystrophic phenotypes characterized by degeneration of embryonic skeletal muscle (discussed in Sect. 8.3.3).

8.2.3 Collagen

Collagen is the most abundant extracellular matrix protein in the mammalian body and consists of elongated fibrils found in fibrous tissues and also the cornea, cartilage, bone, blood vessels, gut, and discs of the spinal column. There are 28 distinct mammalian collagen family members (Ricard-Blum 2011). The zebrafish genome encodes at least 22 collagen isoforms including 10 representative collagen family members (type I, II, IV, V, VI, VII, VIII, IX, X, and XI). *Collagen* mRNA expression patterns during zebrafish development correlate with known sites of protein function in mammals. For example, *collagen9a3* and *collagen27a1a* are detected in the jaw cartilage, while *collagen1a1a* is abundantly expressed in the

epidermis, somites, and pectoral fins (Thisse et al. 2001). The type IV collagen *collagen4a5* is expressed throughout the notochord at mid-somitogenesis stages and within the midbrain–hindbrain boundary, eye lens, otic placode, spinal cord, and epidermis after 24 h post-fertilization (Thisse et al. 2004; Xiao and Baier 2007). Notably, unlike fibronectin and laminin (Latimer and Jessen 2010), few *collagens* are expressed during the time period of gastrulation (shield-stage/6 h post-fertilization through tail bud stage/10 h post-fertilization) and those that appear to be restricted to axial tissue (Thisse et al. 2001, 2004; Rauch et al. 2003). Despite the number of *collagen* genes, few zebrafish *collagen* mutants have been identified. Mutation of the *dragnet* locus results in disruption of the type IV collagen gene *collagen4a5* (Xiao and Baier 2007). Homozygous *dragnet* mutant embryos display a retinal ganglion cell axonal targeting defect whereby axons project abnormally into a deeper layer of the tectum, the stratum opticum. It was shown by RNA in situ hybridization that *collagen4a5* expression in the epidermis/basement membrane is directly juxtaposed to the stratum opticum (Xiao and Baier 2007). Disruption of *collagen4a5* alters the basement membrane such that extracellular matrix proteins (e.g., heparan sulfate proteoglycans) are mislocalized, tectum structure is altered, and axonal targeting is defective (Xiao and Baier 2007). Disruption of the *collagen8a1* gene in *gulliver* mutant embryos produces a mild phenotype characterized by distortion and undulation of the notochord (Gansner and Gitlin 2008). The *collagen8a1* gene transcript is strongly expressed in the notochord throughout early embryogenesis (Gansner and Gitlin 2008). Though Laminin1 function within the basement membrane is important for notochord development (Parsons et al. 2002; Pollard et al. 2006), the notochord of *gulliver* mutant embryos is much more reminiscent to that observed in embryos with defects in the extracellular matrix protein Fibrillin-2 (Gansner et al. 2008) and copper homeostasis (Mendelsohn et al. 2006). Indeed it was shown that *collagen8a1* mutant embryos are sensitized to lysyl oxidase cuproenzyme inhibition suggesting that collagens and laminins have distinct functions in formation of the extracellular matrix surrounding the developing notochord (Gansner and Gitlin 2008).

8.2.4 Proteoglycans

Extracellular matrix proteoglycans constitute a protein core that is covalently linked to glycosaminoglycan chains (chondroitin, dermatan, heparan, or keratan sulfate) and, whether secreted or membrane bound, play a variety of roles during development. Proteoglycans represent a large number of proteins that make up a significant portion of the extracellular matrix. Heparan sulfate proteoglycans (HSPGs) in particular have been shown to regulate the availability and action of secreted morphogens (Lin 2004). In the zebrafish myotome, for example, hedgehog-induced expression of Engrailed is required to define specific muscle fiber populations. In an elegant study, Dolez and colleagues demonstrated that Lamininγ1 functions to restrict Engrailed expression to a central domain of the

myotome (Dolez et al. 2011). Mechanistically, Lamininγ1 modulates cellular responsiveness to secreted bone morphogenetic protein by affecting HSPGs present in the extracellular matrix (Dolez et al. 2011). In another recent zebrafish study, antisense morpholino-mediated knockdown of the keratan sulfate proteoglycan lumican was shown to cause an ocular enlargement defect reminiscent of axial myopia in humans, a disease characterized by disrupted arrangement and structure of collagen fibrils (Yeh et al. 2010). Together, these examples illustrate how different proteoglycans can regulate diverse biological processes during zebrafish development. The functions of additional zebrafish proteoglycans will be discussed in Sects. 8.3.1, 8.3.6, 8.3.7, and 8.3.8 (for a detailed discussion of syndecans, see Chap. 1).

8.2.5 Integrins

Integrin glycoproteins are type I transmembrane receptors for extracellular matrix proteins including fibronectin, laminin, and collagen. Integrins form heterodimers through interactions between 18 alpha and 8 beta subunits and function at the cell surface to form adhesive attachments between cells and the extracellular matrix (reviewed by Hynes 2002; Schwartz and Ginsberg 2002). Integrins influence multiple developmental processes including cell adhesion, migration, cell survival, proliferation, and gene expression (reviewed by Danen and Sonnenberg 2003). A notable aspect of integrin function is to facilitate extracellular matrix assembly and turnover. In this process, integrin binding to matrix proteins creates tension on the extracellular matrix fibrils that promotes further integrin binding and cross-linking of fibrils (Schwarzbauer and Sechler 1999). In one example of outside-in signaling, integrin engagement of extracellular matrix actually regulates the localization and synthesis of matrix metalloproteinases that are capable of affecting matrix remodeling and turnover (Galvez et al. 2002; Gonzalo et al. 2010) (see Sect. 8.2.5). The zebrafish genome contains at least 17 integrin or integrin-like genes including genes encoding nine alpha subunits and eight beta subunits. Notably, the zebrafish has multiple *integrinβ1* homologs including full-length and truncated paralogs that could potentially share the roles of the β1 subunit from other vertebrates or have additional functions such as altered ligand-binding (Mould et al. 2006). Using recombinant proteins and biochemical binding assays, it was demonstrated that zebrafish Integrinα5 can form a functional heterodimer with human β1 integrin. Interestingly, zebrafish Integrinα5 (with different β1 subunits) was able to bind zebrafish but not human fibronectin (Mould et al. 2009). It is suggested that amino acid differences in the putative integrin-binding site between human and zebrafish fibronectin (asparagine and glycine, respectively) may explain this difference (Mould et al. 2009). The zebrafish integrins exhibit diverse expression patterns in the developing embryo including the notochord, somites, pharyngeal arches, pectoral fins, pronephros, and dental epithelium (Thisse et al. 2004; Ablooglu et al. 2007). A comparison of *fibronectin1* and *fibronectin1b* mRNA

localization with its integrin receptor subunits *integrinα5* and *integrinβ1* demonstrates distinct and overlapping expression domains in adaxial cells, posterior tail bud, presomitic mesoderm, notochord, and somites (Thisse et al. 2001, 2004; Julich et al. 2005). Loss of *integrinα5* in both mouse and zebrafish produces defects in somitogenesis and the maintenance of somite boundaries (Goh et al. 1997; Julich et al. 2005; Koshida et al. 2005) (see Sect. 8.3.2). By contrast, loss of Integrin αvβ1b function was shown to affect dorsal forerunner cell migration during gastrulation, thus disrupting the formation of Kupffer's vesicle and left–right body asymmetry (Ablooglu et al. 2010).

8.2.6 Matrix Metalloproteinases

Matrix metalloproteinases (MMPs) belong to the metzincin superfamily of zinc-dependent endopeptidases and are capable of cleaving both extracellular matrix and non-extracellular matrix targets such as receptors, ligands, and adhesion molecules. The general structural features of MMP proteins include a propeptide followed by catalytic domain that is connected to a hemopexin domain via a hinge region. Most MMPs are secreted as inactive zymogens requiring proprotein convertase activity to remove the N-terminal propeptide domain for activation (reviewed by Zucker et al. 2003). Membrane-tethered MMPs are characterized by either a glycosylphosphatidylinositol (GPI)-anchor or a type I transmembrane domain for localization to the cell surface. Unlike secreted MMPs, membrane-tethered MMPs are activated in the trans-Golgi network by convertases (reviewed by Sternlicht and Werb 2001) or by alternative mechanisms such as auto-proteolysis (Rozanov et al. 2001). MMPs are inhibited by soluble proteins called tissue inhibitors of metalloproteinases (TIMPs).

Since their first identification 50 years ago, 24 MMPs have been identified in humans along with 4 TIMPs. Recently, Wyatt et al. reported the first thorough sequence and phylogenetic analysis of zebrafish MMPs and TIMPs (Wyatt et al. 2009). While the zebrafish genome has a similar complement of *mmp* and *timp* genes as compared to humans, the complexity of this repertoire is lower. For example, zebrafish appear to lack orthologs of human MMP1, 3, 7, 8, 10, 12, 26, and 27 and TIMP1 (Wyatt et al. 2009). Notably, each of the six membrane-tethered MMPs are represented in the zebrafish genome including additional isoforms that likely arose after duplication of the teleost genome (Taylor et al. 2003). The differences in MMPs present in humans and zebrafish might be due to differences in the type and/or spatial distribution of specific extracellular matrix substrates. Gene expression patterns have been described for only four zebrafish MMPs (*mmp2*, *mmp9*, *mmp13*, and *mmp14*) (Thisse et al. 2001, 2004; Zhang et al. 2003a, b; Yoong et al. 2007; Coyle et al. 2008). Zebrafish *mmp2* exhibits low expression in the early embryo (before somitogenesis) and a dynamic pattern at later stages localizing to the somites, pectoral fins, epidermis, otic vesicle, pharyngeal arches, and jaw cartilage (Thisse et al. 2004). By contrast, *mmp9* expression is

restricted to the notochord, myeloid cells (similar to *mmp13a*), and potentially a site of hematopoiesis termed the intermediate cell mass (Yoong et al. 2007). Zebrafish *mmp14a* and *mmp14b* are broadly expressed during gastrulation and at later stages localize to the pectoral fins, jaw cartilage, neurocranium, and neuromasts of the head (Coyle et al. 2008).

During development, the nature of the extracellular matrix is a key determinant of multiple cell behaviors including motility, where matrix acts as both a physical barrier to directed migration and a scaffold that cells move on and through. Unlike fibronectin, collagens are broadly expressed at later developmental stages (beyond 24 h post-fertilization) and thus have the potential to influence numerous cell migration events (Thisse et al. 2001, 2004; Rauch et al. 2003). Zebrafish lack the collagenases MMP1 and MMP8 but have two MMP13 homologs (*mmp13a* and *mmp13b*), a collagenase with overlapping and distinct cleavage substrates (Lynch and Matrisian 2002; Wyatt et al. 2009). Interestingly, *mmp13a* exhibits a highly restricted embryonic expression pattern localizing to macrophages (Thisse et al. 2001). Interstitial migration of immune system cells occurs in response to inflammation. In an elegant study Zhang et al. demonstrated that macrophage migration after acute injury is dependent on zebrafish MMP13a (Zhang et al. 2008). It will be important to determine how the MMP13a zymogen is activated and to identify the extracellular matrix substrate(s) involved in this process.

Studies of tumor cell migration have demonstrated a critical role for membrane-tethered MMPs (MMP14/MT1-MMP, MMP15/MT2-MMP, and MMP16/MT3-MMP) during both invasion of collagen and transmigration through type IV collagen- and laminin-containing basement membranes (Hotary et al. 2000, 2006). Notably, these three membrane-tethered MMPs also cleave fibronectin (Lynch and Matrisian 2002). The abundant expression of fibronectin and laminin in zebrafish gastrula-stage embryos (Latimer and Jessen 2010) suggests that membrane-tethered MMPs might play a significant role during this developmental process. Indeed, MMP14 was shown to regulate planar cell polarity underlying convergence and extension gastrulation cell movements (Coyle et al. 2008) (see Sect. 8.3.1).

8.2.7 Adamalysins

Adamalysins (ADAMs) belong to the metzincin superfamily of membrane-bound proteases and contain a disintegrin and metalloprotease motif with or without thrombospondin-like repeats (ADAM/ADAMTS) (reviewed by van Goor et al. 2009). Additional domains include a prodomain, a cysteine-rich domain, an epidermal growth factor-like motif, transmembrane, and cytoplasmic domains. ADAMs are unique because they possess both adhesion and protease domains. Similar to MMPs, ADAM proteases are produced as zymogens requiring activation by proprotein convertases. The human genome contains 23 ADAM genes, but only around half of these genes encode functionally active metalloproteases

(van Goor et al. 2009). The major role of ADAM metalloproteases is to cleave extracellular matrix proteins and to shed multiple proteins from the cell surface including ligands for the epidermal growth factor receptor (Sahin et al. 2004). The zebrafish genome encodes at least 11 ADAM and 8 ADAMTS metalloproteases as well as additional isoforms that, as described above for MMPs, likely arose after duplication of the teleost genome (Taylor et al. 2003). Very little expression data exist for zebrafish ADAMs metalloproteases. Expression of *adam23a* exhibits widespread expression in the central nervous system (Rauch et al. 2003). By contrast, *adam8a* mRNA is restricted to anterior mesoderm during early embryogenesis and localizes to the neural tube, macrophages, kidney, intermediate cell mass, and cardiovascular system later in development (Thisse et al. 2004; Iida et al. 2010). It is perhaps notable that both zebrafish *adam8a* and *mmp13a* are expressed in macrophages as recent data have suggested that like MMP13, ADAM8 might regulate the recruitment of blood cells to sites of inflammation (Zarbock and Rossaint 2011). A recent study in zebrafish by Iida et al. in zebrafish nicely provided a link between ADAM8 function and the onset of erythroid blood cell circulation after release from the endothelial cells of the vessel lumen (Iida et al. 2010). Here, knockdown of *adam8* using antisense morpholino oligonucleotides caused a paucity of circulating blood cells while not affecting heartbeat, development of the cardiovascular system, or erythroid cell fate determination. Using an in vitro assay, Iida et al. showed that the mucin-type adhesion protein P-selectin glycoprotein ligand-1, a protein known to undergo ectodomain shedding from the cell surface, was cleaved by zebrafish ADAM8 (Iida et al. 2010). Further studies are needed to determine whether P-selectin glycoprotein ligand-1 represents a physiological substrate for ADAM8 and whether there are additional substrates such as integrins.

8.3 Extracellular Matrix and Zebrafish Morphogenesis

8.3.1 Gastrulation

Gastrulation is the process whereby the three embryonic germ layers (ectoderm, mesoderm, and endoderm) are established and is accompanied by dynamic morphogenetic cell movement behaviors that shape the body plan into a recognizable embryo with anterior–posterior and dorsal–ventral axes (reviewed by Jessen and Solnica-Krezel 2005; Solnica-Krezel 2005). Critical roles for extracellular matrix proteins have long been recognized for amphibian gastrulation (Boucaut and Darribere 1983; Lee et al. 1984) (see Chap. 3). These and other studies have shown that fibronectin interactions with Integrin α5β1 receptors is required for polarized membrane protrusive activity underlying convergent extension cell movements (Marsden and DeSimone 2003; Davidson et al. 2006). Loss of fibronectin or integrin activity produces misshapen frog gastrula embryos characterized by shortened and broadened body axes (Skoglund and Keller 2010). In contrast to

frog, the first thorough analysis of fibronectin and laminin protein expression during the zebrafish gastrulation time period (6–10 h post-fertilization) was published in 2010 (Latimer and Jessen 2010).

Fibronectin and laminin proteins are first detected by immunolabeling at approximately 65 % epiboly (7.3 h post-fertilization) and localize to the epiblast–hypoblast boundary (Latimer and Jessen 2010). As gastrulation proceeds, fibronectin and laminin protein expression and fibrillogenesis increases, and a new expression domain appears between the mesendodermal cells and the underlying yolk syncytial layer (Fig. 8.1). Fibrils of fibronectin also extend between individual cells and align with membrane protrusions produced by deep mesodermal cells (Latimer and Jessen 2010). Together, these data indicate that, similar to the frog gastrula, zebrafish embryos form "layers" of extracellular matrix at tissue boundaries (ectoderm–mesoderm) and beneath and surrounding deep mesodermal cells. The precise role of these matrices during zebrafish gastrulation is unclear but undoubtedly influences polarized cell behaviors necessary for convergence and extension cell movements. Indeed, antisense morpholino oligonucleotide-mediated knockdown of both *fibronectin1* and *fibronectin1b* produces zebrafish embryos with phenotype characteristic of defective convergence and extension (Latimer and Jessen 2010). An elegant study by Charles Little's research group utilized the early chick embryo to demonstrate that a fibronectin-containing extracellular matrix can actually move in concert with epiblastic cells (Zamir et al. 2008). These data provide new challenges to our understanding of the role of the extracellular matrix and morphogens during gastrulation cell movements and represents an important area for future zebrafish research.

Demonstration of a role for matrix metalloproteinases during zebrafish gastrulation has provided new insight into the relationship between cell polarity and extracellular matrix remodeling. It was shown that knockdown of membrane type 1 matrix metalloproteinase (Mmp14) function disrupts planar cell polarity and convergence and extension cell movements (Coyle et al. 2008). Mmp14 also exhibits a strong genetic interaction with certain components of the planar cell polarity signaling system including the HSPG and Wnt co-receptor Glypican4 (Coyle et al. 2008). It was subsequently shown that another polarity protein, Vang-like 2 (Vangl2), regulates endocytosis and cell surface levels of Mmp14 and that Mmp14 could function downstream of Vangl2 to influence convergence and extension cell movements (Williams et al. 2012). Significantly, it was shown that *vangl2*/*trilobite* mutant embryos (Solnica-Krezel et al. 1996; Jessen et al. 2002) have increased matrix metalloproteinase activity and decreased fibronectin (Williams et al. 2012). Several studies have demonstrated a complex relationship between MMP14 activity and integrin function, often in polarized cells (Gonzalo et al. 2010). Further work is needed to understand the role of pericellular proteolysis and extracellular matrix remodeling during zebrafish gastrulation including the requirements for integrin receptors and cell–extracellular matrix adhesion.

Proper cellular interactions with extracellular matrix proteins have been linked to the directed migration of endodermal cells during zebrafish gastrulation. Endodermal cells form a sparse noncontiguous layer beneath the mesoderm (Warga and

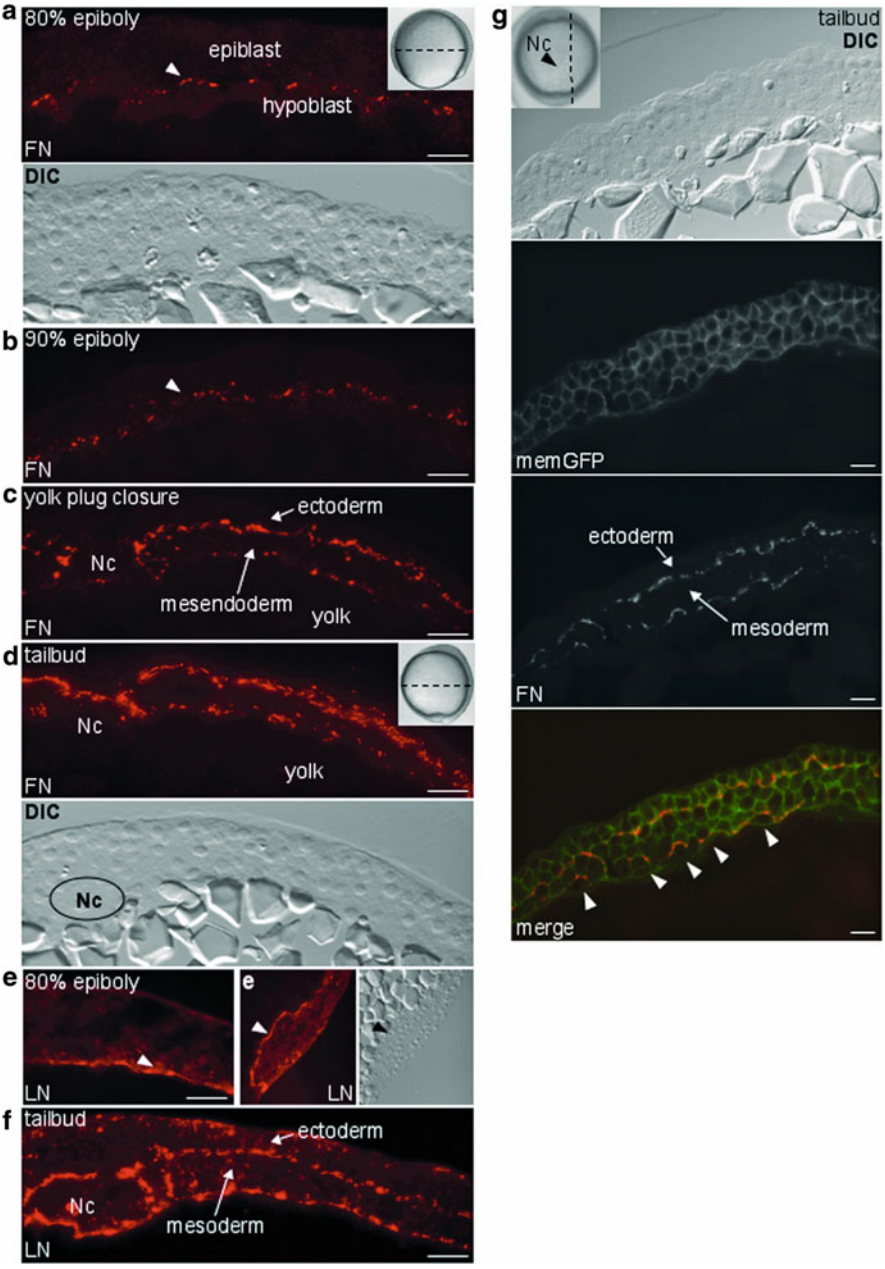

Fig. 8.1 Images of fibronectin (FN) and laminin (LN) expression during zebrafish gastrulation. (**a**) Fibronectin and laminin expression in cross-sectioned (**a**–**f**) and sagittally sectioned (**e** and **g**) gastrula-stage embryos. *Insets* in (**a**) and (**d**) show the cross-section plane (*horizontal lines*), while the *inset* in panel (**g**) depicts the sagittal-section plane (*vertical line*); *arrowhead* in (**g**) marks the embryonic midline or notochord (Nc). (**a** and **b**) At 80 % and 90 % epiboly fibronectin localizes to

Nusslein-Volhard 1999) and likely interact with the deepest layer of fibronectin present in the gastrula embryo (Latimer and Jessen 2010). Directed migration of endodermal cells towards the dorsal midline requires the chemokine Cxcl12b (expressed in the mesoderm) and its receptor Cxcr4a (expressed in the endoderm) to inhibit abnormal anterior migration (Nair and Schilling 2008). Mechanistically, it was shown that disruption of chemokine signaling affected integrin–fibronectin interactions and cell adhesion (Nair and Schilling 2008). Consequently, the extracellular matrix is required for endodermal cell tethering to the mesoderm and thus its proper directed movement during convergence and extension.

Connections between gastrulation cell movements and extracellular matrix also include proteins that modify both the structure and assembly of matrix components. For example, β1,4-galactosyltransferase was shown to regulate convergence and extension movements (Machingo et al. 2006). Galactosyltransferases catalyze the addition of monosaccharides to growing polysaccharide chains, thereby producing specific glycosidic linkages that can differentially affect the function of carbohydrates present in the extracellular matrix. It was shown that laminin is improperly galactosylated in β1,4-galactosyltransferase knockdown zebrafish embryos, suggesting a role for laminin glycoside chains during cell migration (Machingo et al. 2006). Decorin is another proteoglycan component of the extracellular matrix that has been linked to convergence and extension cell movements and jaw cartilage formation (Zoeller et al. 2009). A key function of decorin is to bind collagens and fibronectin where it can regulate different aspects of extracellular matrix structure and rate of fibrillogenesis. It would therefore be interesting to examine changes in extracellular matrix assembly in *decorin* loss of function embryos.

8.3.2 Somitogenesis

Somites are epithelial segmental structures that form transiently in bilateral pairs along the anterior–posterior embryonic body axis (reviewed by Holley 2007). Somites are derived from mesenchymal presomitic mesoderm, and a molecular

Fig. 8.1 (continued) the epiblast–hypoblast boundary, *arrowheads*. (**c**) At yolk plug closure two fibronectin domains are visible, the ectoderm–mesoderm boundary (formally epiblast–hypoblast) and the deeper mesendoderm–yolk boundary. Fibronectin is also observed adjacent to the Nc. (**d**) By the end of gastrulation, fibronectin assembly becomes more fibrillar and continues to define germ layer tissue boundaries. (**e**) Laminin expression at 80 % epiboly localizes to the deep mesendoderm–yolk boundary, *arrowhead*. (**e**) Notably at this stage, in sagittally sectioned embryos, laminin is observed underneath internalized hypoblast cells near the blastoderm margin (*arrowheads*). (**f**) By the end of gastrulation, laminin expression demarcates tissue boundaries similar but not identical to fibronectin. (**g**) Fibronectin expression in membrane–GFP-injected wild-type embryos. *Arrowheads* denote deep membrane–GFP-labeled mesendodermal cells. Scale bars: 20 μm. Adapted from Latimer and Jessen (2010) with permission from Elsevier

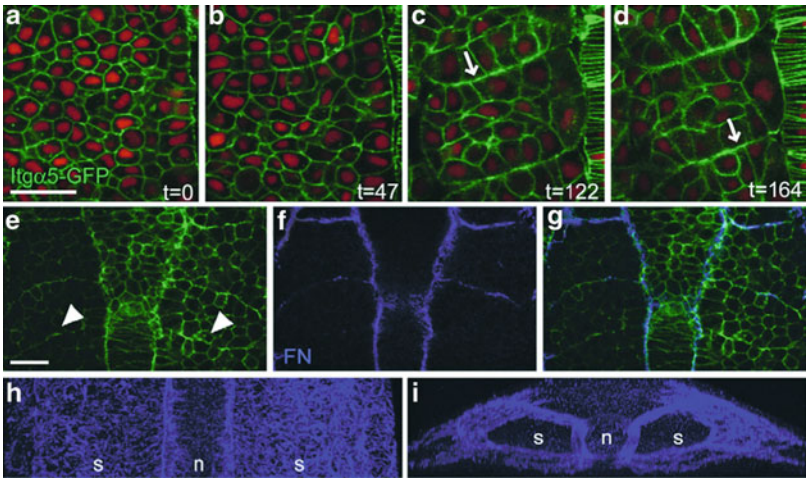

Fig. 8.2 Integrinα5-GFP (Itgα5-GFP) clustering and fibronectin (FN) matrix assembly during somitogenesis in zebrafish. (**a**–**d**) Four time points (indicated in minutes) showing Integrinα5-GFP localization during somite formation. Integrinα5-GFP (*green*) is distributed along the cortex in mesenchymal presomitic cells (**a**) but clusters to the basal side of columnar border cells (**b**–**d**, *arrows*). Nuclei are *red*. (**e**–**g**) Integrinα5-GFP (**e**), fibronectin (**f**), and overlay (**g**). Nascent borders show Integrinα5-GFP clustering (*arrowheads* in **e**) but no fibronectin immunostaining. (**h**, **i**) Three-dimensional reconstruction showing fibronectin matrix along the surface of the paraxial mesoderm. (**a**–**h**) Dorsal views, anterior is *up*. (**i**) Rotation of (**h**) showing a transverse view of the presomitic mesoderm, dorsal is *up*. Embryos are at the 8- to 10-somite (**a**–**d**, **h**, **i**) or 5- to 6-somite (**e**–**g**) stage. *n* notochord, *s* somites. Scale bars: 30 μm. Adapted from Julich et al. (2009) with permission from The Company of Biologists Ltd

oscillator termed the segmentation or somite clock controls their formation. In zebrafish this clock is regulated by Notch signaling with additional roles for several other proteins/pathways including Eph/Ephrins (Julich et al. 2005, 2009). The expression of zebrafish fibronectin (Fig. 8.2) and laminin at somitic boundaries has been recognized for almost a decade (Crawford et al. 2003). Furthermore, the expression of Integrinα5 (Fig. 8.2), paxillin, and focal adhesion kinase at intersomitic furrows strongly suggests that cell–extracellular matrix adhesion plays a key role in somite boundary morphogenesis (Henry et al. 2001; Crawford et al. 2003; Julich et al. 2005). Indeed, it has long been appreciated that *fibronectin* and *Integrina5* knockout mice exhibit defects in somitogenesis (George et al. 1993; Yang et al. 1999). In 2005, two papers reported that loss of zebrafish *fibronectin* and *integrinα5* regulate anterior somitogenesis but do not influence the Notch-dependent oscillator clock (Julich et al. 2005; Koshida et al. 2005). It was proposed that Integrinα5-mediated adhesion to fibronectin is required for the assembly of intersomitic extracellular matrix and necessary for both epithelialization and maintenance of somite boundaries. Notably, *integrinα5* mutant embryos have undetectable levels of phosphorylated focal adhesion kinase indicating that formation of cell–extracellular matrix adhesions is disrupted (Koshida et al. 2005). It is known that presomitic mesodermal cells undergo morphological changes to become

polarized epithelial cells that border nascent somite boundaries (Henry et al. 2000; Barrios et al. 2003) and that aspects of this organization are lost in *integrinα5* and *fibronectin1* mutant embryos (Koshida et al. 2005). Based on standard models of fibronectin fibrillogenesis as a cell-mediated process (Mao and Schwarzbauer 2005), it is likely that interactions between Integrinα5β1 expressed by adaxial cells and secreted fibronectin dimers triggers cross-linking and further integrin–fibronectin binding (Koshida et al. 2005). However, it is unknown how cell–extracellular matrix interactions and potentially outside-in integrin signaling promote epithelialization of somitic boundary cells. Recent work has shown that Eph/Ephrin signaling regulates the initial clustering of Integrinα5 and that this event precedes extracellular matrix formation (Fig. 8.2) and is independent of fibronectin binding (Julich et al. 2009). Interestingly, cell surface Integrinα5 on adjacent paraxial mesodermal cells non-autonomously inhibits Integrinα5 clustering and subsequent fibronectin binding and extracellular matrix assembly along somitic boundaries (Julich et al. 2009). Derepression induced by Ephrin B2a reverse signaling initiates Integrinα5 clustering and subsequent fibronectin binding, thus providing a mechanism to restrict extracellular matrix assembly to specific tissue surfaces (Julich et al. 2009).

8.3.3 Skeletal Musculature

At later developmental stages (after 24 h post-fertilization), the zebrafish myotome forms and is composed of skeletal muscle fibers derived from the somites. Loss of fibronectin disrupts myogenesis as evidenced by muscle fiber disorganization and an uncoupling of fast-twitch and slow-twitch fiber lengths (Snow et al. 2008). The zebrafish system has emerged as a useful model for interrogating the function of integrins and the collagen- and laminin-containing basement membrane required for muscle contraction (Hall et al. 2007; Postel et al. 2008; Jacoby et al. 2009; Kim and Ingham 2009). In the skeletal basement membrane, laminin forms two important linkages, one to the dystrophin glycoprotein complex and the other to integrins of the subsarcolemmal focal adhesion complexes. These linkages are important for transmitting the force generated by muscle contraction across the sarcolemma, and their disruption is thought to play a key role in the pathogenesis of muscular dystrophy (Carmignac and Durbeej 2012). The zebrafish *candyfloss* (*lamininα2*) mutant exhibits a degenerative muscle phenotype, first detected at 36 h post-fertilization, that is characterized by detachment and retraction of muscle fibers from the myoseptum between each somite (Hall et al. 2007). Mechanistically, it is thought that Lamininα2 acts within the extracellular matrix itself to promote the stability of muscle attachments. Without Lamininα2 function, and upon mechanical load-induced stress, muscle fiber detachment occurs followed by apoptosis (Hall et al. 2007). Mutation of the zebrafish *softy* locus (*lamininβ2*) produces a similar muscle detachment phenotype at 3 days post-fertilization (Jacoby et al. 2009). However, while homozygous *lamininα2*/*candyfloss* mutant embryos largely fail to

survive, the majority of *lamininβ2/softy* homozygotes survive to maturity (Jacoby et al. 2009). This was attributed to the formation of ectopic fiber terminations in *softy* mutants characterized as ectopic myoseptum-like structures that supported the attachment of fibers (Jacoby et al. 2009). Analysis of *candyfloss/softy* double mutant embryos demonstrated that *laminina2* is epistatic to *lamininβ2* with the double mutant having a phenotype that is most similar to *laminina2/candyfloss* (Jacoby et al. 2009). While *laminina2* and *lamininβ2* co-localize in myotendinous junctions, identification of other laminin isoforms expressed during muscle development will contribute greatly to our understanding of extracellular matrix proteins and muscular dystrophy (Sztal et al. 2011).

8.3.4 Directed Migration of Hindbrain Motor Neurons

The migration of zebrafish hindbrain branchiomotor neurons has become an important system for studying complex interactions between migrating cells and their environment including components of the extracellular matrix (Chandrasekhar 2004; Bingham et al. 2010). The facial branchiomotor neurons (FBMNs) arise in rhombomere 4 beginning at 16 h post-fertilization and migrate tangentially to rhombomeres 6 and 7 over the course of 4–6 h per neuron (reviewed by Chandrasekhar 2004). The entire process ends by 48 h post-fertilization. The early zebrafish hindbrain is a pseudostratified neuroepithelium that is polarized along both the apical–basal axis and the planar axis (Chandrasekhar 2004). In the last decade, accumulating data demonstrate a requirement for planar cell polarity (PCP) proteins during FBMN migration. Disruption of *vang-like 2* (*vangl2*) (Bingham et al. 2002; Jessen et al. 2002), *prickle1a* (Carreira-Barbosa et al. 2003), *prickle1b* (Rohrschneider et al. 2007; Mapp et al. 2010, 2011), *frizzled3a* (Wada et al. 2006), or *celsr2/Drosophila flamingo* homolog (Wada et al. 2006) results in impaired directed migration of hindbrain FBMNs. Additional proteins required for FBMN migration include transient axonal glycoprotein 1, a cell adhesion molecule expressed in FBMNs (Sittaramane et al. 2009), and Nhsl1b, a protein similar to the human Nance-Horan syndrome protein and the *Drosophila* Scribble-interacting protein Guk-holder (Walsh et al. 2011). Current data indicate that PCP proteins can exhibit cell nonautonomous (Jessen et al. 2002; Wada et al. 2005, 2006) or cell autonomous (Jessen et al. 2002; Rohrschneider et al. 2007; Mapp et al. 2011; Walsh et al. 2011) functions during FBMN migration. In terms of the former, it is thought that PCP-dependent effects on the neuroepithelium provide a permissive microenvironment for directed cell migration along straight trajectories. An important clue to the role of the neuroepithelium environment is provided by the observation that loss of *laminina1* (*bashful*) disrupts FBMN migration (Paulus and Halloran 2006; Sittaramane et al. 2009). Ventral to the neuroepithelium is a laminin-containing basement membrane (Fig. 8.3). In an elegant study by Grant and Moens, it was shown that loss of *laminina1* causes FBMNs to aberrantly mismigrate ventrally where they "escape" from the hindbrain

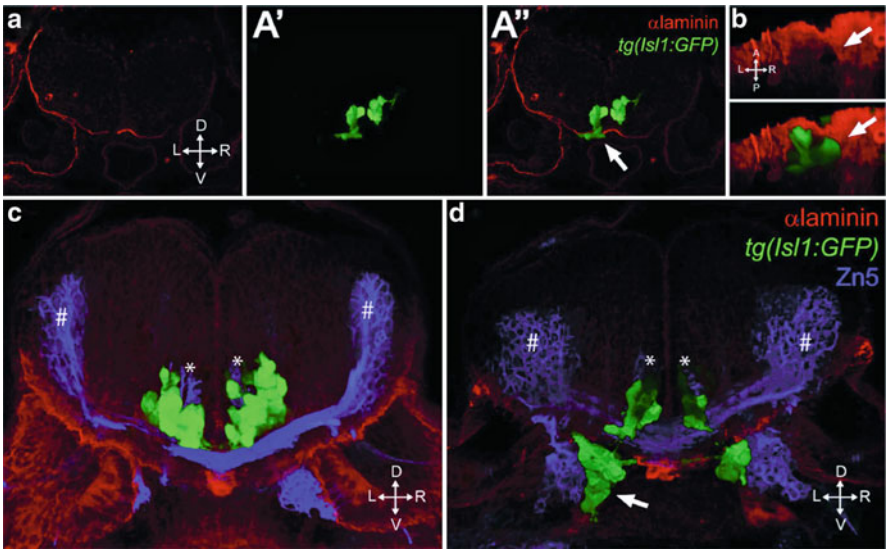

Fig. 8.3 FBMNs exit the hindbrain through holes in the ventral laminin-containing basement membrane that can be phenocopied by laminin knockdown. (**a**–**a**″) Single XY section of a vibratome cross-section through a 48-h post-fertilization aPKCλ+ζ, *tg(islet1:GFP)* double morphant stained for laminin (*red*). Ventrally mismigrating FBMNs exit the hindbrain through a hole in laminin (*arrow*). (**b**) Ventral view of a three-dimensional reconstruction of the same coronal section showing the laminin hole and the mismigrating FBMNs (*arrows*). (**c**, **d**) Three-dimensional reconstructions of 70 μm thick vibratome cross-sections through 48-h post-fertilization wild-type (**c**) and laminin*α* 1 morpholino-injected *tg(islet1:GFP)* embryos (**d**) stained for laminin (*red*) and Zn5/Alcama (*blue*) showing that laminin knockdown results in ventrally mismigrated FBMNs (*arrow*) that exit the hindbrain while leaving abducens motor neurons (*asterisks*) and commissural interneurons (*hash symbols*) unaffected. ZN5/Alcama-staining neurons outside the hindbrain are the sensory neurons of the acoustic nerve (nVIII). Adapted from Grant and Moens (2010) with permission from BioMed Central

by passing through holes or discontinuities in the neuroepithelial basement membrane structure (Fig. 8.3) (Grant and Moens 2010). Furthermore, basement membrane laminin was shown to promote centrosome reorientation and increased posterior FBMN migration indicating that this extracellular matrix protein also acts as a substrate for migration (Grant and Moens 2010). Neuroepithelial basement membrane holes are also detected at 48 h post-fertilization after knockdown of PAR–aPKC complex proteins (Grant and Moens 2010), previously shown to influence epithelial cell polarity directly and also indirectly by maintaining the basolateral localization of proteins including the Lgl–Dlg–Scrib complex. Notably, zebrafish Scribble1 function is required for FBMN migration (Wada et al. 2005). Utilizing mosaic cell transplantation analyses, it was shown that aPKC functions cell non-autonomously, suggesting that it acts in the neuroepithelium surrounding FBMNs (Grant and Moens 2010). Taken together, these data support the notion that maintenance of the laminin-containing ventral basement membrane of the hindbrain neuroepithelium is important for FBMN directed migration to caudal

rhombomeres. Additional work will be needed to identify potential requirements for other extracellular matrix proteins, their receptors, and metalloproteases.

8.3.5 Gut-Looping Morphogenesis

Extracellular matrix remodeling can influence multiple aspects of organogenesis including migration, cell–cell interactions, and branching morphogenesis. Utilizing zebrafish, it was shown that regulation of extracellular matrix remodeling is essential for the asymmetric migration of the lateral plate mesoderm necessary for gut-looping and correct spatial organization of liver and pancreas (Yin et al. 2010). By examining the expression of the transcription factor *hand2* (using the transgenic line *Tg*(*hand2*:EGFP)), the authors identified a novel cell rearrangement occurring in the lateral plate mesoderm that is regulated by left/right gene expression. Significantly, whereas in wild-type embryos laminin expression diminishes along the path of lateral plate mesoderm migration, in *hand2* mutant embryos laminin deposition/expression persists, an effect not due to altered gene (*laminin* or *integrin*) expression (Yin et al. 2010). Unlike the heart primordium where *hand2* mutants exhibit disorganized fibronectin deposition, loss of *hand2* does not disrupt fibronectin distribution in the gut-looping region (Trinh et al. 2005; Yin et al. 2010). Notably, partial loss of *laminin* in a *hand2* mutant background suppressed the lateral plate mesoderm migration defects, while inhibition of matrix metalloproteinase (MMP) activity using the broad-spectrum inhibitor GM6001 recapitulated aspects of the *hand2* mutant phenotype (Yin et al. 2010). Of the *mmp* genes tested, the membrane-tethered *mmp14a* was decreased in *hand2* mutants, while expression of the Mmp inhibitors *timp2a* and *timp2b* was increased. Knockdown of *mmp14a* using antisense morpholino oligonucleotides produced a gut-looping phenotype in wild-type embryos (Yin et al. 2010). Together, these data strongly implicate Hand2 as a regulator of Mmp activity, extracellular matrix remodeling, and cell migration during the early stages of organogenesis in zebrafish.

8.3.6 Heart Development

The vertebrate heart initially forms as a simple tube that is derived from bilateral fields of mesoderm that fuse at the midline. Through a complex series of morphogenetic events that include rightward looping, chamber specification, and valve formation, this simple tube becomes a four-chambered heart (reviewed by Stainier 2001). During the early stages of this process in zebrafish (14-somite stage or ~16 h post-fertilization), bilateral cardiac progenitors initiate migration from the anterior lateral plate mesoderm to the midline where they fuse (21-somite stage or ~19.5 h post-fertilization) to form an intermediate structure referred to as the cardiac cone

(Stainier 2001; Glickman and Yelon 2002; Trinh and Stainier 2004; Schoenebeck and Yelon 2007). The cardiac cone undergoes elongation to form the primitive bilayered heart tube comprised of an outer layer of myocardium and inner sheet of endocardial progenitors (Rohr et al. 2006; Holtzman et al. 2007). In zebrafish, bilateral cardiac progenitors form continuous polarized epithelia as they migrate towards the midline (Trinh and Stainier 2004). Cardiomyocyte precursors undergo directed collective migration between the endoderm and the extraembryonic yolk syncytial layer and require interactions with both of these cell populations and the surrounding extracellular matrix for proper cell migration and heart tube formation (Alexander et al. 1999; Kikuchi et al. 2000; Dickmeis et al. 2001; Trinh and Stainier 2004; Arrington and Yost 2009).

Genetic analyses of mutations that perturb primary heart field fusion indicate that disruption of the migration or differentiation of myocardial progenitors may result in failure of midline fusion and formation of two separate hearts, referred to as cardia bifida (Chen et al. 1996; Reiter et al. 1999, 2001; Yelon et al. 2000). Proper myocardial progenitor migration requires the adjacent primitive endocardium and endoderm (Alexander et al. 1999; Kikuchi et al. 2000; Dickmeis et al. 2001; Holtzman et al. 2007) and deposition of extracellular matrix proteins (Trinh and Stainier 2004; Arrington and Yost 2009). Although the precise composition of the extracellular matrix during heart formation is unclear, recent studies indicate that cardiomyocyte progenitors migrate along a basement membrane containing the extracellular matrix proteins fibronectin, laminin, and sulfated proteoglycans (Trinh and Stainier 2004; Sakaguchi et al. 2006; Arrington and Yost 2009; Langenbacher et al. 2012). The prominent role that cell–extracellular matrix interactions play during heart formation is evidenced by cardiac malformations in zebrafish with loss of *fibronectin1*/*natter*, the proteoglycan *syndecan 2*, or *sphingosine 1-phosphate*, a lipid mediator that is required for cell–extracellular matrix interactions (Kupperman et al. 2000; Trinh and Stainier 2004; Sakaguchi et al. 2006; Matsui et al. 2007; Osborne et al. 2008; Arrington and Yost 2009; Kawahara et al. 2009). During cardiac progenitor migration, fibronectin is dynamically expressed: initially fibronectin is deposited in the lateral plate mesoderm, and later, during cardiac cone formation, fibronectin protein is maintained and surrounds the migrating bilateral myocardial progenitors at the ventral midline between the endoderm and endocardial cell layers (Trinh and Stainier 2004). The characteristic cardia bifida phenotype in *natter* mutant embryos results from the disruption of *fibronectin1* and subsequent loss of polarity and epithelial integrity during myocardial progenitor migration (Trinh and Stainier 2004). During embryogenesis *fibronectin1*/*natter* mutants fail to undergo mediolateral expansion of the anterior lateral plate mesoderm, while posterior lateral plate mesoderm is unaffected (Trinh and Stainier 2004), indicating that the requirement for *fibronectin1* includes more global roles during morphogenesis of anterior lateral plate mesoderm in addition to migration of myocardial progenitors.

The expression and deposition of fibronectin and laminin in the early zebrafish embryo is controlled in part by the extraembryonic yolk syncytial layer, the multinucleated exterior cytoplasm of the yolk cell (D'Amico and Cooper 2001;

Sakaguchi et al. 2006). Several genes of the *mix* family of transcription factors are expressed in the extraembryonic yolk syncytial layer and have been implicated in deposition of extracellular matrix including *mix-type homeobox gene 1* (*mxtx1*) and *bonnie and clyde* (*bon*/*mixer*) (Alexander et al. 1999; Kikuchi et al. 2000; Sakaguchi et al. 2006). Sakaguchi and colleagues demonstrated that antisense morpholino oligonucleotide-mediated knockdown of *mxtx1* causes cardia bifida and further showed that a genetic interaction exists between *mxtx1* and *fibronectin1*/*natter* (Sakaguchi et al. 2006). Interestingly, the loss of extraembryonic *mxtx1* and impaired migration of myocardial progenitors was associated with decreased embryonic Fibronectin1 expression in the lateral plate mesoderm, indicating an essential role for yolk syncytial layer proteins in regulation of the extracellular matrix during myocardial migration. Additionally, laminin deposition was impaired in *mxtx1* morpholino-injected heterozygous *fibronectin1*/*natter* embryos, and knockdown of *lamininc1* exacerbated embryonic phenotypes in both *fibronectin1*/*natter* and *mxtx1* morpholino-injected embryos (Sakaguchi et al. 2006). Although knockdown of *lamininc1* or loss of either *lamininβ1*/*grumpy* or *lamininγ1*/*sleepy* alone is not sufficient to disrupt heart tube formation (Parsons et al. 2002; Sakaguchi et al. 2006), it is likely that combined interactions of individual extracellular matrix proteins are crucial for cardiac development.

In contrast to the yolk syncytial layer-specific expression of *mxtx1*, *bon*/*mixer* is expressed in the yolk syncytial layer, mesoderm, and endoderm during early heart development. Expression of *bon*/*mixer* is essential for early endoderm differentiation and myocardial progenitor fusion at the midline (Kikuchi et al. 2000). However, due to the requirement of endoderm in cardiomyocyte progenitor migration, it is unclear whether *bon*/*mixer* function is also required within the yolk syncytial layer for embryonic extracellular matrix deposition similarly to *mxtx1*.

Recently, the Na+, K+ ATPase *atp1a1* was identified as a possible downstream effector of *mxtx1* in the yolk syncytial layer (Langenbacher et al. 2012). Similar to *mxtx1* and *fibronectin1*/*natter* mutant embryos, tissue-specific morpholino-induced knockdown of *atp1a1* function in the yolk syncytial layer inhibits expression of *fibronectin1* mRNA and extracellular matrix proteins surrounding cardiac progenitors. In addition, although cardiac fusion occurs normally in *atp1a1* knockdown embryos, myocardial cells exhibit defective heart tube elongation (Langenbacher et al. 2012). Analysis of embryonic and extraembryonic knockdown demonstrated that *atp1a1* functions non-cell autonomously to regulate heart tube elongation and cell autonomously to control cardiac contraction during development (Langenbacher et al. 2012).

The basic helix–loop–helix transcription factor Hand2 is also required during cardiac fusion to negatively regulate fibronectin deposition (Yelon et al. 2000; Trinh et al. 2005; Garavito-Aguilar et al. 2010). Zebrafish *hand2* mutant embryos have disrupted cardiomyocyte progenitor migration and cardia bifida (Yelon et al. 2000). Although *hand2* is expressed throughout the primary heart field (Yelon et al. 2000), transplantation experiments indicate that it functions non-cell autonomously during myocardial progenitor migration. Myocardial progenitors in *hand2* mutant embryos exhibit loss of epithelial polarity and have altered expression of

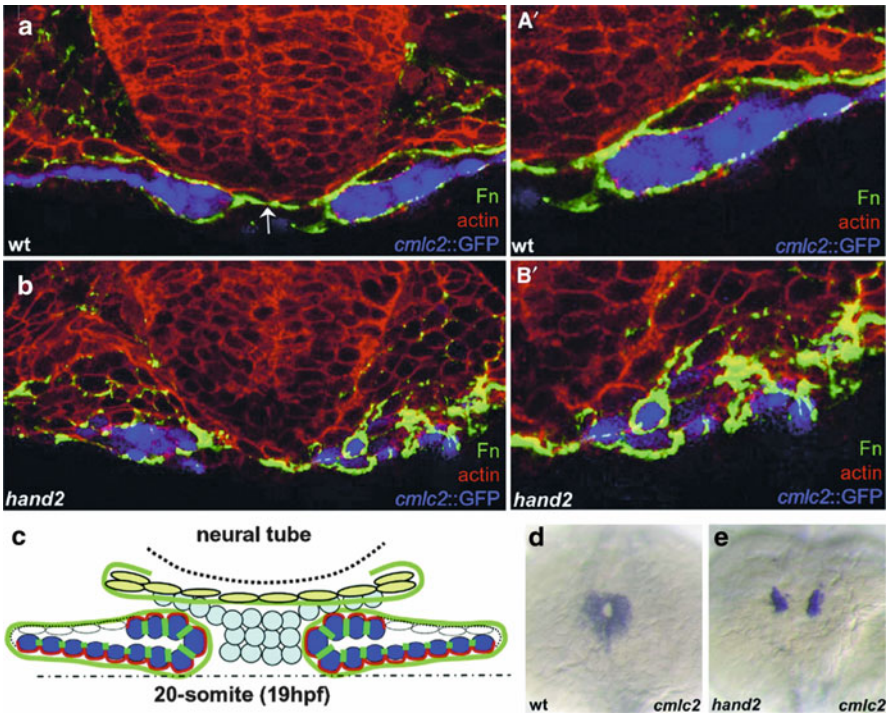

Fig. 8.4 Defective fibronectin deposition in hand2 mutants. (**a** and **b**) Transverse sections of *cmlc2:GFP* (false colored *blue*) transgenic embryos immunostained for β-catenin (labeling actin, *red*) and for fibronectin (*green*) in wild-type (**a** and **a′**) and *hand2* (**b** and **b′**) mutant embryos at the 20-somite stage; dorsal is to the *top*. (**a′**) and (**b′**) show magnified views of the lower right corners of (**a**) and (**b**), respectively. In wild-type embryos (**a** and **a′**), fibronectin is deposited around the basal surface of the myocardial epithelia (*blue*) and at the midline between the endoderm and endocardial precursors (*arrow*). (**b** and **b′**) In *hand2* mutants, fibronectin deposition is disorganized and no longer restricted to the basal surface of the myocardial precursors. (**c**) The myocardial epithelia are diagrammed in transverse sections showing the arrangement of the myocardial precursors (*dark blue* cells), endocardial precursors (*light blue*), actin (*red*), aPKCs and ZO-1 (*green*) in the myocardial precursors, and fibronectin (*lime green*) deposition. (**d**) and (**e**) show *cmlc2* expression; dorsal views, anterior is to the *top*. (**d**) In wild-type embryos, the myocardial precursors have fused to form the cardiac cone. (**e**) In *hand2* mutants, the myocardial precursors are reduced in number and fail to migrate to the midline and fuse. Modified from Figs. 1, 2, and 4 from Trinh et al. (2005) with permission from Elsevier

fibronectin and disorganized matrix deposition (Fig. 8.4) (Trinh et al. 2005; Garavito-Aguilar et al. 2010). Consistent with the notion that Hand2 restricts the levels of fibronectin, heterozygosity for both *fibronectin1* in *hand2* partially rescues cardiac progenitor migration and primary heart field fusion but not epithelial cell polarity (Garavito-Aguilar et al. 2010).

Cell–fibronectin interactions and fibrillogenesis are regulated by heparin-binding domains on fibronectin that are known to bind glycosaminoglycan chains on cell surface proteoglycans (McDonald et al. 1987; Hocking et al. 1994;

Christopher et al. 1997; Bultmann et al. 1998; Schwarzbauer and Sechler 1999). The heparan sulfate proteoglycan Syndecan-2 mediates assembly of extracellular matrix proteins to facilitate migration of cardiac progenitors. Syndecan-2 is expressed throughout the embryo and is required for left–right patterning signals from the ectoderm to the adjacent cardiac mesoderm (Kramer and Yost 2002) (see Chap. 1). Syndecan-2 is also expressed in the extraembryonic yolk syncytial layer where it is required for fibronectin and laminin deposition and assembly throughout the embryo (Arrington and Yost 2009). Interestingly, embryonic and extraembryonic forms of Syndecan-2 are functionally distinct. Embryonic morpholino-mediated knockdown of *syndecan-2* has no effect on myocardial migration, whereas knockdown of *syndecan-2* specifically within the extraembryonic yolk syncytial layer inhibits cardiac cone formation and results in loss of fibronectin fibrillogenesis and reduced assembly of the laminin-containing basement membrane adjacent to cardiomyocyte progenitors (Arrington and Yost 2009). These functional differences are likely due to unique posttranslational processing and glycosaminoglycan chain modifications on extraembryonic Syndecan-2.

Although not required for heart formation, retinol-binding protein 4 (Rbp4) also regulates expression of *fibronectin1* in the yolk syncytial layer (Li et al. 2007). Interestingly, *rbp4* expression is restricted to the ventrolateral domain of the yolk syncytial layer, and yolk-specific morpholino-mediated knockdown of *rbp4* results in reduced expression of *fibronectin1* in the ventrolateral yolk and formation of two liver buds (Li et al. 2007). These data indicate a role for fibronectin in liver progenitor migration and distinct regional regulation of extracellular matrix protein deposition within the embryo by the yolk syncytial layer during zebrafish embryogenesis.

8.3.7 Vascular Development

During embryogenesis blood vessels are generated as a result of two distinct processes, vasculogenesis and angiogenesis (reviewed by Weinstein and Lawson 2002). Vasculogenesis is the process whereby blood vessels are formed de novo from mesoderm-derived endothelial progenitors that undergo migration, differentiation, vascular lumen formation, and organization into a primitive vascular plexus. In angiogenesis, new blood vessels arise from the branching and expansion of existing vasculature. While cell–extracellular matrix interactions are thought to have a prominent role in both of these processes during blood vessel development, few studies have directly examined regulation of extracellular matrix proteins.

Formation of the major axial vessels in zebrafish, the dorsal aorta and posterior cardinal vein, involves midline-directed migration of angioblast precursors from the lateral plate mesoderm and their coalescence to form an initial vascular cord (Torres-Vazquez et al. 2004; Jin et al. 2005) and subsequent tube formation (Kamei et al. 2006). Using a *flk1:EGFP* transgene, Jin and colleagues demonstrated that fibronectin is deposited around individual angioblasts as they migrate over the

endoderm towards the midline and that deposition of extracellular matrix is increased after coalescence and tube formation (Jin et al. 2005). Unlike heart formation, the endoderm is dispensable for fibronectin assembly and angioblast migration towards the midline. Studies examining loss of individual extracellular matrix gene products including *fibronectin1/natter*, *fibronectin1b*, *collagens*, and *laminins* demonstrated later vascular remodeling defects, but failed to detect defects in initial formation of the major axial vessels, suggesting redundancy among extracellular matrix components (Parsons et al. 2002; Trinh and Stainier 2004; Pollard et al. 2006; Snow et al. 2008; Huang et al. 2009; Bignon et al. 2011).

Endothelial cells and their supporting vascular smooth muscle cells and pericytes generate a complex basement membrane that is composed of fibronectin, laminins, collagens, microfibrils, and heparan sulfate proteoglycans. Failure to assemble and remodel this basement membrane results in defective endothelial cell migration and adhesion. Intersegmental vessels emerge from the dorsa aorta and undergo dorsal migration between the somites. Several studies suggest that laminins have an important role in intersegmental vessel growth. In zebrafish *lamininβ1/grumpy* or *lamininγ1/sleepy* mutant embryos, the dorsal aorta is formed normally, but formation of the notochord and intersegmental blood vessels is severely impaired (Parsons et al. 2002). Intersegmental vessels develop normally in *lamininα1/bashful* mutant embryos or wild-type embryos injected with *lamininα4* morpholinos. However, combined knockdown of *lamininα4* in *lamininα1/bashful* mutants disrupts migration and formation of intersegmental endothelial sprouts indicating redundant functions for Laminin α isoforms (Pollard et al. 2006). Loss of the transcription factor *foxc1* results in defects in vascular basement membrane integrity (as indicated by loss of laminin deposition) and arterial specification that resulted in disrupted morphogenesis of dorsal aorta and axial vessels (Skarie and Link 2009). These defects were phenocopied by combined injection of suboptimal doses of *foxc1* and *lamininα1* morpholinos, indicating Foxc1 functions to maintain basement membrane integrity.

The extracellular matrix is extensively modified and remodeled during vascular development. The heparan sulfate proteoglycan Syndecan-2 is important for cell-mediated assembly and rearrangement of fibronectin and laminin into fibrils (Klass et al. 2000). Chen and colleagues showed that embryonic expression of *syndecan-2* is essential for intersegmental vessel formation (Chen et al. 2004). A further study indicated that heparan sulfate proteoglycans require the modifying enzyme *heparan sulfate 6-O sulfotransferase 2* for later branching morphogenesis of the posterior cardinal vein (Chen et al. 2005). Additionally, the extracellular matrix cross-linking enzyme lysyl oxidase-like protein 2 is required for intersegmental vessel formation, capillary formation in vivo, and collagen IV assembly in vitro (Bignon et al. 2011). Loss of the secreted extracellular matrix-associated protein Egfl7/VE-statin causes defects in the extension and junctional arrangements of endothelial cells (Parker et al. 2004; De Maziere et al. 2008) indicating a role for extracellular matrix proteins in endothelial cell polarity.

Recent studies have also supported a role for microfibrils during vascular development. Morpholino-mediated knockdown of the extracellular matrix

microfibril component Fibrillin-1 results in dilation of the posterior cardinal vein and altered endothelial plexus formation (Chen et al. 2006). Similarly, mutations in *fibrillin-2*/*puff daddy* disrupt venous plexus and axial vessel formation (Gansner et al. 2008). Additionally, both overexpression and morpholino knockdown experiments demonstrated that the levels of *microfibril-associated glycoprotein-1* (*magp1*) are critical for vascular development (Chen et al. 2006). Vascular defects in *magp1* morpholino-injected embryos are exacerbated by inhibition of integrin signaling, indicating that microfibrils, and specifically *magp1*, may mediate cell–extracellular matrix interactions during vascular formation (Chen et al. 2006). This hypothesis is supported by experiments showing that loss of integrin-linked kinase in both mice and zebrafish results in lethal vascular defects (Friedrich et al. 2004).

8.3.8 Craniofacial Morphogenesis

Formation of the craniofacial skeleton requires complex cell–extracellular matrix interactions to regulate the migration, differentiation, and condensation of neural crest-derived ectomesenchyme within the pharyngeal arches (reviewed by Kulesa et al. 2010). Cranial neural crest differentiation is determined in part through epithelial–mesenchymal interactions and modulation of transcription factor expression. Chondrocyte cell fates are specified by increased Sox9 expression that promotes an osteochondroprogenitor fate (Spokony et al. 2002; Mori-Akiyama et al. 2003) and downregulation of Sox10 and Foxd3, allowing for mesenchymal differentiation (John et al. 2011; Mundell and Labosky 2011). In the pharyngeal arches, chondrocyte progenitors condense to form the spatially distinct primordia of craniofacial bones and cartilage. Both mesenchymal condensation and chondrocyte differentiation are intimately linked to the expression and secretion of numerous extracellular matrix proteins including fibrillar and non-fibrillar collagens and proteoglycans that surround each chondrocyte (Thisse et al. 2001; Baas et al. 2009; Fang et al. 2010; Dale and Topczewski 2011).

Chondrocyte progenitor cells are dependent on correct deposition and assembly of extracellular matrix proteins for proliferation, differentiation, and cartilage morphogenesis. Zebrafish with mutations in *sox9a*/*jellyfish* exhibit defects in craniofacial cartilage morphogenesis and decreased expression of *colagen2a1a* encoding the α3 chain of collagen XI (Yan et al. 2002). These data highlight the importance of collagen proteins in both the specification and structural properties of chondrocytes during endochondral bone formation. Similarly, morpholino-mediated knockdown of *collagen11a1*, encoding the α1 chain of the cartilage collagen XI, resulted in disrupted chondrocyte stacking and pharyngeal cartilage malformations (Baas et al. 2009).

Several studies have suggested that increased collagen deposition also disrupts craniofacial morphogenesis. Mannose-6-phosphate is a key recognition residue for the sorting of lysosomal hydrolases into lysosomes. Disruption of

mannose-6-phosphate biosynthesis in zebrafish results in reduced *sox9* expression in chondrocyte progenitors, excessive type II collagen deposition, and increased proteolytic activity of cathepsins and MMP13 (Flanagan-Steet et al. 2009; Petrey et al. 2012). Although the precise role of mannose-6-phosphate in chondrocyte specification is unclear, inhibition of Cathepsin K activity restored levels of protease activity and partially rescued collagen deposition and craniofacial defects in mannose-6-phosphate-deficient embryos (Petrey et al. 2012). Additionally, loss of the cilia ion channel gene *polycystin2* or combined knockdown of *polycystin1* paralogs not only results in increased collagen deposition and cartilage and craniofacial defects but also causes broader defects throughout the embryo including dorsal axis curvature, notochord malformations, and pronephric cyst formation (Mangos et al. 2010). It was also shown that loss of Mmp14a function produces a severe defect in jaw cartilage formation (Coyle et al. 2008), perhaps due to increased extracellular matrix protein deposition.

Recent studies have demonstrated that components of the coat protein II (COPII) complex, involved in protein trafficking between the ER and Golgi, are essential for proper deposition of extracellular matrix proteins and craniofacial development (reviewed by Melville and Knapik 2011). Loss of function mutations in the COPII components *sec23a*/*crusher* and *sec24d*/*bulldog* result in the accumulation of extracellular matrix proteins in the ER and reduced deposition of type II collagen and matrillin (Lang et al. 2006; Sarmah et al. 2010). Notably, these global cellular defects in COPII-mediated ER to Golgi transport specifically manifest in craniofacial defects, likely due to the high levels of extracellular matrix secretion required for chondrogenesis. Morpholino-mediated knockdown of *sec13*, encoding an outer component of the COPII coat, disrupts collagen and proteoglycan deposition and results in craniofacial skeletal defects similar to those observed in *sec23a*/*crusher* mutant embryos (Townley et al. 2008). This study indicates that coupling between the outer (Sec13/31) and inner (Sec23/24) components of the COPII coat is required for craniofacial cartilage development (Townley et al. 2008). Additionally, mutations in the transcription factor *creb3l2*/*feelgood* disrupt expression of select COPII component genes and result in craniofacial defects linked to reduced trafficking of type II and IV collagen. Interestingly, secretion of laminins, glycosaminoglycans, and certain glycoproteins was unaffected in *creb3l2*/*feelgood* mutants (Melville et al. 2011). Similarly, transport of the membrane-associated extracellular matrix interacting proteins Integrin β1 and cadherins was also unaffected in *sec24d*/*bulldog* mutants (Sarmah et al. 2010). Together, these findings indicate that chondrocytes have increased sensitivity to defects within the COPII secretory pathway that result in reduced deposition of specific extracellular matrix components and impaired chondrocyte maturation.

Proteoglycans are a major component of the extracellular matrix surrounding chondrocytes during craniofacial development, and several recent studies have demonstrated that genes involved in proteoglycan synthesis are essential for craniofacial morphogenesis (Eames et al. 2010, 2011; Moro et al. 2010; Wiweger et al. 2011). Proteoglycan formation requires UDP xylose to initiate attachment of glycosaminoglycans such as heparan or chondroitin sulfates to core proteoglycans.

Zebrafish with homozygous mutations in *UDP xylose synthase 1*(*uxs1*) have loss of proteoglycans, and increased transcription of *sox9* and *collagen2a1a*, but severely reduced deposition of Collagen2a1 protein (Eames et al. 2010; Wiweger et al. 2011), suggesting that proteoglycans are involved in the secretion or assembly of collagen and chondrocyte specification. Similarly, loss of function mutations in the glycosaminoglycan-catabolizing enzyme iduronate-2-sulfatase results in impaired craniofacial cartilage patterning and loss of *sox10*-expressing neural crest-derived progenitors (Moro et al. 2010).

8.4 Concluding Remarks

The study of extracellular matrix assembly, remodeling, and degradation during zebrafish embryonic development has begun to yield important insight into a variety of morphogenetic processes. The utility of zebrafish for confocal microscopy affords researchers the opportunity to discern extracellular matrix structure in relation to any cell population that can be fluorescently labeled. With the recognition that extracellular matrix proteins influence most aspects of embryogenesis, the continued identification and functional analysis of extracellular matrix interacting proteins and modifying enzymes becomes critical.

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Chapter 9
The Role of HA and Has2 in the Development and Function of the Skeleton

Peter J. Roughley and Pierre Moffatt

Abstract Hyaluronan (HA) is present throughout the body, including all the bones and cartilages of the skeleton, where it may fulfill both a structural and metabolic role depending on its molecular size. In mammals, HA is produced by three hyaluronan synthases (Has), of which Has2 is the predominant form in cartilage and bone. HA can be degraded by hyaluronidases (Hyal) and free radicals. Mammals possess five hyaluronidases, of which Hyal1 and Hyal2 are thought to be predominant in cartilage and bone. The structural role of HA in cartilage is dependent on its ability to form proteoglycan aggregates, whereas its metabolic role involves intracellular signaling induced by interaction with receptors such as CD44 and RHAMM. Such signaling differs between high-molecular-weight HA and its fragments. HA and its fragments play a major role in endochondral bone formation and possibly intramembranous bone formation, as they can regulate the differentiation and action of chondrocytes, osteoblasts, and osteoclasts. Cartilage-specific depletion of HA synthesis has been studied in floxed Has2 mice that have been crossed with mice expressing Cre under control of either the Prx1 or the Col2a1 promoter. Such deletion of Has2 gene expression results in a chondrodysplastic phenotype, in which all endochondral bones of the skeleton are severely truncated. The phenotype is characterized by severely impaired longitudinal growth of the bones due to abnormal organization and differentiation within the growth plates, particularly in the process of chondrocyte hypertrophy. The Col2a1-driven mice also exhibit defective modeling of the endochondral bone.

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9.1 Introduction

Many bones in the appendicular and axial skeleton are formed from a hyaline cartilage precursor, which subsequently develop and grow by endochondral ossification. The remaining bones of the skeleton, particularly those of the skull, do not involve a cartilage precursor and arise directly and grow by intramembranous bone formation. Hyaline cartilage persists in the endochondral bones at their growth plates during juvenile development and as articular cartilage at the bone surface in diarthrodial joints, where it allows smooth motion. In contrast, the more restricted motion of the spine is provided by the fibrocartilaginous intervertebral discs.

Cartilage and bone function depend on the unique structure of their extracellular matrix (ECM). In mature articular cartilage and intervertebral disc, the ECM provides the ability to resist compressive loading, and in the growth plate, it provides the scaffold that maintains the unique cellular organization responsible for long bone elongation. The cartilage ECM is characterized by the presence of proteoglycan aggregates that are formed via the interaction of hyaluronan (hyaluronic acid, HA) with numerous aggrecan molecules. While aggrecan is absent in bone, HA is present, though it is not thought to play a structural role as in cartilage but rather may participate in cell signaling. Irrespective of whether HA fulfills a structural or metabolic role in the skeleton, it is essential for the normal development and function of both cartilage and bone.

9.2 HA Structure and Metabolism

9.2.1 HA Structure and Distribution

HA was first identified in the vitreous humor of the eye in 1934 (Meyer and Palmer 1953), but its structure was not elucidated until 1954 (Weissmann et al. 1954). It is a linear polysaccharide composed of repeating disaccharides of D-glucuronic acid (GlcA) and *N*-acetyl D-glucosamine (GlcNAc), which are linked by β(1–3) and β(1–4) bonds, respectively (Fig. 9.1), and is a member of the glycosaminoglycan (GAG) family. HA is distinguished from other GAGs, such as chondroitin sulfate (CS), dermatan sulfate (DS), keratan sulfate (KS), heparan sulfate (HS), and heparin, by its long chain length, lack of sulfation, and mode of synthesis (Fraser et al. 1997). A typical HA molecule may possess in excess of 10,000 disaccharide units with a molecular weight of over 5 MDa, whereas the sulfated GAGs rarely exceed 50 kDa. HA is synthesized as a free polysaccharide chain at the plasma membrane of the cell, unlike the sulfated GAGs which are all synthesized on a protein primer within the Golgi. Its chain elongation is also reported to occur at the reducing terminus, rather than the nonreducing terminus as occurs in the sulfated GAGs (Prehm 2006).

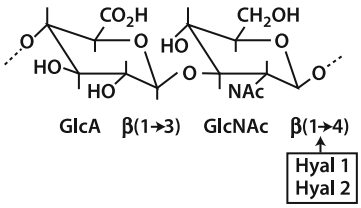

Fig. 9.1 The structure of HA. The figure illustrates the structure of glucuronic acid (GlcA) and *N*-acetyl-glucosamine (GlcNAc) that form the repeating disaccharide unit of HA and their interaction by β1 → 3 and β1 → 4 bonds. The figure also depicts the cleavage site of HA by mammalian hyaluronidases (Hyal1 and Hyal2)

HA is present in all vertebrates but has so far not been detected in invertebrates. It is however produced by some species of bacteria. HA is a ubiquitous molecule in vertebrates, being present in all tissues and body fluids (Fraser et al. 1997). In man, the abundance of HA is highest in the umbilical cord, a tissue often used for its preparation, and synovial fluid, where it is present at concentrations greater than 1 mg/ml. Among the body's connective tissues, HA is most abundant in skin, vitreous, and cartilages of the skeleton. In the rat, about 27 % of the HA present in the body is found in the skeleton and its supporting structures (Reed et al. 1988). The tissue abundance of HA varies throughout life, but it is present from the embryo to the adult. HA can have many diverse functions throughout life, which depend on both its abundance and molecular weight. HA abundance can also vary with the site within a tissue. In the growth plate, its abundance increases from the proliferative to the hypertrophic zone (Matsui et al. 1991), where it is thought to contribute to cell hypertrophy in addition to its more conventional role in proteoglycan aggregate formation within the ECM (Pavasant et al. 1996).

9.2.2 HA Synthesis

HA is produced by hyaluronan synthase (Has) residing at the cell membrane (Weigel et al. 1997). Three distinct mammalian Has have been described, Has1, Has2, and Has3 (Shyjan et al. 1996; Watanabe and Yamaguchi 1996; Spicer et al. 1997), and while all produce HA of an identical composition, it can differ in its rate of synthesis, chain length, and the ease with which it can be released from the cell surface depending on the Has involved (Itano et al. 1999). Has1, Has2, and Has3 are integral membrane proteins, with a similar size and structure (Weigel and DeAngelis 2007). Each possesses seven transmembrane or membrane-associated domains and a large cytoplasmic domain containing the active site for HA synthesis (Fig. 9.2). Although the active conformation of Has at the cell membrane has not been defined precisely, there is evidence that at least Has2 is capable of forming homodimers and heterodimers with Has3 and that this and ubiquitination are important for maximal activity (Karousou et al. 2010). Synthesis takes place from

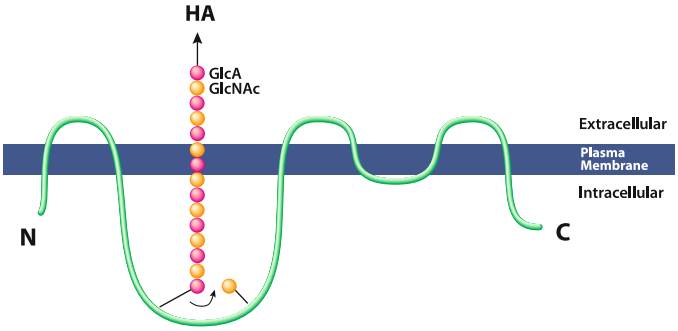

Fig. 9.2 HA synthesis via Has. Has is depicted as an integral plasma membrane enzyme with multiple intracellular, extracellular, and transmembrane domains. The largest intracellular domain possesses the catalytic site for HA synthesis. HA is synthesized in the cytosol by the alternating addition of glucuronic acid (GlcA) and *N*-acetyl glucosamine (GlcNAc), with the resulting growing polymer being extruded directly from the cell

cytoplasmic UDP-glucuronic acid and UDP-*N*-acetyl glucosamine. The monosaccharides are added to the reducing end of the nascent HA chain in the cytoplasm, and the growing chain is continually extruded into the extracellular space through a pore in the plasma membrane formed by the enzyme. The newly synthesized HA may be retained at the cell surface via interaction with Has or a HA receptor such as CD44, or it may be released and diffuse away from the cell. It is not clear what determines when release occurs. In cartilage, proteoglycan aggregate formation occurs via the interaction of aggrecan and link protein with the extracellular HA.

All Has genes are expressed by chondrocytes (Hiscock et al. 2000; Nishida et al. 1999; Recklies et al. 2001), with Has2 having the highest expression level and being principally responsible for HA production in all cartilages. It is less clear which Has is predominant in bone and whether osteoblasts, osteoclasts, and osteocytes are all involved in HA production. All three Has are expressed during embryonic development, albeit at different times and in different locations (Tien and Spicer 2005). Has2 appears to be by far the most important Has during embryonic development, as mice lacking Has2 gene expression die during mid-gestation (Camenisch et al. 2000), whereas those lacking Has1 or Has3 gene expression are viable and appear normal. Mice lacking both Has1 and Has3 also appear normal, though there is reported to be accelerated wound healing (Mack et al. 2012). Conditional inactivation of the Has2 gene in cartilage (Matsumoto et al. 2009; Moffatt et al. 2011) or over expression of Has2 in limb bud mesoderm (Li et al. 2007) results in severe chondrodystrophic phenotypes. This illustrates both the crucial role that Has2 plays in cartilage development and endochondral bone formation and the need for HA production to be regulated during limb development.

9.2.3 HA Degradation

In vivo, HA can be degraded by the action of hyaluronidases (Hyal) and free radicals (Stern et al. 2007). Six hyaluronidases have been described in mammals—Hyal1, Hyal2, Hyal3, Hyal4, HyalP1, and PH20, though PH20 is a testicular enzyme confined to the acrosome of sperm and in humans HyalP1 in an inactive pseudogene (Csoka et al. 2001). All the active hyaluronidases are endo-β-*N*-acetyl hexosaminidases, that utilize hydrolysis to cleave HA at the β(1–4) bonds (Fig. 9.1). The enzymes are also able to degrade CS via cleavage of the β(1–4) bond between *N*-acetyl galactosamine and glucuronic acid, albeit with lower efficiency than they cleave HA. The exception may be Hyal4, which may possess a greater chondroitinase activity.

Hyal1, Hyal2, Hyal3, and Hyal4 have been shown to be expressed in both cartilage and bone (Bastow et al. 2008). However, Hyal1 and Hyal2 are ubiquitously expressed and are considered to be the major hyaluronidases in somatic tissues. The two enzymes are distinct in their cellular location and the size of the HA fragments that they produce. Hyal1 is a lysosomal enzyme, whereas Hyal2 is a glycosylphosphatidylinositol (GPI)-anchored enzyme on the outer surface of the plasma membrane. Hyal2 may be responsible for the initial degradation of extracellular HA, which is then internalized for subsequent degradation by Hyal1. Hyal2 has a limited capacity to degrade HA and produces fragments of about 20 kDa (50 disaccharide units). In contrast, Hyal1 results in more extensive degradation and fragments of smaller size. Hyal2 may also be released from the cell membrane and function in the more remote ECM, where it has been postulated to play a role in HA degradation during inflammation (Durigova et al. 2011). Genetic ablation of Hyal1 (Martin et al. 2008), Hyal2 (Jadin et al. 2008), or Hyal3 (Atmuri et al. 2008) in the mouse has very few phenotypic consequences on development, reproduction, and aging. In the skeleton, only subtle changes were observed in the Hyal1 and Hyal2 knockout mice: a slight loss of articular cartilage proteoglycan for Hyal1 and the appearance of an extraosseous structure in the frontonasal process and abnormally shaped cervical vertebrae for Hyal2.

HA is also susceptible to nonenzymic degradation by free radicals generated during cell metabolism (Stern et al. 2007). Superoxide is produced by many cells via NADPH oxidase and may undergo dismutation to form hydrogen peroxide. Neither superoxide nor hydrogen peroxide by themselves degrades HA, but when present together or when hydrogen peroxide is present with a transition metal, rapid degradation of HA may occur via the production of hydroxyl radicals. Superoxide also plays a role in the conversion of nitric oxide to peroxynitrite, which can also result in the degradation of HA. Because of the relatively short half-life of free radicals, HA near the cell surface will be most susceptible to radical-mediated degradation.

It is now clear that HA fragments can have distinct functions compared to the high-molecular-weight HA generated by Has (Stern et al. 2006; Noble 2002). For example, high-molecular-weight HA plays a structural role, is anti-inflammatory

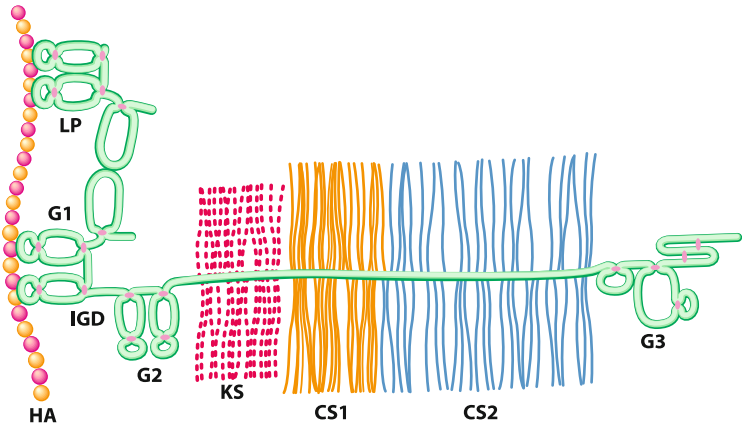

Fig. 9.3 The structure of proteoglycan aggregates. The figure depicts the interaction of aggrecan with HA and the stabilization of the interaction by a link protein (LP). The disulfide-bonded globular regions (G1, G2, and G3) of the aggrecan core protein and the interglobular domain (IGD) are indicated. The sites of substitution by chondroitin sulfate (CS) and keratan sulfate (KS) chains are also depicted together with the location of the two subdomains for CS substitution (CS1 and CS2)

and antiangiogenic, and impedes cell differentiation, whereas HA fragments are pro-inflammatory and pro-angiogenic. Even the fragments may differ in function among themselves, depending on their molecular size, with the small HA tetrasaccharides being anti-apoptotic. HA fragment function may not be confined to the extracellular environment, as intracellular HA fragments may also influence cell metabolism (Hascall et al. 2004).

9.3 HA Functional Roles

9.3.1 HA in ECM Structure

HA forms the backbone of the proteoglycan aggregates that characterize all cartilages (Hascall 1988). A typical proteoglycan aggregate is composed of a central filament of HA with up to 100 aggrecan molecules radiating from it, with each interaction being stabilized by the presence of a link protein (LP) (Fig. 9.3) (Morgelin et al. 1988). The aggrecan core protein possesses three globular regions (Doege et al. 1987, 1991; Watanabe et al. 1995; Hering et al. 1997), termed G1, G2, and G3. The G1 region is responsible for the interaction with HA (Watanabe et al. 1997) and is separated from the G2 region by a relatively short interglobular domain (IGD) (Flannery et al. 1998) The G3 region is separated from the G2 region by a long GAG-attachment region substituted with CS and KS chains. The G3 region is essential for normal trafficking of the newly synthesized aggrecan through

the cell (Zheng et al. 1998), while the GAG chains provide the molecule with the osmotic properties essential for tissue swelling (Hascall 1988). In addition to aggrecan, the proteoglycan aggregates may contain versican, which belongs to the same hyalectin family as aggrecan (Wight 2002). The versican core protein possesses terminal globular regions analogous to the G1 and G3 regions of aggrecan but contains far fewer CS chains in its central domain. In both cartilage and intervertebral disc, versican is present at a lower abundance than aggrecan (Sztrolovics et al. 2002), and its contribution to tissue function is not clear.

The large size of the proteoglycan aggregates results in their entrapment by the collagen framework of the tissue, and the high charge conferred by the GAGs results in tissue swelling and water retention. Proteoglycan aggregate function is impaired by glycolytic cleavage of the HA or proteolytic degradation of aggrecan, as occurs in the arthritides. Proteolytic processing by aggrecanases and matrix metalloproteinases (MMPs) (Sztrolovics et al. 1997; Lark et al. 1997; Hughes et al. 1995) results in the accumulation of aggregates enriched in G1 regions, which do not possess the osmotic properties associated with more intact molecules. The G1 regions remaining bound to HA may accumulate in the ECM for many years (Maroudas et al. 1998; Sivan et al. 2006). While the size of HA in the proteoglycan aggregates does decrease with age (Holmes et al. 1988), it is partially protected from both free radical and hyaluronidase cleavage by the presence of the link proteins (Rodriguez and Roughley 2006). Aggrecan fragments not possessing a G1 region are no longer bound to HA and may diffuse within the tissue. These GAG-rich fragments are rapidly lost from articular cartilage but may be retained for many years in the intervertebral disc (Roughley et al. 2006).

Proteoglycan aggregate function can also be perturbed by impaired synthesis, resulting in decreased size or abundance of the HA or decreased abundance or charge of the aggrecan. The consequence of such defects of aggrecan on skeletal development is illustrated by the chondrodystrophic phenotypes that result when gene mutations occur in aggrecan itself—spondyloepiphyseal dysplasia in humans (Gleghorn et al. 2005; Tompson et al. 2009), cartilage matrix deficiency (cmd) in mice (Watanabe et al. 1994; Krueger et al. 1999), and nanomelia in chickens (Primorac et al. 1994; Li et al. 1993)—or in the molecules responsible for its sulfation (Wallis 1995; Superti-Furga et al. 1996; Karniski 2001). Mutations in the versican gene also result in heritable disorders in man (Mukhopadhyay et al. 2006), but the phenotype extends beyond the skeleton, as expected for its expression in many extraskeletal tissues. To date, no human skeletal dysplasia has been attributed to HA deficiency due to a mutation in a Has gene, possibly because of the ubiquitous nature of HA and the lethal consequences that its deficiency could have on embryonic development.

9.3.2 HA Receptors and Signaling

HA should not be viewed as merely the scaffold upon which proteoglycan aggregate formation occurs, as it is a multifunctional molecule (Lee and Spicer 2000) that can

interact with many other HA-binding proteins (Day and Prestwich 2002) and so influence cell metabolism (Fig. 9.4). HA has been shown to interact with several unrelated proteins generically called hyaladherins. Two of the most studied are CD44 and RHAMM (receptor for hyaluronan-mediated motility, also called CD168), which exist in many different isoforms through alternative splicing. CD44 is an integral cell surface membrane protein that contains a "link" module and can be substituted with a GAG chain on its extracellular domain, depending on whether the appropriate exon is present or not. The standard CD44 variant encodes a ubiquitously expressed protein, whereas all other isoforms, possessing variable extensions within the extracellular portion of the molecule, generally have a more restricted tissue and cell-type expression pattern. CD44 is thought to act as a cell adhesion molecule and to serve as a clearance receptor for internalization and degradation of HA (Knudson et al. 2002). RHAMM can be found both intracellularly and extracellularly, even though it does not possess a signal peptide sequence and is not targeted to the typical secretory pathway (Maxwell et al. 2008). It is generally accepted that RHAMM is present extracellularly at the cell membrane, where it can interact with CD44. While HA binding to CD44 occurs through its link domain, binding to RHAMM involves a basic region.

CD44 and RHAMM do not present inherent catalytic activity and thus signal indirectly through clustering and interaction with various membrane co-receptors, cytoskeletal components, and cytoplasmic molecules (Aruffo et al. 1990; Slevin et al. 2007; Turley et al. 2002). Some of the intracellular effects mediated by CD44 and RHAMM are through modulation of the phosphorylation status of a variety of kinases. For instance, CD44 is known to engage in protein–protein interaction with and modulate the activity of several membrane receptors possessing intrinsic kinase activity (MAPK, Src, ErbB2, EGFR, c-Met, PDGFR, VEGFR, TGFB-R). The effects of CD44 are extremely complex, being cell-type specific and dependent on the isoform produced and whether its interaction is with high molecular weight or fragmented HA. Accordingly, downstream effectors solicited by CD44 activation are numerous. Apart from its interaction with other membrane receptors, CD44 is known to associate with the ezrin/radixin/moesin (ERM) protein complex. ERM is localized just beneath the plasma membrane of cells and is believed to link the plasma membrane with the cytoskeleton through interaction with cell surface receptors and actin filaments. CD44 and other cell adhesion molecules (CD43, ICAM-1, syndecans, and L-selectin) bind to ERM triggering actin filament reorganization and subsequently affecting various processes, such as cell adhesion and motility, signaling, phagocytosis, and apoptosis.

CD44 has also been shown to signal directly after its nuclear translocation. Cleavage of the cell surface CD44 by metalloproteases was reported to generate a soluble fragment and a 25 kDa ectodomain, which undergoes further juxtamembrane processing to liberate an intracellular domain (ICD) (Okamoto et al. 2001; Lammich et al. 2002; Murakami et al. 2003). Upon stimulation of cell signaling through protein kinase C and Ca^{2+} activation, intramembrane gamma-secretase cleavage at the transmembrane domain of CD44 releases the 72-residue ICD, which is found to accumulate in the nucleus. Nuclear CD44 ICD

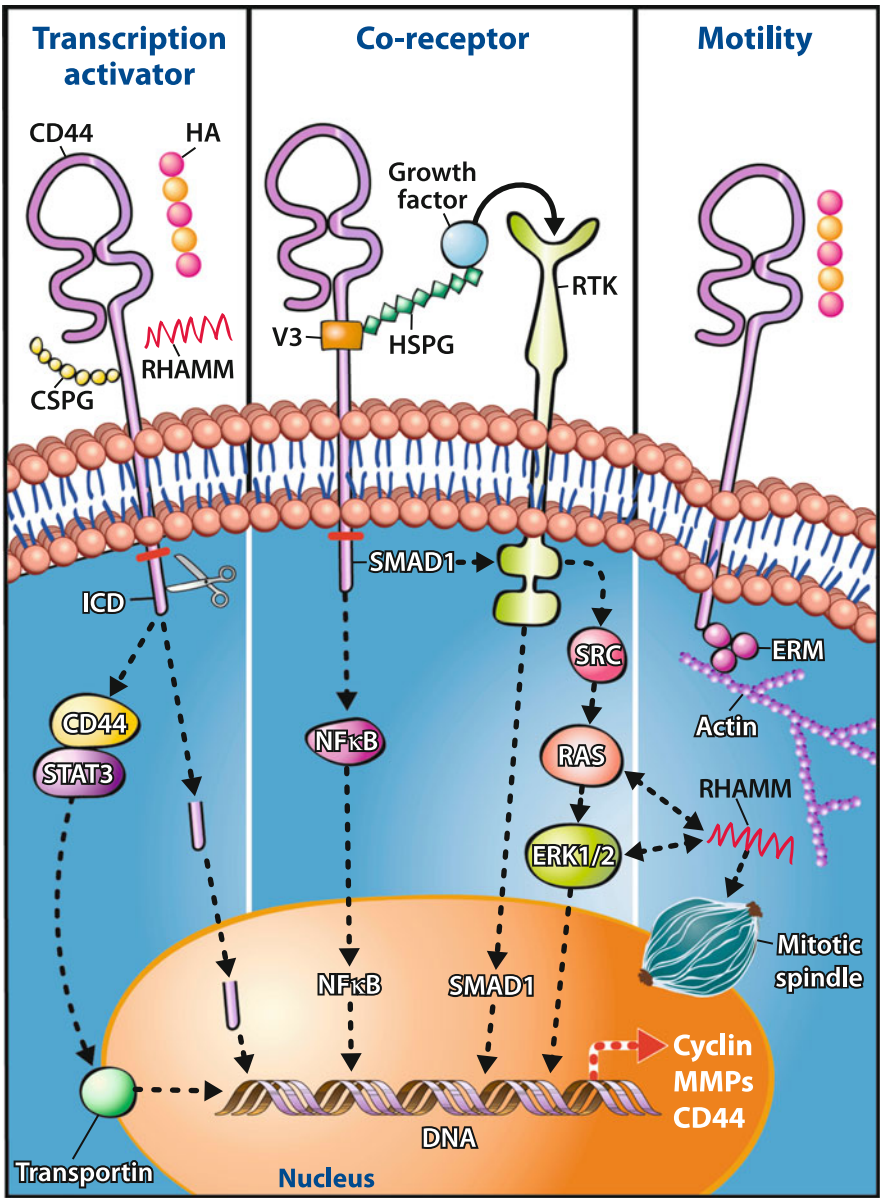

Fig. 9.4 Schematic representation of signaling functions of HA through CD44 and RHAMM. CD44 can signal directly or indirectly by interaction with a diverse repertoire of other molecules. CD44 or its intracellular domain (ICD) can translocate to the nucleus and modulate gene expression (*left*). CD44 can also act as a co-receptor "presenting" growth factors or transducer proteins to their corresponding receptor tyrosine kinase (RTK) (*middle*). Ensuing is a cascade of phosphorylation events that culminate in transcriptional regulation of gene expression. The intracellular portion of CD44 also tethers with the ezrin/radixin/moesin complex (ERM) and the actin network to promote cell motility (*right*). Intracellular RHAMM can participate in cell proliferation/division events through interaction with kinases and the mitotic spindle

has been reported to activate a TPA responsive element luciferase reporter construct. The CD44 gene itself has been shown to be a target of the ICD transcriptional activity, thus forming a feed-forward loop. Recently, the entire CD44 molecule was also shown to be present in the nucleus. The mechanism leading to the nuclear accumulation of full length CD44 has been shown to depend on its active endocytosis and transport from the cell membrane and on interaction with the nuclear membrane transportin molecule (Janiszewska et al. 2010). Although the mechanisms involved are still not fully understood, they seem to be independent of HA binding. In addition, the full length CD44 molecule has been shown to be internalized and to interact with cytosolic STAT3 and indirectly with the P300 acetyltransferase (Lee et al. 2009). The complex was found to migrate into the nucleus to regulate expression of cyclin D1 and promote cell proliferation. Other target genes also regulated by the CD44/STAT3/P300 complex include those for MMP2, VEGF, and BCL. The CD44 ICD has also been shown to be a docking site for SMAD1 (Peterson et al. 2004). Treatment of bovine articular chondrocytes with BMP7 induced the translocation of CD44-associated SMAD1 to the nucleus with transcriptional activation of a SMAD-binding element reporter plasmid. The appearance of nuclear SMAD1 was dependent on HA-CD44 interaction, as it was abolished by hyaluronidase treatment. It was proposed that SMAD1 binding to the ICD of CD44 would anchor it close to the plasma membrane for rapid presentation to the type I BMPR upon BMP7 stimulation (Andhare et al. 2009). However, the mechanism governing the CD44-SMAD1 interaction was not defined but was suggested to involve a phosphoserine in the CD44 ICD.

Incubation of articular chondrocytes with low molecular weight HA fragments has also been shown to stimulate gene expression through different signaling pathways (Fieber et al. 2004; Ohno et al. 2005, 2006; Schmitz et al. 2010). Phosphorylation and activation of Akt and NFkB stimulate distinct anabolic and catabolic responses, respectively, as reflected by the induction of Has2 and MMP gene expression. Although the upstream signaling cascades have not been clearly identified, the effects of the HA fragments appear to be partly related to HA displacement from CD44. It has been proposed that HA fragment binding results in "declustering" of CD44 and signaling through cytoskeletal (ERM) reorganization and possibly activation of kinases such as PKC and NFkB. It is not known whether degradative HA fragments are produced in the growth plate and whether they would be at a sufficient concentration to have any significant impact on either chondrocytes or osteoblasts.

RHAMM is supposedly expressed at very low levels, and its distribution appears mostly limited to injured tissues and pathologic cases involving inflammation and cancer (Fieber et al. 1999). Intracellular RHAMM can interact directly with ERK and is associated with mitotic spindle and microtubule assembly, where it interacts with BRCA1 (Tolg et al. 2010; Maxwell et al. 2011). Hence, RHAMM has been ascribed as a tumor-susceptibility gene in breast cancer, and its elevated expression and effects on the disorganization of the mitotic spindle have been postulated as one of the putative mechanisms involved in tumorigenicity. The HA-binding properties

of RHAMM and the interaction with CD44 have also been linked to the neoplastic transforming capability of RHAMM.

Because of the relatively wide tissue distribution of CD44 and RHAMM and their roles in many seemingly crucial aspects of cell differentiation and activity, such as proliferation, migration, and invasion, it was anticipated that their gene deletion would be detrimental to life. Surprisingly, the knockout mouse models for CD44 and RHAMM did not display any overt defects under normal conditions (Protin et al. 1999; Schmits et al. 1997). However, several studies have documented subtle to more severe defects when the mice, or isolated knockout cells, are subjected to different insults, either in vivo or in vitro. For instance, the CD44 and RHAMM knockout mice presented striking problems in repair processes after bleomycin-induced lung damage (Teder et al. 2002), angiogenesis (Cao et al. 2006), and skin wound injury (Tolg et al. 2006). Excessive accumulation of HA was found in the lungs of CD44 KO mice, suggesting clearance problems. RHAMM deficient fibroblasts have impaired motility and blunted signaling through ERK1/2 phosphorylation. Moreover, arthritis was found to be exacerbated in CD44 knockout mice, when induced by collagen immunization (Nedvetzki et al. 2004) or TNF-α overexpression (Hayer et al. 2005). Given the close relationship in terms of function for CD44 and RHAMM, it is perhaps not surprising that RHAMM was shown to functionally substitute for the absence of CD44. ICAM-1, another molecule with adhesion properties, was also found to compensate for the lack of CD44 in vivo and act as a substitute co-receptor for c-Met in CD44 null mice (Olaku et al. 2011).

9.4 HA in Chondrogenesis and Osteogenesis

9.4.1 HA in Chondrogenesis and Endochondral Bone Formation

Chondrogenesis (Fig. 9.5) begins in mice with mesenchymal cell condensation at about embryonic day 9.5 (E9.5) in a process that is driven by bone morphogenetic proteins (BMPs) (Barna and Niswander 2007). By E10.5, the templates for most skeletal elements have been formed, and the mesenchymal cells within the condensations differentiate into chondrocytes to form the cartilaginous anlagen of the skeleton, under stimulation by Sox9, Sox5, and Sox6 (Bi et al. 1999; Lefebvre et al. 1998; Han and Lefebvre 2008). Chondrocyte differentiation is accompanied by a change from type I collagen (Col1) production to type II collagen (Col2) and aggrecan. In contrast, the cells at the periphery of the condensations differentiate to form the surrounding perichondrium. The embryonic cartilages grow by chondrocyte proliferation until about E13.5, when cells in the center of the future bones cease proliferation and undergo a transformation to first prehypertrophic and then hypertrophic chondrocytes, which are characterized by their expression of type X collagen (Col10) and the calcification of their surrounding ECM (Karsenty et al.

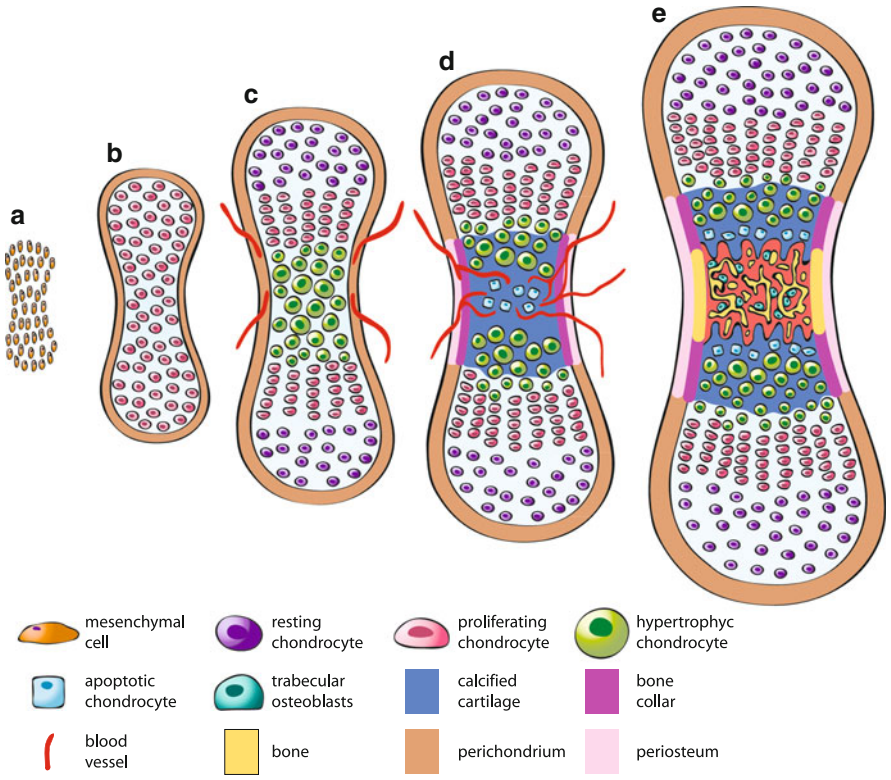

Fig. 9.5 Development of a fetal long bone. The figure depicts mesenchymal condensation (**a**); chondrocyte and perichondrial differentiation (**b**); the development of proliferative, maturation, and hypertrophic zones and the onset of vascularization into the perichondrium (**c**); the formation of the periosteal bone collar and vascular invasion of the calcified hypertrophic cartilage (**d**); and replacement of the calcified hypertrophic cartilage by bone (**e**)

2009). At the same time as chondrocyte hypertrophy occurs, cells in the perichondrium differentiate into osteoblasts, under stimulation by Runx2 (Ducy et al. 1997), and enclose the hypertrophic cells with a bone collar. Vascular endothelial growth factor (VEGF) production by the hypertrophic chondrocytes stimulates blood vessel ingrowth (Gerber et al. 1999; Dai and Rabie 2007) and the arrival of osteoblastic and osteoclastic precursor cells. As the hypertrophic chondrocytes die, osteoblasts deposit bone on the calcified cartilage and a primary center of ossification is formed. This process of endochondral ossification is recapitulated later in the epiphyses of many long bones by the formation of secondary centers of ossification and continues throughout juvenile life within the growth plates. In all cases, chondrocyte proliferation and hypertrophy are controlled by a variety of regulatory factors, including fibroblast growth factor receptor 3 (FGFR3), Indian hedgehog (Ihh), and parathyroid hormone-related peptide (PTHrP) (Ornitz and Marie 2002; Lanske et al. 1996; Vortkamp et al. 1996; St Jacques et al. 1999).

HA is associated with many stages of chondrogenesis and endochondral ossification (Bastow et al. 2008). In the developing limb bud, Has2 secretes the HA required to facilitate mesenchymal cell migration (Toole et al. 1972; Kosher et al. 1981), and the formation of precartilaginous condensations is associated with the expression of CD44 by the mesenchymal cells and interaction with the HA (Rousche and Knudson 2002). Subsequent removal of the HA by hyaluronidase cleavage then occurs to allow chondrocyte differentiation (Li et al. 2007). During endochondral ossification, an increase in HA production is associated with chondrocyte hypertrophy and appears to be necessary to facilitate cell swelling (Pavasant et al. 1996). Hypertrophy is also associated with aggrecan loss from the surrounding ECM (Matsui et al. 1991), and this may be mediated by hyaluronidase cleavage of the proteoglycan aggregates (Buckwalter et al. 1987). HA fragments generated in the hypertrophic zone may serve to terminate the hypertrophic process by suppressing the expression of Runx2 (Tanne et al. 2008). The HA fragments may also facilitate vascular invasion, as such fragments are known to be angiogenic (West et al. 1985; Pardue et al. 2008).

The temporal and region-specific expression pattern of the various components of the Has2/HA/CD44/RHAMM cascade in the forming and developing skeleton has not been extensively studied. During early development in the chick limb, the expression of Has2 by in situ hybridization was highest in the region corresponding to the apical ectodermal ridge (AER) and was excluded from the condensing mesenchyme (Li et al. 2007). This result is consistent with HA being produced mostly by cells of the AER, where it is proposed to maintain the underlying layer of mesenchymal cells in a proliferative and non-differentiating state. When out of reach of this HA-rich environment, condensation of mesenchymal cells becomes favorable and acts as one of the events initiating the chondrogenic program. Therefore, a gradient of HA diffusion could help control limb bud outgrowth and patterning. The role of CD44 in the developing limb bud appears to be related to that of Has2 and HA. The standard CD44 isoform is expressed throughout the limb bud, but many different splice variants are restricted to the AER (Sherman et al. 1998). Neutralization of CD44 with an anti-CD44 antibody caused diminished proliferation of mesenchymal cells and reduced the growth of the limb bud in mice. This may be related to the capacity of FGF8 to bind to or be sequestered by the V3 variant of CD44 which possesses a HS chain. Thus, CD44 serves an important function for FGF8 presentation and indirectly regulates the proliferation of neighboring mesenchymal cells.

Little is also known about the role of Has2, HA, RHAMM, and CD44 during later limb development in the establishment of the growth plate and in longitudinal growth. In situ hybridization for Has2 gene expression in the mouse femur and tibia at birth showed that highest expression was localized to the prehypertrophic chondrocytes, with some signal in the articular surfaces (Dy et al. 2010). Very little if any signal was detected elsewhere in the long bones, including regions of the primary spongiosa where osteoclasts, osteoblasts, and osteocytes are present. Considering that HA can be detected throughout the growth plate, it is possible that levels of Has2 mRNA were below the detection limit inherent to in situ

hybridization. These low levels may still produce sufficient Has2 protein to generate considerable amounts of HA. Unfortunately, no studies have reported on the localization of the Has2 protein in the growth plate. At present, it is unclear why a high level of Has2 expression is needed by the prehypertrophic cells, but it is possible that this relates to a unique function of HA in the pericellular environment of these cells and that this differs from its role in the rest of the growth plate.

In developing mouse long bones, CD44 expression appears to be restricted to the chondro-osseous junction (Jamal and Aubin 1996; Nakamura and Ozawa 1996; Noonan et al. 1996). At this interface, both osteoclast and osteoblast precursors express cell surface CD44. As growth and development proceed, osteocytes have also been identified as expressing high levels of CD44 (Hughes et al. 1994; Nakamura et al. 1995). Even though CD44 appears to be expressed in cultured articular chondrocytes, it was not detected in vivo in growth plate chondrocytes. If true, this would suggest that the effects of HA in the growth plate chondrocytes are likely not mediated through CD44 interaction. Consistent with studies conducted in the mouse and rat, CD44 in chick joints showed high levels in the articular fibrocartilage and weak expression in the epiphyseal chondrocytes (Dowthwaite et al. 1998). RHAMM just showed slightly lower expression in epiphyseal than articular chondrocytes. To our knowledge, the specific role of RHAMM in the skeleton in vivo has not been studied and could represent an alternative molecule mediating HA signaling. A recent study has shown that RHAMM overexpression in MC3T3 osteoblasts slightly enhanced ERK signaling and inhibited differentiation and subsequent mineralization (Hatano et al. 2011). While CD44 knockout mice do not possess histomorphometric defects associated with osteoblast and osteoclast parameters (de Vries et al. 2005), a μCT analyses revealed a subtle reduction in endocortical bone resorption associated with reduced RANKL production (Cao et al. 2005).

9.4.2 HA in Intramembranous Bone Formation

In contrast to endochondral bone formation, intramembranous ossification occurs without the formation of an initial cartilaginous template (Hall and Miyake 1992; Dunlop and Hall 1995), but in both cases, the eventual synthesis of mineralized bone occurs through the same process. The cranial vault, mandible, and parts of the clavicle typify bony elements formed purely through an intramembranous process, and periosteal bone development which forms the thickened cortical midshaft of long bones is also intramembranous in nature. Intramembranous bones arise directly through differentiation of mesenchymal cells, initially compacted in sheets or membranes, into osteoblasts. Although less well understood, the process of intramembranous bone formation seems to share many developmental cues and signals (BMPs, TGFβ, FGFs, Wnt, Runx2, Sox9) with endochondral bone formation. It has even been proposed that a chondrocyte-like cell, presenting a gene expression signature normally found in chondrocytes, is detected as an intermediate

stage within the sequence of events leading to fully differentiated osteoblasts (Abzhanov et al. 2007).

The role of HA during intramembranous bone formation in vivo is unknown. However, osteoblasts do express Has2 message, and HA is also known to influence the behavior of both osteoclasts and osteoblasts in vitro. HA may participate in osteoclast binding to the ECM (Prince 2004), and HA fragments induce bone resorption (Ariyoshi et al. 2005). In contrast, HA has been reported to be an inhibitor of osteoblast differentiation (Falconi and Aubin 2007).

9.4.3 Has Expression in Chondrogenic and Osteogenic Cell Lines

The ATDC5 cell line has been used extensively to study chondrogenesis, as in culture it can recapitulate the differentiation from a mesenchymal cell to a chondrocyte. Has2 has been shown to be expressed at low levels in non-differentiated cells and to increase three- to fourfold with differentiation to chondrocytes. Has1 expression is 2–3 orders of magnitude lower than Has2, suggesting that Has1 is unlikely to compensate for a lack of Has2. shRNA-mediated knockdown of Has2 gene expression in ATDC5 cells using a lentiviral delivery system induces a sustained knockdown and results in up to 67 % reduction in HA production. ATDC5 cells with the highest degree of knockdown display an altered morphological appearance and a dramatic reduction in Alcian blue staining indicative of proteoglycan depletion in the ECM (Fig. 9.6). Thus, failure to produce normal HA levels impairs chondrogenesis and reduces cartilage ECM formation.

The MC3T3-E1 cell line is commonly used to study osteogenesis, as in culture it can recapitulate the differentiation from an osteoblast precursor to a mature osteoblast producing mineralized bone. The expression levels of Has1 and Has2 in differentiating MC3T3-E1 osteoblasts were relatively constant, but the absolute levels of Has2 expression were 2–3 orders of magnitude higher than Has1. Thus, Has2 is likely the major contributor to HA production by osteoblasts in osteoid during bone formation and modeling.

9.5 Has2 Knockout Mice

9.5.1 Prx1-driven Has2 Knockout Mice

The early embryonic lethal nature of the global Has2 knockout mouse prevents the role of HA production by Has2 in skeletal development from being studied. To overcome this problem, two cartilage-specific knockout mice have been generated using the Cre-loxP system. The first of these utilized a mouse line with a floxed exon 2 in the Has2 gene, which was crossed with a line expressing Cre recombinase

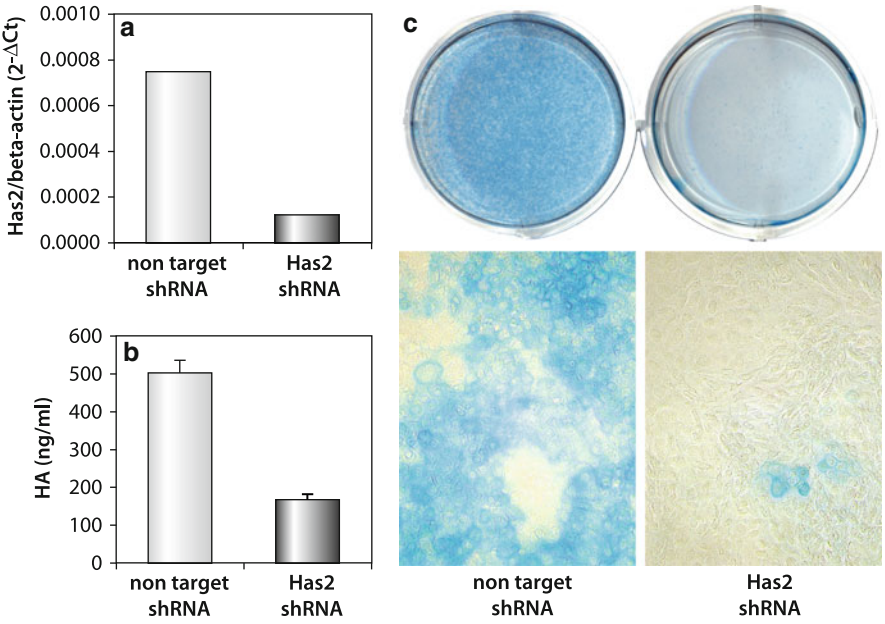

Fig. 9.6 Knockdown of Has2 expression in ATDC5 cells. Stable pools of ATDC5 cells expressing the lentiviral shRNA were analyzed after 14 days for Has2 message levels (**a**) and HA secretion in the culture media during the last 24 h (**b**). Alcian blue staining of the day 14 cultures reveals considerably less proteoglycan production in the shRNA expressing cells (**c**)

driven by a Prx1 enhancer (Matsumoto et al. 2009). The Prx1 enhancer results in Cre expression in the early limb bud mesenchyme and a subset of craniofacial mesenchyme (Logan et al. 2002). As such, it allows gene function to be studied in the developing limbs of the appendicular skeleton but does not result in gene excision in the axial skeleton. Exon 2 of the Has2 gene contains the start codon and two transmembrane domains, and its excision generates a null allele. The knockout mouse limbs show no evidence of Has2 message bearing exon 2, and HA content is barely detectable.

By E16.5, all bones of both the hindlimbs and forelimbs of the mutant Prx1-Cre-Has2 mice are extremely short compared to wild-type mice, and Alcian blue staining of the cartilaginous regions is very much reduced compared to wild-type mice. This suggests that the aggrecan content of the cartilage is severely diminished, as might be expected if the HA content of the cartilage is depleted and HA is essential for aggrecan retention via proteoglycan aggregate formation. In support of this conclusion, aggrecan expression is not itself altered in the mutant mice, confirming that the aggrecan depletion is due to loss from the ECM rather that lack of production by the chondrocytes.

The limb phenotype of the mutant mice indicates that longitudinal bone growth is perturbed in the absence of HA. It is therefore not surprising that the growth plates of the developing bones are abnormal, with the normal columnar cellular

organization being perturbed and the disorganized cells being more densely packed. Both the cellular disorganization and the dense packing could be related to the loss of aggrecan from the ECM. Indeed, mice lacking aggrecan, or possessing unstable proteoglycan aggregates due to the absence of link protein, exhibit a similar growth plate disorganization (Watanabe and Yamada 2002).

The abnormality in the mutant growth plates is not restricted to cellular organization but also involves cell differentiation, as the normal progression from resting to hypertrophic chondrocytes is perturbed. While there is little difference in chondrocyte proliferation in the wild-type and mutant growth plates, subsequent chondrocyte hypertrophy within the mutant growth plates is impaired. This results in a reduction in the number of hypertrophic chondrocytes. At least in part, this appears to be due to a reduction in the expression of Indian hedgehog (Ihh), which is produced by the prehypertrophic chondrocytes and initiates hypertrophy. Thus, the phenotype of the mutant mice due to the absence of HA in the growth plates is not only due to impairment of its structural role in the ECM but also to impairment in its role in cell signaling.

In addition, the mutant mice exhibit a patterning defect in the proximal phalanges of some digits, though the link between such a specific patterning defect and HA depletion is unclear. The mutant mice also exhibit impaired joint cavitation, a process that is driven by HA (Dowthwaite et al. 2003). In the normal mouse, HA is elevated in the interzone between two developing bones, and its production results in a cell-free space that drives cavity formation in this region. It is therefore not surprising that in the absence of HA, the cells of the interzone remain closely packed. These changes are probably independent of a depletion in HA production by chondrocytes but reflect the early expression of Prx1 and perturbation of HA production by the mesenchymal stem cells prior to their patterning or differentiation into various skeletal elements.

9.5.2 Col2a1-Driven Has2 Knockout Mice

The second cartilage-specific Has2 knockout mouse was generated by crossing the floxed Has2 mice described above with mice possessing Cre under control of the Col2a1 promoter (Moffatt et al. 2011; Roughley et al. 2011). Excision of the floxed exon 2 in the Has2 gene will therefore occur later in skeletogenesis than with the Prx1-driven mice. Thus, while the Prx1-Cre-Has2 mouse undergoes excision in the mesenchymal stem cells of the limb buds, the Col2-Cre-Has2 mouse is expected to involve only committed chondrocytes. However, unlike the Prx1-Cre-Has2 mouse, which only generates a phenotype in the limbs, the Col2-Cre-Has2 mouse is expected to also involve the intervertebral discs and vertebrae of the spine. Heterozygous Col2-Cre-Has2 mice appeared normal at birth but showed a slightly diminished growth rate with minimal skeletal abnormality. In contrast, homozygous knockout animals developed in utero but died near birth and exhibited a severe chondrodystrophic phenotype, with abnormalities throughout the skeleton,

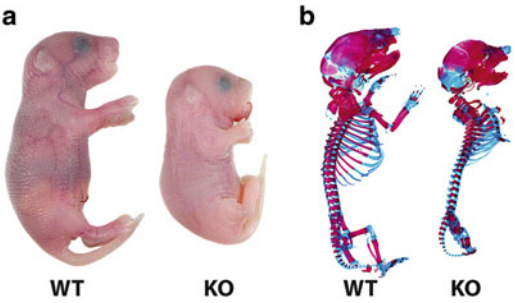

Fig. 9.7 Appearance and skeleton of Has2 knockout mice. Wild-type mice (WT) and Has2 knockout mice (KO) at E18.5 are compared for their external appearance (**a**) and for the structure of their skeletons (**b**). Note the severe chondrodysplasia in the knockout mice, with shortened body length, limbs, snout, and rib cage. Cartilage is stained with *Alcian blue* and bone with *Alizarin red*

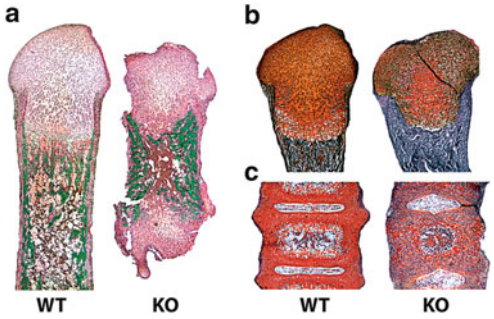

Fig. 9.8 Histological analysis of the long bones and spine. Non-decalcified sections of femurs from wild-type mice (WT) and Has2 knockout mice (KO) were stained with Goldner stain (**a**). The femurs of the Has2 KO mice possess a thick cortical bone adjacent to the periosteum, with little evidence for modeling at the endosteal surface. Decalcified sections of femurs (**b**) and spines (**c**) from wild-type mice and Has2 knockout mice were stained with Safranin O. There was little evidence of an organized growth plate at the femoral epiphysis, and the formation of the vertebral primary center of ossification and the appearance of the IVDs were abnormal in the KO mice

including the limbs, spine, and rib cage (Fig. 9.7). The severely compressed rib cage, with its detrimental consequence on lung development, may account for the premature death of these mice compared to the Prx1-Cre-Has2 mice.

The long bones of the limbs of the mutant mice were short and wide, and while the cartilaginous anlagen for the bones appeared to develop normally, there was little evidence of further growth during embryonic development or for bone modeling within the diaphysis (Fig. 9.8). In the wild-type bones, a distinct primary center of ossification with adjacent growth plates had formed in the diaphysis by E15.5, whereas in the mutant, the primary center of ossification had yet to form, though hypertrophic cells were present. By E18.5, the diaphysis of the wild-type bones possessed a cortical rim with a well-formed trabecular center, whereas the mutant bones possessed an extensively mineralized tissue throughout the diaphysis,

suggesting that the initial mineralized bone matrix had not been modeled. Perhaps surprisingly, these defects appear to be more severe than those reported for the long bone of the Prx1-Cre-Has2 mouse. The reason for the impaired modeling is not clear, as there was no apparent deficiency in vascular invasion or osteoclasts to account for such a defect. However, both osteoblast and osteoclast functions are influenced by the presence of HA in their surrounding ECM, and their attachment to a HA-deficient cartilage matrix may promote osteoid formation and impair bone resorption.

The increased mineralization in the mutant diaphysis could also be due to excessive periosteal bone formation. This raises the intriguing possibility of decreased production of HA by the osteoblasts themselves in the Col2-Cre-Has2 mouse. In support of this possibility, Col2-driven lacZ expression has been transiently detected in the periosteal osteoblasts (Nakamura et al. 2006). Col2 has also been shown to be transiently expressed during intramembranous bone formation of the skull (Abzhanov et al. 2007), suggesting that cranial vault osteoblasts might also be affected in the Col2-Cre-Has2 knockout mice. Interestingly, the heads of the Has2 knockout mice appear shorter with a dome-shaped cranial vault (Fig. 9.7). Alternatively, lack of HA in the cartilage could create an environment where signaling events from chondrocytes to periosteal osteoblasts are disturbed. In this respect, it is interesting to note that the presence of CS can regulate the diffusion of Ihh between prehypertrophic chondrocytes and the periosteum (Cortes et al. 2009). CS localization in the growth plate cartilage is dependent on proteoglycan aggregate formation, which would be prevented in the absence of HA.

To fully assess the role of diminished HA production by osteoblasts on bone formation and turnover, osteoblast-specific knockout mice will be needed. They could be generated using an osterix(Osx)-Cre (Rodda and McMahon 2006) or a bone-specific Col1a1-Cre (Dacquin et al. 2002) mouse line to excise the floxed Has2 gene in the osteoblast lineage. The Osx-Cre line should excise Has2 early in committed osteoblast precursors but, at the same time, should not affect chondrogenesis. Such studies can show whether deletion of Has2 only in osteoblasts affects both intramembranous ossification and endochondral ossification. Indirectly, it could also indicate whether the presence of HA in osteoid has any influence on osteoclast remodeling activity.

At E18.5, there was also no evidence for normal growth plate formation and organized endochondral ossification in the mutant long bones, as expected in view of the lack of linear growth (Fig. 9.8). As with the Prx1-Cre-Has2 mice, there did not appear to be a deficiency in cell proliferation in the region where the growth plate should be, but the proliferating chondrocytes appeared unable to organize into linear columns. The cells did however begin to undergo hypertrophy, though the resulting cells were smaller than normal hypertrophic chondrocytes, more irregular in shape, and appeared to die prematurely. They also did not efficiently calcify their surrounding ECM. Thus, the absence of HA affects the formation, function, and fate of the hypertrophic chondrocytes and impairs the normal progression from endochondral cartilage to endochondral bone.

Both the vertebrae and intervertebral discs of the spine also appeared abnormal at E18.5 (Fig. 9.8). All vertebrae were reduced in height, and there was no evidence of an organized primary center of ossification with surrounding proliferative and hypertrophic chondrocytes. As with the mutant long bones, chondrocyte proliferation and hypertrophy surrounding the primary centers of ossification in the mutant vertebrae occurred in a haphazard and incomplete manner, with evidence for excessive mineralized tissue in its center. Development of the mutant intervertebral discs appeared to be delayed, with an increased abundance of large vacuolated cells (presumably notochordal cells) in their nucleus pulposus and remnants of the notochord persisting between adjacent discs.

In contrast to the Prx1-Cre-Has2 mouse, aggrecan was still abundant in the epiphyseal cartilage ECM despite the depletion in HA synthesis, though in both mice the amount of ECM was drastically reduced. In the absence of HA, aggrecan would be expected to diffuse from the tissue, and therefore it is likely that some HA persists in the Col2-Cre-Has2 mouse. Such HA might have been produced by mesenchymal cells in the cartilage anlagen of the bones prior to chondrocyte differentiation and then persist in the developing cartilage. However, such residual HA is insufficient to maintain normal function.

9.5.3 Inducible Has2 Knockout Mice

While HA production via Has2 gene expression is essential for normal cartilage and long bone development in the fetus (Moffatt et al. 2011; Matsumoto et al. 2009), it is not known whether a decrease in Has2 activity or HA production must be present from the onset of chondrogenesis for a pathologic phenotype to develop or whether depletion at later stages of development can also be problematic. Deficient HA synthesis by growth plate chondrocytes in the juvenile could impair long bone and vertebral growth, and deficient HA synthesis by articular chondrocytes and intervertebral disc cells in the adult could result in premature joint degeneration.

To address this issue, an inducible cartilage-specific Has2 knockout mouse, in which Has2 expression can be inactivated specifically in cartilage at various stages of postnatal development and growth, is needed. For this purpose, a mouse expressing the Cre transgene under control of both the Col2 promoter (for tissue specificity) and doxycycline administration (for temporal selectivity) has been generated (Grover and Roughley 2006). The inducible Col2-rtTA-Cre mouse can be crossed with the floxed Has2 line to generate the conditional knockout line. Although such mice develop a phenotype similar to that of the Col2-Cre-Has2 mice when fed doxycycline from the moment of conception, no major phenotype has been observed when such mice have been fed doxycycline postnatally.

One of the underlying premises upon which the predicted outcomes for postnatal HA depletion are based is that HA turnover is taking place in cartilage throughout life at a sufficient rate for diminished HA synthesis to deplete HA levels. If this is not the case, then no phenotype would be observed. It is certainly possible that HA

turnover in the remote cartilage ECM could be slow and this could have contributed to the residual Safranin O staining present in the Col2-Cre-Has2 knockout mice. However, HA turnover is thought to be rapid at the cell surface (Morales and Hascall 1988), and depletion at this site might be expected to influence cell signaling and result in an abnormal phenotype irrespective of whether ECM changes occur or not.

It is also possible that postnatal administration of doxycycline is not able to promote complete excision of the floxed Has2 alleles in all chondrocytes, either because of poor delivery into larger avascular cartilages or because the activity of the Col2 promoter decreases with age. An alternative strategy to circumvent the latter problem would be the use of an alternative inducible Cre mouse line, such as the Agc1-Cre-Tet mouse, which uses an aggrecan gene enhancer to drive Cre expression (Han and Lefebvre 2008), or the aggrecan-Cre-ERT2 mouse, which possesses an inducible Cre construct within the 3′-UTR of the aggrecan gene (Henry et al. 2009). Both of these lines allow Cre expression in mature cartilage. The lack of a phenotype does, however, raise the question as to what level HA must be depleted in order for an abnormal phenotype to develop?

9.6 Disorders Due to Impaired HA Metabolism

Even though Has gene mutations have not been associated with skeletal disorders in humans, perturbation in Has expression and HA production has been associated with several nonskeletal disorders, particularly malignancies (Adamia et al. 2008; Ghosh et al. 2009; Yamane et al. 2010). Furthermore, in the Shar-Pei dog, a mutation upstream of the Has2 gene results in increased expression of Has2 and increased HA production, resulting in the characteristic thickened skin and a predisposition for periodic fever syndrome (Olsson et al. 2011). This latter feature may be a consequence of the pro-inflammatory nature of increased HA fragment generation during hyaluronidase-mediated turnover of the HA. An inability to degrade HA to small fragments, due to Hyal1 deficiency, results in mucopolysaccharidosis type IX (Triggs-Raine et al. 1999), which does show skeletal abnormalities leading to short stature.

It is also possible that deficient HA production could be associated postnatally with skeletal problems involving impaired osteogenesis, such as delayed bone formation during fracture healing and distraction osteogenesis, which both involve endochondral ossification. This could be due to abnormalities in HA production or fragmentation. In such cases, there is the intriguing possibility of using local HA administration to enhance bone repair. Local administration of HA has been used clinically in several situations for many years, including eye surgery and the arthritic joint (Laurent and Fraser 1992).

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