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Microstructured extracellular matrices in tissue engineering and development

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Microscale heterogeneity in the extracellular matrix (ECM) provides spatial information that allows tissues to develop and function properly *in vivo*. This heterogeneity in composition (chemistry) and structure (geometry) creates distinct microenvironments for the cells that comprise a tissue. In response, populations of cells can coordinate their behaviors across micrometer-to-millimeter length scales to function as a unified whole. We believe techniques to mimic the microscale heterogeneity of the ECM *in vitro* will revolutionize studies that examine how large groups of cells interact. Micropatterned ECMs used for engineering perfused microvascular networks and functional epidermis and for understanding symmetry-breaking events in epithelial morphogenesis illustrate potential applications in tissue engineering and development.

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Introduction

The extracellular matrix (ECM) environments of most tissues and organs are inherently heterogeneous [1]. This heterogeneity, whether chemical or structural in nature, is critical for proper tissue form and function. For example, a dense basal lamina that separates epithelial and endothelial cells from the underlying interstitial ECM is required for correct cellular polarity and differentiation [2]. Likewise, periodic or fractal ECM geometries are needed for efficient transport in absorptive and secretory tissues [3]. For the past thirty years, investigations of the behavior of cultured cells and tissues have relied heavily on the use of homogeneous two-dimensional (2D) and three-dimensional (3D) ECMs. Although these systems have shed light on the basic biology of cell adhesion, the differences between normal and malignant cells, and the mechanisms underlying tissue-specific gene expression [4], they cannot replicate the complex structure of ECMs *in vivo*, in which bends, folds, channels and branches — at the size scale of groups of cells — are plentiful [5]. Thus, the cooperative behavior of cells across large length scales (e.g. in morphogenesis, physiology and tissue engineering) has been nearly impossible to study *in vitro*, other than with explanted tissues.

What if researchers could create artificial ECMs that replicate the heterogeneity of native ones? For example, what if one could engineer a collagen gel so that it contained a branching network that mimicked the scale and shape of actual glands *in vivo*? What studies would these designer ECMs enable? This review describes recent uses of microstructured ECMs (ECMs that possess texture at the 5–1000 μm scale) in tissue engineering and studies of development, and provides evidence that cells grown in these ECMs exhibit unique behaviors not present in homogeneous cultures. Examples of microstructured ECMs designed for microvascular and epidermal tissue engineering and for recapitulation of epithelial development *in vitro* illustrate these ideas.

Why introduce structure into ECMs?

Traditional view: dominance of chemistry

The ECM consists of glycoproteins (such as collagen, fibronectin and laminin), proteoglycans and glycosaminoglycans that undergo self-assembly as well as cell-directed assembly to form a complex organized meshwork [6]. Besides serving as a scaffold to which cells adhere, ECMs act as reservoirs that sequester and release growth factors and other molecules that affect cellular behavior [7]. ECM molecules and their receptors are required during development, because null mutations in either generally lead to embryonic or perinatal lethality, or to severe abnormalities shortly after birth [8,9]. Many ECMs also play central roles in homeostasis [2], wound repair [10], and diseases such as atherosclerosis [11] and cancer [12,13], partly through mechanical alterations [14••].

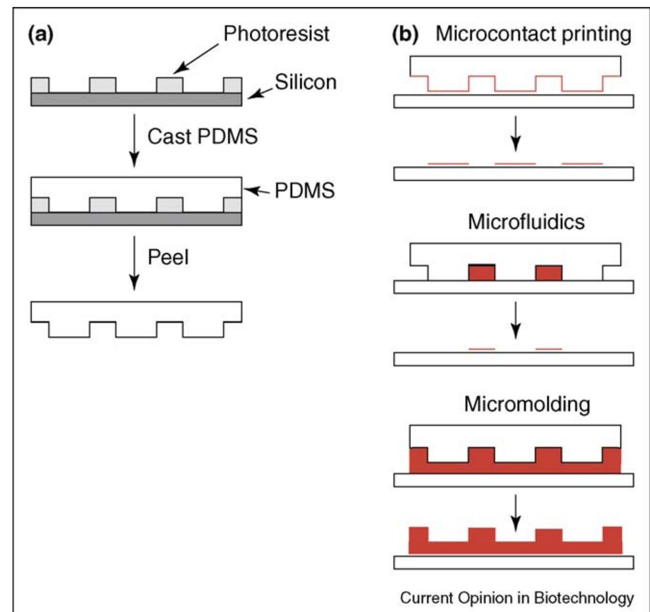
Whereas the composition and structure of the ECM varies depending on the tissue and organ, in general ECMs are compliant viscoelastic materials with bulk properties of hydrogels [15]. Reconstitution of ECMs *in vitro* often proceeds by gelation of a liquid mixture of collagens and other proteins; hence, the final gels are invariably homogeneous at the micrometer scale (although not at the nanometer scale) [16,17]. Extensive studies of cellular behavior in these homogeneous gels have understandably favored the view that biochemical composition plays the dominant role in ECM functionality.

Emerging view: importance of form

In contrast to the bulk hydrogels traditionally used in culture models, ECMs within native tissues contain as much architecture as their constituent cells. Organs are built of tissues in which the cells and ECMs take the form of sheets (e.g. squamous epithelia, basal lamina), tubes (e.g. vessels, bronchioles, glands), branches (e.g. blood and lymphatic vessels, lung, kidney and mammary epithelia), folds (e.g. dermal papillae, intestinal villae), and bends (e.g. vessels).

Form is often viewed as the output of a biological process but it also directly affects cellular behavior, in part by controlling the magnitude and distribution of mechanical stresses within the tissue [18•]. For example, angiogenesis is believed to occur preferentially at the convex walls of microvessels where mechanical forces are often greatest *in vivo* [19]. As we and others have shown through manipulating the shapes of cultured cells, form also exerts a strong effect on cellular behavior *in vitro*: the shape of a cell controls several functions, including proliferation, apoptosis, glucose metabolism, RNA processing, tissue-specific gene expression and differentiation, and stem cell commitment [20–28]. Similarly, individual cells within a contiguous aggregate display different behaviors that result from the interplay between cellular location, overall aggregate shape, and mechanical forces [29••,30,31]. Thus, form can be viewed as an independent determinant of ECM functionality.

Figure 1



Schematic of soft lithography as applied to biological materials. **(a)** Elastomeric poly(dimethylsiloxane) (PDMS) stamps are cured against a photolithographically created silicon master. Peeling the stamp leaves a bas-relief of the original pattern. **(b)** PDMS can then be coated with ECM and stamped against a solid substratum to transfer the protein (microcontact printing), sealed against a solid substratum to create channels into which a liquid solution of ECM is perfused (microfluidics), or used as a mold against which ECM hydrogels are cured (micromolding).

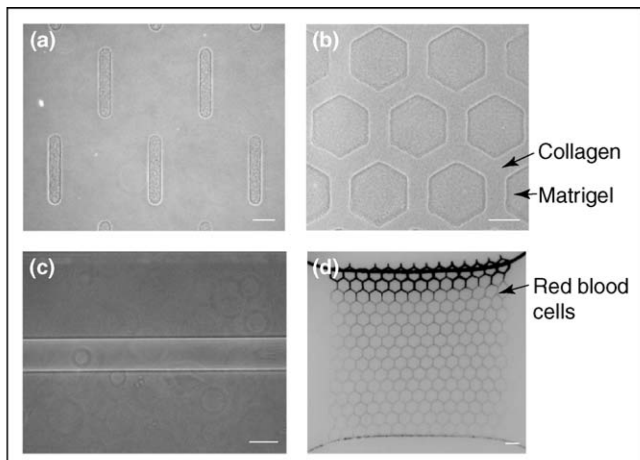
Methods for patterning ECMs

To build *in vitro* systems that faithfully reproduce the structure of tissues probably requires the synthesis of ECMs with microscale heterogeneity. To date, several synthetic schemes have been developed to form 2D patterns of matrix proteins on rigid substrata. Early work used photolithography — a light-based patterning technique akin to high-resolution photography — to indirectly control where proteins could adsorb on glass or silicon [32]. More recently, 'soft' lithographic techniques, originally developed by Whitesides and colleagues [33], that use elastomeric stamps to pattern ECM have grown in popularity, largely because of ease of use (Figure 1). Patterning usually takes place through contact printing or adsorption in microfluidic channels; with stamps that have multiple levels of features, it is possible to generate complex 2D mosaics and gradients of ECMs [34,35]. Because cells grown on these patterned substrata often behave differently from cells in homogeneous monolayer culture, 2D patterns have provided a useful tool for investigating the role of microenvironment in basic cell biology [28].

In contrast to the extensive work in 2D patterning of adsorbed or printed ECMs, only recently have investigators focused on recapitulating the 3D architecture of ECM gels. Initial attempts used light to photopolymerize small organic molecules into hydrogels and required specialized chemistries [36]. To synthesize 3D patterned ECMs consisting of natural proteins, we and others have recently developed several techniques to mold macromolecular gels [37,38•,39]. These methods rely on molds that are treated so that their surfaces are non-adherent; liquid precursors (e.g. an acid extract of type I collagen or cold matrigel) that are gelled against these molds detach easily to yield gels with sharply defined features with $<1\ \mu m$ resolution (Figure 2a) [37].

In addition to introducing surface texture onto a gel, lithographic techniques can be used to form monolithic gels that contain internal surfaces, such as cavities, channels and networks (Figure 2). Formation of internal patterns requires the use of sacrificial materials, which are initially embedded in a gel and then removed to yield an open internal space. Examples of such sacrificial materials include paraffin [40], matrigel [41] and gelatin (AP Golden and J Tien, unpublished). Another strategy for patterning ECMs in 3D relies on stacking or direct ink-jet printing of microstructured gels to form multilayered laminates that localize distinct populations of cells to different planes or areas [37,42]. These methods attempt to build ECMs and tissues layer by layer, and still need to address issues of resolution, speed and alignment.

Figure 2



Images of microstructured gels. **(a)** Arrays of posts in type I collagen. **(b)** Arrays of matrigel embedded in type I collagen. **(c)** A cylindrical channel in fibrin. **(d)** An open network in type I collagen, perfused by a suspension of red blood cells. Scale bars refer to 100 μm in (a–c) and 500 μm in (d). [Image in (b) adapted from [41] with permission].

Much work remains to be done to replicate the full diversity of tissues in these *in vitro* systems. In particular, the engineering of ECMs suitable for modeling tissues with scant stroma and high cellularity (e.g. renal medulla) remains a challenge. Nevertheless, several groups have begun to exploit the unique architectures of these microstructured ECMs for tissue engineering and development.

ECMs for tissue engineering

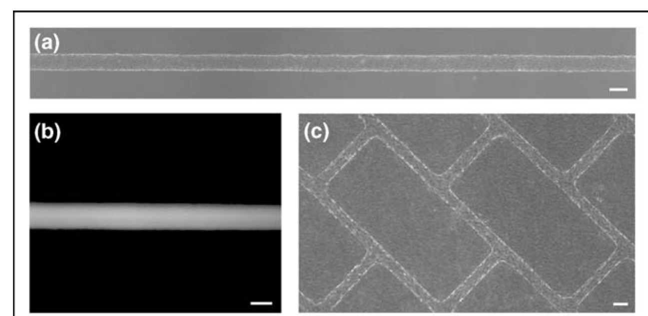
A promising use of patterned ECMs lies in tissue engineering, as shown below by recent examples in engineering functional microvessels and epidermis *in vitro*. Efforts in tissue engineering are currently biased towards designing synthetic biomaterials (e.g. scaffolds) and growth factors, with the expectation that specific molecules can direct cells seeded within them to achieve tissue-specific function and histology [43•,44]. Although this approach can form tissues with simple laminated organization (skin, cornea and arteries), it has difficulty in forming functional complex tissues. For example, vascular endothelial cells seeded within bulk ECM gels or synthetic polymers will form random cords, but these cords do not coalesce into an open microvascular network, even in the presence of large amounts of growth factors [45]. The absence of flow in homogeneous constructs *in vitro* can predispose endothelial cords to apoptosis. As a result, several groups have used various strategies to enhance survival of engineered microvascular tissues, such as transfection with anti-apoptotic genes [46] and introduction of mesenchymal cells [47].

How can microstructured ECMs help in forming functional microvessels? As originally envisioned by Vacanti and colleagues [48], using an ECM with pre-formed channels that could be perfused upon cell seeding would enhance the stability of the construct. This approach provides immediate exposure to shear stress and chemical factors, and thus should avoid the regression observed with cells seeded in bulk gels. Using patterned ECMs, we have recently demonstrated that perfused endothelial tubes remained patent for weeks without any observable changes in cellular organization (Figure 3). These tubes were formed by seeding endothelial cells through ECM gels that have open channels spanning the gels [38•]. Over time, these endothelial tubes developed functional behaviors typical of microvessels *in vivo*, such as barrier function and support of leukocyte adhesion. Thus, the use of microstructured ECMs can enhance the stability and functionality of engineered microvessels.

An appropriate 3D organization appears to play a role beyond simply providing ready perfusion. In fact, perfusing embedded endothelial cells via interstitial flow does not lead to formation of stable vessels (GM Price and J Tien, unpublished). The combination of perfusion and correct polarization, both of which stem from the presence of an open channel, may be required for stable vessels to develop.

Use of microstructured ECMs has also led to enhanced differentiation in engineered epidermis: Toner, Pins and colleagues [49,50•] reasoned that ECMs that mimic the wavy geometry of rete ridges (the interdigitations between epidermis and dermis) should provide seeded keratinocytes with topographic cues present in native skin, and thus should lead to more functional tissue, compared with cells seeded on flat ECMs. To test their hypothesis, they created a synthetic 'basal lamina' by polymerizing collagen or gelatin against an undulating silicone elastomer surface. When cultured on top of these

Figure 3



Images of perfused microvessels and microvascular networks comprised of microstructured type I collagen gels and human dermal microvascular endothelial cells. **(a)** An eight-day-old endothelial tube. **(b)** A solution of fluorescently labeled albumin, perfused through a five-day-old endothelial tube. The cells form a strong barrier that confines the protein to the luminal compartment. **(c)** A six-day-old patterned microvascular network. Scale bars refer to 100 μm.

ECM membranes, keratinocytes conformed to the micrometer scale ridges on the surface and showed enhanced stratification and expression of differentiation markers in deep undulations.

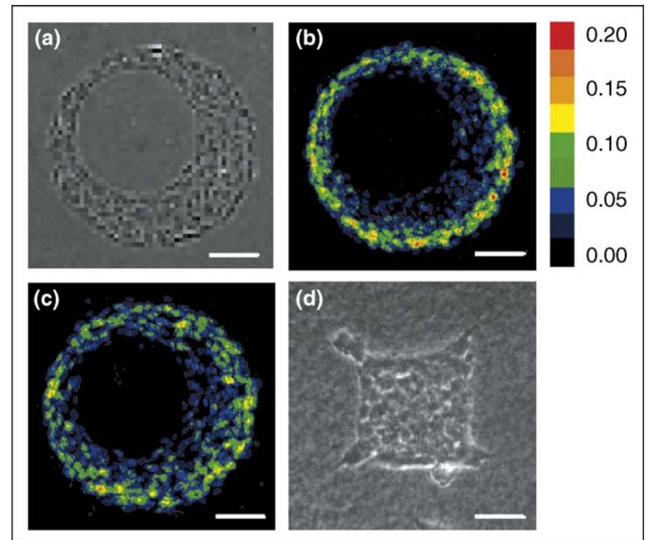
ECMs for the study of development

A second application of patterned ECMs is in the study of tissue development. A better understanding of the mechanisms that direct development and functional differentiation will not only suggest strategies to treat developmental defects, but will also advance efforts to engineer complex, functional tissues. A large subset of tissues and organs — kidney, lung, pancreas and prostate, salivary and mammary glands — develop by a process called branching morphogenesis [51]. This process starts with local invagination of an epithelial sheet to form a primary placode or bud [52]. This primary structure then undergoes reiterative bifurcation and/or lateral branching at non-random sites. The specific distribution of branch sites and lengths is unique for each tissue and generates tissue-specific 3D geometries.

How can microstructured ECMs aid in the study of development? Culture models developed by Bissell and colleagues [53] and *in vivo* studies have established that morphogenesis and functional differentiation of epithelial cells are both critically dependent on the ECM, and have proven the importance of three-dimensionality in biological signaling [54]. In particular, collagen and fibronectin fibrils accumulate at sites of clefting and branching in embryonic salivary gland, lung and kidney [55–59]. Despite the recognition that branching morphogenesis takes place in a complex heterogeneous 3D ECM, the biochemical requirements for branching in the mammary gland have been defined using *in vitro* models of mammary epithelial cells cultured within homogeneous gels of collagen I or matrigel [60–62]. These models have helped determine the signals that are absolutely required for branching to occur. Nevertheless, cells in homogeneous gels branch randomly rather than in the characteristic arborized pattern of the gland *in vivo*. Use of these gels has thus provided a limited understanding of the signals that determine sites of branching in the mammary gland and other organs.

We and others have recently begun to use microstructured ECMs to examine how the placement of epithelial cells can, by itself, control the development of form [18,29••,63]. Numerical simulations and experimental cultures both suggest that the form of the pre-existing tissue instructs several of the symmetry-breaking events during morphogenesis [29••,64], a concept introduced as early as the late 1800s by Wilhelm His [65]. For instance, by culturing cells on large (100–1000 μm) 2D islands of ECM, it was found that whether individual cells within an epithelial sheet proliferate depended on their position within the sheet and its overall geometry [29••]. Slight asymmetry in the shape of the sheet of cells altered the pattern of proliferation (Figure 4). These events require integrity of the cellular sheet, because disrupting connections between the cells leads to unpatterned proliferation. We speculate that, in 3D, these spatial variations induce feed-forward events that magnify and preserve an original slight heterogeneity so that a stable, complex tissue can emerge. To test this hypothesis, we have studied the response of epithelial cells within patterned cavities in collagen gels to morphogens. Stereotyped sites of invasion developed in these 3D cultures, indicating that tissue structure not only affects proliferation, but migration as well (CM Nelson and MJ Bissell, unpublished). These results suggest that the 3D architecture of a tissue can alter further morphogenesis and support the idea that form should be viewed as an independent effector of development. Microstructured ECMs thus allow us to address how form affects its own evolution in the presence of morphogens.

Figure 4



Images of proliferation and invasion in structured ECMs. **(a)** Phase contrast image of cells cultured on 2D pattern of asymmetric (off-center) annulus pattern. **(b)** Colorimetric image of proliferation on 2D asymmetric pattern, generated by stacking images from 50 samples to show the frequency of cells proliferating as a fuction of space. A pixel value of 0.20 indicates that 20% of cells at that location proliferated. **(c)** Abrogation of shape-induced selectivity in proliferation by disruption of cell–cell adhesion using a dominant-negative cadherin construct. Proliferation becomes more uniform across the monolayer with this treatment. **(d)** Epithelial cells cultured within cubic cavities migrate specifically from the vertices. Scale bars refer to 100 μm in (a–c) and 50 μm in (d). [Images in (a–c) reproduced from [29••] with permission, copyright 2005, National Academy of Sciences, USA.]

Conclusions

The development of methods to deliberately introduce microscale variations in culture models has enabled the possibility of testing how ECM aids in the proper

development of tissue form and function. ECM is not simply a glue that binds cells into tissues, nor is it just a reservoir of immobilized growth factors, proteases and matrix proteins. Heterogeneity in the ECM — whether in terms of geometry or chemical composition — appears to provide signals designed to integrate the behavior of populations of cells across large distances. Although in their infancy, the applications of microstructured ECMs to the study of tissue engineering and development seem especially promising, and could one day lead to a rational, quantitative description of morphogenetic processes. In retrospect, it is not surprising that, even in a material as well studied as type I collagen [16], there are ways to enhance functionality simply by adding structure.

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