Control of Capillary Growth and Differentiation by Extracellular Matrix*

Use of a Tensegrity (Tensional Integrity) Mechanism for Signal Processing

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This article reviews the mechanism by which extracellular matrix (ECM) molecules regulate cell growth and differentiation during tissue development. ECM is not a newcomer to the field of morphogenesis. It is well accepted that both basement membranes and collagenous stroma play key roles during tissue formation. However, it is unclear where in the morphogenetic cascade ECM molecules exert their effects and, more important, how matrix molecules convey regulatory information that can alter cell function. These questions serve as the focus for this article.

For the purpose of this review, I will concentrate on the process of capillary development, or what is known as "angiogenesis." Angiogenesis is an excellent system for analysis of the molecular basis of morphogenesis because isolated capillary endothelial cells retain the ability to reorganize into branching tubular networks when cultured *in vitro*.[1] Also, many *in vivo* neovascularization models exist, such as the rabbit cornea and chick chorioallantoic membrane, that can be used to determine the physiologic relevance of results obtained from studies with cultured cells. Further, angiogenesis is an important process to study because knowledge of the mechanism of capillary growth control could have widespread clinical applications. For example, angiogenic stimulators might be useful in clinical situations where healing is limited by insufficient vascular ingrowth (*eg*, wounds, burns, skin ulcers, myocardial infarcts). Angiogenesis inhibitors also might be used to treat diseases such as cancer that depend on sustained neovascularization for their continued growth and development. Interestingly, one of the first examples of angiostatic therapy was recently introduced into pulmonary medicine: interferon-alpha-2a was successfully used to induce regression of vascular lesions in patients with pulmonary capillary hemangiomatosis.[2] Our hope is that similar types of antiangiogenic therapies may become common in the future.

CONTROL IN THE LOCAL TISSUE MICROENVIRONMENT

In general, past research on the mechanism of tissue development focused on the identification of soluble growth factors that can initiate or trigger tissue morphogenesis. This was especially true in the field of capillary development. Enormous progress has been made in this area in the past 10 yr, with many different "angiogenic factors" now sequenced and cloned.[3] One of the best characterized angiogenic molecules is the heparin-binding mitogen, basic fibroblast growth factor (FGF). FGF has been shown to stimulate capillary endothelial cell growth *in vitro* and neovascularization when implanted *in vivo*.[4,5]

Nevertheless, the growth-promoting action of soluble angiogenic factors such as FGF is not sufficient to explain how functional capillaries develop. For example, for a capillary branch to form in response to angiogenic stimulation, some mechanism must exist whereby 1 or 2 endothelial cells along the preexisting vessel can respond to angiogenic stimulation by growing while neighboring differentiated endothelial cells, only microns away, remain quiescent (Fig 1). The same growth stimulation without any additional regulatory controls would produce a disorganized cluster of cells. Furthermore, the observation that growing capillary sprouts, quiescent differentiated tubes, and regressing vessels all coexist within the same microenvironment during later stages of capillary development[6] indicates that once again, endothelial cell sensitivity to angiogenic factors must vary greatly over small distances. Establishment of local differentials of cell growth and differentiation serves as the basis for pattern formation in all developing systems.

How can cell growth and differentiation be selectively turned on and off in a tissue that is saturated with soluble mitogens? Our first clue that ECM may play a part in this local control mechanism came from review of past studies which analyzed the process of capillary branch formation in more detail.[7,8] Local breakdown of basement membrane (the specialized capillary ECM) was found to be one of the first events occurring during initiation of angiogenesis (see Fig 1). ECM-degrading enzymes most likely mediate this proc-

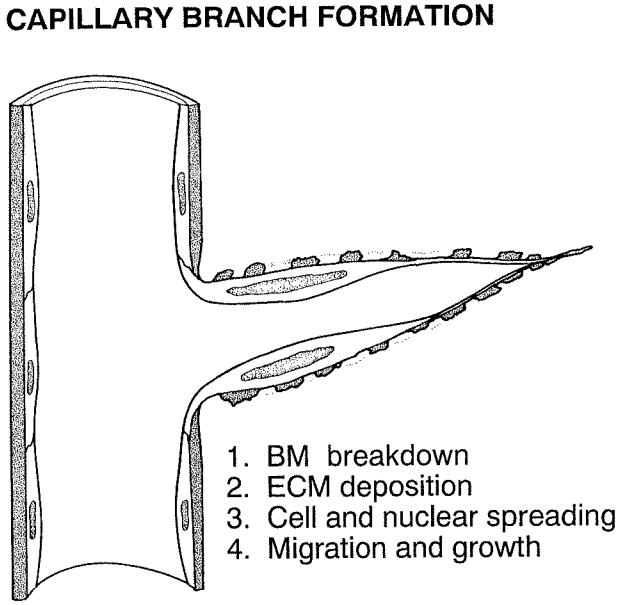

FIGURE 1. Local control of endothelial cell responsiveness to soluble mitogens during capillary branch formation.

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ess since FGF also stimulates production of plasminogen activator and collagenases by endothelial cells *in vitro*.[9] Once a hole is formed in the basement membrane, neighboring endothelial cells (as well as their nuclei) greatly increase in size as the cells extend long processes into surrounding connective tissue. Cell spreading as well as subsequent migration and growth appear to be mediated by *de novo* deposition of ECM molecules.[10,11] This supposition is supported by immunofluorescence studies that clearly delineate a rudimentary ECM scaffolding along the endothelial cell-stromal interface at all times during capillary development.[12-14] Finally, remodeling of this ECM scaffolding into an intact basement membrane (*ie*, formation of a continuous basal lamina at the electron microscopic level) correlates closely with cessation of growth and formation of functional capillary tubes. Accumulation of intact basement membrane also may play a central role during establishment of cell polarity within the forming endothelium.[15]

Mechanochemical Switching Between Growth and Differentiation

Are changes of ECM composition or integrity responsible for the observed alterations of endothelial sensitivity to soluble mitogens? To answer this question, we sought to devise an *in vitro* angiogenesis model in which cell-ECM interactions could be controlled in a defined manner.[16] In these studies, which were performed in collaboration with Dr Judah Folkman, capillary endothelial cells were plated at high density (2-5 x 10^4 cells/cm^2) on nonadhesive bacteriologic dishes precoated with defined densities of purified ECM molecules such as fibronectin (FN) or type IV collagen. All experiments were carried out in chemically defined, serum-free medium consisting of Dulbecco's modified eagle medium supplemented with transferrin (5 μg/ml), high-density lipoprotein (10 μg/ml), and a saturating amount of recombinant FGF (2 ng/ml). Defined medium was used because the presence of certain serum components (*eg*, FN, vitronectin, different growth factors) would greatly complicate this type of study.

Using this approach, we found that we could selectively switch capillary endothelial cells between programs of growth and differentiation in the presence of a soluble mitogen (FGF) simply by varying the ECM coating density (Fig 2, top). On high FN densities (>500 ng/cm^2), cells spread extensively and formed many cell-cell contacts. Cells exhibited high growth rates on these highly adhesive dishes, but tubes were never observed. When cells were plated at similar densities on low FN densities (<100 ng/cm^2), individual cells attached and formed cell-cell contacts; however, they could not spread, and therefore they remained as round aggregates throughout the course of the experiment. No tubes were observed, and none of these cells entered S phase when analyzed by ^{3}H-thymidine autoradiography even though they were exposed to the same concentration of FGF. When cells were plated on a moderate FN density (100-500 ng/cm^2), cells initially extended and formed multiple cell-cell contacts over the first 6 h of culture. However, this was followed by multicellular retraction in the next 18 to 36 h, culminating in formation of tubular networks that often covered the entire surface of the 35-mm culture dish. Interestingly, cells within retracted tubes ceased growing, whereas neighboring cells that remained adherent and spread on the dish continued to synthesize DNA. Thus, the mechanical alterations that turned on capillary differentiation also switched off growth and induced quiescence locally. In other words, using substrata of varying mechanical integrity, we were able to establish local differentials of capillary endothelial cell growth *in vitro* which were similar to those previously observed *in vivo*.

These results suggested that tensile forces generated within the intracellular cytoskeleton of individual cells and exerted on extracellular attachment sites (*ie*, both on ECM

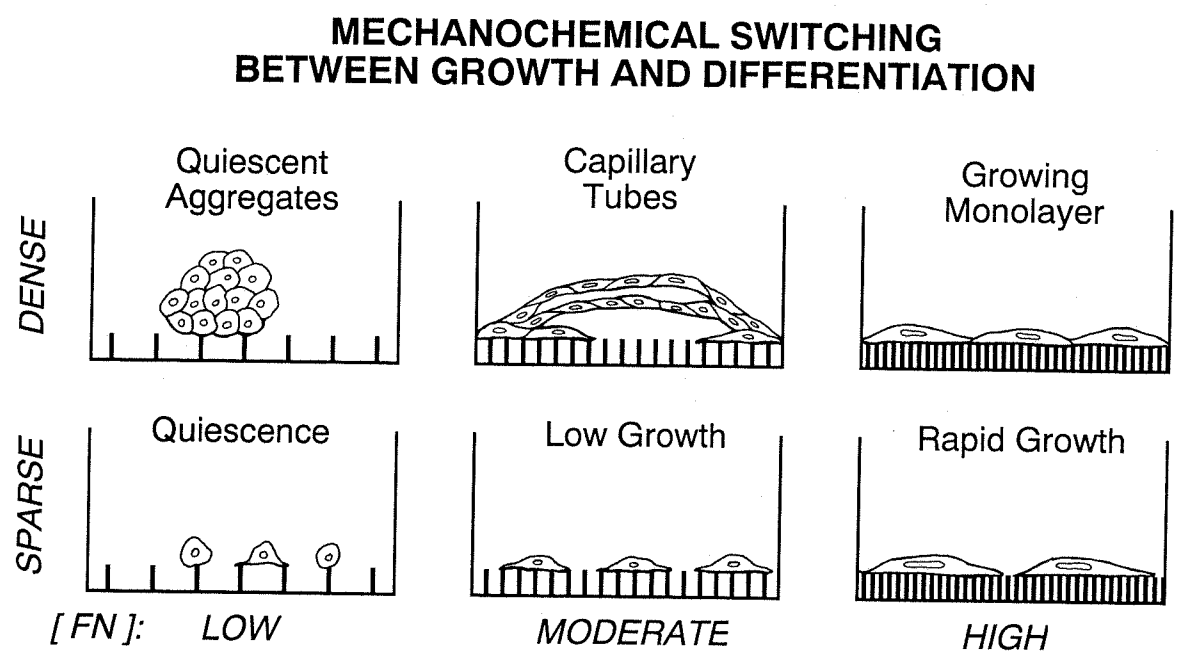

Figure 2. Mechanochemical switching between growth and differentiation in cultured capillary endothelial cells.

and on neighboring cells) may regulate capillary organization. Highly adhesive dishes effectively resisted these forces and promoted both cell spreading and growth. Low FN densities, which were only minimally adhesive, could not resist the forces generated by large multicellular clusters and thus these aggregates often completely detached themselves from the surfaces of the dishes. In contrast, moderately adhesive dishes both supported cell extension and permitted partial multicellular retraction. Tube formation resulted when the forces exerted by the cell aggregates were balanced by an ECM density that could provide appropriate mechanical resistance. The importance of this balance of forces was made clear in an experiment in which we used higher cell plating numbers to overcome the inhibitory effects of the high FN coating density. Under these conditions, adherent monolayers formed as well as groups of overlying cells that only contacted and exerted tractional forces on underlying cells. This approach resulted in amplification of cell-generated forces (much as the alignment of muscle cells does in muscle) while the mechanical integrity of the adhesive substratum was held constant. Accordingly, this cell multilayer retracted until a new balance of forces was regained. In the case of capillary endothelial cells, this equilibrium form is manifested as a branching tubular network. Interestingly, this appears to be specific for endothelial cells. Although other cells types, such as hepatocytes, increase their differentiated function (*eg*, secretion of albumin) when allowed to retract on these dishes, they do not form tubes.

ECM Molecules Control Cell Growth by Modulating Cell Shape

What kind of mechanism could explain how this might work? One major alteration that was induced by multicellular retraction was a change of cell shape. Cells tended to shorten and become more round when they reorganized into tubes. Thus, we first addressed the question of whether these tension-dependent changes of cell shape were the cause of this local growth differential. To do this, we cultured the cells using the same FGF-containing medium and ECM-coated dishes as we had used previously. However, lower cell plating numbers were utilized so that we could separate the effects of ECM from those induced by cell-cell contact formation (Fig 2, bottom). Using this approach, we found that endothelial cell sensitivity to the proliferative effects of FGF increased in an exponential fashion as FN coating densities were raised and cell spreading was promoted.[17] Similar results were obtained using increasing concentrations of soluble RGD-peptides to progressively dislodge cell surface integrin receptors from their FN points of attachment and thereby progressively induce cell retraction. Furthermore, cells could not grow in the presence of either high concentrations of soluble FN (which can bind cell surface integrin receptors) or FN-coated microbeads (which both bind and cluster integrins), if cell spreading was prevented. Taken together these results suggest that it is the extent to which an endothelial cell stretches that determines its ability to enter S phase when stimulated by FGF.

Growth Signaling Across Transmembrane Integrin Receptors

How might FN stimulate cell growth by promoting cell extension? The first explanation that commonly comes to mind is that spreading of the cell surface results in exposure of increased numbers of FGF receptors. However, this does not occur. FN-dependent changes of cell shape do not alter the number of cell surface FGF receptors or change their affinity. Another possibility is that ECM molecules, such as FN, directly activate intracellular chemical signaling pathways similar to those used by soluble mitogens as a result of binding to ECM receptors on the cell surface (*eg*, members of the integrin class of transmembrane receptors). Working in collaboration with Dr Martin Schwartz, we have recently found that this is indeed the case. FN regulates growth by activating the cell surface Na^+/H^+ antiporter and raising intracellular pH in capillary endothelial cells.[18] Interestingly, activation of the Na^+/H^+ antiporter appears to be a common signaling pathway that is shared by most, if not all, peptide growth factors. However, it is not known how antiporter activation stimulates cell cycle progression.

Recently we have been able to separate the effects of FN on the antiporter from its effect on cell shape. Experiments with FN-coated microbeads (4.5 μm diameter), integrin chain-specific antibodies, and Fab fragments of these antibodies revealed that clustering of the β1 and α5 integrin chains of the FN receptor is sufficient to activate the Na^+/H^+ antiporter in round cells.[19] However, while integrin clustering can promote endothelial cell cycle progression through early G1 (as indicated by activation of the Na^+/H^+ antiporter), it is not sufficient to promote entry into S phase. As described above, endothelial cells will not grow when bound to FN-coated microbeads, if spreading is prevented. Thus, large-scale changes of cell shape also appear to be required for cells to progress further through G1 and into S.

Mechanical Signal Processing via a Tensegrity Mechanism

How could changing cell shape or physically stretching a cell alter its growth rate? One possibility is that ECM molecules convey regulatory information by activating a mechanical or structural signaling system inside the cell.[20] This hypothesis emerged from studies with three-dimensional cell models built of sticks and elastic string that were constructed according to the rules of an architectural system that is based on tensional integrity and for this reason has been termed "tensegrity." As first defined by Buckminster Fuller,[21] a tensegrity structure is a system comprised of compression-resistant elements that need not physically touch; rather, they are pulled up and open by a *continuous* series of tension elements. This is a highly efficient architectural system because mechanical loads are distributed evenly over *all* of its structural elements. This situation is in direct contrast to most man-made structures, which commonly have many structural elements that are not load-bearing (*eg*, office walls in a skyscraper). Importantly, cell models may be built using this system (Fig 3) because cells, like tensegrity structures, generate tensile forces within their continuous cytoskeleton and maintain structural integ-

rity independent of gravity. The tensegrity cell models and the theory behind the use of a cellular mechanism for transducing mechanical signals into chemical information in cells and tissues have been previously described.[20,22-26]

An important implication of the tensegrity paradigm is that cell shape is determined through a dynamic balance between mechanical forces that are generated within the intracellular cytoskeleton and resisted by extracellular forces at points of attachment to ECM as well as to other cells. Tensegrity structures, much like cells, change shape and take on new minimum energy forms, depending on the adhesivity and relative rigidity of the attachment substratum (see Fig 3). Both cells and tensegrity structures spread on highly adhesive rigid foundations, yet they physically retract when plated on malleable substrata that cannot resist cell-generated mechanical loads (*eg*, Silastic rubber, collagen gels, Matrigel). Adherent tensegrity models also spontaneously round up and "leap off the dish" when their attachments are released, just as cultured cells do after the addition of trypsin or soluble RGD-containing peptides.[17]

Additional studies with "nucleated" tensegrity cell models demonstrated that the shape of both the cell and nucleus will extend in a coordinated fashion when the cell is allowed to spread on a rigid foundation.[20,23] Key to this coordination is the presence of a continuous series of structural interconnections stretching from the cell surface to the tensegrity nucleus (additional tensile threads were used for this purpose in our models). The same coordination between cell and nuclear structure can be seen in living endothelial cells extending on ECM-coated dishes[27] as well as in epithelial tumor cells spreading on intact basement membrane.[15] Additional studies with the nucleated cell models revealed that when the cell's attachment points are released, both the tensegrity cell and its nucleus retract and take on more rounded forms. However, nuclear retraction lags behind cell retraction owing to the elastic quality of the filamentous network that connects the nucleus to the cell periphery. Living endothelial cells exhibit the same behavior during trypsinization.[23] First the cell periphery retracts, then a few seconds later the nucleus begins to shrink, with full retraction occurring within seconds. Thus, both living cells and nuclei, much like tensegrity structures, exist in a state of isometric tension and have a large amount of potential energy.

These results suggest that a structural system may exist within cells for direct communication of regulatory information, in the form of mechanical forces, from the cell surface to the nucleus. But do cells utilize a tensegrity mechanism for organizing their cytoskeleton? This point awaits experimental confirmation. However, data from many studies are consistent with this model. For example, Heidemann and co-workers have shown that the shape of PC12 neurites is determined through a balance of mechanical forces that are generated within contractile microfilaments and resisted by both intracellular microtubules and extracellular attachment points.[28,29] Localized tension and compression elements also have been shown to mediate shape determination in retinal photoreceptors[30] and in plant protoplasts.[31] In support of a tensegrity mechanism for regulation of nuclear structure, recent studies show that intermediate filaments physically interlink the nucleus with the cell periphery[32,33] and that nuclear pore complexes contract when cells round up and become quiescent.[34,35] The tensegrity concept is also consistent with the loss of structural regulation that is observed during cancer development.[20,22,36,37]

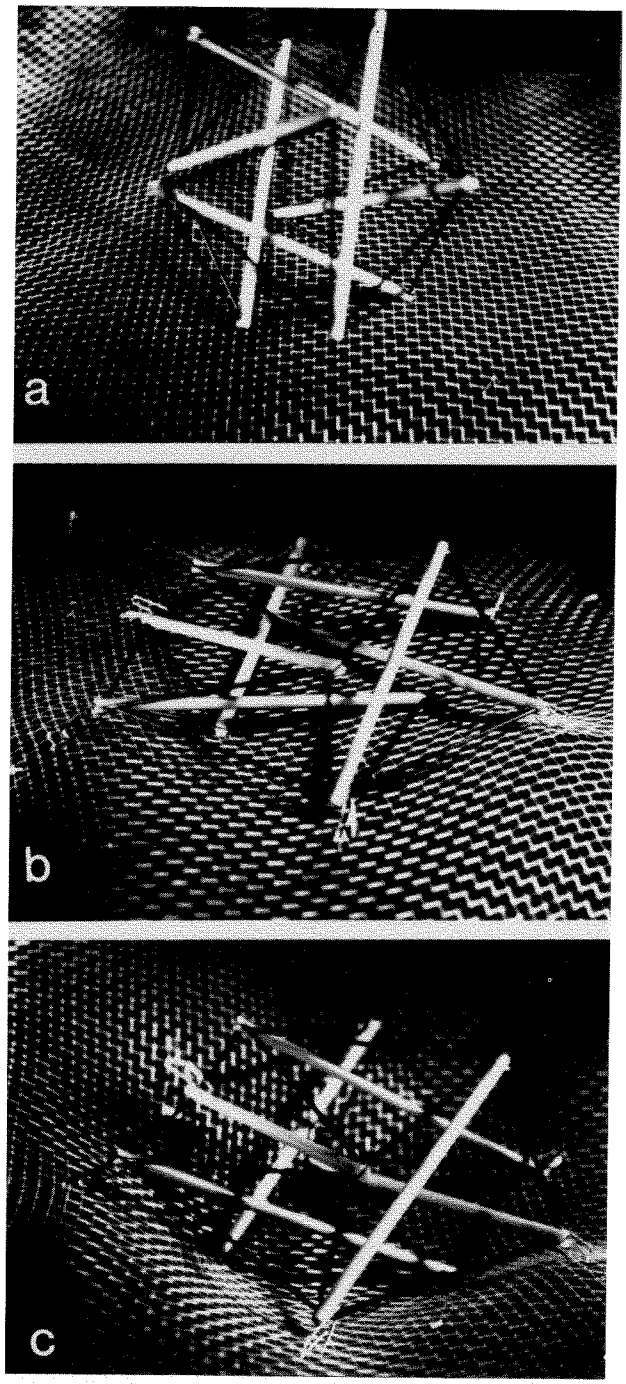

FIGURE 3. Studies with tensegrity cell models. Photographs show an unattached cell (*a*), a spread cell adherent to a rigid anchoring foundation (*b*), and a retracted cell attached to a malleable foundation (*c*). The shape of the tensegrity structure, much like that of a cell, is determined by the adhesivity and structural integrity of its attachment foundation. (See Ingber and Jamieson[20] and Ingber and Folkman[23] for additional studies with a similar model that contains a tensegrity nucleus.)

The tensegrity models are not meant to describe a precise

cytoskeletal morphology; rather, they are visual representations of an integrated structural organization system which we believe cells utilize. Whether or not specific types of cytoskeletal filaments (*eg*, microtubules, microfilaments, intermediate filaments) are represented by rigid struts or tensile threads is not critical. In fact, cell-generated tensile loads may be shifted from internal compression-resistant struts and onto extracellular attachment sites, thereby negating the need for internal struts at certain times. Interestingly, a transfer of mechanical forces from internal microtubules to both intracellular actin filaments and ECM attachment points is observed within fibroblasts treated with microtubule depolymerizers.[38] Furthermore, the same type of polymer (*eg*, actin) may serve different roles at different times and in different locations in the cell. For example, in addition to producing contractile forces, actin-containing microfilaments can act as either semirigid struts,[39] tensile "stiffeners,"[40] viscoelastic gels,[41] or elastic solids,[41,42] depending on their configuration (*eg*, fibers vs gels) and their complement of actin-associated proteins. The stability of microtubules similarly can vary greatly depending on whether or not tubulin undergoes posttranslation modification. It is also important to clarify that we do not believe that individual cytoskeletal filaments are highly elastic. Rather, we use elastic elements in our models in an attempt to model dynamic changes of cytoskeletal filament polymerization as well as energy-dependent shortening and severing/renucleation events (*eg*, sliding of different microfilaments within the same contractile bundle, microfilament breakage and reassembly induced by gelsolin). Thus, a living cell may best be thought of as an *energy-dependent* tensegrity system, whereas cells depleted of energy (or cell-free cytoskeletal networks depleted of severing proteins[42]) appear to resemble semirigid geodesic structures in which triangulated struts carry both tensile and compressive loads.

What is clear from analysis of tensegrity structures is that the physical changes which these structurally integrated networks undergo precisely model the behavior of living cells. However, we are still left asking the question: how could a tensegrity mechanism be used to transduce mechanical forces into biochemical information inside the cell? Key to this mechanism is the fact that mechanical stresses can only be transmitted over structural elements that are physically interconnected. Thus, transmembrane ECM receptors and associated cytoskeletal interconnections (*eg*, actin-associated proteins such as talin, α-actinin, and vinculin) may experience physical forces that nearby soluble molecules cannot recognize. Mechanical forces may then be transduced into biochemical alterations inside the cell as a result of alterations of *local* thermodynamic parameters along this system of structurally interconnected molecules. For example, Gibbs free energy and activation energy terms can be altered as a result of changes of architectural stresses that can be translated into changes of pressure and volume through the use of stress tensors. In this manner, forces generated within contractile microfilaments and resisted by occupied ECM receptors may feed back to change the equilibrium length of cytoskeletal polymers and thus, regulate cytoskeletal filament assembly. Assembly of microfilaments, microtubules, and intermediate filaments is sensitive to tension in cultured cells,[29,43,44] and a thermodynamic model of cytoskeletal regulation that incorporates the tensegrity mechanism for complementary force interactions has recently been described.[25]

Alterations of cytoskeletal polymerization could then alter cell growth and differentiation in a variety of ways. Lateral transmission of physical forces through cytoskeletal interconnections in the cell cortex might alter the distribution and function of membrane proteins (*eg*, "stretch-activated" ion channels, growth factor receptors, integrins). It is also possible that force-induced changes of actin polymerization could modulate chemical signaling pathways inside the cell. For example, a rapid increase in actin polymerization in the cell cortex might release gelsolin and profilin, two different actin-binding proteins that can bind intermediates in the phosphatidylinositol pathway and thereby interfere with their turnover.[45,46] Large-scale changes in cytoskeletal filament polymerization could also affect cell function by altering intracellular transport rates, redistributing organelles, or changing the distribution of any one of the many elements of the cell's metabolic machinery that are physically associated with the cytoskeleton.[32]

Both cytoskeletal filament assembly and direct transmission of physical forces through the cytoskeletal network may alter DNA synthesis as well as the expression of differentiation-specific genes by altering nuclear structure. Nuclear enlargement commonly precedes entry into S phase[27,47] suggesting that large-scale changes of nuclear structure may be required for DNA synthesis initiation rather than being the result of DNA replication. Physical extension of the nuclear protein matrix, the proteinaceous framework that serves to orient much of the nuclear replicative machinery,[26,37,48,49] may release mechanical constraints for DNA unwinding, a step that is rate-limiting for initiation of DNA replication.[50] Tension-dependent changes of nuclear structure may also rearrange genes and DNA regulatory proteins (*eg*, DNA polymerases, topoisomerases, steroid-binding proteins), increase access of transcriptional factors to promoter binding sites, promote nuclear pore extension and nucleocytoplasmic transport, and facilitate incorporation of new structural/regulatory proteins within the nuclear matrix scaffolding. However, stretching the nucleus does not directly stimulate growth. Rather, nuclear structural alterations appear to permit entry into S phase only if the correct set of soluble cues (*eg*, growth factors) and intracellular signals (*eg*, activation of the Na^+/H^+ antiporter) is also present. For example, nuclear spreading can occur in the G2 phase of the cell cycle without initiating a new round of DNA synthesis.

Thus, utilization of an integrated cytoskeletal network that is structurally continuous may be critical for information processing in cells. Mechanical signal propagation in this type of structural continuum would be expected to be much more rapid than any type of chemical signal transmission. Whether or not cells use this information for regulating metabolic functions remains to be demonstrated. However, given the incredible efficiency of biologic systems, we believe it highly unlikely that the cell would not use an information processing system of this type, especially since a continuous structural network already appears to exist inside the cell.

Implications for Tissue Development

Most textbooks on cell biology describe tissues in terms

of their composition and focus on the characteristics of the specific molecules, organelles, and other specialized structures that constitute the cell. What is lost in this type of description is the importance of structural relationships. Tissue function is a direct expression of tissue *pattern*. This is made clear when function is lost due to tissue disorganization, for example during tumor formation.[20,22,36] Thus, to understand the mechanism by which morphogenesis is controlled, we must first identify the rules for structural order and stability that function in tissues as well as in cells. The tensegrity model presents a mechanism for integrating structure and function in biologic tissues. ECM, as a physiologic anchoring scaffolding, is in a critical position because it interconnects intracellular cytoskeletal assemblies with other structural networks outside the cell. Thus, contact formation between ECM molecules and specific cell surface integrin receptors may provide a gating function for transmission of signals that regulate cell growth and differentiation. Our studies with capillary endothelial cells suggest that this regulatory information is to a large degree mechanical in nature. ECM molecules convey morphogenetic signals by binding and clustering cell surface integrin receptors and by physically resisting cytoskeletal tension that is applied to these receptors. The mechanism by which resultant changes in physical force distributions are transduced into biochemical information is unclear. However, we believe that the tensegrity mechanism presented in this article can serve as an excellent starting point for experiments designed to answer this question.

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