CHAPTER 7

MECHANOCHEMICAL SWITCHING BETWEEN GROWTH AND DIFFERENTIATION BY EXTRACELLULAR MATRIX

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INTRODUCTION

Tissue engineering has as its main goal the fabrication of artificial tissues for use as replacements for damaged body structures. Great advances have been made in terms of the developing prosthetic devices that can repair structural defects (e.g., vascular grafts) and even replace complex mechanical behaviors (e.g., artificial joints). However, the challenge for the future is to develop tissue substitutes that restore the normal biochemical functions of living tissues, in addition to their structural features. To accomplish this feat, we must first establish precise design criteria for tissue fabrication. These design features should be based on a thorough understanding of the molecular and cellular basis of tissue regulation. They also must take into account the important role that insoluble extracellular matrix (ECM) scaffolds and mechanical stresses play in tissue formation and repair. This latter point is critical since the spatial organization of cells and the mechanical constraints imposed on them as they grow appear to actively regulate tissue development.[1,2]

The goal of this brief chapter is not to provide an extensive review of literature in the field of ECM biology or tissue development. Rather, I will summarize the known functions of ECM and describe some recent insights we have made relating to how ECM regulates cell growth and differentiation during tissue morphogenesis. Our analysis of this regulatory mechanism should be of particular interest to the tissue engineer, because it has led to the identification of critical chemical and mechanical features of ECM that are responsible for control of the growth and function of many cell types. In addition, I hope to introduce the reader to some unanswered puzzles in developmental biology which, if deciphered, could provide powerful new approaches to tissue regeneration and repair.

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EXTRACELLULAR MATRIX STRUCTURE AND FUNCTIONS

Composition and Organization

One of the most critical elements of tissue engineering is the ability to mimic the ECM scaffolds that normally serve to organize cells into tissues. ECMs are composed of different collagen types, large glycoproteins (e.g., fibronectin, laminin, entactin, osteopontin), and proteoglycans that contain large glycosaminoglycan side chains (e.g., heparan sulfate, chondroitin sulfate, dermatan sulfate, keratan sulfate, hyaluronic acid). While all ECMs share these components, the organization, form, and mechanical properties of ECMs can vary widely in different tissues depending on the chemical composition and three dimensional organization of the specific ECM components that are present. For example, interstitial collagens (e.g., types I, III) self assemble into a three dimensional lattice which, in turn, binds fibronectin and proteoglycans. This type of native ECM hydrogel forms the backbone of loose connective tissues, such as dermis. In contrast, basement membrane collagens (types IV, V) assemble into planar arrays; when these collagenous sheets interact with fibronectin, laminin, and heparan sulfate proteoglycan, a planar ECM results (i.e. the "basement membrane"). The ability of tendons to resist tension and of cartilage and bone to resist compression, similarly result from local differences in the organization and composition of the ECM.

In Vivo Foundation for Cell Anchorage

The first and foremost function of ECM in tissue development is its role as a physiological substratum for cell attachment. This feature is easily visualized by treating whole tissues with ECM-degrading enzymes (collagenase, proteases); cell detachment and loss of cell and tissue morphology rapidly result. Cells that are dissociated in this manner can reattach to an artificial culture substrate (e.g., plastic, glass). However, adhesion is again mediated by cell binding to ECM components that are either experimentally immobilized on the culture surface, deposited de novo by the adhering cells, or spontaneously adsorbed from serum (e.g., fibronectin, vitronectin).[3-9] In fact, standard tissue culture plates are actually bacteriological (nonadhesive) plastic dishes that have been chemically treated using proprietary methods to enhance adsorption of serum- and cell-derived ECM proteins. To summarize, cells do not attach directly to the culture substrate (i.e. plastic or glass), rather they bind to intervening ECM components that are adsorbed (or derivatized) to these substrates. For this reason, cell adhesion can be prevented by coating normally adhesive culture surfaces with polymers that prevent protein adsorption, such as poly(hydroxyethylmethacrylate).[10]

Spatial Organizer of Polarized Epithelium

Living cells exhibit polarized form as well as function (e.g., basal nuclei, supranuclear Golgi complex, apical secretory granules in secretory epithelia). Dissociated cells lose this normal orientation when cultured on standard tissue culture substrata or on interstitial connective tissue. In the case of epithelial cells, normal polarized form is often restored if the cells synthesize and accumulate their own ECM or if they are cultured on exogenous basement membrane (i.e. the specialized epithelial ECM).[11-13] These types of studies suggest that basement membrane normally serves to integrate and maintain individual cells within a polarized epithelium. Clearly, there are many intracellular and intercellular determinants of polarized cell form and function

(e.g., cytoskeletal organization, organelle movement, junctional complex formation). However, anchorage to ECM appears to provide an initial point of orientation and stability on which additional steps in the epithelial organization cascade can build. ECM may regulate the orientation of other cell types (e.g., chondrocytes, osteoclasts) as well.

Scaffolding for Orderly Tissue Renewal

All tissues are dynamic structures that exhibit continual turnover of all molecular and cellular components. Thus, it is the maintenance of tissue pattern integrity that is most critical to the survival of the organism. Maintenance of specialized tissue form requires that cells that are lost due to injury or aging must be replaced in an organized fashion. Importantly, orderly tissue renewal has been shown to depend on the continued presence of insoluble ECM scaffoldings which act as templates that maintain the original architectural form and assure for accurate regeneration of pre-existing structures.[14] For example, when cells within a tissue are killed by freezing or treatment with toxic chemicals, all of the cellular components die and are removed, however, the basement membranes often remain intact. These residual ECM scaffoldings assure for correct repositioning of cells (e.g., cell polarity) and restore different cell types to their correct locations (e.g., muscle cells within muscle basement membrane, nerve cells in nerve sheaths, endothelium within vessels, etc.) in addition to promoting the cell migration and growth that are required for repair of all the component tissues (see below). Conversely, loss of ECM integrity during wound healing results in disorganization of tissue pattern, and thus, scar formation. Uncoupling between basement membrane extension and cell doubling also leads to disorganization of tissue morphology during neoplastic transformation.[15]

Establishment of Tissue Microenvironments

Specialized ECMs often establish a physical boundary between neighboring tissues. For example, the basement membrane normally restricts mixing between the epithelium and underlying connective tissue and compromise of basement membrane integrity is indicative of the onset of malignant invasion when seen in the context of tumor formation.[15] The ECM boundary also may regulate macromolecular transport between adjacent tissues given that the basement membrane provides the semi-permeable filtration barrier in the kidney glomerulus.[16] However, little is known about this potential function of the ECM in the local tissue microenvironment

SEQUESTRATION, STORAGE, AND PRESENTATION OF SOLUBLE REGULATORY MOLECULES

ECMs also may modulate tissue growth and morphogenesis through their ability to bind, store, and eventually release soluble regulators of morphogenesis. For example, basic fibroblast growth factor (FGF), a mitogen for fibroblasts, smooth muscle cells, and endothelial cells has been identified within ECMs deposited by cells cultured in vitro[17] and within basement membranes in certain normal tissues (e.g., cornea) in vivo.[18] The low growth rate observed in most normal tissues may result from sequestration of mitogens whereas release of these stored factors (stormones), due to injury or hormonally-induced changes in ECM turnover, may help to switch growth on locally. Binding of other types of regulatory molecules to the endothelial basement membrane (e.g., plasminogen activator inhibitor;[19]) also may play a role in tissue physiology (e.g., blood coagulation, cell migration).

Regulator of Cell Growth, Differentiation and Apoptosis

Most normal (nontransformed) cells only grow when attached and spread on a solid substrate.[10] Cells attach and spread in vitro either by depositing new ECM components or by binding to exogenous ECM.[3-9] In fact, cell spreading and growth can be suppressed by inhibiting ECM deposition in vitro using drugs.[4-6] Cell growth stimulated by soluble mitogens also has been shown to vary depending on the type of ECM component used for cell attachment (e.g., collagen versus fibronectin;[3,8,9]) as well as on the mechanical properties of the ECM (e.g., malleable gel versus rigid ECM-coated dish;[20-24]). Furthermore, the substrates that promote growth tend to suppress differentiation and vice versa. For examples, many cells proliferate and lose differentiated features when cultured on attached type I collagen gels that can resist cell tension and promote cell spreading. In contrast, the same cells cease growing and increase expression of tissue-specific functions (e.g., albumin secretion in hepatocytes, milk secretion and acinus formation by mammary cells, capillary tube formation by endothelial cells) if cultured on the same gels that are made flexible by floating them free in medium or on attached ECM gels that exhibit high malleability (e.g., basement membrane gels, such as Matrigel). Under these conditions, the cells exert tension across their adhesions resulting in contraction of the ECM gel and cell rounding which, in turn, shut off growth and turn on differentiation-specific gene functions. The differentiation-inducing effects of these malleable ECM substrates also can be suppressed by making the gels rigid through chemical fixation,[20,23] thus confirming the critical role of cell-generated mechanical forces in this response.

While local changes in ECM turnover may promote tissue remodeling, large scale breakdown of the ECM may force the same growing tissues to undergo involution. Many cultured cells rapidly lose viability and undergo programmed cell death (i.e., apoptosis) when detached from ECM and maintained in a round form in suspension.[25] Loss of basement membrane integrity is also observed in regions of tissues that are actively regressing[26,27] and growing tissues (e.g., capillaries, mammary gland) can be induced to involute using pharmacological agents (e.g., proline analogues) that inhibit ECM deposition and lead to basement membrane dissolution in vivo.[27-29] Recent transgenic mice studies confirm that growing tissues can be made to involute by shifting the endogenous proteolytic balance such that total ECM breakdown results.[30] These findings suggest that local changes in ECM composition and flexibility may regulate cell sensitivity to soluble mitogens and thereby, control cell growth, viability, and function in the local tissue microenvironment.

PATTERN FORMATION THROUGH ECM REMODELING

Mesenchymal Control of Epithelial Pattern

Probably the greatest insight into the role of ECM in tissue development comes from analysis of embryogenesis. In the embryo, genesis of a tissue's characteristic form (e.g., tubular versus acinar) and deposition of ECM are both controlled by complex interactions between adjacent epithelial and mesenchymal cell societies. The epithelial cell is genetically programmed to express tissue-specific (differentiated) functions and to deposit the insoluble basement membrane which functions as a common attachment foundation that both separates adjacent tissues and stabilizes tissue form.[31,32] However, while production

of tissue-specific cell products (cytodifferentiation) is determined by the epithelium, tissue pattern (histodifferentiation) is usually directed by the surrounding mesenchyme. For example, when embryonic mammary epithelium is isolated and combined with salivary mesenchyme, the mammary epithelial cells take on the form of the salivary gland although they still produce milk proteins.[33] However, the specificity of these epithelial-mesenchymal interactions can vary widely from organ to organ. For instance, embryonic salivary epithelia specifically require salivary mesenchyme for successful development while pancreatic epithelia will undergo normal cytodifferentiation and histodifferentiation in response to mesenchyme isolated from a variety of embryonic tissues.[34]

Tissue Patterning through Localized ECM Remodeling

The complex tissue patterns that are generated through epithelial-mesenchymal interactions result from the establishment of local differentials in tissue growth and expansion in a microenvironment that is likely saturated with soluble mitogens. The classic work on salivary gland development by Bernfield and coworkers revealed that the epithelium imposes morphological stability through production of its basement membrane whereas the mesenchyme produces local changes in tissue form, specifically by degrading basement membrane at selective sites.[31,35-38] An increased rate of cell division is observed in the tips of growing lobules that also exhibit the highest rate of ECM breakdown and resynthesis (i.e., highest turnover rate). At the same time, the mesenchyme slows basement membrane turnover and suppresses epithelial cell growth in the clefts of the glands. This is accomplished by secretion of fibrillar collagens that slow ECM degradation locally and thereby, promote basement membrane accumulation in these regions. Similar local coupling between ECM turnover, cell growth rates, and tissue expansion is observed in many other developing tissues, including growing capillary blood vessels.[39]

It is important to note that increased ECM turnover involves enhanced rates of matrix synthesis as well as degradation. In fact, net basement membrane accumulation must result for epithelial tissues to grow and expand laterally and thus, the local rate of ECM synthesis must be greater than that of degradation in these high turnover regions. If the rate of ECM degradation is significantly greater than synthesis, then net basement membrane dissolution results. As described above, this would lead to cell death and tissue regression rather than expansion.

Role of Mechanical Stresses

Before ending the discussion of the role of ECM in pattern formation, it is critical to emphasize that while chemical regulators mediate tissue morphogenesis, the signals that are actually responsible for dictating tissue pattern are often mechanical in nature. The pattern-generating effects of compression on bone, shear on blood vessels, and tension on muscle are just a few examples. Mechanical stresses are also important for embryological development, however, internal cell-generated forces appear to play a more critical role. For example, mechanical tension that is generated via actomyosin filament sliding within the cytoskeleton of the cells that compose the embryo play a key role during gastrulation.[40] In fact, the pattern of development can be experimentally altered by applying external stresses to the embryo using micropipettes.[41] Tensile forces that are generated internally within mesenchyme and transmitted across ECM are also likely required for

the "condensation" of mesenchyme that commonly precedes formation of new organ rudiments. In this context, it is interesting to note that the pattern-generating capabilities of mesenchyme isolated from different developing tissues have been shown to correlate with differences in their ability to exert mechanical tension on external substrates (e.g., microbeads;[42]). More in-depth discussion of the role of cell-generated mechanical stresses in embryogenesis and wound healing can be found elsewhere.[1,2]

MECHANOCHEMICAL SWITCHING BETWEEN GROWTH AND DIFFERENTIATION

Given the pivotal role that ECM plays in tissue development, we set out to analyze how changes in cell-ECM interactions might act locally to regulate cell sensitivity to soluble mitogens and thereby, establish the growth differentials that are required for tissue morphogenesis. To accomplish this, we developed a simplified in vitro model system which retained only the minimal determinants necessary for maintenance of the physiological functions of interest (i.e., cell growth and differentiation).[43-45] To determine the effects of varying cell-ECM contacts directly, we pre-coated bacteriological Petri dishes that were otherwise nonadhesive with different densities of purified ECM molecules, such as fibronectin, laminin, or different collagen types. Quiescent, serum-deprived cells were plated on these dishes in chemically-defined medium that contained a constant and saturating amount of soluble growth factor.

Initially, we used capillary endothelial cells and found that DNA synthesis and cell doubling rates increased in an exponential fashion as the density of immobilized ECM ligand was raised and cell spreading was promoted.[43,44] When higher cell plating numbers were used to promote cell-cell interactions as well as cell-ECM contact formation, the capillary cells could be switched between growth and differentiation (capillary tube formation) in the presence of saturating amounts of soluble mitogen (FGF) simplifying by varying the ECM coating density.[44] Specifically, when plated on a high ECM density (e.g., > 500 ng/cm^2 fibronectin), the cells attached, spread extensively, formed many cell-cell contacts, and organized into a planar cell monolayer. When the same cells were plated on a low ECM density (< 100 ng/cm^2), the cells attached but they could not spread and thus, only cell clumps or aggregates were observed. When the same capillary cells were plated on a moderate density, cells first attached, spread, and formed cell-cell contacts as they did on the higher ECM densities. However, the tensile forces generated by the cells appeared to overcome the resistance provided by their relatively weak ECM adhesions and thus, the cell aggregates began to retract over a period of hours until a mechanical equilibrium was attained. Under these conditions, formation of an extensive network comprised of interconnected capillary tubes resulted. Many of these capillary tubes became elevated above the culture surface, although the network remained adherent to the culture dish at discrete points through interconnected multicellular aggregates. The importance of mechanical forces for this switching between growth and differentiation was confirmed by demonstrating that similar capillary tube formation could be induced on the high ECM density that normally promoted spreading and growth, simply by increasing the cell plating numbers and thereby amplifying the level of cell tension.

More recently, we have used this system to demonstrate similar shape (stretch)-dependent switching between growth and differentiation in other cell types. For example, we were able to show that the

growth and differentiation of primary rat hepatocytes could be controlled independently of cell-cell contact formation by varying cell-ECM contacts and cell spreading using the method described above.[45] Additional studies confirmed that ECM exerts its regulatory effects at the level of gene expression[45,46] and that these effects are mediated at least in part through modulation of the cytoskeleton.[47] These results are consistent with those from other laboratories which demonstrate that malleable ECM gels (e.g., Matrigel, native collagen gels) that promote cell rounding also induce differentiation and suppress growth whereas the opposite effects are produced when these gels are fixed and made rigid.[20-24]

Altering cell-ECM contacts by varying ECM coating densities appears to influence cell function via two distinct, but integrated, mechanisms. First, increasing the local density of immobilized ECM ligand promotes clustering of transmembrane ECM receptors on the cell surface which are known as "integrins".[48] We found that integrin clustering, in turn, activates a number of different chemical signaling pathways (e.g., tyrosine phosphorylation, inositol lipid turnover, Na^+/H^+ exchange) that are also utilized by growth factor receptors to alter cellular biochemistry and gene expression.[49-52] Activation of these signaling pathways likely plays an important role in control of cell differentiation and survival, however, integrin-dependent chemical signaling alone is not sufficient to explain how cells are induced to enter S phase and proliferate.[43,46] A second mechanism that involves tension-dependent changes in cell shape and cytoskeletal organization also comes into play.

To demonstrate the importance of cell shape directly, we adapted a technique for forming spontaneously assembled monolayers (SAMs) of alkanethiols[53] to create micropatterned surfaces containing adhesive islands with defined shape and position on the cell (micrometer) scale.[54] This method initially involves fabrication of an flexible elastomeric stamp that exhibits the particular surface features of interest using photolithographic techniques. The topographic high points on the stamp (e.g., 60 x 60 μm plateaus raised above recessed intervening regions) are coated with an alkanethiol ink and the stamp is then apposed to a gold-coated surface. The alkanethiol forms SAMs covering only the regions where the stamp contacts the surface (i.e., 60 x 60 μm squares). Then the surrounding uncoated regions are filled with a SAM composed of similar alkanethiols that are conjugated to poly(ethylene glycol) (PEG) that prevents protein adsorption. The result is a chemically-defined culture surface that is completely covered with a continuous SAM of alkanethiols. However, this surface contains local adhesive islands of defined geometry that support protein adsorption surrounded by nonadhesive (PEG-covered) boundary regions that do not. These substrates were then coated with a high density of purified ECM protein, such as laminin. Using this technique, cell position and shape could be precisely controlled because the cells only attach to the ECM-coated adhesive islands. In fact, we could even engineer square and rectangular cells exhibiting 90° corners using this approach.[54]

Using this micropatterning method, we then asked if cells are restricted to a small size similar to that produced on a low ECM coating concentration, but the local density of immobilized integrin ligand is increased 1000-fold, which is the critical functional determinant: the ECM density or cell shape? The answer was that it was cell shape. Cells remained quiescent on these small adhesive islands coated with a high ECM density, even though the cells were stimulated with high concentrations of soluble growth factors.[54] Furthermore, cell growth

(DNA synthesis) increased in parallel as the size of the adhesive island was increased, thus confirming that cell shape was the critical governor of this response. Furthermore, inhibition of hepatocyte growth on the small islands was accompanied by a concomitant increase in albumin secretion. Thus, cell shape and function could be engineered simply by altering the geometry of the adhesive substrate.

SUMMARY

In summary, our work has shown that the development of functional tissues, such as branching capillary networks, requires both soluble growth factors and insoluble ECM molecules. The ECM appears to be the dominant regulator, however, since it dictates whether individual cells will either proliferate, differentiate, or die locally in response to soluble stimuli. This local control mechanism is likely critical for the establishment of local cell growth differentials that mediate pattern formation in all developing tissues.

Analysis of the molecular basis of these effects revealed that ECM molecules alter cell growth via both biochemical and biomechanical signaling mechanisms. ECM molecules cluster specific integrin receptors on the cell surface and thereby activate intracellular chemical signaling pathways,[49-52] stimulate expression of early growth response genes (e.g., *c-fos, jun-B*),[46] and induce quiescent cells to pass through the G_0/G_1 transition. However, in addition, the immobilized ECM components must physically resist cell tension and promote changes of cell shape[43-45,54] and cytoskeletal organization[47,55] in order to promote full progression through G_1 and entry into S phase. Studies with living and membrane-permeabilized cells confirm that changes in cell shape result from the action of mechanical tension which is generated within microfilaments and balanced by resistance sites within the underlying ECM.[1,2,56] Taken together, this work suggests that the pattern-regulating information ECM conveys to cells is both chemical and mechanical in nature. Thus, design of future artificial ECMs for tissue engineering applications must take into account both of these features.

THE FUTURE

Early tissue engineering efforts by reconstructive surgeons and material scientists started with a knowledge of the clinical need and of the mechanical behavior of connective tissues on the macroscopic scale and worked backwards. The long term goal for the field is to design and fabricate tissue substitutes starting from first principles. This include mechanical principles as well as an in depth understanding of the molecular and biophysical basis of tissue regulation. Clearly, given the potent and varied functions of the ECM, fabrication of artificial ECMs will play a central role in all of these future efforts. We and others have already begun to explore the utility of synthetic bioerodible polymers as cell attachment substrates,[57,58] and the potential usefulness of immobilized synthetic ECM peptides for controlling cell growth and function.[46,59] These materials offer a major advantage in terms of biocompatibility since the artificial substrates completely disappear over time and thus, the implanted donor cells become fully incorporated into the host. They also provide great chemical versatility as well as the potential for large scale production at relatively low cost. In addition, use of synthetic chemistry reduces the likelihood of batch to batch variation during large-scale production, a problem which can potentially complicate use of purified ECM components. For these reasons, polymer chemistry and novel fabrication techniques will likely lead to development of more effective tissue substitutes.

However, if we understood the fundamental principles that guide ECM remodeling and pattern formation in tissues, perhaps tissue engineering might take a different approach in the future. For example, one could envision entirely new methods of clinical intervention if we understood how tissue-specific mesenchyme generates tissue pattern; how ECM turnover is controlled *locally*; or how compressing or pulling tissues alter their growth and form. This knowledge could lead to methods for identifying and isolating relevant "pattern-generating" cells; for developing pharmacological modifiers of ECM remodeling that may be incorporated in local regions of implants to promote or suppress tissue expansion locally; and for fabricating artificial ECMs with the correct mechanical properties to switch on or off the function of interest (e.g., growth vs. differentiation or apoptosis) at a particular time or place. These are the just a few of the challenges for the future.

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