Developmental Biology 341 (2010) 126–140

Contents lists available at ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/developmentalbiology

Review

The extracellular matrix in development and morphogenesis: A dynamic view

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ARTICLE INFO

Article history:
Received for publication 23 September 2009
Revised 16 October 2009
Accepted 17 October 2009
Available online 23 October 2009

ABSTRACT

The extracellular matrix (ECM) is synthesized and secreted by embryonic cells beginning at the earliest stages of development. Our understanding of ECM composition, structure and function has grown considerably in the last several decades and this knowledge has revealed that the extracellular microenvironment is critically important for cell growth, survival, differentiation and morphogenesis. ECM and the cellular receptors that interact with it mediate both physical linkages with the cytoskeleton and the bidirectional flow of information between the extracellular and intracellular compartments. This review considers the range of cell and tissue functions attributed to ECM molecules and summarizes recent findings specific to key developmental processes. The importance of ECM as a dynamic repository for growth factors is highlighted along with more recent studies implicating the 3-dimensional organization and physical properties of the ECM as it relates to cell signaling and the regulation of morphogenetic cell behaviors. Embryonic cell and tissue generated forces and mechanical signals arising from ECM adhesion represent emerging areas of interest in this field.

Introduction

In 1981, as a student in the venerable "Physiology Course" at the Marine Biological Laboratory in Woods Hole, one of us (DWD) publicly admitted to a burgeoning interest in the extracellular matrix (ECM) and was challenged by a prominent cell biologist to justify why anyone would want to work on "that stuff". At the time, many had come to view the ECM and the connective tissues in which it is found in abundance, as having a necessary but largely passive structural role—or as the aforementioned cell biologist so colorfully offered, "the styrofoam packing material" of cells and tissues. A lot has changed since 1981 let alone 1959 when the first issue of *DB* graced our libraries' shelves. And while the ECM is still acknowledged to perform its pedestrian but necessary function as a scaffold that "fills the spaces" between cells and tissues, we have also come to appreciate its widespread functional importance and dynamic roles in diverse cellular processes.

Several key observations culminating in the discovery of the integrins (and other ECM receptors) in the mid 1980s, altered forever our view of the ECM and its involvement in normal physiology and homeostasis, disease progression and development (Dzamba et al., 2001). Integrins were a key piece of a puzzle that led ultimately, to a rich mechanistic understanding of the physical linkages between intracellular and extracellular compartments that serve to mediate adhesion, resist mechanical stress, and facilitate the bidirectional flow of cell signals.

A successful systematic overview of the ECM and its importance in developmental processes is a daunting if not presumptuous task so we have chosen instead to focus on a relatively few select examples across multiple systems that best serve to illustrate key concepts and mechanisms. However, Table 1 is provided to summarize ECM loss-of-function phenotypes from multiple systems, with citations to the original sources of information. This review is organized according to general functional processes critical for developmental events (Fig. 1) instead of by ECM molecule, developmental stage or by organism. Our goal was to stress concepts related to ECM functions in development and to offer speculative insights related to areas of current or emerging interest.

We begin with a brief primer on ECM molecules many of which will be discussed throughout this review. It is important to consider that the extracellular compartment contains a variety of ECM components, the composition and organization of which changes throughout development beginning with fertilization. For example, some oocytes and eggs are invested with extensive ECM of maternal origin (e.g., zona pellucida in mammals, jelly coats of amphibians and sea urchins) and at fertilization additional matrix is secreted and assembled as a consequence of the cortical reaction. Whether of maternal or later zygotic origin, ECMs are modified and remodeled throughout development. These dynamic rearrangements and compositional differences are critical to understanding ECM functions at key points in development.

A primer on ECM molecules

ECM is composed of several distinct families of molecules with disparate evolutionary origins. These include glycosaminoglycans and proteoglycans, collagens and non-collagenous glycoproteins.

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doi:10.1016/j.ydbio.2009.10.026

Table 1
ECM loss-of-function phenotypes.

ECM	Isoform/component	Loss-of-function phenotypes	Citations
Fibronectin		Embryonic lethal (~E10.5). Cardia bifida, defects in mesoderm specification, axis elongation, neural tube morphogenesis, myocardial precursor migration and yolk sac vasculature	[m]George et al., 1993, [m]Georges-Labouesse et al., 1996, [x]Davidson et al., 2006, [d]Trinh and Stainier, 2004, [g]Linask and Lash, 1988a, [g]Linask and Lash, 1988b
Laminin	α1	Embryonic lethal (E6.5). Extraembryonic tissue developmental defects, epiblast polarization defects, compromised parietal and visceral endoderm differentiation, induction of apoptosis, axis elongation and eye defects	[m]Alpy et al., 2005, [m]Miner et al., 2004, [m]Schéele et al., 2005, [d]Zinkevich et al., 2006
	α2	Post-natal death (5 weeks). Muscular dystrophy	[m]Guo et al., 2003, [m]Miyagoe et al., 1997, [d]Hall et al., 2007
	α3	Post-natal death (3 days). Severe skin blistering	[m]Ryan et al., 1999
	α4	Viable (small increase in deaths after birth). Defects in synaptic specialization, haemorrhages, cardiovascular defects.	[m]Patton et al., 2001, [m]Thyboll et al., 2002, [d]Knöll et al., 2007
	α5	Embryonic lethal (before E17). Exencephaly, syndactyly, extraembryonic tissue disorganization, and fin formation defects	[m]Miner et al., 1998, [d]Webb et al., 2007
	β1	Embryonic lethal (E5.5). Defects in extraembryonic tissue development, gastrulation, implantation, notochord differentiation and eye formation.	[m]Miner et al., 2004, [d]Parsons et al., 2002, [d]Gross et al., 2005
	β2	Post-natal death (15-30 days). Growth arrest, neuromuscular junction and renal defects	[m]Noakes et al., 1995a, [m]Noakes et al., 1995b
	β3	Death just after birth. Severe skin blistering	[m]Kuster et al., 1997
	γ1	Embryonic lethal. Notochord differentiation and eye defects	[m]Smyth et al., 1999, [d]Parsons et al., 2002, [d]Gross et al., 2005
	γ2	Post-natal death (5 days). Severe skin blistering	[m]Meng et al., 2003
Collagen	ColI	Embryonic lethal (E12-14). Aortic rupture and severe tissue integrity defects	[m]Liu et al., 1995, [m]Löhler et al., 1984, [m]Schnieke et al., 1983
	ColII	Death at birth. Cartilage formation defects	[m]Li et al., 1995a, [m]Aszódi et al., 2001
	ColIII	Post-natal death (2 days). Growth retardation, reduced life-span, skin blistering, blood vessel rupture	[m]Liu et al., 1997
	ColIV	Embryonic lethal (E10.5–11.5). Defects in basement membrane integrity and Reichert's membrane integrity, growth retardation (*col4α1/α2*) Renal failure (*col4α3/α4/α5*)	[m]Pöschl et al., 2004, [m]Cosgrove et al., 1996, [m]Miner and Sanes, 1996, [m]Rheault et al., 2004
	ColV	Embryonic lethal (E10). Collagen fibril assembly defects, compromised skin and connective tissue integrity	[m]Wenstrup et al., 2004, [m]Andrikopoulos et al., 1995
	ColVI	Viable. Joint degeneration, musculoskeletal abnormalities, lower body weight, decreased bone density	[m]Alexopoulos et al., 2009
	ColVII	Post-natal death (2 weeks). Cutaneous blistering.	[m]Heinonen et al., 1999
	ColVIII	Viable. Notochord and eye defects	[m]Hopfer et al., 2005, [d]Gansner and Gitlin, 2008
	ColIX	Viable. Noninflammatory degenerative joint disease, cartilage maintenance defects, abnormal fin vascular plexus development.	[m]Aszódi et al., 2001, [m]Fässler et al., 1994, [d]Huang et al., 2009
	ColX	Viable. Defects in growth plate development, trabecular morphology, bone architecture and craniofacial skeleton.	[m]Kwan et al., 1997, [m]Chung et al., 1997
	ColXI	Death at birth. Compromised chondrocyte differentiation, severe cartilage defects in limbs, ribs, mandible and trachea.	[m]Seegmiller et al., 1971, [m]Li et al., 1995b
	ColXII	Viable. Periodontal ligament and skin matrix architecture abnormalities (not null)	[m]Reichenberger et al., 2000
	ColXIV	Viable. Defects in fiber and fibril assembly in tendons	[m]Ansorge et al., 2009
	ColXV	Viable. Mild muscular and cardiovascular defects, compromised notochord and somite differentiation.	[m]Eklund et al., 2001, [d]Pagnon-Minot et al., 2008
	ColXVII	Post-natal death (2 weeks). Severe blisters and erosions, hair loss, growth retardation.	[m]Nishie et al., 2007
	ColXVIII	Viable. Abnormal blood vessels in eyes, neuromuscular junction defects, synapse disorganization.	[m]Fukai et al., 2002, [c]Ackley et al., 2003
	ColXIX	Post-natal death (3 weeks). Malnourished, smooth muscle functional defects, smooth muscle transdifferentiation in esophagus inhibited.	[m]Sumiyoshi et al., 2004
Elastin		Post-natal death (4 days). Obstructed, stiff and tortuous arteries.	[m]Li et al., 1998, [m]Wagenseil et al., 2009
Fibrillin	Fbn1	Post-natal death (3 weeks). Cardiovascular defects	[m]Pereira et al., 1997
	Fbn2	Viable. Syndactyly and defective mesenchymal differentiation, notochord morphogenesis.	[m]Arteaga-Solis et al., 2001, [m]Chaudhry et al., 2001, [x]Skoglund et al., 2006
Fibulin	Fibulin-1	Perinatal lethal. Haemorrhages, blood loss, vascular, lung and kidney defects	[m]Kostka et al., 2001
	Fibulin-3	Viable. Reduced reproductivity, early on-set aging.	[m]McLaughlin et al., 2007
	Fibulin-4	Perinatal lethal. Severe elastinopathy in lungs and vasculature	[m]McLaughlin et al., 2006
	Fibulin-5	Viable. Loose skin, excessive abdominal folds, expanded lungs, vascular anomalies	[m]Yanagisawa et al., 2002, [m]Nakamura et al., 2002

(continued on next page)

Table 1 *(continued)*

ECM	Isoform/component	Loss-of-function phenotypes	Citations
Vitronectin		Viable and normal	[m]Zheng et al., 1995
Tenascin	Tn-C	Viable. CNS defects and abnormal locomotive behavior, neural crest migration defects	[m]Fukamauchi et al., 1996, [m]Forsberg et al., 1996, [g]Tucker, 2001
	Tn-R	Viable. Defects in perineural nets and optic nerve.	[m]Weber et al., 1999
	Tn-X	Viable and normal	[m]Matsumoto et al., 2001
Perlecan		40% embryonic lethal (E10.5), 60% death after birth. Defective cephalic development, broad and bowed long bones, narrow thorax, craniofacial abnormalities, severe cartilage defects	[m]Arikawa-Hirasawa et al., 1999
Versican		Embryonic lethal (E10.5). Heart defects (mouse mutant *hdf* maps to versican locus), neural crest migration defects	[m]Mjaatvedt et al., 1998, [a]Stigson et al., 1997
Aggrecan		Viable. Cleft palate, short limbs, tail and snout, cartilage defects (mouse mutant *cmd* maps to aggrecan locus).	[m]Watanabe et al., 1994
Neurocan		Viable. Mild defects in synaptic plasticity	[m]Zhou et al., 2001
Brevican		Viable. Mild defects in long-term potentiation maintenance	[m]Brakebusch et al., 2002

[m] = *M. musculus*.
[d] = *D. rerio*.
[x] = *X. laevis*.
[g] = *G. galus*.
[c] = *C. elegans*.
[a] = *A. mexicanum*.

Glycosaminoglycans (GAGs) are linear unbranched polymers of repeating disaccharides composed of a hexosamine and a uronic acid. These molecules have remarkable physical properties attributable to the abundance of carboxyl, hydroxyl and sulfate groups that define individual GAGs (e.g., chondroitin-, dermatin-, keratin- and heparan-sulfates). As such they are polyanionic molecules and their electrostatic properties make them "osmotically active." Their net negative charge attracts Na++ and, thus, draws water in causing the interstitial spaces in which GAGs reside to swell. This swelling can open up pathways that promote the invasion and migration of cells as has been suggested for the non-sulfated GAG hyaluronan (HA), which is correlated with, for example, cancer metastasis and initiation of cell migration (Toole, 2001). Thus, the regulated expression of hyaluronan synthases and other enzymes involved in GAG assembly can have important developmental consequences (Camenisch et al., 2000; Spicer et al., 2002). With the exception of hyaluronan, all GAGs are covalently linked to core proteins to form proteoglycans (PGs). HA does, however, assemble with aggregating PGs and these interactions are critical for the formation of pericellular matrices, the unique physical and hydrodynamic properties of which influence morpho-

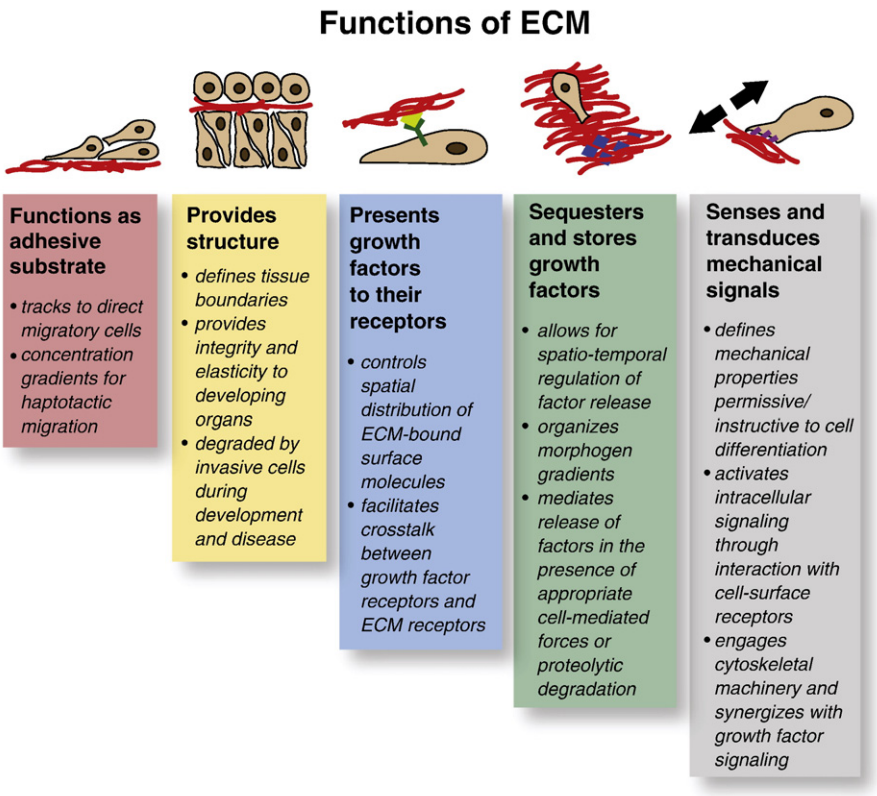

Fig. 1. Summary of ECM functions in development. The ECM is multi-functional and can influence multiple biochemical and mechanical processes simultaneously. This figure illustrates different functional states of the ECM and their biological contexts. The five categories are not mutually exclusive. When interpreting ECM loss-of-function phenotypes, one should consider that multiple processes may be compromised thus specific roles of individual ECM components are difficult to glean. A couple of important properties of ECM are not illustrated in this cartoon. First, ECMs are highly dynamic and can be modified by the cells that come into contact with them creating a bi-directional mode of cell–matrix communication. Second, ECM–ECM interactions vary the chemical and mechanical composition of the extracellular microenvironment. In this review, we incorporate several examples of how the functions of ECM are utilized during embryonic development.

genetic cell behaviors and regulate the diffusion of many secreted growth factors and morphogens. The transmembrane receptor for HA, CD44, also participates in the assembly of the pericellular matrix and the subsequent propagation of cell signals.

ECM glycoproteins include both the collagens and a diverse array of non-collagenous proteins such as laminins, tenascins, and fibronectin. A great deal is now known about collagen structure and function. Collagens are the most abundant proteins in the animal kingdom and as a general property, function to limit the distensibility of tissues owing to the enormous tensile strengths of collagen fibrils. A triple-helical organization of component pro-α-chains defines the collagens and contributes to the unique physical properties of these ECM proteins. There are now 28 known collagens and these are the products of 49 distinct collagen α-chain gene products (Gordon and Hahn, 2010). Collagens are broadly classified into both fibrillar and non-fibrillar forms and can also be assembled into reticular networks and sheets. The organization, distribution and density of fibrils and networks varies with tissue type, and the direction and magnitude of forces to which a given tissue is subjected. Heritable mutations and acquired (e.g., dietary) disruptions in the expression or function of individual collagens and/or proteins involved in collagen synthesis, processing and assembly have been known for many years (Kuivaniemi et al., 1991). One of the first insertional mutations to be identified in mouse was in the α1(I) collagen gene. The mutation is embryonic lethal (Schnieke et al., 1983) but surprisingly, embryos lacking collagen I reach a late stage of development when they die suddenly from aortic rupture (Löhler et al., 1984), in keeping with the central role of fibrillar collagen in limiting tissue distensibility.

Non-collagenous glycoproteins of the ECM are represented by several families of proteins with diverse origins. Many of these ECM molecules are composed of multiple chains each encoded by distinct (e.g., laminin trimer) or single (e.g., fibronectin dimer) genes. Further variation in protein sequence, structure and function can occur through alternative splicing of expressed transcripts as in the case of fibronectin, splice variants of which are subject to developmental stage- and tissue-specific expression (Astrof et al., 2007; DeSimone et al., 1992; Ffrench-Constant and Hynes, 1989). While the structures of ECM glycoproteins and the phylogenetic relationships of the genes that encode them vary widely, many share analogous functions and common structural motifs. One example is the adhesive Arg-Gly-Asp (RGD) sequence that resides within a hydrophilic loop of fibronectin, vitronectin, tenascin and other ECM proteins. RGD sequences are critical for recognition and binding to many integrins although it is important to point out that not all ECM glycoproteins have functional RGD sequences and not all integrins bind RGD. This review focuses primarily on the functions of non-collagenous ECM glycoproteins in development but a thorough overview of these ECM proteins and their receptors is not practical; the reader is instead referred to a number of other reviews on this subject (Barczyk et al., 2010; Durbeej, 2010; Dzamba et al., 2001; Ramirez and Sakai, 2010).

Together, these distinct groups of ECM molecules provide capacity for enormous functional complexity. The architecture and assembly of ECM in embryonic interstitial spaces may provide the structural integrity needed to promote and, in some cases, restrict cell movements, limit the diffusion of morphogens and provide binding sites for a number of families of cell surface receptors for ECM, including the integrins and syndecans. Thus, the ECM can be thought of as comprising a "morphogenetic language or code" that is interpreted by the cells that come in contact with it. This "sensing" of embedded information in the ECM by specialized receptors at the cell surface can have a profound influence on cell behaviors by affecting not only pedestrian adhesive functions but also cell polarity, migration and other intracellular signals that regulate cell survival, proliferation and differentiation. As we shall see, short and long range physical forces generated as a consequence of morphogenetic movements can also alter the availability of cell-interactive and other functional domains embedded within the matrix and regulate a cell's response to the extracellular environment. These data demonstrate that our current knowledge of ECM bears little resemblance to earlier characterizations of a "passive" matrix holding cells and tissues in place. The following sections provide a sampling of the widespread functions of ECM in development and highlight a diversity of mechanisms that directly or indirectly depend upon the actions of matrix molecules and their cellular receptors.

ECM in cell and tissue migration

The most familiar developmental function attributed to ECM is arguably cell migration. Dynamic ECM–integrin binding interactions are known to facilitate cycles of cell adhesion and deadhesion to substrate. When these cycles are combined with a contractile cytoskeleton to generate traction forces on an ECM substrate, cell locomotion occurs. Isolated migratory cells *in vitro* often display a preference for specific ECM molecules depending on the repertoire and binding activities of the integrins expressed by those cells. It would be easy to conclude that ECM is little more than "sticky-stuff" that depends on the right ECM–integrin combination for migration to proceed. However, ECM can interact with cells through associated growth factors or cytokines, intracellular signaling, mechanotransduction and cross-regulation with other cell-surface receptors, each of which may also regulate cell migration. In this review, we will consider two case-studies that highlight different ways ECM can influence cell migration: myocardial precursor migration in the formation of the zebrafish heart tube and neural-crest migration. Additional interesting examples reviewed elsewhere include primordial germ cell migration (Raz, 2004) and neuronal migration in the development of the cerebellum (Porcionatto, 2006).

Migration of cardiac precursor cells

The vertebrate heart is first assembled as a linear bilayered tube composed of an inner endothelial layer and outer muscular layer (Glickman and Yelon, 2002; Stainier, 2001). Myocardial precursors initially start to differentiate at the lateral regions on both the right and left side of the embryo and progressively migrate toward the midline before fusion with endocardial cells and formation of the heart tube. Failure of myocardial precursor migration leads to the condition cardia bifida where two separate hearts form in lateral positions. Several zebrafish mutations that result in cardia bifida have been identified including *natter*, which maps to a mutation in the *fibronectin* gene that causes loss of fibronectin expression during myocardial precursor migration (Trinh and Stainier, 2004). Fibronectin knockdown by a low dose of antisense morpholino also results in cardia bifida (Matsui et al., 2007).

There are two major tissue locations where fibronectin is expressed during myocardial precursor migration. Fibronectin is deposited on (1) the basal surface of the lateral plate mesoderm that contains the myocardial precursor cells, and (2) at the midline between the endoderm and endocardial precursors (Trinh and Stainier, 2004). Cardia bifida can be partially rescued in fibronectin minus morphant embryos by direct injection of exogenous fibronectin into the midline region (Matsui et al., 2007) indicating that midline deposition of fibronectin may play a role in directing myocardial precursor migration to the center. Analysis of an endocardial mutant *clo* where midline fibronectin deposition was absent but fibronectin around the lateral plate mesoderm was unaffected, resulted in delayed myocardial migration but not complete inhibition of the migration process itself (Trinh and Stainier, 2004). Thus midline fibronectin may be important for the timing of myocardial precursor migration but not strictly required for migration per se. Cardia bifida was also observed in embryos lacking Mtx1, a transcription factor controlling fibronectin expression (Sakaguchi et al., 2006).

However, Mtx1 depletion also resulted in reduced laminin deposition (Sakaguchi et al., 2006), thus, the ECM-dependent defect in myocardial precursor migration may depend on multiple ECM proteins.

It is clear from the *natter* mutant that fibronectin also plays a role in organizing the polarity of myocardial precursor cells. These cells migrate as a coherent mass to form polarized epithelia (Trinh and Stainier, 2004). In the *natter* mutant, apical and junctional markers aPKC and ZO-1 became mislocalized, and the organization of the myocardial precursors into polarized epithelia was disrupted (Trinh and Stainier, 2004). Cell–ECM interactions in this tissue are important for specification of polarity, which in turn is important for migration.

Interestingly, the *natter* mutation genetically interacts with another cardia bifida mutation, *miles-apart* (Matsui et al., 2007), which encodes a lysosphingolipid G-protein coupled receptor (Kupperman et al., 2000). Cultured zebrafish cells from the *miles-apart* mutant show decreased adhesion to fibronectin but not laminin *in vitro*, which can be rescued by addition of sphingosine phosphate 1 (SP1), the ligand for the *miles-apart* receptor (Matsui et al., 2007). Additionally, a mutation in Spns2, a multipass transmembrane protein that is important for export of SP1 also causes cardia bifida (Kawahara et al., 2009). However, the mechanism by which ECM and SP1 coordinate to regulate migration is still unknown. The lessons learnt from studies of myocardial precursor migration are that ECM can influence cell migration through regulation of adhesion and polarity, providing/maintaining temporal cues, influencing the presence of other matrix proteins such as laminin and crosstalk with signaling factors and their receptors.

Neural crest cell migration

Another important migratory population of cells within the developing embryo is the neural crest. Neural crest cells are induced at the border of the neural plate and the non-neural ectoderm (Knecht and Bronner-Fraser, 2002). During neural tube closure, neural crest precursors are incorporated into the dorsal neural tube from where they delaminate and migrate in streams to far reaches of the embryo. Derivatives of neural crest include neurons and glia, craniofacial cartilage and bone, pigment cells, connective tissue and sympathoadrenal cells (Knecht and Bronner-Fraser, 2002). Over the years, numerous factors have been identified as important regulators of neural crest migration including, but not restricted to BMPs and their antagonists, Wnts, Hox genes, E-cadherin, Ephrins, Eph receptors, MMPs and ECM (Christiansen et al., 2000).

A conditional knockout of β1 integrin in mouse neural crest precursor cells resulted in severe neuronal defects that were ultimately lethal one month after birth (Pietri et al., 2004). Migration of neural crest cell derivatives such as Schwann cells was defective in addition to other abrogated processes that included delayed maturation of Schwann cells and defective axo-glial segregation (Pietri et al., 2004). However there were also changes in the patterns of deposition for fibronectin, laminin, tenascin and collagen IV. This highlights the difficulty in deciphering the roles of ECM *in vivo* because perturbations of one factor can lead to a cascade of changes involving multiple cell–ECM interactions.

The mechanisms responsible for directed migration of subsets of neural crest cells are complex. Neural crest migrates along ECM and individual ECM molecules support different migratory behaviors. The neural crest streams of mice lacking *laminin α5* are expanded (Coles et al., 2006). This suggests that laminin α5 may function normally to restrict migration into narrow streams. Other ECM components have been shown to attract or repel migrating neural crest. Implanted micromembranes of PG/M-versican isoforms V0 and V1 attract neural crest cells whereas micromembranes of aggrecan cause migratory cells to arrest near the implant (Perissinotto et al., 2000). Expression patterns of these two types of proteoglycans are largely complimentary and non-overlapping suggesting that sub-populations of neural crest cells may migrate on versican-containing matrix and avoid aggrecan-containing matrix.

Cell migration speed obeys a bell-curve distribution relative to concentration of substrate ECM (Palecek et al., 1997), with maximal migration speed achieved at an intermediate concentration of ECM that facilitates both adhesion and deadhesion of cell–substrate contacts. One way in which directed cell migration occurs is by haptotaxis where cells migrate along a gradient of ECM molecule concentration. Cells move away from regions of low ECM concentration where adhesion is weak, and move toward regions of higher ECM concentration where migration will slow or arrest if adhesive strength is too great. In general, gradients of ECM concentration in the paths of migratory cells have been difficult to confirm. One example is that of tenascin, which is expressed in the avian neural tube in a dorsoventral gradient visualized by immunostaining (Bronner-Fraser, 1988). When antisense Tenascin-C morpholinos were electroporated into the neural tube, defects in neural crest migration were observed (Tucker, 2001) supporting the idea that tenascin may provide an adhesive substrate that facilitates haptotactic migration for at least some stretches of the neural crest migratory pathway.

Composition of ECM may also dictate the migration speed of cells that come in contact with it. Individual ECM molecules can have differing effects on the adhesion and/or rate of migration in different cell types. For example, cranial neural crest cells migrate faster than trunk neural crest cells on laminin although both cell types migrate at the same speed on fibronectin (Strachan and Condic, 2003).

Degradation of ECM substrates by proteases provides another important mechanism for the regulation of cell migration. Proteases from both the matrix metalloproteinase (MMP) and ADAM families are implicated in neural crest migration. For example, ADAM13 protease activity is required for normal cranial neural crest cell migration in *Xenopus* (Alfandari et al., 2001). ADAM13 degrades fibronectin *in vitro*, however, more recent work also demonstrates involvement in neural crest induction through modulation of Ephrin B signaling (Wei and DeSimone, unpublished observations). Although MMPs and ADAMs are known to degrade ECM components, they also target for cleavage components of several signaling pathways including various growth factor receptors, Eph/Ephrins, and notch and delta. So once again, observed migration defects following loss-of-function of a given protease known to degrade ECM could also be regulating the activities of additional non-ECM substrates to affect adhesion and migration.

ECM in branching morphogenesis

The development of branched organs involves the invasion of epithelial buds and tubes into surrounding embryonic mesenchyme rich in ECM, and this process is key to building many composite tissues. Branching also provides an interesting example of the multiple roles played by ECM in morphogenesis. Proteoglycans, GAGs, collagens and many other ECM glycoproteins have all been implicated as important regulators of mammary gland, salivary gland, kidney, gut and lung development. The branching units are surrounded by microenvironments of ECM that change in composition and spatial distribution over time. This dynamic characteristic makes it challenging to establish functional roles for individual ECM components.

Manipulations that either reduce or promote ECM molecule deposition often inhibit branching. In mammary gland cultures, for example, both the addition of collagenase and the stimulation of collagen expression by TGFβ can perturb branching and ductal growth (Fata et al., 2004; Silberstein et al., 1990; Wicha et al., 1980). Similarly, a function blocking antibody directed against the laminin α1 G-domain inhibits branching of *ex vivo* salivary gland organ cultures as does addition of synthetic laminin α1 G-domain peptides that are competent to bind to syndecan-1, its relevant cell-surface receptor

(Patel et al., 2006). The presence or absence of a given ECM component cannot be viewed simply as providing "stop or go" signal for branching morphogenesis. ECMs can serve alternatively as substrates for cell adhesion, barriers to invasion that require degradation for branching to proceed, ligation of cell surface receptors that impact cell signaling, sequestration and/or presentation of growth factors and cytokines, and as mechanical cues vital to the branching process. Thus a given ECM component may have functions that both promote and attenuate cell behaviors important for branching depending upon the spatiotemporal context of its expression. It is the combinatorial effect of multiple ECM molecules as well as the dynamic regulation of ECM accumulation and degradation that instruct the morphogenesis of branching.

Expression and modification of the ECM microenvironment

Simple analysis of the heterogeneity of ECM expression patterns along branching structures provides some clues as to the function of a given ECM molecule. The branching units of the mammary gland system are the terminal end buds that accumulate a thick ECM around bud flanks composed mostly of collagen IV, laminin 1, laminin 5 and heparin sulfate proteoglycans (Fata et al., 2004), whereas a thinner ECM rich in hyaluronic acid is present at the end bud tips (Silberstein and Daniel, 1982). Reduced collagen around the end bud tips may be just as important as the enhanced accumulation of collagen around the flanks. A fibrous ECM surrounding the ducts helps maintain its tubular organization (Hinck and Silberstein, 2005). In contrast, mammary gland cultures exposed to a β1 integrin activating antibody that enhances binding to collagen inhibited branching (Alford et al., 1998), suggesting that migratory epithelial cells at the end bud tips may normally be required to reduce adhesion to ECM in order to invade the mesenchyme.

However, it is important to point out that fibrous ECM is not simply a physical barrier requiring degradation or modification in regions where cell migration is to occur. The mammary gland expresses discoidin domain receptors (DDR), receptor tyrosine kinases that become phosphorylated when ligated by fibrillar collagen but not denatured collagen fragments (Vogel et al., 2001). Loss of DDR1 causes defects in mammary gland branching in part due to hyperproliferation suggesting that activation of DDR1 normally promotes a quiescent state (Vogel et al., 2001). In this case, a collagen rich ECM at bud flanks is required for a signaling function. Interestingly, loss of DDR1 also causes increased collagen deposition around the ducts, which implies that DDR1 function and collagen expression and/or accumulation are linked. Additionally, this also suggests that the amount, and not just composition of ECM may also be important to the regulation of branching morphogenesis.

Degradation of ECM may serve other roles in addition to removing a matrix "barrier". For example, collagen cleavage produces biologically active fragments such as tumstatin and endostatin that can in turn, regulate migration, proliferation, and cell survival (Ortega and Werb, 2002). ECM degradation can also release important signaling molecules such as Areg, Wnts, TGFβ and FGF, which have been shown to regulate branching (Sternlicht et al., 2006).

Analyses of ECM expression patterns in branching organs highlight the difficulty of analyzing contributions of ECM to morphogenesis. The developing kidney expresses unique and transient combinations of ECM proteins and their receptors in various sub-compartments of the organ (reviewed in Kanwar et al., 2004). However the requirement for such exquisite patterns of expression is not clear because these patterns vary widely between species. Additionally, many single gene knockout mouse models for ECM components do not result in kidney defects *in vivo*, suggesting that there is extensive functional compensation possible in this and other organ systems. Nevertheless, native ECM composition and organization provides the context within which pro-branching contributors operate. Data obtained using the mammary gland system have shown that blocking the function of α3β1 integrin, a laminin and collagen receptor, has opposing effects depending on the ECM present in the culture. Branching is enhanced in collagen I gels (Berdichevsky et al., 1994) but inhibited when mammary glands are cultured on laminin-rich basement membrane gels (Stahl et al., 1997). This demonstrates that the function of the same integrin can likely vary depending on the ECM composition of the microenvironment.

Finally, differential expression of integrin and non-integrin ECM receptors has been proposed to regulate branching by maintaining pro-migratory and pro-proliferative signals at the growing end bud tips while maintaining constraining and anti-proliferative signals at the bud flanks and ducts (Fata et al., 2004). However, data on the expression patterns of ECM receptors (as opposed to many ECM molecules) in branched organs, unfortunately, remains limited.

The importance of ECM architecture to branching

If all required proliferative, migratory, polarity and survival cues could be supplied to cells in the absence of ECM, it is difficult to imagine that they would default to anything other than a growing ball of tissue. ECM provides both elastic and rigid elements that likely participate in the propagation and/or resistance of forces needed to sculpt tissues into functioning organ structures. On a macro scale, ECM–cell interactions likely contribute mechanical stiffness for morphogenesis to proceed normally. When grown in mechanically loaded collagen gels, mammary epithelial cells fail to express β-casein and differentiate (Paszek and Weaver, 2004). Furthermore, using collagen and basement membrane gels calibrated for specific elasticities, it was demonstrated that highly rigid ECM inhibits branching and instead promotes cell spreading and focal adhesion formation (Paszek et al., 2005).

On a micro-scale, local anisotropies in the distribution of tension could determine where and when branch points occur. Hinck and Silberstein (2005) suggested that asymmetric induction of sulfated GAGs (SGAGs) at the mammary gland terminal end bud could be responsible for altering branch direction and/or end bud bifurcation. Deposition of SGAGs followed by accumulation of collagen I creates a thickened and relatively inelastic ECM (compared to the collagen-free end bud tip) that acts as a girdle around the end bud at a time when constriction to ductal dimensions occurs. In the salivary gland, lung and kidney similar models for initiation of branching have been proposed. Fibronectin, which stimulates branching when supplied exogenously, transiently accumulates at early clefts (Sakai et al., 2003). Human salivary gland epithelial cells exposed to pre-aggregated cellular fibronectin induced the formation of local cell–matrix complexes including integrins and the cytoskeletal protein paxilin while downregulating cell-surface E-cadherin in adjacent cells (Sakai et al., 2003). Fibronectin may function to form clefts by converting cell–cell adhesions to cell–matrix adhesions. Additionally, local depositions of fibronectin may function to promote assembly of collagen III, which has been shown to accumulate where clefts form (Nakanishi et al., 1988) perhaps to provide a rigid support to stabilize the cleft.

The role of ECM in branching morphogenesis is not limited to just structural support. ECM binding to cell-surface receptors can induce signaling cascades that lead to transcription of important growth factors. In the developing kidney, ligation of α8β1 by the ECM protein nephronectin is important for induction of glial cell-line derived neurotrophic factor (GDNF) which is a key regulator of uteric bud formation and branching (Linton et al., 2007). Genetic deletions of α8 integrin and nephronectin cause kidney agenesis, which can be rescued by deleting *Sprouty*, an antagonist of the GDNF receptor RET (Linton et al., 2007), which further supports the argument that the role of nephronectin is to stimulate GDNF expression. In the mammary gland system, ECM–β1 integrin interactions are upstream

of a well-defined signaling cascade for β-casein transcription and therefore mammary epithelial cell differentiation. Additionally, ECM may function to sequester morphogens and other secreted proteins that can be released by proteolysis. Examples of these ECM functions are further discussed later in this review.

Individual ECM molecules in the microenvironment may also impact the expression and/or accumulation of other bioactive ECM molecules. For example, mammary epithelial cells on laminin down-regulate expression of fibronectin. Levels of fibronectin are observed to change during mammary gland branching; it is more abundant during proliferative stages but is subsequently down-regulated during the growth arrest stage preceding acinar differentiation (Williams et al., 2008). Thus the spatio-temporal regulation of one ECM component can influence the abundance of other components that may provide additional cues for morphogenesis.

Structural contributions of ECMs

Throughout morphogenesis, motile cells undergo changes in shape, protrusive activity and polarity while exerting force on neighboring cells and tissues in order to generate structures such as tubes, rods, sheets and cavities. ECM can play important structural roles in these processes by contributing anisotropies in the extracellular microenvironment or by defining tissue boundaries as discussed for branching morphogenesis.

During elongation of the chordate embryonic axis, extension of the notochord, somitic mesoderm and neural tube all occur in the anterior-posterior direction (reviewed in Keller et al., 2000). Regulation of axis elongation involves multiple mechanisms including non-canonical Wnt/PCP signaling, cytoskeletal remodeling/contractility, differential cell adhesion and ECM. In *Xenopus*, fibronectin, fibrillin and laminin are each expressed as fibrillar matrices in slightly different patterns around the notochord and presomitic mesoderm (Davidson et al., 2004; Fey and Hausen, 1990; Skoglund et al., 2006). Knockdown of fibronectin or fibrillin by antisense morpholinos in *Xenopus* causes defects in convergent extension of the notochord and presomitic mesoderm (Davidson et al., 2006; Skoglund et al., 2006) although in the case of fibronectin the importance of fibrils is less clear (Rozario et al., 2009; discussed further below). Structural support for notochord elongation may rely on other ECM proteins such as fibrillin (Gansner et al., 2008; Skoglund et al., 2006; Bette Dzamba, personal communication).

Within the notochord, at least two distinct cell behaviors can be observed during convergent extension. Notochord cells adopt a spindle-shaped morphology and make bipolar protrusions, which are proposed to "tug" on neighboring cells and drive mediolateral intercalation behavior. Cells at the notochord–somite boundary become quiescent as they contact the ECM at the boundary provoking speculation that a "boundary capture" mechanism may be at play to resolve the separation between the notochord and presomitic mesoderm and inhibit notochordal cells from breaching the boundary. A study of notochord morphogenesis in the tunicate *Ciona* has identified laminin as an important player in notochord elongation potentially through a boundary-capture mechanism (Veeman et al., 2008). The mutant *chongmague* which maps to *laminin*α*3/4/5* is defective in axis elongation with notochordal cells failing to recognize the notochord-somite boundary and becoming dispersed in the larval tail (Veeman et al., 2008). A laminin loss-of-function study in *Xenopus* has not been reported. However, there may be a role for laminin in somite morphogenesis. Morpholino knockdown of dystroglycan, a laminin receptor, causes reduced somitic cell size, number and integrity (Hidalgo et al., 2009). Distroglycan morphants also exhibited a marked reduction in deposition of laminin around the somites. Knockdown of integrin α6 subunit containing laminin receptors also causes defects in axis elongation by abrogating neural extension and neural tube closure (Lallier and DeSimone, 2000; Lallier et al., 1996). To complicate matters further, the zebrafish laminin mutants *grumpy, sleepy* and *bashful* cause defects in notochord differentiation (Parsons et al., 2002; Pollard et al., 2006). Thus laminin may play multiple roles in organization and extension of the notochord, somites and neural tube that are crucial to proper embryo elongation.

Collagens play major roles in providing structural stiffness and cohesiveness to tissues and their ECMs. Loss of collagen III, V, VII and XVII compromise the integrity of skin, vasculature, connective tissue and eyes (Andrikopoulos et al., 1995; Heinonen et al., 1999; Liu et al., 1997; Nishie et al., 2007). Collagen III and V are both needed to facilitate the assembly of collagen I (the most abundant collagen protein) into fibrils and fibers. The loss of such fibers and fibrils cause devastating consequences such as skin blistering and blood vessel rupture (Andrikopoulos et al., 1995; Liu et al., 1997). Some laminin chains have also been implicated in functioning as a structural component critical to the integrity of skin. Mutations in laminin β3 and γ2 result in poorly developed hemidesmosomes that lead to severe skin blistering (Kuster et al., 1997; Meng et al., 2003).

Contributions of elastic tissues

While tissue stiffness imparted by ECM plays an important role in morphogenesis, it is clear that tissue "pliability" and elasticity are also critical. Some tissues respond to physical forces acting upon them by folding, bending or stretching and in the case of the walls of muscular arteries and alveoli, elastic recoil is essential for proper tissue and organ functions. The elastic matrices (including elastic fibers and sheets) that impart these tissue properties are composed of the ECM protein elastin as well as unbranched microfibrils that are thought to form scaffolds important for the nucleation and assembly of elastin (reviewed in Mithieux and Weiss, 2005). Microfibrils can be composed of many different proteins including fibulins, fibrillins, microfibril-associated glycoprotein-1 (MAGP-1), emilin and vitronectin. Although elastin comprises ~90% dry weight of formed elastic fibers, the microfibrillar components are critical for function. For example, fibulins were found to be important regulators of tissue elasticity. Loss of fibulin-4 or -5 causes reduced elasticity in the lungs, skin and vasculature leading to malformations such as loose skin and contorted aortas (McLaughlin et al., 2006; Nakamura et al., 2002; Yanagisawa et al., 2002).

In developing arteries, elastin is organized into concentric sheets or elastic laminae that are in turn associated with collagen fibrils and layers of smooth muscle cells (Wagenseil and Mecham, 2009). Homozygous null elastin knockout mice (*Eln* −/−) die shortly after birth from obstructed arteries (Li et al., 1998). Interestingly, loss of elastin leads to overproliferation and disorganization of smooth muscle in the vessel wall. These arteries become stiff and tortuous, and cellular infiltration into the lumen occurs, which is the cause ultimately for blocked arteries in these embryos (Li et al., 1998; Wagenseil et al., 2009). Interestingly, heterozygous elastin knockout mice (*Eln* +/−) survive to adulthood with normal cardiac function but with higher blood pressure (Wagenseil et al., 2009). Increased deposition of elastin or collagen was not observed in *Eln* +/− mice. Instead, the arteries form in the presence of low levels of elastin with increased smooth muscle cell differentiation. The result is an increase in arterial wall thickness, which may represent an adaptive response to decreased circumferential wall stress relative to normal wild-type vessels (Wagenseil et al., 2009). These studies suggest a physiological adaptive feedback mechanism between hemodynamics and vascular remodeling that is further dependent on the elastic matrix, which may explain why *Eln*+/− mice are viable.

ECM and tissue asymmetry

The process of amniote gut looping provides one interesting example of how the combinatorial effects of ECM and cell and tissue

generated forces can produce local anisotropies that facilitate morphogenesis. The characteristic counter-clockwise turning of the intestine is established by left–right asymmetric cellular and molecular events early in the formation of the gut. A leftward tilt is initiated in the primitive gut by several different processes that vary across species. Left–right asymmetry in the amniote gut is caused by cellular asymmetries in the dorsal mesentery; a structure that suspends the primitive gut from the body wall composed of mesenchymal cells wrapped in a layer of epithelial cells (Davis et al., 2008). On the right side, the epithelial cells are flattened and cuboidal while the mesenchymal cells are sparsely distributed. On the left side the epithelial cells are columnar and the mesenchymal cells are densely packed (Davis et al., 2008). Unlike in *Xenopus*, no asymmetries in cell proliferation, cell death or cell migration dyamics were observed in the developing avian gut (Davis et al., 2008).

In silico modeling revealed that leftward tilting within the dorsal mesentery could be achieved under conditions where, on the right side, ECM volume is increased and cell–cell adhesion is decreased, whereas on the left side, ECM volume is decreased and cell–cell adhesion is increased (Kurpios et al., 2008). Indeed, left–right asymmetries in ECM composition are observed within the dorsal mesentery with increased GAGs and basement membrane components on the left side and increased hyaluronan on the right side (Kurpios et al., 2008). Hyaluronan attracts water and promotes the swelling of matrix and additionally can inhibit cell–cell adhesion by forming a porous coat around cells (Brown and Papaioannou, 1993; Haddon and Lewis, 1991). Thus the asymmetric deposition of hyaluronan on the right side of the dorsal mesentery has the ability to create conditions that promote leftward tilting. Left–right asymmetric expression of the cell adhesion molecule N-cadherin was also observed. N-cadherin expression is regulated by the transcription factor Pitx2, which is preferentially expressed on the left side (Kurpios et al., 2008). Misexpression of N-cadherin in the dorsal mesentery results in changes in ECM composition that are inhibitory to the leftward tilt (Kurpios et al., 2008) indicating that there is cross-regulation between cell–cell and cell–ECM interactions in order to create optimal conditions for gut looping.

ECM in growth factor signaling

ECM can bind soluble/secreted factors, maintain them in the extracellular spaces and thereby function as a repository. The consequence of such interactions may be to restrict or promote access of ligands to cognate cell-surface receptors, to modulate the spatial distribution of a diffusable morphogen, or to sequester factors for subsequent release. Indeed, there is much speculation about the potential role of ECM in regulating extracellular signaling though it remains an understudied question.

Role of ECM in TGFβ activation

The best understood example of the role of ECM involvement in growth factor signaling comes from studies of the latent TGFβ binding proteins (LTBPs). Secreted TGFβ covalently dimerizes with its cleaved latency-associated propeptide (LAP), in an inactive complex. LAP binds LTBPs, which contain binding sequences for various integrins (i.e., RGD) and other ECM molecules like fibrillin, vitronectin and fibronectin. TGFβ–LAP–LTBP–ECM interactions form the so-called large latent complexes (LLCs) that are maintained in the extracellular space, inaccessible to cell-surface TGFβ receptors (reviewed in Wipff and Hinz, 2008).

A number of different pathways have been described to explain how TGFβ can be released from LLCs. Proteolytic cleavage of ECM and LTBPs in LLCs by BMP-1, MMPs, plasmin, urokinase, elastase, thrombin and cathepsin can relieve the inhibition of TGFβ (Wipff and Hinz, 2008). In some cases, the LTBP–ECM interaction occurs via heparin sulfate (Chen et al., 2007) and thus is subject to regulation by glycosidases as well. Spatial-temporal regulation of protease expression or secretion could control when and where active TGFβ is released. In some systems, the proteolytic cleavage is dependent on the presence of specific integrins suggesting that the proteolysis and ECM–integrin interactions are linked. It is possible that integrins provide a common docking site that brings the proteases and ECM into close proximity (Wipff and Hinz, 2008). Thus modulation of the affinity of integrins for relevant ECM binding may influence the degree of TGFβ signaling. Another contribution that integrins may make is to present the cell-surface receptor to the active ligand; αvβ3 integrin has been shown to interact with the receptor TGF-β-RII upon stimulation with active TGFβ1 in lung fibroblasts (Scaffidi et al., 2004).

Another interesting TGFβ activation strategy involves the mechanical coupling of integrin–ECM linkages. Integrins are linked intracellularly to cytoskleletal elements and associated contractile machinery, and extracellularly to ECM. Thus contractility of a cell bound to a rigid ECM generates force between a matrix molecule and its receptor. Such forces may uncouple LLCs so as to free TGFβ. The small GTPase RhoA has well established roles in contractility and has been shown to promote TGFβ activation from LLCs (Jenkins et al., 2006). Additionally, interactions of the actin cytoskeleton with the integrin cytoplasmic tail are necessary for TGF-β activation (Munger et al., 1999). These observations suggest that TGFβ signaling may be "tuned" by mechanical inputs affected by the composition and rigidity of the ECM, and the motility/movement of cells and tissues in contact with ECM.

TGFβ activation from LLCs has been implicated as a major regulatory event in cardiac development. LTBPs are expressed in developing hearts of murine and avian embryos and LTBP knockdowns result in severe cardiac (Ghosh and Brauer, 1996; Todorovic et al., 2007) and pulmonary (Sterner-Kock et al., 2002) defects. Interestingly, the contractility of myofibroblasts themselves can release latent TGFβ (Wipff et al., 2007). The cardiac system is a particularly intriguing model for the study of how cellular contractility and linkage to ECM may influence the level of growth factor signaling although little of this work has yet been done *in vivo*.

Latent TGF-β activation has also been implicated in bone development where LTBP-fibrillin co-localization occurs in embryonic long bones (Dallas et al., 2000). In addition, exogenously supplied LTBPs were shown to shift sensitivity of *Xenopus* ectoderm for the mesoderm inducer activin, a TGFβ-superfamily member (Altmann et al., 2002). This suggests that mesoderm induction may depend on latent-TGF-β activation though this has not yet been demonstrated *in vivo*.

ECM, syndecans and other growth factors

ECM participates in FGF signaling during the development of the mouse salivary mandibular gland (SMG). FGF10-FGFR2b signaling is crucial for development of the SMG. FGF10 binds the receptor with higher affinity when present in a ternary complex with heparan sulfate (Kan et al., 1999; Pantoliano et al., 1994). Basement membrane around the SMG was found to contain perlecan-heparan sulfate, which can be released by proteolysis by heparanase (Patel et al., 2007). Interestingly, addition of exogenous heparanase to *ex vivo* SMG cultures enhanced branching (Patel et al., 2007). Thus ECM can sequester a co-factor like heparan sulfate that is released upon expression of an appropriate endoglycosidase like heparanase.

The non-integrin ECM receptor, syndecan is known to be important in growth factor signaling. Syndecans bind growth factor ligands through their heparan- and chondroitin-sulfate glycosaminoglycan side chains (Carey, 1997). Expression of different syndecan isoforms is often spatially and temporally regulated through development suggesting that these transmembrane proteoglycans play key signaling roles. However, syndecans bind to a large variety of growth

factors including FGF, PDGF, EGF, HGF and VEGF (Carey, 1997) and thus surface expression of syndecan may indicate the activation of multiple cell signaling pathways. Syndecans may increase affinity for growth factor binding to their receptors (Carey, 1997; Oehrl and Panayotou, 2008) by sequestering ligands close to the cell surface and increasing the effective concentration of available ligand. Furthermore, there have been examples of syndecans displaying different affinities for specific ligands. In the developing mouse neurepithelium, syndecan-1 switches from binding bFGF to aFGF in a temporally regulated manner (Nurcombe et al., 1993).

Syndecan-2 has been shown to play an important role in the specification of left/right asymmetry in *Xenopus*. Dominant-negative and targeted morpholino knockdown of syndecan-2 causes heterotaxia (reversed heart/gut looping) (Kramer and Yost, 2002). Heterotaxia was dependent on gycoasminoglycan side-chain addition to syndecan-2 and could be rescued by expression of the growth factor Vg-1 (Kramer and Yost, 2002). Syndecan-2 coimmunoprecipitates with Vg-1 suggesting that binding of Vg-1 by syndecan-2 is an important step in left/right asymmetry specification.

The ability of ECM to bind growth factors may also regulate chemotactic events. In the *Xenopus* gastrula, mesodermal cells migrate along an assembled fibronectin matrix on the animal cap ectoderm toward the animal pole of the embryo. Disassociated mesoderm cells or cell aggregates migrate in a random fashion when plated on plasma fibronectin *in vitro* suggesting that some directional cue has been lost. However, on substrates conditioned with matrix from an intact animal ectoderm, mesoderm aggregates migrate directionally toward the animal pole as they would *in vivo* (Nagel et al., 2004). This directional migration was perturbed when a dominant negative PDGFRα receptor was expressed in the mesoderm or dominant negative PDGFA ligand was expressed in the animal ectoderm prior to substrate conditioning (Nagel et al., 2004). Additionally, a truncated form of PDGFA lacking the matrix binding domain was unable to support directional migration. Thus the ECM from the ectoderm likely binds PDGF to set up a chemotactic gradient to guide cell migration.

ECM may also sequester other proteins (that aren't growth factors themselves) but that modulate signaling pathways. Cyr61 was discovered as a matrix-associated protein during *Xenopus* gastrulation. It appears to "buffer" Wnt-signaling activity; enhancing the canonical Wnt pathway when the Wnt ligand is present in low abundance but inhibiting the pathway when Wnt is present in excess, through yet unknown mechanisms (Latinkic et al., 2003). All these examples indicate that ECM provides a rich signaling environment important for a range of developmental decisions.

ECM in differentiation

In addition to roles in morphogenesis through regulation of cell adhesion, motility and polarity, ECM also functions in the specification of cell fates. The spatio-temporal regulation of ECM expression and deposition suggests that ECM may provide permissive and even instructive differentiation signals. In the mouse limb bud, myogenic differentiation occurs as laminin, collagen IV and nidogen (entactin) expression increases whereas fibronectin expression decreases (Godfrey and Gradall, 1998). While this is correlative, it suggests that different ECM molecules may have opposing functions in the specification of particular cell fates. Entactin was shown to promote myogenic differentiation *in vitro* (Neu et al., 2006) though a function for fibronectin as a suppressor of myogenic fate has not been reported.

Such observations have prompted a plethora of studies investigating which ECM components induce differentiation of adult stem cells, embryonic stem cells and multipotent embryonic precursor cell populations *in vitro*. The physiological relevance of these studies is often difficult to surmise because complimentary ECM loss-of-function manipulations either produce multiple defects in addition to defects in differentiation or mild and subtle defects because of compensation by other ECM components. Nevertheless, such studies have provided us with insights into the relationship between the microenvironment and specification of cell fates.

For example, it was long believed that embryonic stem cells from the inner cell mass of the mouse blastocysts were no longer competent to make trophoblastic cells that are crucial in invading the maternal uterine wall and establishing the placenta. However, microarray analysis of embryonic stem (ES) cells plated on collagen IV uncovered expression of trophoblastic markers (Schenke-Layland et al., 2007). The authors confirmed that ES cells were able to differentiate into a throphoblastic lineage on a feeder layer coated with collagen IV but not laminin, fibronectin or collagen I. This suggests that the inner cell mass is indeed competent to differentiate into throphoblasts given the right environmental cues.

While it is attractive to imagine that specific ECM molecules dictate particular cells fates, not surprisingly, the reality is more complex. Laminin alone has been shown to promote specific fates in different tissues. Mouse and human neural stem cell precursors differentiate into neurons, astrocytes and specific glia on laminin but not fibronectin (Flanagan et al., 2006). At the same time, multipotent embryonic lung cells can be induced to differentiate into smooth muscle cells on laminin as well (Nguyen and Senior, 2006; Relan et al., 1999). However, not all laminin isoforms are competent to induce cell differentiation (Nguyen and Senior, 2006) suggesting that there may indeed be exquisite specificity in how ECM influences differentiation.

How might ECM influence cell fate decisions *in vivo*? One obvious answer has already been considered; that morphogens and cytokines are stored or displayed by ECM components and cells receive these signals as they come into contact with assembled ECM or when the associated factors are released by proteolysis. There are certainly examples of cross-talk between growth factor signaling and ECM that result in cell fate specification. The loss of tendon cell differentiation in *Drosophila* following PS integrin knockouts can be rescued by upregulation of EGF signaling suggesting an interesting cross-regulation between the ECM and EGF (Martin-Bermudo, 2000). Tenascin-C promotes glial cell differentiation via regulation of Wnt signaling (Ruiz et al., 2004). Syndecan-4 has also been shown to influence neural induction in *Xenopus* via FGF and ERK signaling pathways (Kuriyama and Mayor, 2009).

In recent years, a new and more direct connection between ECM and cell fate specification has come to light. Physical and mechanical cues from the microenvironment transmitted through the ECM–integrin interaction may directly influence nuclear events (reviewed in Wang et al., 2009). Thus, a new and exciting field of mechanotransduction that involves ECM and its role in cell fate specification has begun to emerge.

Contributions of physical properties of ECMs

In an elegant series of experiments, Engler et al. (2006) demonstrated that mesenchymal stem cell (MSC) fates can be determined by the stiffness of the ECM substrate. MSCs plated on collagen gels "tuned" to mimic the elasticity of brain, muscle or bone tissues gave rise to neurogenic, myogenic and osteogenic cell fates, respectively. Some computational models predict that signal transduction by mechanical force can operate significantly faster than that of soluble growth factor signaling pathways (Na et al., 2008). This brings up the interesting question of whether rapid response to force generation in embryos could serve to regulate some cell fate decisions. A number of molecular players have been implicated in mechanisms of mechanotransduction involving ECM. Integrins coupling to the cytoskeletal network can extend to the nuclear scaffold via the LINK complex, composed of nesprins, sun and lamin proteins (Wang et al., 2009). The nuclear lamin scaffold in turn, may influence nuclear organization, chromatin modification, transcriptional regulation and

mRNA processing. Specific nuclear scaffold proteins such as RUNX have also been implicated in regulation of differentiation (Stein et al., 2007). RUNX is a negative regulator of the segment polarity gene, *engrailed* in *Drosophila* and the thymocyte specification factor CD4 in mice (Durst and Hiebert, 2004). Also, nuclear membrane association of RUNX is a developmentally regulated process that plays a role in osteoblast specification (Lindenmuth et al., 1997).

Physical forces acting on the ECM itself can affect how cells receive cues from the environment. Stretching of fibronectin reveals cryptic integrin binding sites and also influences the affinity for integrin ligation and clustering (Baneyx et al., 2002; Vogel, 2006). Such changes may be expected to influence adhesivity of the cells that contact a stretched matrix, but also has been shown to influence differentiation. A fibronectin fragment containing a Leu 1048 to Pro mutation in the cell binding region stabilizes the conformation of this fragment to favor high affinity binding specifically to α5β1. This fragment was further shown to promote osteogenic differentiation of MSCs (Martino et al., 2009). The biological activity of this fragment of fibronectin could be reversed using a function blocking antibody against α5β1 but not αvβ3. How the conformation of ECM molecules may be affected *in vivo* by physical forces is an intriguing question. It may be dependent on the composition of ECMs and dynamic reciprocal interactions with the very cells that are receiving matrix-derived signals.

Additionally, ECM may indirectly affect differentiation by regulating cell shape. Micropatterned ECM substrates have been used to constrain the shapes of cells in culture (e.g., rounded vs. elongated or spread cells) and under such conditions cell shape was shown to influence cell fate (Guilak et al., 2009; McBeath et al., 2004). In fact, mesenchymal stem cells grown in laminin culture conditions that favor elongated cells downregulate the small GTPase RhoA and differentiate into smooth muscle cells (Beqaj et al., 2002). When cultured to enrich for rounded cells, cytoplasmic RhoA levels were increased and the transcription factor SRF was excluded from the nucleus, which in turn inhibited expression of smooth muscle markers. Differentiation was restored in these cells by plating on laminin-2.

Mammary gland differentiation

Perhaps the best-studied example of ECM effects on gene expression comes from studies of the mammary gland. Acinar differentiation and expression of milk proteins requires interaction of mammary epithelial cells with a conducive 3-D ECM microenvironment (Nelson and Bissell, 2006). Laminin-1 (but not fibronectin, collagen I or collagen IV) induces β-lactoglobulin and β-casein transcription though integrin-dependent phosphorylation of the prolactin receptor that is an upstream regulator of the transcription factor STAT5 (Streuli et al., 1995a,b). Specific DNA sequences in the promoter region of β-casein have been identified as ECM-response elements that on their own regulate transcription in a laminin-dependent manner (Schmidhauser et al., 1992). This intriguing finding has led to the hypothesis that cis-regulatory elements may exist for specific matrix molecules (Nelson and Bissell, 2006) though other examples have yet to be uncovered.

ECM dynamics and the role of cell and tissue forces

The physical state of the ECM is highly dynamic during development. Cells assemble and remodel ECM in ways that affect not only matrix composition but also its 3-D organization. Differences in ECM density, composition and architecture can influence profoundly cell behaviors. We have already discussed evidence for the involvement of specific ECM molecules in differentiation and motility and the importance of ECM structure in imparting tissue stiffness. Given these functional properties, the question of how and when matrix is assembled and remodeled in the embryo becomes an important consideration. For example, Rozario et al. (2009) experimentally separated simple fibronectin synthesis and deposition at cell surfaces from the assembly of more complex fibrillar structures during Xenopus gastrulation. In the absence of fibrillar fibronectin, epiboly and radial intercalation were disrupted but convergent extension and mediolateral intercalation progressed normally even though the latter process is also known to require fibronectin–integrin adhesion and signaling (Davidson et al., 2006; Marsden and DeSimone, 2003). While this study is one of the few to address the functional consequences of assembly state to specific developmental events *in vivo*, a number of cell culture experiments in recent years serve to highlight the importance of "matrix-topography" to cell physiology (Cukierman et al., 2001; Mao and Schwarzbauer, 2005) and migration (Doyle et al., 2009). More work needs to be done in this area but based on these studies there is ample reason to suggest that cellular mechanisms regulating matrix assembly may serve as normal checkpoints for the progression of morphogenetic and/or cell fate decisions. In addition, tissue boundaries are outlined by assembled ECM leading to a physical separation that is likely critical to maintain tissue identity and integrity, to facilitate morphogenesis, and to regulate signaling interactions between tissues.

ECM assembly and remodeling is regulated during embryogenesis

ECM assembly is complex and mechanisms of assembly vary depending upon the matrix molecules in question and the cells that produce, assemble and remodel them. The dynamic nature of the assembly process is particularly evident during embryogenesis; *de novo* accumulation of matrix and subsequent remodeling occurs throughout and accompanies the formation of emerging tissues including branched organs as discussed earlier. Matrix assembly is often *coincident* with the initiation of a morphogenetic movement. For example, the assembly of fibronectin fibrils along the blastocoel roof in amphibians precedes mesendoderm migration (Lee et al., 1984; Nakatsuji et al., 1985; Winklbauer, 1998), which in turn requires fibronectin adhesion (Boucaut et al., 1984; Davidson et al., 2002). In Xenopus, mesendoderm cells remodel the assembled matrix as they pass over it (Davidson et al., 2004) and it has been suggested that this is involved in regulating the velocity of mesendoderm migration (Rozario et al., 2009).

Because the physical assembly state of the ECM is clearly critical to cell response and function, it is important to consider the possibility that the assembly process, in itself, is subject to developmental control as discussed above. Recently, Dzamba et al. (2009) demonstrated that in the absence of Wnt/PCP signaling fibronectin matrix assembly is inhibited in the blastocoel roof of Xenopus embryos. In this case, cells "assemble" matrix at their edges but subsequent elaboration of fibrils across cell surfaces fails to occur. Interestingly, the mechanism by which integrin dependent fibrils are assembled requires cadherins, cortical actin assembly and the activation of myosin contractility in response to Rac and Pak kinase activity (Dzamba et al., 2009). These data also suggest that tissue generated stresses resulting from morphogenetic movements may contribute to the spatial assembly of matrices *in vivo*. Studies done with zebrafish embryos provide evidence of an additional mechanism for the regulation of fibronectin matrix assembly along tissue boundaries. Jülich et al. (2009) show that Eph/Ephrin signaling clusters α5β1 integrins along cell borders and de-represses integrin trans-inhibitory signals to permit the proper spatiotemporal deposition of fibronectin matrix during somitogenesis. These data suggest a mechanism for self-organization of the ECM at emergent tissue boundaries.

ECM on the move?

Morphogenesis involves short- and long-range movements of cells and tissues and most, if not all, of these movements involve

interactions with ECM. The prevalent view based largely on what has been inferred from cell culture studies, is that cells "respond" to ECM, for example, by moving towards or away from ECM molecules that are assembled in the extracellular compartment. Concentration gradients of deposited ECM, the physical properties of the matrix (e.g., stiffness, elasticity, viscosity) and the molecular composition of the matrix influence a given cell's response to the ECM and regulate specific behaviors.

In cell culture, ECM is typically immobilized on glass or plastic surfaces and under these conditions, when cells engage the ECM with their integrins and exert actomyosin-dependent contractile forces they translocate. One provocative alternative interpretation, however, is that the migration of cells *in vitro* may not always reflect true "migration" *per se* but instead, a co-opting of the integrin receptors and contractile machinery that are normally involved in matrix remodeling. If the matrix cannot be remodeled because it is stuck to an artificial substrate as it is produced then the consequence will be increased cell motility and restricted ECM displacement. It is perhaps ironic that the near universal cell-biology textbook example of a migratory cell is the mammalian fibroblast, the principal *in vivo* function of which is to synthesize, assemble and turnover ECM in connective tissues.

So, what is the state of the ECM in embryonic tissues that undergo dramatic and well-established movements and cellular rearrangements *in vivo*? Do cells always move with respect to a matrix that is largely "fixed" in place? Until recently, such questions have been difficult to address for technical reasons; both the ECM molecule(s) and the migratory cells in question have to be imaged in live embryos and tissues over time. Zamir and colleagues (Czirok et al., 2006; Zamir et al., 2005, 2008) successfully developed both the imaging and computational methods needed to accomplish this using the optically favorable avian embryo. Using fluorescently-tagged fibronectin or fibrillin as fiduciary markers they imaged by time-lapse both the assembled ECM and epiblastic cell movements during primitive streak formation. Astonishingly, a remarkable degree of correspondence between both epiblastic cell and sub-epiblastic ECM displacements was observed as morphogenesis proceeded in these embryos. A similar correlation between fibronectin fibrils and cell movements has also been reported for *Xenopus* gastrula-stage mesoderm and ectoderm explants (Davidson et al., 2008). The most parsimonious explanation for these observations is that migratory cells and tissues "carry their ECM with them" under some circumstances. Whether this is more likely to occur during early morphogenesis as ECM is first being assembled (i.e., in many cases by the same cells undergoing cell/tissue movements) remains to be established. It is clear, however, that models of cell and tissue motility based primarily on cell adhesion to ECM must take into account the realities of a "substrate" that is not necessarily fixed in place. It will be of great interest to determine the degree to which ECM is displaced in later stage migratory events such as neural crest and germ cell migration.

If the ECM is important for cell and tissue movements as so many studies have demonstrated, then a "mobile ECM" may be seen as offering a challenge to our thinking about the likely cellular mechanisms involved. Similarly, if ECM-dependent motile behaviors do not always involve the generation of traction forces and the active "pulling or pushing" against fixed matrix elements, how do cells move? Some clues may come from recent studies of the involvement of fibronectin in *Xenopus* convergent extension. When fibronectin or integrin $\alpha5\beta1$ function is perturbed in *Xenopus* embryos, axis elongation is reduced and the normally bipolar protrusive activity of mesoderm undergoing mediolateral intercalation movements is disrupted (Davidson et al., 2006; Marsden and DeSimone, 2003). In the absence of fibronectin the mesoderm becomes multipolar protrusive and these cells will converge toward the midline but instead of driving extension in the anterioposterior direction, the affected cells overlap and underlap one another and, thus, the tissue thickens. The net result is "convergent thickening" instead of convergent extension (Davidson et al., 2006). These observations suggest that integrin occupancy by its ligand fibronectin may help regulate protrusive activity. While non-canonical wnt planar cell polarity signaling is essential for bipolar protrusive activity (Wallingford and Harland, 2001; Wallingford et al., 2000), lack of integrin occupancy in these cells leads to increased frequency and randomization of cellular protrusions (Davidson et al., 2006). Interestingly, increased fibronectin assembly and/or binding at the mesoderm cell surface are associated with a decrease in protrusive activity (Davidson et al., 2008, 2006). One intriguing possibility is that the role of the ECM in this system is to promote or attenuate integrin signals important for protrusive activity and possibly the regulation of cadherin adhesion and traction along cell surfaces to facilitate cell intercalation behaviors. Thus, integrin–cadherin "crosstalk" may play a significant role in the regulation of both the cell–cell and cell–ECM adhesive activities involved (Dzamba et al., 2009).

Summary

It is clear that the ECM impacts a number of cellular functions that are critical for normal development and morphogenesis. Advances in the cell biology of ECM and ECM receptors have provided new and important ways of thinking about the roles of matrix in development beyond simple adhesion and space filling properties. Cell signaling through ECM can impact cell fate decisions, cell proliferation and survival, and other specialized functions. An emergent area of interest and importance includes the mechanical contributions of ECM architecture to these processes. Future studies will be needed to establish how tissue generated forces are influenced by ECM and, in turn, how these forces might regulate ECM assembly and function.

Acknowledgments

We apologize to the many colleagues whose deserving work we were unable to include in this review due to constraints on length and scope. We are grateful to our colleagues Bette Dzamba, Greg Weber and Maureen Bjerke for reading the manuscript and providing helpful comments. Special thanks goes to Dr. Charlie Little for many inspired conversations about ECM over the years and for suggesting the concept of ECM as "morphogenetic language." One of us (TR) wishes to point out that the "Embryology Course" at the MBL in Woods Hole *also* has a long and venerable history of inspiring many scientists including those who have worked on ECM in embryos. The authors acknowledge gratefully research support from the National Institutes of Health (HD26402).

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