Review

Design of three-dimensional biomimetic scaffolds

Shawn C. Owen,[1,2] Molly S. Shoichet[1,2,3,4]

[1]Department of Chemical Engineering and Applied Chemistry, University of Toronto, 200 College Street, Toronto, Ontario, Canada M5S 3ES
[2]Terrence Donnelly Centre for Cellular and Biomolecular Research, 160 College Street, Toronto, Ontario, Canada M5S 3E1
[3]Institute of Biomaterials and Biomedical Engineering, University of Toronto, 164 College Street, Toronto, Ontario, Canada M5S 3G9
[4]Department of Chemistry, University of Toronto, 80 Saint George Street, Toronto, Ontario, Canada M5S 3H6

Received 24 February 2010; revised 24 March 2010; accepted 25 March 2010
Published online 1 July 2010 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/jbm.a.32834

Abstract: A detailed understanding of the biophysical features that affect cell growth and development is important in guiding the design of biomimetic scaffolds. The cellular microenvironment is a network of structural and functional components that provide mechanical and chemical stimuli, which influence cell function and fate. Important developmental signals are conveyed to cells through interactions with neighboring cells, the extracellular matrix (ECM), and growth factors. Currently, there are number of approaches to create 3D tissue models *in vitro* that allow for control over cell adhesion, the physical properties of the surrogate matrix, and the spatial distribution of growth factors. This review describes some of the most significant biological features of the ECM, and several engineering methods currently being implemented to design and tune synthetic scaffolds to mimic these features. © 2010 Wiley Periodicals, Inc. J Biomed Mater Res Part A: 94A: 1321–1331, 2010.

Key Words: extracellular matrix, scaffold materials, tissue engineering, biological models, regenerative medicine

INTRODUCTION

Two-dimensional (2D) cell culture is commonly used to study cell function and behavior, providing a basic method to explore biological mechanisms, cell differentiation, and therapeutic efficacy before moving into more complex, *in vivo* models. However, cells cultured in traditional 2D models fail to provide an accurate representation of cells *in situ* as they lack the contextual cues found in the native three-dimensional (3D) tissue.[1,2] Several factors in the cellular microenvironment provide important signals to cells, including interactions with neighboring cells, the extracellular matrix, and soluble factors.

The extracellular matrix (ECM) is a heterogeneous composition of proteoglycans, proteins, and signaling molecules that was originally known for its role in providing structural support to cells and as a milieu for cell migration. Recent investigations of the ECM have clarified its role beyond an inert background to an active component in cell signaling.[2–4] Beginning with embryogenesis and continuing throughout adulthood, the ECM influences cell differentiation, proliferation, survival, and migration through both biochemical interactions (cell adhesion, presentation of growth factors) and mechanical cues[5] (stiffness, deformability). Successful understanding of ECM signals will facilitate the ability to guide cell behavior and evaluate complex intracellular signaling pathways.

Considering the impact of the ECM on cellular behavior, a multidisciplinary paradigm shift is underway towards the development of biomimetic 3D cell culture systems that incorporate ECM molecules to recapitulate the native environment more accurately than 2D systems.[6,7] Current 3D models are made from polysaccharides, collagens, synthetic biomaterials, spheroids of other cells, peptides, cell fragments, or decellularized ECM from living tissue.[8] There are a number of excellent reviews which discuss these natural and synthetic scaffolds.[9–14]

Among the scaffolds currently in use, the majority are either simple or ill-defined. Simple matrices include synthetic polymers such as poly(ethylene glycol) (PEG) and poly(vinyl alcohol) (PVA), or naturally derived polymers such as chitosan and collagen. As these scaffolds are often made from only one or two components, the physicochemical properties can be controlled; however, many of these polymers have limited cellular recognition and, therefore, natural cell–matrix adhesions may be limited or completely absent. Any variation in adhesion will alter the signaling mechanisms that are important to many cellular processes.

At the other extreme are complex, ill-defined matrices such as Matrigel[15] or reconstituted tissue.[16] These scaffolds provide factors that impact cell function; however, the inherent complexity of these scaffolds makes it difficult to understand cell signaling. Batch-to-batch variability hinders the reproducibility of experiments. Problems with biocompatibility prevent implantation of the scaffolds into human patients, and the mechanical properties of these scaffolds are not

Correspondence to: M. S. Shoichet; e-mail: molly.shoichet@utoronto.ca

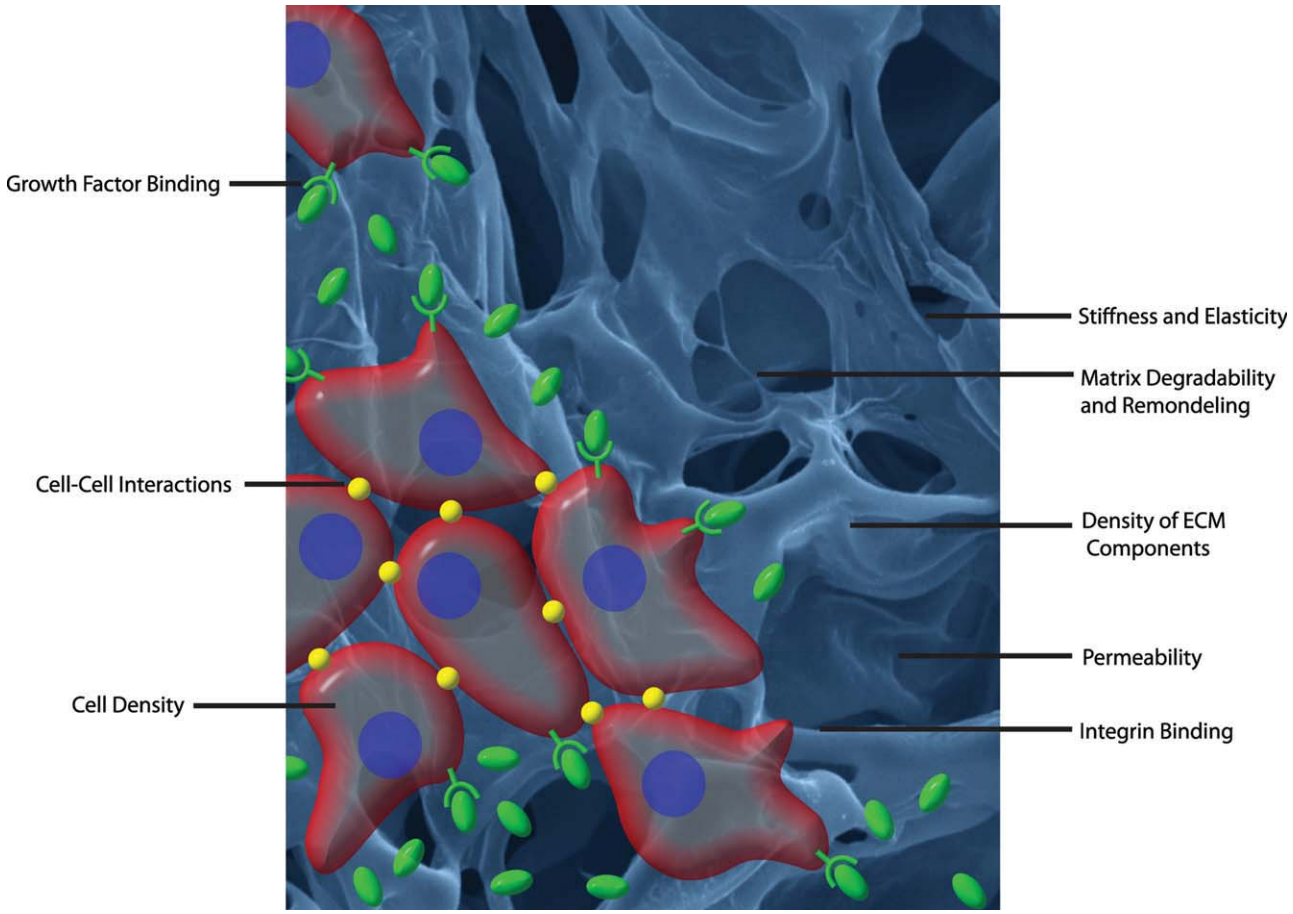

FIGURE 1. The complex 3D cellular environment provides mechanical and biochemical signals that guide cell function. The components of the ECM dictate the stiffness of matrix and the types of cell–matrix adhesions. The matrix composition also determines the ease of nutrients to diffuse to tissues, and the ability of cells to migrate through the matrix. Nonstructural factors such as cell density, cell–cell interactions, and bound or secreted signaling proteins are important in guiding cell differentiation and function. Image copyright (2010) by Karyn Ho and Anne Hsieh. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

easily manipulated. As such, the direct effect of these current surrogate ECMs on cell behavior remains ambiguous.

Appreciation of the complexity of the cell response to ECM signaling has stimulated the development of 3D scaffolds that imitate a range of ECM properties. 3D models can overcome the constraints of current 2D models by incorporating both mechanical and biochemical components directly into the matrix. In the first part of this review, we outline several biological features of the cellular environment that are important in guiding cell fate. Later, we discuss noteworthy engineering advancements in designing scaffolds with tunable components to allow control of matrix factors that affect cell function.

DEFINING THE NATURAL BIOPHYSICAL AND CHEMICAL PROPERTIES OF THE EXTRACELLULAR MATRIX

All cells reside in a complex microenvironment that is tailored to guide their physiological functions. As shown in Figure 1, the complex 3D cellular environment provides mechanical and biochemical signals that are important in guiding cell growth and function. Composition of the ECM dictates matrix stiffness, nutrient diffusion to tissues, and cell–matrix interactions, including cell adhesion and migration. Nonstructural factors, such as cell density, cell–cell interactions, and bound or secreted signaling proteins, are also important in guiding cell differentiation and function.

Structural elements of the ECM include a hydrated meshwork of laminin, collagen, elastin, entactin (nidogen), proteoglycans, fibronectin, and various other constituents.[17] The more fibrous components (e.g., collagen and elastin) provide architectural rigidity and tension for the cells, while the non-fibrous components (predominantly glycosaminoglycans) regulate turgor pressure, form intimate intracellular connections, and modulate the binding, display, and activity of growth factors.[18]

The cellular environment is paramount: during embryogenesis and differentiation into the three primary germ cell layers; in complex tissue and organ formation; throughout adulthood in maintaining homeostasis; and in response to insult.[19] During early development highly organized chemical gradients in the ECM guide cell migration to form the gastrula. Cell differentiation is further directed through morphogenesis and organogenesis by both cell–matrix and cell–cell interactions.[20] Most cells in the body are maintained in

a quiescent state following embryogenesis; however, proliferation and differentiation of some specialized cells (such as hematopoietic progenitor cells) are continually regulated by ECM interactions. In addition, the ECM has been shown to be instrumental in physiological response to wounding and infection.[21,22] The vital instructive cues in the cellular microenvironment include cell binding interactions, mechanical and structural support, and the presentation of regulatory molecules.

Cellular adhesion to the ECM

The matrix environment in which cells are grown influences the type and extent of cellular adhesion, which in turn affects cell proliferation.[23] Integrins are the primary cell surface receptors that are responsible for cell–matrix adhesion (Fig. 2). They are composed of two transmembrane units—a large α subunit and a smaller β subunit—that form non-covalent heterodimers in the presence of extracellular Ca^{2+}.[24] Various combinations of α- and β-subunits allow for the formation of 24 different heterodimers, which determine ligand specificity. Although some redundancy exists between integrin pairs and their respective ligands, the loss of almost any integrin has deleterious effects.[25] Most importantly, integrins not only act as anchors to the ECM but also transduce mechanochemical signals to the cell via intracellular transduction. Initial binding of integrins often leads to the clustering of additional specialized adhesive proteins and local remodeling of cytoskeletal and cytoplasmic proteins.[3] The resulting focal adhesions sensitize cells to mechanical stimuli, including the rigidity and elasticity of the ECM.

Integrins bind a number of "insoluble" components of the ECM including laminin, elastin, and hyaluronan, among others.[24] The types and concentrations of these insoluble factors provide signals that are disseminated by the integrin family, promoting activation of diverse cytoplasmic proteins to control a number of cellular processes: differentiation, survival/apoptosis, cell polarity, gene regulation, actin organization, proliferation, and cell migration.[2,24–26] For example, the polarity of epithelial cells is essential in tissue organization for structural formation (such as ductal arrangement), and the directionality of product secretion (such as lactation). It has been shown that epithelial cell integrins must interact with a laminin-rich basement membrane to form the proper architecture and achieve normal cell function.[27,28] Cells cultured in a 2D environment lack basal and apical membrane differentiation, while cells cultured in a 3D matrix may present appropriate integrins to maintain polarity.[29]

An impressive list of integrins and their associated cytoplasmic proteins was arranged by van der Flier and Sonnenberg;[24] however, no comprehensive index has been tabulated that links the extracellular binding of integrin dimers with their respective cellular function. Progress in elucidating the role of each integrin and its downstream regulation of cell behavior will aid in the design of more specialized ECM surrogates that are specific to a desired cellular outcome. The use of defined ECM scaffolds will provide greater insight into the impact of integrin–matrix interactions.

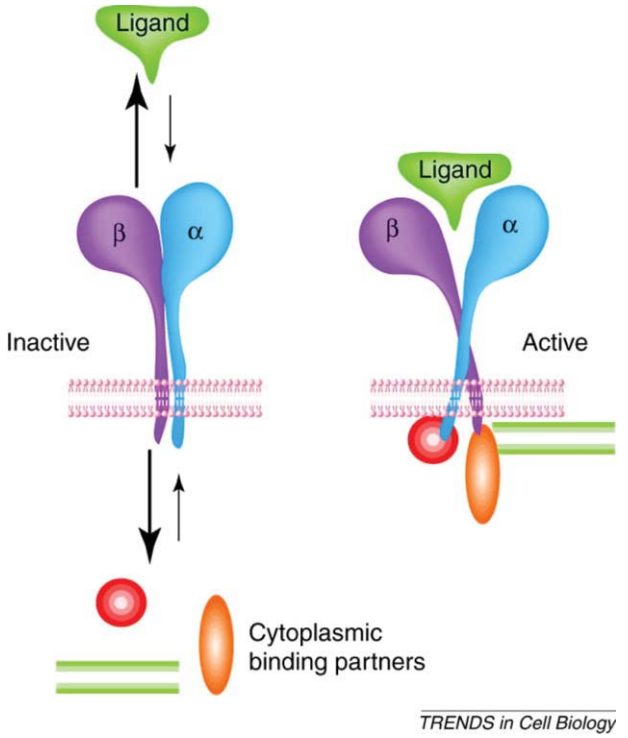

FIGURE 2. Integrins are composed of two transmembrane units—a large α subunit and a smaller β subunit—that form noncovalent heterodimers that have high affinity for EMC ligands. Integrins not only act as anchors to the ECM but also transduce mechanochemical signals to the cell via intracellular transduction that lead to the remodeling of cytoskeletal and cytoplasmic proteins. (Reproduced with permission from Schwartz MA, Trends Cell Biol, 2001, 11, 466–470, Elsevier). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Density and stiffness of the ECM

Cells are not only sensitive to ECM adhesion but also to its density and stiffness. For example, cultured fibroblasts exhibited significantly different migration patterns when the density of the matrices was changed.[30] When ECM density was increased, by increasing concentrations of collagen, the migration of fibroblasts was reduced. Thus, an inverse correlation between matrix density and cell migration was observed while matrix ligand and integrin receptor concentrations were held constant.

Important work by Discher and colleagues demonstrated the importance of matrix elasticity on stem cell fate.[31] Mesenchymal stem cells (MSCs) were cultured on collagen-coated gels that mimicked the elasticity of various tissues. The MSCs responded to gel elasticity by differentiating into lineages that corresponded to the stiffness of the native environment (Fig. 3). For example, MSCs cultured on soft gels (~0.1–1 kPa), to mimic brain elasticity, developed a neuronal morphology, with filopodia branching and spreading. More importantly, the RNA profile of these cells showed an increased expression of the neuronal progenitor marker, nestin, and the neuron marker, βIII tubulin. Interestingly, medium stiffness gels (8–17 kPa), which mimic striated muscle elasticity, promoted differentiation to myogenic cells,

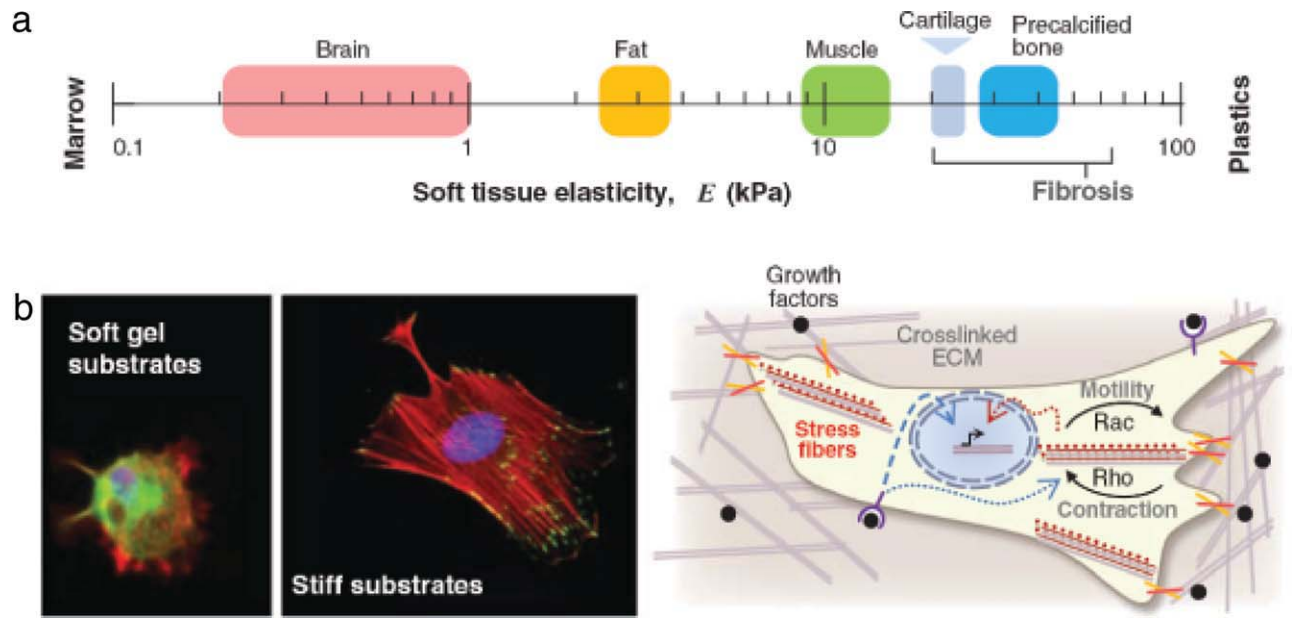

FIGURE 3. (a) Scale of tissue elasticity ranging from the softest (brain) to stiffest (bone). (b) Cells cultured on gels that mimic a soft tissue environment anchor less strongly to the substrate than cells cultured on gels that mimic a stiff tissue environment (left). Signals from growth factors bound to the ECM affect cell function by mediating gene expression through various kinases such as Rho and Rac (right). (Reproduced with permission from Discher DE, Mooney DJ, Zandstra PW, Science, 2009, 324, 1673–1677, AAAS). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

and the gels with the highest stiffness (25–40 kPa) to mimic bone elasticity, enhanced osteogenic differentiation. Similar results have been shown in other studies.[32,33]

Dependence on matrix rigidity has likewise been observed for mammary epithelia[34] and glioblastomas.[35] Increasing matrix stiffness disrupts cell morphology, and leads to increased proliferation. As shown in Figure 3(b), increasing ECM rigidity elevates the activity of the Ras homolog gene family member A (RhoA), which subsequently induces cytoskeletal tension, decreases cell–cell contact, disrupts cell polarity, and increases growth rate.

Permeability of the ECM

In addition to rigidity and elasticity, other architectural features of the ECM are also important in dictating cell behavior. Metabolic activity requires access to nutrients and the removal of waste, both of which are primarily a function of diffusion. The porosity and permeability of the ECM directly affect the extent and nature of diffusion, and therefore influence cell processes.

To grow properly, cells require an ECM that permits the diffusion of nutrients and waste. Research has suggested that the diffusion of oxygen is limited to ~100 μm from the source, even in highly vascularized tissue such as the liver.[36] The diffusion of other nutrients (proteins or steroid hormones) is dependent on the tortuosity and elimination pathways of the tissue. High cell density and dense ECM composition reduces the supply of nutrients to the interior of multilayered tissues, and prevents the removal of deleterious waste compounds. This phenomenon is very common in solid tumors that develop necrotic cores as a result of poor diffusion.[37] As such, the overall permeability of the ECM affects the diffusion of nutrients and, consequently, affects cell differentiation and function.

The pore sizes found in natural tissue are ideal for the arrangement of cells and function of tissues in each specific environment. Perhaps the most studied system linking ECM permeability and porosity with cellular function is osteogenesis. The matrix of bone tissues is a highly organized framework of fibers (collagen, elastin), proteoglycans, and proteins, not unlike other tissues; however, the bone matrix also contains a high concentration of inorganic material (hydroxyapatite) which contributes to the stiffness of bone tissue. Bone can be classified as either highly porous and spongy or as compact and lamellar, with each containing a dominant cell type—osteoblasts and osteoclasts, respectively. Osteogenesis begins in the bone marrow where progenitor cells differentiate into osteoblasts. These cells are responsible for the generation and mineralization of the bone matrix by secretion of type I collagen and hydroxyapatite. Interestingly, as osteoblasts secrete these components into the ECM, the matrix porosity and permeability dramatically decreases, the levels of growth factors decrease, and the trapped osteoblasts are induced to differentiate into osteoclasts.[38] In contrast to osteoblasts, osteoclasts mainly function to breakdown bone and reabsorb minerals. The biomimetic scaffold designed to study regeneration and repair of bone (or any other tissue), must recapitulate the natural topography of that tissue to maintain proper cell functioning.

The impact of porosity on cell growth and proliferation has also been demonstrated using synthetic scaffolds. Using

centrifugation to process polycaprolactone (PCL) scaffolds, Lee and coworkers fabricated scaffolds with a gradient of pore diameters ranging from ~88–405 μm, and then examined the interaction of cells (chondrocytes, osteoblasts, and fibroblasts) with the scaffolds *in vitro*.[39] Chondrocyte and osteoblast growth was greatest in scaffolds with pore sizes 380–405 μm, while fibroblast growth was greatest in scaffolds with pore sizes 186–200 μm. Scaffolds with pore size 290–310 μm encouraged the greatest degree of tissue infiltration resulting in bone formation *in vivo*.

Degradation and remodeling of the matrix

Following binding to the ECM, cells respond to the environment by releasing different proteases. The type of concentration of protease released depends on the composition of the ECM and its sensitivity to enzymatic degradation. In this manner, cells are defined by their environment, but also simultaneously remodel it. Seminal work by Bissell et al. has termed this type of cell–matrix synergy as "dynamic reciprocity."[40]

Most cells reside in a state of homeostasis, reaching full development at the end of embryogenesis.[41] Some cells, however, go through significant physiologic changes at much later stages of development, requiring remodeling of the cell environment. Among these are cells of the mammary gland, which branch into ducts and terminal lobular units (acini) during puberty, and again change during pregnancy, finally reaching a fully developed state only after parturition. Epithelial mammary cells initially respond to hormone secretion and the elasticity of their environment by growing small projections. This is followed by remodeling of their environment through secretion of proteases, such as matrix metalloproteinease (MMP), and enzymes, such as hyaluronidases.[42] Degradation of the ECM changes the local modulus, decreases the number of cell–matrix adhesions, and also results in the release of ECM fragments that may possess biological activity. The cues that result from degradation are relayed back to the cell, guiding subsequent behavior and function. Thus, the ability of cells to remodel their environment, in concert with hormonal cues and reciprocal signaling, allows for proper functional development.

Cell–cell interactions

The cellular microenvironment includes cell–cell interactions where cell density alone can influence cell function.[43,44] Moreover, different cell types invariably influence cell function. Cell–cell interactions are instrumental in recapitulating the native environment and promoting the morphogenesis of functional tissue. For example, mammary epithelial cells *in situ* maintain physical contact with neighboring myoepithelial cells via a combination of connections, including adherens and gap junctions.[45] Adherens junctions generate the polarization of epithelial cells, leading to the development of basal and apical membranes that are required for proper secretory function. Early investigations by Okada showed that co-cultures of myoepithelial and epithelial cells, formed penetrating tubes into collagen gels, but monocultures of either cell lacked structural correctness (Fig. 4).[46] Similar co-dependency between myoepithelial (arising from the mesoderm) and epithelial cells (arising from the ectoderm) is seen in all glandular and vascular tissue.[45]

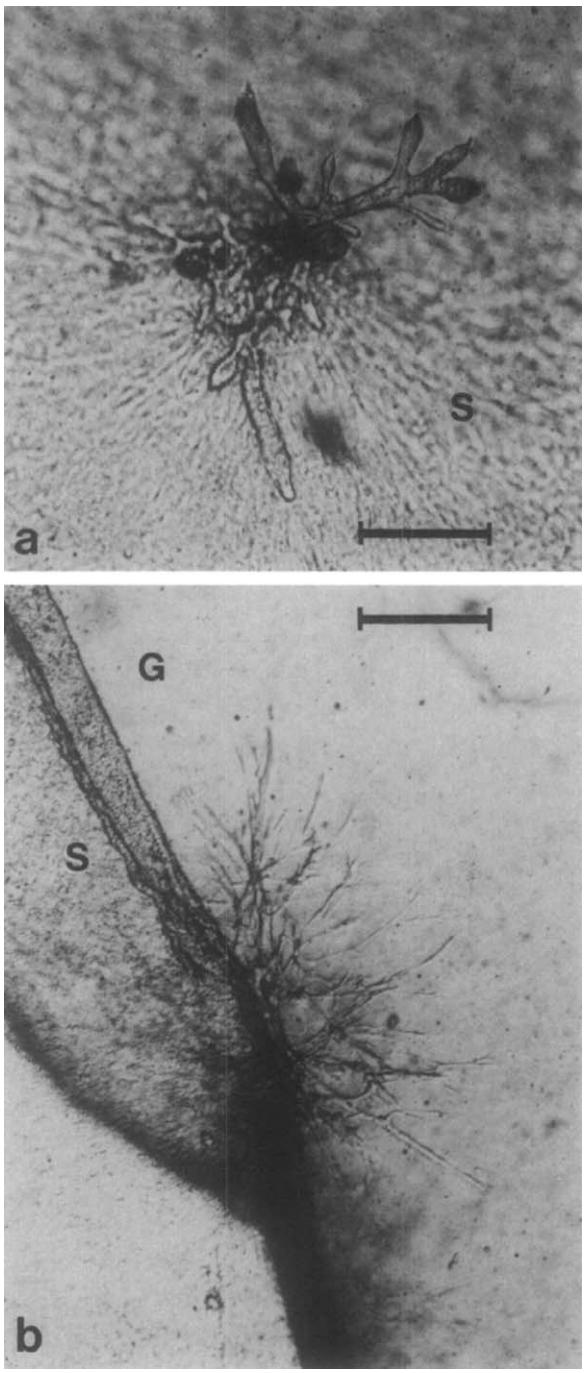

FIGURE 4. Light micrographs of two types of outgrowths seen from co-cultures of myoepithelial and epithelial cells on floating collagen gels. (a) Twelve days after culture, blunt outgrowths were seen from the cell sheet, (b) 6 days after culture, pointed outgrowths were seen originating at the edge of the cell sheet. Scale bar: 200 μm. (Reproduced with permission from Bennett DC, Armstrong BL, Okada SM, Dev Biol, 1981, 87, 193–199, Elsevier).

The importance of cell–matrix and cell–cell interactions is apparent in the brain where neurons are surrounded by the ECM and glial cells (oligodendrocytes and astrocytes). Astrocytes guide neuron migration during development, and promote the myelination activity of oligodendrocytes which act as insulating conduits to route synaptic signals.[47] In addition, astrocytes provide biochemical support to neurons by supplying nutrients and regulating the concentration of ions in the extracellular space. Importantly, there is dynamic, bi-directional signaling between astrocytes and neurons. Glutamate released from astrocytes influences the transmission of signals between neurons at synaptic junctions; conversely neuronal activity stimulates glial fibrillary acidic protein (GFAP) production in astrocytes.[48]

Cell–cell interactions are also required for appropriate phenotypic growth. Bhatia et al. have shown that hepatocytes co-cultured with fibroblasts restore the appropriate hepatocellular phenotype.[49] Further work has also shown the dependency of liver sinusoidal endothelial cells (LSECs) on neighboring cells.[50] Analysis demonstrated that the expression of characteristic cell surface markers and proliferation were optimized when LSECs were maintained in co-cultures of both hepatocytes and fibroblasts. The stimulatory cues that arise from these intimate neighbors include physical junctions as well as secreted paracrine chemical factors, all of which are instrumental in determining cell fate.

The cellular environment is paramount in guiding cell growth and function. The combination of the structural characteristics of the matrix, the types of cell–matrix adhesions, as well as other factors such as cell–cell interactions, and bound or secreted signaling proteins are all important aspects of the cellular environment that must be regulated for proper cell function.

BIOENGINEERING APPROACHES TO TUNE MATRIX PROPERTIES

Appreciating the significance of the cellular environment has led to numerous surrogate scaffolds with the expectation that mixing cells into a porous matrix and adding soluble growth factors will result in functional tissue. However, in many cases, culturing cells in these surrogates has not led to the desired outcome. Successful fabrication of functional tissue analogs requires an awareness of the physical, biochemical, and cellular stimuli of the microenvironment. As such, researchers have begun to modulate many aspects of synthetic matrices in an attempt to overcome the limitations of oversimplified or undefined matrices currently in use.

Controlling the mechanical properties of the scaffold

Understanding the signals that guide cell fate lies at the interface of biology, chemistry, and materials science and comprise the field of tissue engineering.[51] Advancements in biomaterials and engineering have established a large set of tools to develop strategies to control the range of signals that affect tissue form and function. Several approaches have made noteworthy improvements in tuning scaffolds to recapitulate the modulus and ductility of a range of native environments. The most common approach to control scaffold rigidity is by varying the types and ratio of components. In an attempt to direct cartilage regeneration, Kuo et al. cultivated chondrocytes in a ternary, physical mixture of natural and synthetic polymer scaffolds containing PEG/chitin/chitosan.[52] The authors found that the regeneration of cartilaginous material could be controlled simply by adjusting the composition of the hybrid scaffold. A second approach to manipulate the mechanical properties of fully synthetic hydrogel scaffolds was devised by Anseth and colleagues by changing macromer concentrations in copolymer formulations.[53] Photocrosslinking gels based on multi-vinyl macromers of PEG and PLA were fabricated to optimize the compressive modulus of the gel to mimic physiological compressive loads. Varying the concentration of PEG macromer from 10 to 20%, resulted in gels with moduli ranging from 60 to 500 kPa. Similarly, Borzacchiello and co-workers controlled the elastic moduli of natural matrices composed of hyaluronic acid (HA) and collagen by changing the molecular weight of HA in the system.[54] Interpenetrating networks of HA with collagen resulted in firm gels that preserved the important biological and chemical properties of HA. These simple approaches represent an important step in tuning the physical properties of ECM mimetics; however, more research is required to evaluate the effectiveness of these scaffolds to control cell function.

A fascinating approach to modulate the rigidity of an ECM substitute, derived by Chen et al. is to direct control over contractile forces using microfabricated cantilevers.[55] NIH 3T3 fibroblasts were cultured in collagen gels, and the overall stiffness was measured and controlled by anchored cantilevers. Varying the overall rigidity of the scaffold provided a means to regulate changes in the protein expression of embedded cells, whereas changes in the amount of fibrillar actin, fibronectin, and tenascin C reflected responses to mechanical stress. This study highlights the dynamic reciprocity between cellular forces, ECM remodeling, and cellular function. Importantly, the authors present a means to induce stress gradients in tissue scaffolds that can be used to control cell differentiation.

In a separate study, the migration of vascular smooth muscle cells was examined with respect to matrix stiffness using defined polyacrylamide gels with moduli ranging from 5 to 80 kPa.[56] Analysis of cell behavior demonstrated durotaxis—that is cell guidance up a stiffness gradient. Moreover, cells aligned in the direction of the stiffness gradient. Cell morphology varied with the modulus of uniform (gradient free) gels—the extent of cell spreading increased with increasing stiffness.

In addition to meticulous iterations in scaffold design, high-throughput methods and computer simulations have begun to emerge to facilitate the understanding of how materials affect cell function. For instance, Langer and colleagues generated a rapid assay to characterize the interactions of human embryonic stem (hES) cells and a variety of acrylate-based polymers.[57] The group deposited 576 combinations of 25 different acrylate monomers on a layer of

poly(hydroxyethyl methacrylate) (pHEMA) and then hES cells were monitored for changes in cellular morphology, growth, and differentiation. Interestingly, the majority of monomers supported general cell attachment and growth, but also biased differentiation into a cytokeratin-positive, epithelial-like cell. Separately, Huang and Ingber designed computer simulations to model the activation of the cell signaling network in response to general mechanical stimuli, and predict the cell fates that may result.[58] Their results re-emphasize the robust link between cell fate regulation and interactions with physical surroundings, as well as introduce a simple means to conceptualize regulatory signal processing.

Tuning the porosity and permeability of the matrix

The need for a porous, interconnected matrix is apparent; however, defining pore dimensions and the degree of permeability remains a significant challenge. One endeavor by Shoichet and co-workers to control both the pore size and porosity of scaffolds used various concentrations of dextran and PEG.[59] Formulations of 10 wt % dextran with increasing percentages of PEG from 0 to 25 wt %, led to the formation of several scaffold topologies from microporous, to macroporous gel-wall, to macroporous interconnected-beaded structures (Fig. 5). The interconnected-beaded scaffolds contained pores with a median diameter of 41 μm that were connected by narrower channels with a median diameter of 11 μm. An alternative approach from Hollister and colleagues coupled solid free form (SFF) manufacturing with sponge scaffold fabrication to cast an array of materials into porous architectures.[60] Polyglycolide (PGA) and polylactide (PLA) were formed into scaffolds using porogen leaching of NaCl or by solvent evaporation of chloroform. The resulting architecture featured global pore sizes of ~100 μm, and local pore sizes ranging from 10 to 300 μm. Importantly, the architecture of scaffolds can be controlled to manipulate both global and local porosity and pore size. Mikos and co-workers developed a method of controlling the porosity of chitosan scaffolds for osteogenic differentiation by incorporation of lysozyme at the material surface. Incubation with lysozyme degraded the polymer backbone, resulted in the formation of pores, and increased the overall porosity of the scaffold initially at 5–55% after 21 days. Bhatia and colleagues used lithography and microsyringe deposition to control the porosity of poly(DL-lactide-*co*-glycolide) (PLGA) scaffolds, and evaluated the advantages and limitations of each fabrication technique.[61] The majority of research on scaffolds lies in fabrication of the material, for which several reviews have been written.[9,62,63] A core challenge of these technologies is to balance the integrity and mechanical support of scaffolds, while still allowing cell migration, transport of nutrients, and removal of waste.

Regulating matrix degradation

A central feature of several development processes is the ability of cells to degrade and remodel their environment. Susceptibility of the substratum to proteolytic degradation, as well as its capacity to be remodeled, is essential in embryonic development, angiogenesis, ductal formations, and wound healing. In recreating suitable matrices to recapitulate these processes, it is imperative to include components that allow for the natural remodeling of the ECM as seen in the cells' native environment. Efforts by Hubbell and colleagues have led to the development of PEG acrylate polymer scaffolds that are modified with peptide cross linkers.[64,65] The peptide sequences contained in these materials are sensitive to cleavage by specific proteases, such as collegenase and plasmin. By also including cell adhesion proteins, these gels have shown great potential in allowing the migration of smooth muscle cells and fibroblasts.

In a separate approach, Mooney and colleagues explored the influence of matrix degradation rate on myoblast phenotype.[66] Scaffolds were engineered using alginate, and the degradation rate was varied by partial oxidation of alginate before encapsulation of cells. Although the cellular proliferation rate was highest in non-degradable gels, only myoblasts cultured in degradable gels differentiated into multinucleated myofibers. Additional investigation may further clarify the role that scaffold degradation plays on cell differentiation and function.

Beyond degradation of the matrix in the cell microenvironment, the complete removal of scaffolds after the successful *ex vivo* generation of tissue could be advantageous. Scaffold removal would allow for the implantation of generated tissue without the complications associated with physiological responses to the scaffold itself. Pioneering efforts in scaffold removal, or scaffold-less engineered tissues include the work of Okano, Matsuda,[67] Auger,[68] and others.

Incorporation of biochemical signals

Various biochemical cues found in the ECM may include: insoluble components (e.g., laminin, fibronectin); soluble growth factors (e.g., neurotrophin-3, platelet-derived growth factor); and matrix-bound factors (e.g., vascular endothelial growth factor). Inclusion of insoluble biochemical cues is essential for all ECM surrogates. The specific type and concentration of factors should be based on the target cell. Hubbell et al. have constructed fibrin scaffolds while grafting heparin into the matrix during fibrinogen crosslinking.[69,70] Specifically, the transglutaminase enzyme factor XIIIa was used to crosslink individual fibrinogen fibers, and to append a modified heparin protein to the backbone. Natural heparin affinity is then used to bind a number of growth factors, including vascular endothelial growth factor (VEGF), and has been used to guide the morphogenesis of blood vessels. In contrast to the hyperpermeability often observed when free VEGF is used to treat endothelial cells, the low concentrations incorporated into this polymer backbone resulted in the formation of more normal vasculature. Current work by Prestwich has expanded the available ECM scaffold library by incorporating a number of growth factors and cleavable crosslinking agents in hyaluronic acid based matrices.[71–73]

Innovative work by Shoichet and co-workers advanced matrix design to include micropatterned biomolecular gradients into scaffolds for cell guidance.[74] Agarose gels

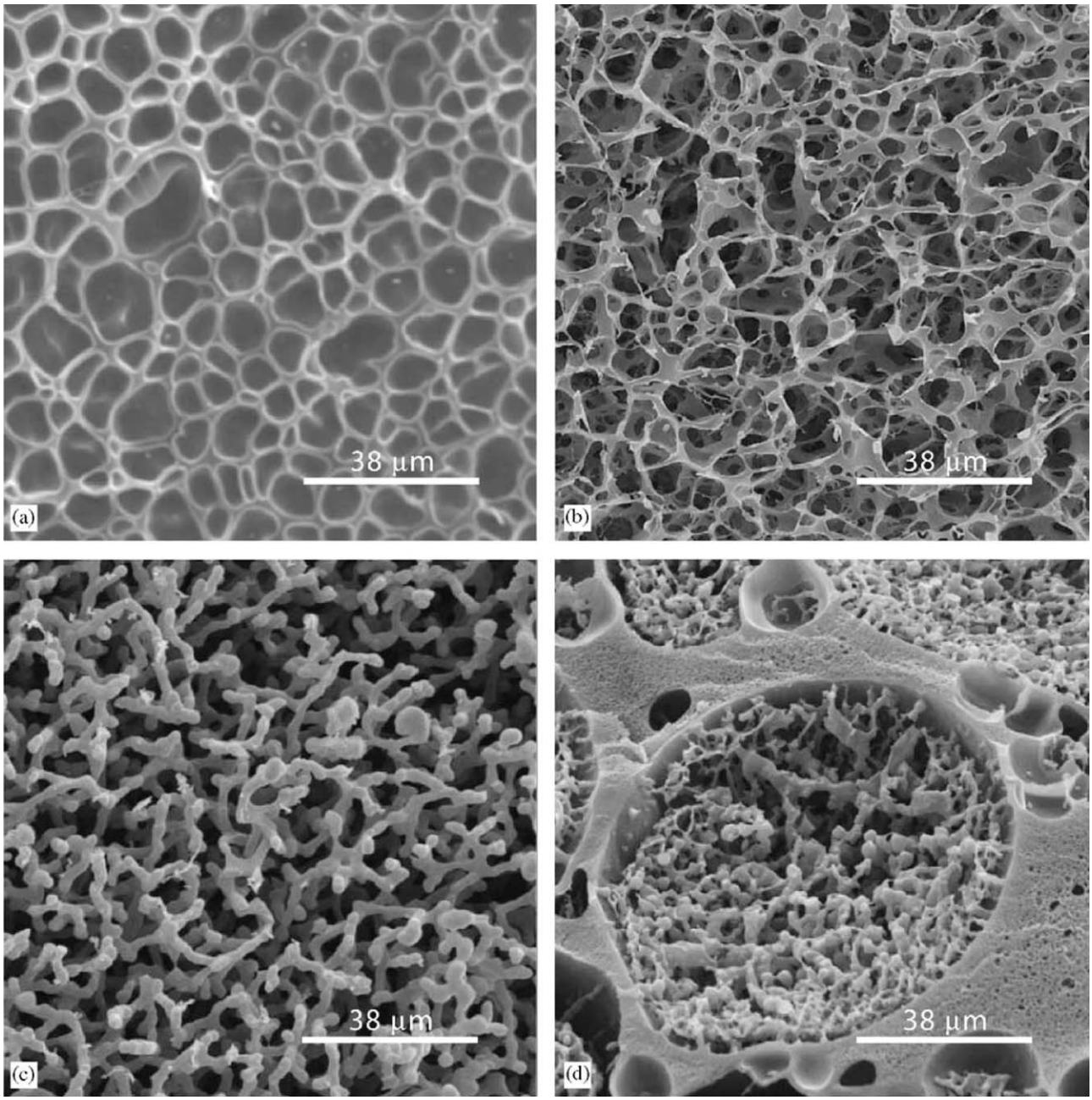

FIGURE 5. Representative scanning electron micrographs of dextran-based scaffolds. Increasing percentages of PEG from 0 to 25 wt %, lead to the formation of several scaffold topologies from microporous, to macroporous gel-wall, to macroporous interconnected-beaded structures. (a) 0 wt %, (b) 2.5 wt %, (c) 5 wt %, and (d) 25 wt %. (Reproduced with permission from Levesque SG, Shoichet MS, Biomaterials, 27, 5277–5285, Elsevier).

modified with a 2-nitrobenzyl protected cysteine yielded free cysteine thiols upon exposure to a conventional He/Ne 325 nm laser source. The exposed thiols reacted readily with maleimide-terminated peptides and proteins, yielding peptide-/protein-modified agarose gels localized throughout specific volumes.[75] The approach has been extended using a two-photon confocal microscope to create more advanced patterned gels, including the production of islands (<20 μm^3) at defined depths that can be linked to create a variety of geometries (Fig. 6). The resulting matrices can be treated with selected proteins and peptides that enhance cell growth and proliferation within complex 3D geometries.[76,77]

An additional example of efforts to control cell fate by the geometric patterning of growth factors has been demonstrated by Ingber and colleagues.[78] Patterned poly(di-methylsiloxane) gels were created by first preparing templates through photolithography of silicone and polymerizing gels to generate defined islands. These specific geometrical polymer islands were then coated with fibronectin, and provided

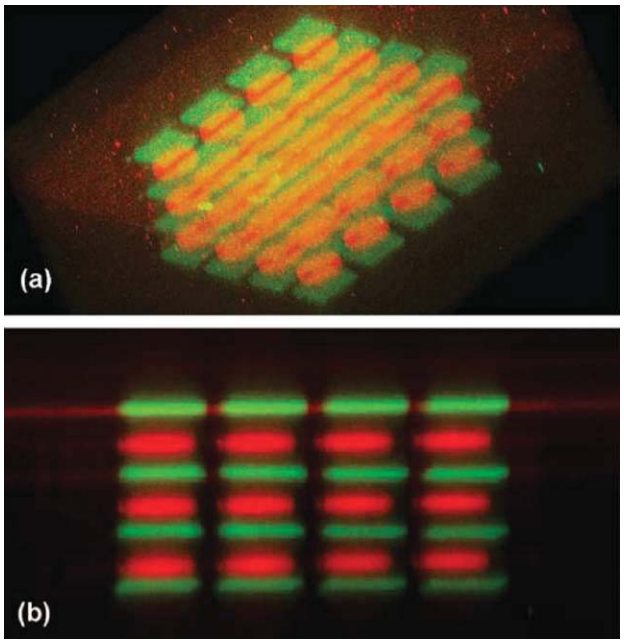

FIGURE 6. (a) Oblique and (b) side view of a hydrogel patterned using muliphoton excitation to activate functional groups. Green squares are 60 × 60 × 18 μm labeled with the green fluorescent dye AF488-Mal. Circles are 50 μm in diameter and are labeled with the red fluorescent dye AF546-Mal. (Reproduced with permission from Wosnick JH, Shoichet MS, Chem Mater 2008, 20, 55–60, American Chemical Society). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

adhesion sites for subsequently treated capillary endothelial cells. The size and shape of immobilized fibronectin islands dictated the degree of cell spreading, and, therefore, guided cell growth or apoptosis.

Multifaceted approaches in developing ECM surrogates

Recent scaffold designs have begun to incorporate both mechanical and biochemical cues to support the phenotypical growth and development of functional tissue. One important aspect of current research is the attention on cell–cell interactions. For example, matrices are being designed to stabilize isolated hepatocytes in co-culture with fibroblasts.[79] The PEG hydrogel-based scaffolds used in these studies consist of photo-patterned adhesive proteins specifically selected based on hepatocyte integrin expression. Patterned hydrogels resulted in higher levels of albumin and urea, indicative of hepatocyte functionality.

In addition to adapting conventional polymers for scaffold synthesis, a number of novel materials are gaining popularity for use as ECM surrogates. Among these are carbon nanotubes,[80] silk nanofibers,[81] and hydrogels.[82] Although many of these materials show promise as the next generation of scaffolds for 3D cell culture, these surrogate extracellular matrices must be tuned to match the mechanical and chemical requirements specific to each target tissue to ensure proper guidance of cell fate.

OUTLOOK

There is significant opportunity to advance the field further through the design of tunable scaffolds and the incorporation of multiple cell-based strategies. For example, little research has succeeded in developing scaffolds with mechanical properties that can be tuned after fabrication. The ability to increase scaffold rigidity after cells have been seeded would allow mechanical properties to be precisely tailored, while avoiding complications during scaffold processing. Another potential improvement would be the capacity to add cells into patterned matrices following scaffold formation. In this manner, sensitive cells would not be exposed to detrimental processes common during scaffold preparation.

Similarly, more research is needed to better understand cell–cell interactions in 3D environments. Further development of co-culture models and methods of cell seeding may improve the use of scaffolds for tissue engineering.

Elucidating the cellular response to environment and application of these mechanisms to the design of biomimetic scaffolds, offers great potential to control stem cell fate and guide tissue regeneration. Techniques that allow for fine tuning of individual aspects of the cellular microenvironment will be essential in developing models to enhance our understanding of the relationship between structure and function and as templates for complex tissues and organs.

ACKNOWLEDGMENTS

The authors are grateful to members of the Shoichet research group for critical review of this manuscript and particularly Karyn Ho for producing Figure 1. They thank NSERC and CIHR for funding through the collaborative health research projects program and the Canada Council for the Arts, Killam Research Foundation.

REFERENCES

1. Schmeichel KL, Bissell MJ. Modeling tissue-specific signaling and organ function in three dimensions. J Cell Sci 2003;116(Part 12): 2377–2388.
2. Cukierman E, Pankov R, Stevens DR, Yamada KM. Taking cell–matrix adhesions to the third dimension. Science 2001;294:1708–1712.
3. Berrier AL, Yamada KM. Cell–matrix adhesion. J Cell Physiol 2007;213:565–573.
4. Lund AW, Yener B, Stegemann JP, Plopper GE. The natural and engineered 3D microenvironment as a regulatory cue during stem cell fate determination. Tissue Eng Part B Rev 2009;15:371–380.
5. Gieni RS, Hendzel MJ. Mechanotransduction from the ECM to the genome: Are the pieces now in place? J Cell Biochem 2008;104: 1964–1987.
6. Prestwich GD. Evaluating drug efficacy and toxicology in three dimensions: Using synthetic extracellular matrices in drug discovery. Acc Chem Res 2008;41:139–148.
7. Griffith LG, Swartz MA. Capturing complex 3D tissue physiology *in vitro*. Nat Rev Mol Cell Biol 2006;7:211–224.
8. Yamada KM, Cukierman E. Modeling tissue morphogenesis and cancer in 3D. Cell 2007;130:601–610.
9. Madurantakam PA, Cost CP, Simpson DG, Bowlin GL. Science of nanofibrous scaffold fabrication: Strategies for next generation tissue-engineering scaffolds. Nanomed 2009;4:193–206.
10. Tibbitt MW, Anseth KS. Hydrogels as extracellular matrix mimics for 3D cell culture. Biotechnol Bioeng 2009;103:655–663.
11. Hoffman AS. Hydrogels for biomedical applications. Adv Drug Deliv Rev 2002;54:3–12.
12. Tsang VL, Bhatia SN. Fabrication of three-dimensional tissues. Adv Biochem Eng Biotechnol 2007;103:189–205.

13. Barnes CP, Sell SA, Boland ED, Simpson DG, Bowlin GL. Nanofiber technology: Designing the next generation of tissue engineering scaffolds. Adv Drug Deliv Rev 2007;59:1413–1433.
14. Shoichet MS. Polymer scaffolds for biomaterials applications. Macromolecules 2010;43:581–591.
15. Kleinman HK, Martin GR. Matrigel: Basement membrane matrix with biological activity. Semin Cancer Biol 2005;15:378–386.
16. Choi JS, Yang HJ, Kim BS, Kim JD, Kim JY, Yoo B, Park K, Lee HY, Cho YW. Human extracellular matrix (ECM) powders for injectable cell delivery and adipose tissue engineering. J Control Release 2009;139:2–7.
17. Laurencin CT, Nair LS. Nanotechnology and Tissue Engineering: The Scaffold. Boca Raton: CRC Press; 2008.
18. Handel TM, Johnson Z, Crown SE, Lau EK, Sweeney M, Proudfoot AE. Regulation of protein function by glycosaminoglycans: As exemplified by chemokines. Annu Rev Biochem 2005;74:385–410.
19. Alison M. The Cancer Handbook. Chichester, West Sussex, England, Hoboken, NJ: John Wiley & Sons; 2007. 1573 p.
20. Dye JF, Lawrence L, Linge C, Leach L, Firth JA, Clark P. Distinct patterns of microvascular endothelial cell morphology are determined by extracellular matrix composition. Endo J Endo Cell Res 2004;11:151–167.
21. Shimizu Y, Rose DM, Ginsberg MH. Integrins in the Immune System. Advances in Immunology, Vol. 72. San Diego: Academic Press Inc; 1999. p 325–380.
22. Schultz GS, Wysocki A. Interactions between extracellular matrix and growth factors in wound healing. Wound Repair Regen 2009; 17:153–162.
23. Bissell MJ. Modeling molecular mechanisms of breast cancer and invasion: Lessons from the normal gland. Biochem Soc Trans 2007;35(Part 1):18–22.
24. van der Flier A, Sonnenberg A. Function and interactions of integrins. Cell Tissue Res 2001;305:285–298.
25. Schwartz MA. Integrin signaling revisited. Trends Cell Biol 2001; 11:466–470.
26. Mooney D, Hansen L, Vacanti J, Langer R, Farmer S, Ingber D. Switching from differentiation to growth in hepatocytes: Control by extracellular-matrix. J Cell Physiol 1992;151:497–505.
27. Nelson CM, Bissell MJ. Of extracellular matrix, scaffolds, and signaling: Tissue architecture regulates development, homeostasis, and cancer. Annu Rev Cell Dev Biol 2006;22:287–309.
28. Streuli CH, Schmidhauser C, Bailey N, Yurchenco P, Skubitz APN, Roskelley C, Bissell MJ. Laminin mediates tissue-specific gene-expression in mammary epithelia. J Cell Biol 1995;129:591–603.
29. Bissell MJ, Radisky DC, Rizki A, Weaver VM, Petersen OW. The organizing principle: Microenvironmental influences in the normal and malignant breast. Differentiation 2002;70:537–546.
30. Zaman MH, Trapani LM, Sieminski AL, Mackellar D, Gong H, Kamm RD, Wells A, Lauffenburger DA, Matsudaira P. Migration of tumor cells in 3D matrices is governed by matrix stiffness along with cell–matrix adhesion and proteolysis. Proc Natl Acad Sci USA 2006;103:10889–10894.
31. Engler AJ, Sen S, Sweeney HL, Discher DE. Matrix elasticity directs stem cell lineage specification. Cell 2006;126:677–689.
32. Saha K, Keung AJ, Irwin EF, Li Y, Little L, Schaffer DV, Healy KE. Substrate modulus directs neural stem cell behavior. Biophys J 2008;95:4426–4438.
33. Leipzig ND, Shoichet MS. The effect of substrate stiffness on adult neural stem cell behavior. Biomaterials 2009;30:6867–6878.
34. Paszek MJ, Zahir N, Johnson KR, Lakins JN, Rozenberg GI, Gefen A, Reinhart-King CA, Margulies SS, Dembo M, Boettiger D. Tensional homeostasis and the malignant phenotype. Cancer Cell 2005;8:241–254.
35. Ulrich TA, Pardo EMD, Kumar S. The mechanical rigidity of the extracellular matrix regulates the structure, motility, and proliferation of glioma cells. Cancer Res 2009;69:4167–4174.
36. McClelland RE, Coger RN. Use of micropathways to improve oxygen transport in a hepatic system. J Biomech Eng Trans Asme 2000;122:268–273.
37. Lenhard RE, Osteen RT, Gansler TS, American Cancer S. Clinical Oncology. Atlanta, GA: American Cancer Society; 2001.
38. Lieberman JR, Friedlander GE. Bone Regeneration and Repair: Biology and Clinical Applications. Totowa, NJ: Humana Press; 2005. 398 p.
39. Oh SH, Park IK, Kim JM, Lee JH. *In vitro* and *in vivo* characteristics of PCL scaffolds with pore size gradient fabricated by a centrifugation method. Biomaterials 2007;28:1664–1671.
40. Bissell MJ, Hall HG, Parry G. How does the extracellular-matrix direct gene-expression. J Theor Biol 1982;99:31–68.
41. Engler AJ, Humbert PO, Wehrle-Haller B, Weaver VM. Multiscale modeling of form and function. Science 2009;324:208–212.
42. Talhouk RS, Chin JR, Unemori EN, Werb Z, Bissell MJ. Proteinases of the mammary gland: Developmental regulation *in vivo* and vectorial secretion in culture. Development 1991;112:439–449.
43. Liu WF, Chen CS. Cellular and multicellular form and function. Adv Drug Deliv Rev 2007;59:1319–1328.
44. Desai RA, Gao L, Raghavan S, Liu WF, Chen CS. Cell polarity triggered by cell–cell adhesion via E-cadherin. J Cell Sci 2009;122: 905–911.
45. Parmar H, Cunha GR. Epithelial-stromal interactions in the mouse and human mammary gland *in vivo*. Endocr Relat Cancer 2004; 11:437–458.
46. Bennett DC, Armstrong BL, Okada SM. Reconstitution of branching tubules from two cloned mammary cell types in culture. Dev Biol 1981;87:193–199.
47. Araque A, Carmignoto G, Haydon PG. Dynamic signaling between astrocytes and neurons. Annu Rev Physiol 2001;63:795–813.
48. Pasti L, Volterra A, Pozzan T, Carmignoto G. Intracellular calcium oscillations in astrocytes: A highly plastic, bidirectional form of communication between neurons and astrocytes in situ. J Neurosci 1997;17:7817–7830.
49. Khetani SR, Bhatia SN. Microscale culture of human liver cells for drug development. Nat Biotechnol 2008;26:120–126.
50. March S, Hui EE, Underhill GH, Khetani S, Bhatia SN. Microenvironmental regulation of the sinusoidal endothelial cell phenotype *in vitro*. Hepatology 2009;50:920–928.
51. Langer R, Vacanti JP. Tissue engineering. Science 1993;260: 920–926.
52. Kuo YC, Ku IN. Cartilage regeneration by novel polyethylene oxide/chitin/chitosan scaffolds. Biomacromolecules 2008;9:2662–2669.
53. Shapira-Schweitzer K, Seliktar D. Matrix stiffness affects spontaneous contraction of cardiomyocytes cultured within a PEGylated fibrinogen biomaterial. Acta Biomaterialia 2007;3:33–41.
54. Xin XJ, Borzacchiello A, Netti PA, Ambrosio L, Nicolais L. Hyaluronic-acid-based semi-interpenetrating materials. J Biomater Sci Polym Ed 2004;15:1223–1236.
55. Legant WR, Pathak A, Yang MT, Deshpande VS, McMeeking RM, Chen CS. Microfabricated tissue gauges to measure and manipulate forces from 3D microtissues. Proc Natl Acad Sci USA 2009; 106:10097–10102.
56. Isenberg BC, DiMilla PA, Walker M, Kim S, Wong JY. Vascular smooth muscle cell durotaxis depends on substrate stiffness gradient strength. Biophys J 2009;97:1313–1322.
57. Anderson DG, Levenberg S, Langer R. Nanoliter-scale synthesis of arrayed biomaterials and application to human embryonic stem cells. Nat Biotechnol 2004;22:863–866.
58. Huang S, Ingber DE. Shape-dependent control of cell growth, differentiation, and apoptosis: Switching between attractors in cell regulatory networks. Exp Cell Res 2000;261:91–103.
59. Levesque SG, Lim RM, Shoichet MS. Macroporous interconnected dextran scaffolds of controlled porosity for tissue-engineering applications. Biomaterials 2005;26:7436–7446.
60. Taboas JM, Maddox RD, Krebsbach PH, Hollister SJ. Indirect solid free form fabrication of local and global porous, biomimetic and composite 3D polymer-ceramic scaffolds. Biomaterials 2003;24: 181–194.
61. Vozzi G, Flaim C, Ahluwalia A, Bhatia S. Fabrication of PLGA scaffolds using soft lithography and microsyringe deposition. Biomaterials 2003;24:2533–2540.
62. Tsang VL, Bhatia SN. Three-dimensional tissue fabrication. Adv Drug Deliv Rev 2004;56:1635–1647.
63. Shin H. Fabrication methods of an engineered microenvironment for analysis of cell–biomaterial interactions. Biomaterials 2007;28: 126–133.
64. Sawhney AS, Pathak CP, Hubbell JA. Bioerodible hydrogels based on phoropolymerized poly(ethylene glycol)-*co*-poly(alpha-hydroxy acid) diacrylate macromers. Macromolecules 1993;26:581–587.

65. Jo YS, Gantz J, Hubbell JA, Lutolf MP. Tailoring hydrogel degradation and drug release via neighboring amino acid controlled ester hydrolysis. Soft Matter 2009;5:440–446.
66. Boontheekul T, Hill EE, Kong HJ, Mooney DJ. Regulating myoblast phenotype through controlled gel stiffness and degradation. Tissue Eng 2007;13:1431–1442.
67. Ohya S, Nakayama Y, Matsuda T. Thermoresponsive artificial extracellular matrix for tissue engineering: Hyaluronic acid bioconjugated with poly(*N*-isopropylacrylamide) grafts. Biomacromolecules 2001;2:856–863.
68. Auger FoA, Berthod Fo, Moulin Vr, Pouliot R, Germain L. Tissue-engineered skin substitutes: From *in vitro* constructs to *in vivo* applications. 2004;39(Part 3):263–275.
69. Schense JC, Hubbell JA. Cross-linking exogenous bifunctional peptides into fibrin gels with factor XIIIa. Bioconjug Chem 1999; 10:75–81.
70. Hubbell JA. Matrix-bound growth factors in tissue repair. Swiss Med Weekly 2006;136:387–391.
71. Vanderhooft JL, Mann BK, Prestwich GD. Synthesis and characterization of novel thiol-reactive poly(ethylene glycol) cross-linkers for extracellular-matrix-mimetic biomaterials. Biomacromolecules 2007;8:2883–2889.
72. Serban MA, Prestwich GD. Modular extracellular matrices: Solutions for the puzzle. Methods 2008;45:93–98.
73. Zhang J, Skardal A, Prestwich GD. Engineered extracellular matrices with cleavable crosslinkers for cell expansion and easy cell recovery. Biomaterials 2008;29:4521–4531.
74. Cao X, Shoichet MS. Photoimmobilization of biomolecules within a 3-dimensional hydrogel matrix. J Biomater Sci Polym Ed 2002; 13:623–636.
75. Wosnick JH, Shoichet MS. Three-dimensional chemical patterning of transparent hydrogels. Chem Mater 2008;20:55–60.
76. Aizawa Y, Leipzig N, Zahir T, Shoichet M. The effect of immobilized platelet derived growth factor AA on neural stem/progenitor cell differentiation on cell-adhesive hydrogels. Biomaterials 2008; 29:4676–4683.
77. Wylie RG, Shoichet MS. Two-photon micropatterning of amines within an agarose hydrogel. J Mater Chem 2008;18:2716–2721.
78. Dike LE, Chen CS, Mrksich M, Tien J, Whitesides GM, Ingber DE. Geometric control of switching between growth, apoptosis, and differentiation during angiogenesis using micropatterned substrates. *In Vitro* Cell Dev Biol Anim 1999;35:441–448.
79. Tsang VL, Chen AA, Cho LM, Jadin KD, Sah RL, DeLong S, West JL, Bhatia SN. Fabrication of 3D hepatic tissues by additive photopatterning of cellular hydrogels. FASEB J 2007;21:790–801.
80. Edwards SL, Werkmeister JA, Ramshaw JAM. Carbon nanotubes in scaffolds for tissue engineering. Exp Rev Med Dev 2009;6:499–505.
81. McClure MJ, Sell SA, Ayres CE, Simpson DG, Bowlin GL. Electrospinning-aligned and random polydioxanone-polycaprolactone-silk fibroin-blended scaffolds: Geometry for a vascular matrix. Biomed Mater 2009;4.
82. Peppas NA, Hilt JZ, Khademhosseini A, Langer R. Hydrogels in biology and medicine: From molecular principles to bionanotechnology. Adv Mater 2006;18:1345–1360.