

Extracellular matrix dynamics in development and regenerative medicine

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Summary

The extracellular matrix (ECM) regulates cell behavior by influencing cell proliferation, survival, shape, migration and differentiation. Far from being a static structure, the ECM is constantly undergoing remodeling – i.e. assembly and degradation – particularly during the normal processes of development, differentiation and wound repair. When misregulated, this can contribute to disease. ECM assembly is regulated by the 3D environment and the cellular tension that is transmitted through integrins. Degradation is controlled by complex proteolytic cascades, and misregulation of these results in ECM damage that is a common component of many

diseases. Tissue engineering strives to replace damaged tissues with stem cells seeded on synthetic structures designed to mimic the ECM and thus restore the normal control of cell function. Stem cell self-renewal and differentiation is influenced by the 3D environment within the stem cell niche. For tissue-engineering strategies to be successful, the intimate dynamic relationship between cells and the ECM must be understood to ensure appropriate cell behavior.

Key words: Biomimetic scaffold, Branching morphogenesis, Extracellular matrix, Fibronectin, Stem cell niche, Stem cells

Introduction

The extracellular matrix (ECM), once thought to function only as a scaffold to maintain tissue and organ structure, regulates many aspects of cell behavior, including cell proliferation and growth, survival, change in cell shape, migration, and differentiation. It is a complex assembly of many proteins and polysaccharides forming an elaborate meshwork within tissues. The primary components are fibrous structural proteins (e.g. collagens, laminins, fibronectin, vitronectin and elastin), specialized proteins [e.g. growth factors, small matricellular proteins and small integrin-binding glycoproteins (SIBLINGS)] and proteoglycans, but the precise composition varies from tissue to tissue.

Within a single tissue, the ECM is constantly being remodeled as cells build and reshape the ECM by degrading and reassembling it; remodeling rates are particularly high during development and wound repair, in response to infectious agents and in many disease states. The ECM can be remodeled in response to signals transmitted by ECM receptors – such as integrins, other laminin receptors and syndecans – or ECM-modifying proteins, such as matrix metalloproteinases (MMPs). ECM remodeling can induce and also be affected by extracellular and cellular tension.

Although ECM assembly is poorly understood, a hierarchy is evident in the assembly process. Integrins are heterodimeric transmembrane receptors that link the ECM to the internal cytoskeleton by binding directly to ECM proteins, such as fibronectin (FN) and laminins, and indirectly to actin via cytoplasmic binding proteins. Integrin-mediated assembly of the ECM glycoprotein FN into fibrils tethered to the cell surface is required for deposition of other ECM proteins, including thrombospondin 1 and type I collagen (Sottile and Hocking, 2002). Assembly of the basement membrane (basal lamina), a specialized form of ECM that underlies epithelial and other cells, is initiated by laminins, which self-assemble into heterotrimers that bind to the cell surface via their cell surface receptors, which

include integrins and other proteins (Li, S. et al., 2003). Collagens are a family of triple-helical proteins that provide tensile strength within the ECM and basement membranes (Kadler et al., 2007); some of these also bind to integrin receptors. The network-forming type IV collagen is a primary component of basement membranes that is internally stabilized by Met-Lys crosslinks (Than et al., 2002), but how this network interacts with others has only recently become clear. Kurban et al. have shown that the von Hippel-Lindau tumor-suppressor protein is required for assembly of the hydroxylated alpha chain of collagen IV (COL4A2) into the ECM (Kurban et al., 2007). Nidogen, which interacts with specific domains of laminin, also links collagen IV with the basement membrane (McKee et al., 2007). It and other extracellular linker proteins are emerging as important players in basement membrane assembly.

The proteolytic cleavage of ECM components represents another mechanism by which the ECM is remodeled. Proteases, including those in the MMP, serine protease (e.g. plasmin, plasminogen activator and uPAR) and cysteine protease (e.g. cathepsins) families, influence matrix dynamics at multiple levels. For example, they convert structural molecules to signaling molecules by releasing small bioactive peptides, release growth factors stored within the ECM and degrade matrix proteins directly. Protease activity must be under exquisite control during normal development and in adult tissues to maintain tissue homeostasis. Not surprisingly, misregulated protease activity is a common characteristic of metastatic tumors and many other disease states.

In recent years, the field of regenerative medicine has developed out of the need to repair or replace damaged tissues. One tissue-engineering strategy is to replace a damaged or deficient organ with an artificial organ composed of cells seeded on a scaffold that functions as an ECM. Recent research has incorporated knowledge of the dynamic nature of the ECM to improve scaffold design. Here we examine current knowledge of ECM remodeling, focusing on

recent findings, and consider how this has impacted such engineering approaches.

ECM synthesis and signaling

Over the past several years, numerous *in vitro* studies have investigated the molecular mechanisms driving ECM remodeling. Many have demonstrated that the properties of cells and their ECM in a two-dimensional (2D) culture differ substantially from those of cells grown in a 3D environment (Cukierman et al., 2001; Roskelley et al., 1994; Streuli et al., 1991). Such studies inspired the recent transition from 2D to 3D cell-culture techniques aiming to recapitulate *in vivo* biology and have significantly impacted our understanding of ECM architecture and its role in tissue remodeling (Pampaloni et al., 2007; Yamada and Cukierman, 2007).

FN assembly into a fibrillar matrix (fibrillogenesis) is crucial for embryonic development (George et al., 1993) and wound healing. FN fibrillogenesis by the heterodimeric integrin $\alpha 5 \beta 1$ (Danen et al., 1995; Nagai et al., 1991; Pankov and Yamada, 2002; Ruoslahti and Pierschbacher, 1987) depends primarily on its binding with specific sites within the secreted FN dimer [the well-known arginine-glycine-aspartate (RGD) sequence and the neighboring modulatory 'synergy site'], which changes the conformation of integrin $\alpha 5 \beta 1$ to activate it in a phosphorylation-independent manner and render it competent for FN assembly. Recent investigations by Takahashi et al. into the necessity of the RGD sequence *in vivo*, however, indicate that, although RGE-knock-in mice, in which the RGD motif is replaced with inactive RGE, have a lethal phenotype, FN fibrillogenesis occurs normally. Further investigation identified a novel integrin-binding site (isoDGR) on FN that is required for integrin- $\alpha v \beta 3$ -mediated fibrillogenesis in an RGD-independent manner (Takahashi et al., 2007).

The binding of FN dimers induces integrin clustering at the cell membrane, followed by the self-association of multiple FN molecules to form a fibrillar ECM (Mao and Schwarzbauer, 2005a; Schwarzbauer and Sechler, 1999). This requires translocation of integrins out of stationary cell-matrix adhesions known as focal contacts into the more dynamic fibrillar adhesions (Pankov et al., 2000). Mao and Schwarzbauer recently reported that HT1080 human fibrosarcoma cells, which require stimulation with integrin activators such as Mn^{2+} to assemble a FN matrix in 2D culture (Sechler et al., 1997), need a 3D architecture alone to stimulate FN assembly (Mao and Schwarzbauer, 2005b). These results indicate that the architecture of the microenvironment influences matrix assembly. In another study, using the same cell line, Winters et al. observed that the activation-independent FN-matrix deposition in 3D culture correlates with decreased levels of Raf1, a MAP kinase kinase in the Erk signaling cascade (Fig. 1), and that inhibition of Raf1, either pharmacologically or with *Raf1*-targeted siRNA, inhibits FN assembly. Raf1 abundance is probably regulated at the level of mRNA stability (Winters et al., 2006). These results indicate that the 3D environment can ultimately affect cellular events at the level of signal transduction, which can in turn influence ECM remodeling.

Activation of integrins by conformational changes is an important regulator of ECM assembly and maintenance. Ligand binding and bivalent cations, such as Mn^{2+} (Sechler et al., 1997), alter the conformation, and hence the activity (i.e. their ability to interact with the ECM), of integrins. Cytoplasmic binding proteins also influence integrin conformation and matrix assembly. Binding of the cytoplasmic actin-binding protein talin to the integrin $\beta 1$ tail is crucial for conformational changes that activate the extracellular domain (Tadokoro et al., 2003). A specific integrin conformation

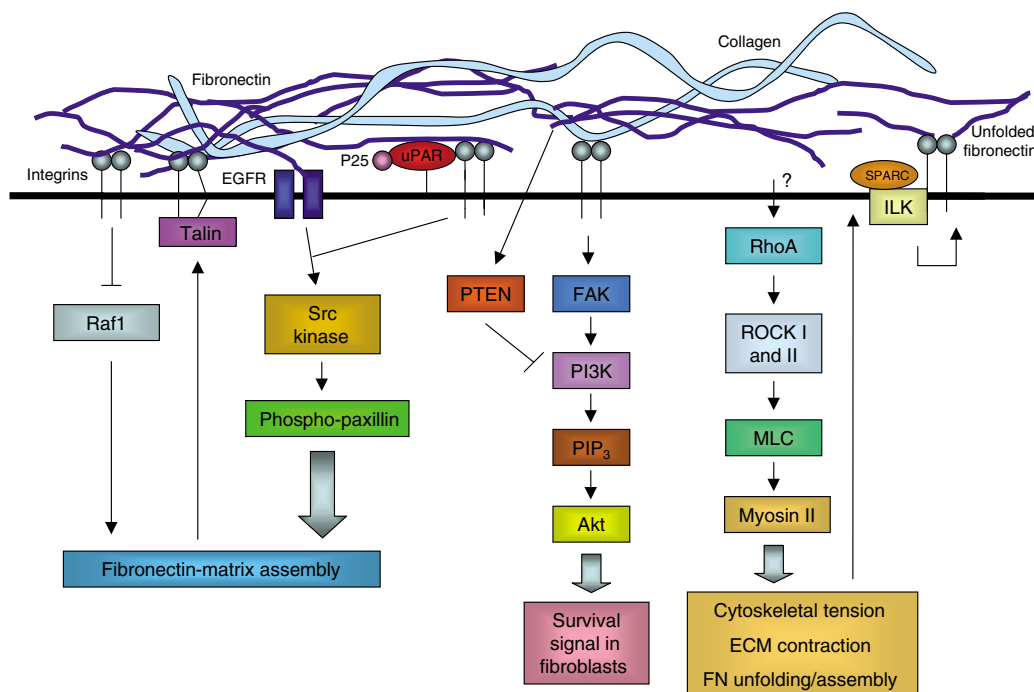


Fig. 1. ECM-mediated effects on matrix remodeling. The 3D matrix environment stimulates integrin-mediated FN assembly through inhibiting a Raf1-mediated signaling pathway. Activation of integrin $\alpha 5 \beta 1$ by uPAR and its P25 ligand also stimulates FN assembly in combination with EGFR via a Src-kinase-mediated pathway. Activation of PTEN by 3D collagen contraction converts a FAK/PI3K-mediated survival signal into a pro-apoptotic signal. Activation of RhoA, presumably ECM-mediated, leads to assembly of a myosin II ring that facilitates FN unfolding during FN fibrillogenesis. SPARC stimulates FN unfolding and assembly using an ILK-dependent pathway.

has been identified that localizes $\alpha 5\beta 1$ integrins into fibrillar adhesions and facilitates FN fibrillogenesis (Clark et al., 2005). Lateral interactions between integrins and other transmembrane proteins have also been identified as regulators of integrin activation. Moreover, Monaghan and colleagues have identified a novel link between integrin $\alpha 5\beta 1$ activity and the urokinase-type plasminogen activator receptor (uPAR, also known as PLAUR) (Fig. 1). The uPAR ligand P25 peptide, through its association with uPAR, stimulates $\alpha 5\beta 1$ activity and FN fibrillogenesis in human skin fibroblasts in a Src-kinase- and epidermal growth factor receptor (EGFR)-dependent fashion (Monaghan-Benson and McKeown-Longo, 2006; Monaghan et al., 2004). In a separate study, Src, together with its substrate paxillin, also promoted prolonged integrin activation and thereby enhanced FN-matrix assembly and maintenance (Wierzbicka-Patynowski et al., 2007). These studies highlight the importance of regulation of integrin activity in ECM assembly.

ECM remodeling and tension

Tension produced by cytoskeletal elements provides an additional mechanism that can regulate matrix dynamics and is required for FN assembly. Because soluble FN is globular when it is secreted (Erickson and Carrell, 1983; Johnson et al., 1999), it must be unfolded into a polymerization-competent state prior to fibril formation. This is mediated in part through binding to integrin $\alpha 5\beta 1$, which is linked via its cytoplasmic domains to many proteins that interface with the cytoskeletal machinery, including focal adhesion kinase (FAK), vinculin and paxillin (DeMali et al., 2003). Baneyx et al. have followed the unfolding of FN dimers during fibril assembly by intramolecular fluorescence resonance energy transfer (FRET) (Baneyx et al., 2002), monitoring loss of FRET as FN dimers incorporate into fibrils. Because cytochalasin D prevents the loss of FRET, the actin cytoskeleton must be crucial for the conformational change. Another recently identified mechanism contributing to FN assembly involves a small matricellular protein: the surface-bound glycoprotein SPARC (also known as osteonectin) (Fig. 1). SPARC, acting via an interaction with integrin-linked kinase (ILK), stimulates FN-induced stress-fiber formation and partial unfolding of FN dimers on the cell surface by enhancing ILK-mediated cytoskeletal contraction via phosphorylation of myosin light-chain phosphatase (Barker et al., 2005). Although the authors suggest that the SPARC-ILK binding is direct and that this complex is exposed on the cell surface, further investigation will be required to define the cellular location of this signaling complex. Nevertheless, together these studies indicate that tension applied through signaling complexes provides the necessary forces for FN assembly at the cell surface.

An important regulator of tension generated through integrin-mediated signaling is the small GTPase family member RhoA (DeMali et al., 2003). RhoA is a well-characterized regulator of stress-fiber formation but also facilitates FN-matrix assembly (Zhong et al., 1998). siRNA-mediated knockdown of downstream effectors of RhoA – Rho kinase (ROCK) I and II – in rat embryonic fibroblasts abolishes FN-matrix assembly, but only ROCK I depletion decreases formation of microfilament bundles (Yoneda et al., 2005) (Fig. 1). Conversely, depletion of ROCK II, but not ROCK I, prevents the phagocytosis of FN-coated beads. A target of ROCK is myosin light chain, and FN-matrix assembly can be rescued in both ROCK I and ROCK II knockdowns by the introduction of phosphomimetic myosin light chain (Yoneda et al., 2007). Total internal reflection fluorescence (TIRF) microscopy

reveals a submembranous pool of myosin II, separate from the myosin II that associates with stress fibers, that is absent in ROCK-II-deficient rat embryonic fibroblasts and appears to contribute to a cortical ring of contractile myosin II (Yoneda et al., 2007) that generates cytoskeletal tension and thus influences ECM remodeling. These results suggest that both ROCK-I-mediated stress-fiber assembly and ROCK-II-mediated FN internalization, each affecting a distinct pool of myosin II, might both play a crucial role in FN-matrix remodeling.

Cellular contractile forces produced by cells that have migrated into a wounded area and synthesize new ECM are an important aspect of the late stages in wound healing (Midwood et al., 2004). Fibroblasts that transition into myofibroblasts in the wound transcriptionally upregulate the smooth muscle isoform of α -actin; this involves transforming growth factor beta 1 (TGF β 1) signaling (Desmouliere et al., 1993) and requires FN (Clark et al., 1997). In such cells, FN also mediates Rho-stimulated cellular contraction. In a wound-healing model in which fibroblasts are seeded on FN-coated polyacrylamide gels, Meckmongkol et al. identified both the FN synergy site and TGF β 1-mediated Rho activation as crucial contributors in FN-mediated cell contraction (Meckmongkol et al., 2007). Bernstein et al. recently identified another player in the myofibroblast transition; they implicated uPAR cleavage as a crucial step in the TGF β 1-mediated transition of fibroblasts into myofibroblasts (Bernstein et al., 2007). Not surprisingly, topical application of FN stimulates wound closure in rats (Kwon et al., 2007). In this model, FN stimulates fibroblast, myofibroblast and macrophage migration, and also stimulates type I collagen deposition to facilitate wound closure, among other effects.

When fibroblasts are cultured in 3D type I collagen matrices, binding of integrin $\alpha 2\beta 1$ to type I collagen initiates a survival signal that involves key transducers of integrin signals: FAK, phosphoinositide 3-kinase (PI3K) and Akt/protein kinase B (PKB) (Tian et al., 2002; Xia et al., 2004). When the 3D collagen matrix contracts, integrins function as mechanotransducers, and Akt is dephosphorylated and thereby inactivated. This converts the survival signal into a pro-apoptotic signal (Tian et al., 2002; Xia et al., 2004). Interestingly, PTEN phosphatase, activated by collagen-matrix contraction, was recently identified as the mediator of this process through its ability to antagonize PI3K (Nho et al., 2006) (Fig. 1). In this way, ECM tension transmitted through integrins can regulate cell fate to remove unneeded cells in the final stages of wound repair.

ECM degradation

The proteolytic cleavage of ECM components represents another mechanism by which the ECM is remodeled and, when misregulated, results in a number of disease states (Koblinski et al., 2000; Mohamed and Sloane, 2006). Several families of proteases operate in the ECM, including MMPs, cysteine proteases and serine proteases. Because the role of MMPs in both development and disease has recently been reviewed (Page-McCaw et al., 2007), we focus primarily on cathepsins and serine proteases such as urokinase plasminogen activator (uPA).

Cathepsins are cysteine proteases that localize predominantly to endolysosomal vesicles but also exhibit extracellular activity. They have been the recent focus of several studies, owing to their emerging role in cancer (Mohamed and Sloane, 2006). Cathepsin B degrades purified type IV collagen, laminin and FN in vitro (Buck et al., 1992), and is upregulated in many tumor cell lines and cancers (Mohamed and Sloane, 2006). The signaling pathways

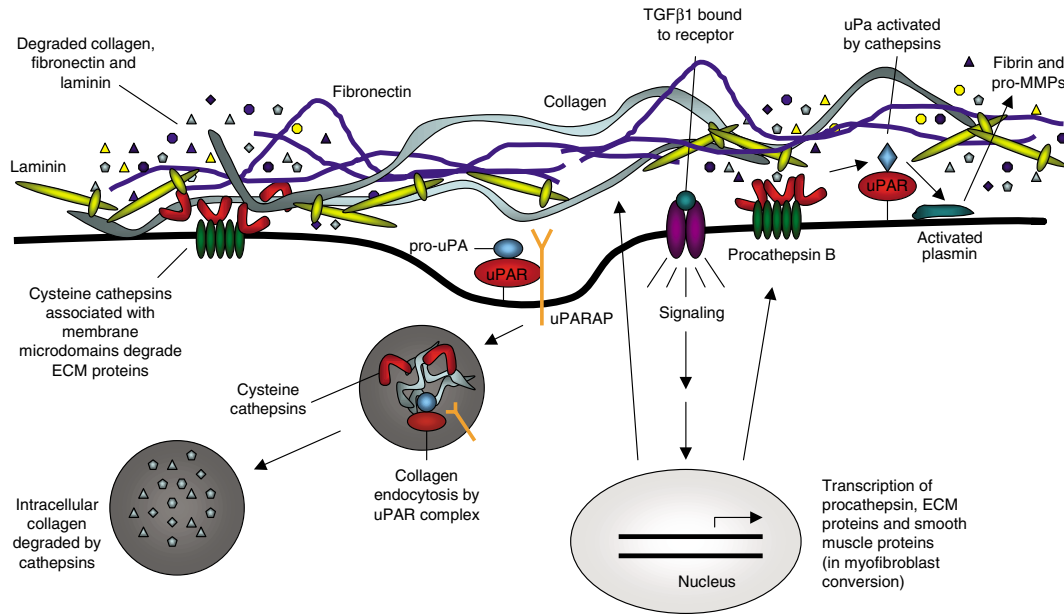


Fig. 2. ECM remodeling by proteolytic degradation. Cathepsins associated with membrane microdomains can degrade ECM proteins such as collagen, FN and laminin. Pro-uPA and uPAR, in complex with uPARAP, mediate the endocytosis of extracellular collagen. Such collagen is then degraded by cathepsins in intracellular vesicles. TGFβ1 signaling triggers transcriptional changes, including the upregulation of procathepsin B, which then participates in further ECM remodeling. Cathepsins also activate pro-uPA, setting off a uPA-mediated cascade of proteolytic cleavage that results in the activation of plasmin, fibrin and pro-MMPs.

regulating cathepsin expression are beginning to be elucidated. Reisenauer and colleagues recently reported an upregulation of cathepsin B expression in three myeloid tumor cell lines (THP-1, MonoMac-1 and MonoMac-6) following TGFβ1 treatment, and such upregulation was associated with increased carcinogenic potential (Reisenauer et al., 2007). This study identifies a link between aberrant TGFβ1 signaling and the proteolytic degradation of matrix components, which occurs during tumor metastasis.

ECM proteolysis is regulated by a complex interplay of proteolytic cascades. This is illustrated by the interaction between cathepsins and the uPA-uPAR system. The serine protease uPA, when bound to its cell surface receptor uPAR, is converted from an inactive zymogen to an active protease, often by cysteine proteases (Fig. 2). Active uPA activates plasmin, which, in turn, cleaves ECM proteins and activates pro-MMPs (Mondino and Blasi, 2004). Recently, Bugge and colleagues demonstrated that pro-uPA and uPAR, in complex with uPAR-associated protein (uPARAP, also known as Endo180 and MRC2), mediate the endocytosis of extracellular collagen. This collagen is then degraded by cysteine proteases in the lysosome (Curino et al., 2005; Engelholm et al., 2003; Kjoller et al., 2004) (Fig. 2). Because intracellular collagen fragments have been detected in many human tumors, this pathway might represent a general mechanism by which cancer cells overcome restrictive barriers, such as the basement membrane. Misregulation of uPA-uPAR signaling has also been implicated in many other pathological states, including antigen-induced arthritis (Busso et al., 1998) and cardiac fibrosis (Heymans et al., 2006; Moriwaki et al., 2004), both of which involve an abnormal accumulation of ECM proteins. In the arthritis model, the absence of uPA prevents fibrin clearance from arthritic joints. Given that fibrin is normally degraded by plasmin, which is activated by uPA, this is not surprising (Busso et al., 1998). Paradoxically, however, it is the overexpression, and not the loss, of uPA that contributes to cardiac fibrosis (Heymans et al., 2006;

Moriwaki et al., 2004). The mechanisms by which protease interactions with the ECM contribute to disease thus remain poorly understood.

ECM remodeling in branching morphogenesis

Branching morphogenesis is a developmental mechanism evident in many organs, including the lung, kidney, prostate, mammary gland and salivary gland, that depends upon basement membrane remodeling. It displays many of the ECM-remodeling mechanisms discussed above. Branching can be studied *ex vivo* in 3D by culturing the embryonic organs. In mouse submandibular salivary gland, kidney and lung, branching requires FN remodeling (Sakai et al., 2003). Fluorescent pulse-chase experiments and live confocal time-lapse microscopy (Larsen et al., 2006) have revealed a FN wedge at the branch initiation site, which is assembled with older FN accumulating at the base of clefts and newer FN assembling behind it. Progressive FN assembly in the cleft could provide a force or organizing structure to drive branching morphogenesis (Fig. 3).

ECM assembly and remodeling is modulated by growth factor signaling in many processes and is particularly evident during branching morphogenesis. FGF7 signaling via an ERK1/2-dependent pathway promotes epithelial budding in submandibular gland organ cultures (Steinberg et al., 2005). By contrast, studies of *ex vivo* adult mouse mammary gland organoids treated with FGF7 show that activation of ERK-1/2 correlates with sustained cell growth without branching and transient suppression of FN expression (Fata et al., 2007). Interestingly, a previous study showed that FGF7 can induce branching in embryonic day 18.5 (E18.5) mammary organoids (Sternlicht et al., 2005). FGF7 thus seems to have different effects in different ages of tissue, and its significance in matrix remodeling during *in vivo* development of either the mammary gland or the salivary gland has not yet been demonstrated. FGF10, which also uses the IIIb isoform of FGF receptor 2

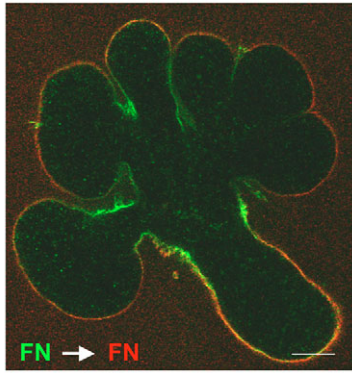


Fig. 3. Live imaging of FN remodeling during salivary gland branching morphogenesis. A live embryonic mouse salivary gland epithelial rudiment was incubated with media containing labeled soluble FN (green), washed and subsequently labeled via incubation with media containing red-labeled FN. Assembled (green) FN accumulates in clefts, whereas newer (red) FN is incorporated distal to the cleft. Scale bar: 100 μm . Adapted from Larsen et al. (Larsen et al., 2006).

(FGFR2IIIb), is more likely to be the relevant growth factor in both cases because mice lacking FGF10 or FGFR2IIIb have defects in mammary and salivary gland development (De Moerloose et al., 2000; Maillieux et al., 2002). Recent work shows that FGF10 stored within the ECM is released by heparanase, an endoglycosylase located in the salivary gland basement membrane, to activate MAPK signaling and branching morphogenesis (Patel et al., 2007).

Multiple ECM proteins are required for the complex process of branching morphogenesis. Laminin $\alpha 5$, for example, was recently shown to function early during branching, acting via integrin- $\beta 1$ -mediated MAPK signaling. However, this appears to sustain cell proliferation in developing buds rather than stimulate branching itself (Rebustini et al., 2007). Important remaining issues thus include how growth factor and ECM signaling pathways coordinate during complex developmental pathways, and how ECM remodeling and cell proliferation are co-regulated.

Branching morphogenesis is also regulated in part by tension between the cell cytoskeleton and the ECM. In the ‘tensegrity’ model, forces generated in developing cells by the cytoskeleton are transmitted across transmembrane integrin mechanoreceptors and opposed by varying levels of stiffness within the ECM (Ingber, 2003a; Ingber, 2003b; Ingber, 2003c). This, in turn, can affect how developing cells proliferate and migrate, as demonstrated by the tendency for branching lung rudiments to project newly branching buds in regions that have a relatively thin basement membrane (Moore et al., 2005). Moore et al. showed that treatment of lung rudiments with a ROCK inhibitor prevents terminal bud formation, which correlates with inhibition of myosin light-chain phosphorylation. Under these conditions, basement membrane thickness remains constant, as opposed to varying (as in untreated rudiments), which indicates that basement membrane contraction has not occurred (Moore et al., 2005). Another recent study linked ROCK activity to ureteric bud development in the kidney (Meyer et al., 2006), but it remains to be seen whether cytoskeletal tension or ECM plasticity is involved. ROCK-mediated control of cytoskeletal tension could therefore be a general mechanism regulating branching morphogenesis.

Remodeling of the ECM by proteolytic degradation is also an essential component of branching morphogenesis, and a number of

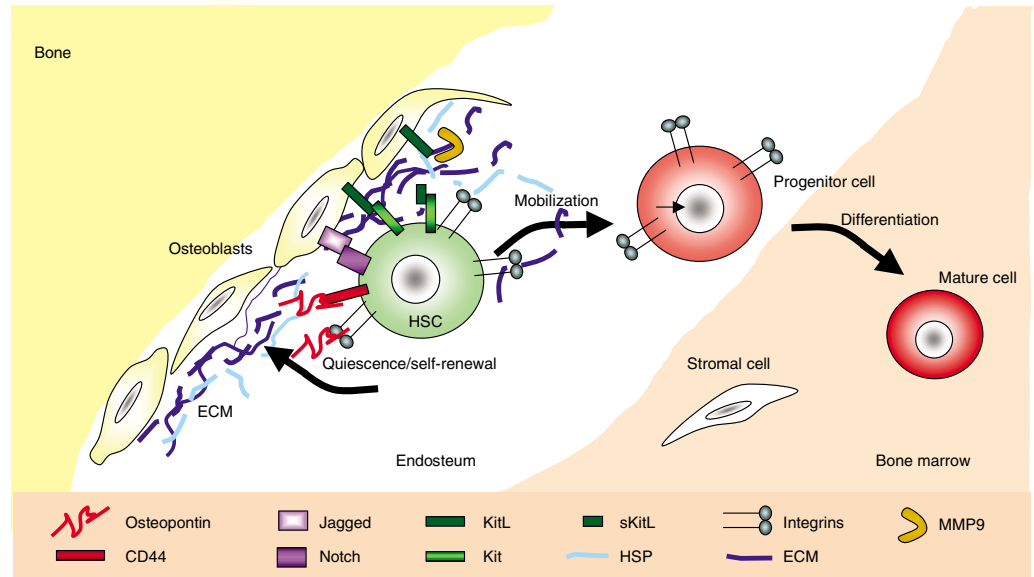
recent studies have demonstrated the involvement of MMP family proteins in many branching organs (Greenlee et al., 2007; Patel et al., 2006). Kheradmand and colleagues recently uncovered a link between the expression of the membrane-inserted MT1-MMP (MMP14) enzyme and EGFR signaling. *MT1-MMP* mRNA is upregulated in response to EGF in wild-type but not *Egfr*^{-/-} mice. One of the major functions of MT1-MMP is the cleavage and activation of pro-MMP2, and *Egfr*^{-/-} mice also exhibit a lower level of active MMP2 as well as both aberrant lung branching and alveolization (Kheradmand et al., 2002). Meyer and colleagues found enhanced MT1-MMP expression at the tips of uterine buds, suggesting that there is local matrix degradation in this region during branching (Meyer et al., 2004). Overexpression of MT1-MMP in the mammary glands of transgenic mice results in development that is aberrant on many levels, including excessive matrix remodeling in the form of fibrosis (Ha et al., 2001). MMP activity during branching is likely to be controlled by tissue inhibitors of metalloproteinases (TIMPs). In fact, in *Timp3*-null lungs, increased MMP activity and FN degradation are detected, correlating with decreased bronchiole branching. Inhibition of MMP activity in these lungs restores FN localization, FAK-mediated signaling and bronchiole branching (Gill et al., 2006).

ECM interactions in the stem cell niche

The stem cell niche is a specialized microenvironment (Schofield, 1978) that helps sustain the stem cell pool within each tissue/organ system (Scadden, 2006). Supporting niche cells, the ECM and growth-modulating factors stored within it, and the physical characteristics of the surrounding 3D structures, all contribute to the niche (Haylock and Nilsson, 2005; Moore and Lemischka, 2006). It is now generally assumed that stem cell niches exist in all adult tissues; however, the exact locations have only been defined in a few tissues (Li and Xie, 2005). The contribution of the ECM to the stem cell niche (Fig. 4) was first recognized following the findings that stromal heparan sulfate proteoglycans (HSPs) participate in the hematopoietic stem cell (HSC) niche (Gupta et al., 1998), and that integrin $\beta 1$ is a stem cell marker that helps restrict stem cells to the epidermal stem cell niche, partially via activation of MAPK signaling (Jensen et al., 1999; Zhu et al., 1999). Integrins are crucial for transmitting many extracellular signals to stem cells; for example, in neuronal stem cells, integrin $\beta 1$ regulates cell proliferation and survival, and migration out of the niche (Leone et al., 2005). The ability of HSCs to travel through the bloodstream to lodge in the bone marrow and in other tissue niches – a multistep, highly regulated process called homing – requires extensive interaction with multiple microenvironments along the way. This involves integrins, selectins and other transmembrane proteins, but has been reviewed recently (Lapidot et al., 2005) and so is not further discussed here.

The HSC niche is perhaps the best characterized stem cell niche (Li and Li, 2006), housing the multipotent HSCs that can self-renew and produce committed progenitor cells that differentiate into blood and other cell types. Recent studies identified the lining of the bone surface, termed the endosteum, as the HSC niche (Calvi et al., 2003; Zhang et al., 2003) and showed that adhesion to osteoblasts (the HSC niche cells) maintains HSC quiescence (Arai et al., 2004). Osteopontin, a phosphorylated glycoprotein produced by osteoblasts that is secreted and incorporated within the ECM, interacts with integrin $\alpha 4$ and $\alpha 5\beta 1$ and the cell surface glycoprotein CD44 on HSCs, functioning as a receptor for hyaluronic acid and other proteins (Vermeulen et al., 1998) within

Fig. 4. ECM-mediated effects on stem cells within the bone marrow stem cell niche. The ECM regulates stem cell attachment and quiescence via integrins and HSPs. Osteopontin participates in regulating quiescence by interacting with integrins and CD44. Notch-jagged and KitL-Kit signaling also maintain quiescence. MMP cleaves the transmembrane form of KitL to create sKitL, which interacts with Kit as a dimer to activate HSCs. Integrins participate in the mobilization of progenitor cells.



the stem cell niche to suppress stem cell expansion (Nilsson et al., 2005; Stier et al., 2005). The transmembrane isoform of the ligand for the Kit tyrosine kinase receptor expressed by HSCs (Andre, 1989), Kit ligand (KitL), also known as stem cell factor (SCF) and steel factor (Huang et al., 1990; Zsebo et al., 1990), is expressed by osteoblasts. It is reported to localize HSCs within the endosteal bone marrow region (Driessen et al., 2003), probably by mediating ECM attachment either directly or indirectly, as suggested by early studies (Kinashi and Springer, 1994).

Although ECM remodeling is involved in the release of stem cells from their niche, as well as their subsequent migration and differentiation in many tissues, the signals regulating these events are only beginning to be understood. Mobilization of quiescent stem cells from the hematopoietic stem cell niche is induced by MMP9, which releases membrane-bound KitL from osteoblasts (Heissig et al., 2002). This soluble Kit ligand (sKitL) can then interact with the Kit tyrosine kinase receptor on HSCs, thereby initiating a signaling cascade resulting in their mobilization. For migration of neural stem cells, laminin has been identified *in vitro* as being an efficient surface along which neural stem cells migrate out of 3D neurospheres, using an $\alpha 6$ -containing integrin (Flanagan et al., 2006).

The ECM also participates in stem cell differentiation. Laminin-332 is sufficient to stimulate osteogenic differentiation of human mesenchymal stem cells (MSCs), acting via an ERK signaling pathway (Klees et al., 2005) requiring FAK (Salasnyk et al., 2007a). Interestingly, FAK inhibits differentiation in other contexts and is a negative regulator of cardiogenesis in mouse embryonic stem cells (ESCs) (Hakuno et al., 2005). Seeding human MSCs on collagen 1 or vitronectin also induces osteogenic differentiation by stimulating ERK-1/2 and the osteogenic transcription factor Runx2 (Cbfa-1) (Salasnyk et al., 2007b). Matrix-binding proteins also affect stem cell differentiation: dentin matrix protein 3 [also known as phosphophoryn (PP)], a SIBLING protein localized in dentin and bone ECM, stimulates mouse embryonic MSCs to differentiate into osteoblasts (Jadlowiec et al., 2004).

Many studies have directly addressed the roles of cell attachment strength and matrix compliance in stem cell fate; the less ESCs attach to a substrate, the more they aggregate and the more efficiently they differentiate into cardiomyocytes. In these cells, aggregation is more crucial for differentiation than are the

biologically active components of the ECM, although production of endogenous collagen is required for ESC maintenance in these cultures (Chen et al., 2007). Changes in cell shape regulate osteogenic differentiation of human MSCs by activating downstream RhoA-ROCK signaling (McBeath et al., 2004), illustrating that cytoskeletal tension also modulates stem cell fate. Engler et al. used soft matrices to mimic brain tissue, stiffer matrices to mimic muscle and rigid matrices to mimic collagenous bone to examine how matrix elasticity contributes to cell specification of naïve MSCs. Similarly to multipotent stem cells, the MSCs cultured on these matrices are initially reprogrammable by the addition of soluble induction factors. However, after several weeks, the MSCs become committed to the lineage dictated by the matrix elasticity (Engler et al., 2006), demonstrating a dominant effect of ECM stiffness on cell fate.

A recent study that profiled gene expression by four types of mesenchymal stem cells revealed that genes whose products are involved in ECM remodeling make up one of the three main classes of genes expressed by all of these stem cells (Tsai et al., 2007). Although few specific mechanisms have so far been defined, it seems likely that the precise regulation of ECM remodeling at specific times functions as an important biological switch that triggers initiation of stem cell differentiation or mobilization within the niche in a tissue-specific manner, but this requires further investigation. Many questions regarding the role of the ECM in regulating cell fate remain to be answered, such as: what is the relative importance of intrinsic cellular cues versus extrinsic microenvironmental influences, what activates or inactivates these signals and how are they integrated?

ECM remodeling and engineered scaffolds

Tissue engineering is an evolving field that seeks to create functioning artificial tissues and organs. Major considerations in tissue engineering include both the type of cell and the substrate to be used. Many strategies use an artificial scaffold that functions as the ECM to facilitate both organization and differentiation of seeded cells into a functional 3D tissue. Successful implantation of artificial bladders in human patients has been achieved by Atala and colleagues (Atala et al., 2006). These artificial bladders are composed of autologous cells (isolated from the patient) seeded on

collagen or composite collagen and polyglycolic acid scaffolds. Other studies are currently focusing on generating artificial cartilage to repair damaged or deteriorated arthritic cartilage (Tuli et al., 2003). For such studies, an understanding of ECM dynamics and cell-ECM interactions is particularly important.

Recent studies have indicated that cells not only detect the composition and stiffness of their substrates but can also detect their geometry. This is also true of artificial substrates: pore sizes in engineered trabecular bone, for example, depend on the initial scaffold geometry (Hofmann et al., 2007). Because it is clear that the ECM can affect cell fate and differentiation, the field is looking towards cell and developmental biology for guidance in the design of such scaffolds. Many successful artificial substrates are biomimetic: they mimic *in vivo* structures. Nanotechnology has made it possible to produce biomimetic synthetic nanofibers, the diameters of which are within the sub-micrometer range (Ashammakhi et al., 2007; Griffith and Swartz, 2006). Recent studies indicate that cells sense and respond differently to such nanofibers. For example, Li et al. showed that chondrocytes secrete increased amounts of ECM when seeded on a nanofiber compared with a microfiber matrix (Li et al., 2006) (Fig. 5a,b). Studies using another culture system have demonstrated that nanotopography strongly influences stem cell differentiation; seeding cells on a nanograting that has a diameter one order of magnitude less than the width of the cell more effectively promotes neuronal differentiation of human MSCs than do other configurations of the same material or retinoic acid (Yim et al., 2007). These studies and others (Griffith and Swartz, 2006) indicate the need for scaffold designs that provide the appropriate mechanical signals.

Because the ECM is dynamic, future tissue engineering might need to mimic changes in its composition and architecture that would normally occur *in vivo*. Many scaffold materials are biodegradable, which has been shown in some studies to be a necessity. For example, in engineered arteries, a minimal amount of residual scaffold is detrimental and compromises the structural integrity of the nascent vessel (Dahl et al., 2007). Although synthetic materials can be degraded, natural materials allow enhanced cell-mediated ECM remodeling. Various forms of collagen have been used as cell scaffolds, such as vitreous collagen as a substrate for corneal fibroblasts (Fig. 5c). In fact, denatured collagen, or collapsed collagen matrices (also known as gelatin), allows more remodeling and matrix deposition than do non-denatured collagen matrices (Abraham et al., 2007). Many investigators have used cell-derived natural scaffolds, which are more amenable to cellular remodeling, for tissue engineering. Ahlfors and Billiar propose that a human-cell-derived matrix scaffold that they generated *in vitro* by growing human dermal fibroblasts in a chemically defined medium for 3 weeks can function as a viable skin-cell scaffold, owing to its high tensile strength and biocompatibility (Ahlfors and Billiar, 2007). Similarly, Jin et al. recently found that a cell-derived scaffold produced by primary porcine articular chondrocytes grown for 3–4 weeks and then freeze-dried before reseeding for 2 days with rabbit articular chondrocytes supports cartilage differentiation when implanted *in vivo* (Jin et al., 2007). Many recent studies have revealed that artificial scaffolds perform best when they are bioresponsive and react to biological stimuli (Leach, 2006) so that they can be manipulated by the cells in a similar way to natural scaffolds.

Another way to mimic the *in vivo* environment is to engineer matrices to contain bioactive molecules that can be used to control cell behavior or allow cells to interact with the substrate and generate

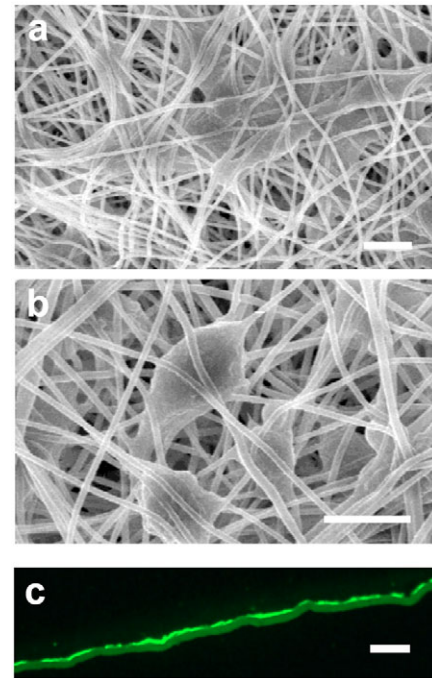


Fig. 5. Cells growing on engineered scaffolds. (a,b) Scanning electron microscopy (SEM) images of mouse fibroblasts grown on PLGA nanofibers for 7 days, as described previously (Li et al., 2002). Images are displayed at: (a) original magnification $\times 1500$ and (b) original magnification $\times 2500$. Scale bar: 10 μm . Images printed with permission from Li et al. (Li et al., 2002). (c) Side view of rabbit corneal epithelial cells immunostained with an anti-cornea-specific cytokeratin K3/12 (clone AE5, green) grown on a vitrified collagen 'vitrigel' sheet (light green). Scale bar: 50 μm . Image courtesy of Winnette McIntosh Ambrose, Toshiaki Takezawa and Jennifer H. Elisseeff.

their own ECM. Tethering of growth factors (e.g. EGF) to a scaffold (Fan et al., 2007) and sustained release of heparin from nanofibers, for example (Luong-Van et al., 2006), have been achieved. The ultimate goal is for scaffolds to release bioactive molecules in the appropriate temporal-spatial manner to control cell differentiation and morphogenesis, which requires us to know this from studies of how this occurs *in vivo*. In some studies, bioactive peptides have been attached to inert synthetic scaffolds to facilitate cell-mediated matrix assembly. In a recent study, a bioactive portion of the FN molecule was attached to an albumin-based nanoparticle scaffold, which allowed cells to make FN fibrils on a non-permissive substrate. Significantly, if the nanoparticles were immobilized, or if contraction was prevented with ROCK inhibitors, FN fibrillogenesis did not occur (Pereira et al., 2007). Another study indicates that cartilage-specific gene expression and ECM production by human ESCs can be stimulated on poly(ethylene glycol)-diacrylate (PEGDA) hydrogels when an RGD peptide is covalently attached to the polymer (Hwang et al., 2006). Although this RGD modification is an oversimplified method for mimicking the ECM, it is sufficient to induce cartilage-specific morphogenesis and differentiation in the presence of TGF- $\beta 1$, including expression of proteoglycans and types I, II and X collagen. Similar strategies might therefore be useful to improve differentiation and matrix synthesis in other tissue-engineering applications.

Novel engineering strategies based on the knowledge that both matrices and cells can self-assemble are also being employed, the assumption being that these processes will occur more efficiently

in vivo. One injectable matrix that can self-assemble into fibers of only 3 nm in diameter is composed of a repeated peptide sequence (Nowak et al., 2002) that forms either an ordered or random matrix, depending on external factors (Haines-Butterick et al., 2007; Nowak et al., 2002). It remains to be seen whether tissue assembly occurs within this peptide matrix in vivo. Cells also have the capacity to self-organize. Many studies have used Matrigel, a reconstituted basement membrane derived from the Engelbroth-Holm-Swarm (EHS) mouse sarcoma (Kleinman et al., 1986) that mimics mechanical and biochemical properties of ECM, to facilitate cellular self-organization. Recent in vitro studies have indicated that both adult human and embryonic mouse salivary gland cells can self-organize in vitro within compliant matrices to form complex 3D structures resembling native differentiated salivary gland tissue (Joraku et al., 2007; Wei et al., 2007). Similarly, fetal pulmonary cells self-organize into alveolar-forming units and express type II alveolar differentiation markers in Matrigel (Mondrinos et al., 2006). However, the epithelial cells do not survive in artificial poly-lactic-co-glycolic acid (PLGA) porous foam or in poly-L-lactic-acid (PLLA) nanofiber matrices, even in the presence of exogenous tissue-specific growth factors. One reason for this difference could be that the epithelial cells can remodel the Matrigel but not the synthetic engineered substrates. These studies point to the need for dynamic interactions between the cells and the scaffold material for successful tissue assembly.

Conclusions and perspectives

ECM remodeling is important in wound repair and in developmental processes such as branching morphogenesis and stem cell homing, maintenance and differentiation. Tissue engineering has progressed to a point at which it is imperative that we understand cell-substrate interactions, and knowledge of matrix remodeling is central to this issue. Accordingly, a focus of current research efforts in tissue engineering is on generating biomimetic and bioresponsive substrates, and on understanding how cells interact with these to assemble their own ECM. Another focus of research is to investigate how the scaffolds can be used to control delivery of signals in a temporal and spatial manner to guide or maintain cell differentiation. For devices that are implanted for long periods and require a steady supply of cells, engineering a stem cell niche within the transplanted device or devising methods to recruit stem cells from the patient are issues to consider.

There are gaps in our knowledge of cell-ECM-scaffold interactions that might be addressed by emerging technologies. New imaging methods under development should address this, including methods for imaging individual cells within 3D aggregates (Keller et al., 2006; Manley and Lelkes, 2006). A novel series of probes known as quenched activity based probes (qABPs) are now being used to detect protease activity directly in live cells by fluorescence microscopy (Blum et al., 2005). Confocal reflection microscopy, which is useful for monitoring ECM structure and can be combined with conventional confocal microscopy (Hartmann et al., 2006), might prove useful for live imaging of ECM remodeling. To investigate the interactions between cells and artificial scaffolds at the level of cell-matrix adhesions, researchers have applied FRET – which is used to study molecular dynamics – to analyze such interactions. FRET can be used to measure changes in the crosslink density of synthetic matrices and changes in cell-matrix adhesions at the level of bonds between integrins and their ligands (Huebsch and Mooney, 2007). As we understand more about how cells interact with and remodel the ECM, rational design of new scaffolds that can

restore a functional microenvironment in vivo to either prolong the effectiveness of therapies, cure disease or replace organs will become a reality.

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