 MCB, vol.1, no.1, pp.53-68, 2004

The Mechanochemical Basis of Cell and Tissue Regulation

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Abstract: This article is a summary of a lecture presented at a symposium on "Mechanics and Chemistry of Biosystems" in honor of Professor Y.C. Fung that convened at the University of California, Irvine in February 2004. The article reviews work from our laboratory that focuses on the mechanism by which mechanical and chemical signals interplay to control how individual cells decide whether to grow, differentiate, move, or die, and thereby promote pattern formation during tissue morphogenesis. Pursuit of this challenge has required development and application of new microtechnologies, theoretical formulations, computational models and bioinformatics tools. These approaches have been used to apply controlled mechanical stresses to specific cell surface molecules and to measure mechanical and biochemical responses; to control cell shape independently of chemical factors; and to handle the structural, hierarchical and informational complexity of living cells. Results of these studies have changed our view of how cells and tissues control their shape and mechanical properties, and have led to the discovery that integrins and the cytoskeleton play a central role in cellular mechanotransduction. Recognition of these critical links between mechanics and cellular biochemistry should lead to novel strategies for the development of new drugs and engineered tissues, as well as biomimetic microdevices and nanotechnologies that more effectively function within the context of living tissues.

keyword: Mechanotransduction, integrins, cytoskeleton, tensegrity, cell engineering, morphogenesis

1 Introduction

This paper is based on an invited lecture I presented at a symposium on "Mechanics and Chemistry of Biosystems" in honor of Professor Y.C. Fung that convened at the University of California, Irvine in February 2004. Our work on mechanobiology, and especially on cellular tensegrity, has always been controversial; this became even more so as we crossed disciplines from biology into engineering. Professor Fung was one of the few established scientists and engineers who saw the potential value of our message early on, and who spoke out in our defense in public forums when our work was openly attacked. I still keep a letter Professor Fung wrote me early in my career, in which he wrote: "I am sure you are on the right track to discover the mystery of growth. Your theory is the best so far". I cannot tell you how validating this was for me as a young scientist. To me, it demonstrated the depth of his commitment in support of new ideas, regardless of how heretical they may seem at first glance. The advancement of science and engineering was his only concern. Thus, it is indeed my pleasure, and my honor, to be able to participate in this wonderful symposium and journal issue in appreciation of Professor Fung's seminal contributions to the field of bioengineering.

My laboratory is interested in the general mechanism of cell and developmental regulation: how cells respond to signals and coordinate their behaviors to produce tissues with specialized form and function. Molecular cell biologists have made great advances in terms of uncovering the biochemical signaling pathways that mediate behavioral control. But if we want to fully understand biological regulation, we also have to consider how these chemical interactions function in the physical context of living tissues. This is critical because it has been known for over a century that mechanical forces also impact tissue development [Wolff, (1892); Thompson (1952)]. The effects of large-scale forces on tissue growth, such as of compression on bone, tension on muscle, and hemodynamic forces on blood vessels, are obvious examples.

What is less clear, however, is that microscale forces also impact the development of all living tissues. For example, we work in the area of vascular development and angiogenesis – the growth of blood capillaries. Most biologists tend to think of vascularization in a relatively

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linear way in which soluble angiogenic factors activate growth signaling cascades in endothelial cells that lead to the progressive elongation of new capillary blood vessels. The reality is that this process is much more complex as neighboring regions of the same growing tissue often simultaneously exhibit different behaviors. For instance, early analysis of tissue remodeling [Clark and Clark (1938)] revealed that while one capillary sprout may grow in response to angiogenic stimulants, another capillary tube right next to it, will remain quiescent and differentiated, while yet another undergoes retraction and dies through a process of programmed cell death known as apoptosis. And all of this happens in a tissue microenvironment that we now know is saturated with many soluble mitogens. Moreover, establishment of similar local differentials of cell growth, differentiation, movement and viability are critical for pattern formation in all developing tissues, and in all species. But how could this work? The answer requires understanding of the importance of cell and tissue micromechanics for control of cellular biochemistry.

1.1 Mechanochemical control hypothesis

The working hypothesis that has driven our work for more than twenty years is that although tissue morphogenesis is genetically-determined and chemically-mediated, the actual process of tissue construction may be regulated *mechanically* [Ingber and Jamieson (1982,1985); Huang and Ingber (1999)]. This idea was based on early observations of developing epithelium [Bernfield and Banerjee (1978)] and capillaries [Ausprunk and Folkman (1977)] which showed that tissue expansion is initiated by a local increase in turnover of the basement membrane directly beneath the subset of cells that will subsequently form the new bud or branch (Fig. 1). Basement membrane is a specialized extracellular matrix (ECM) – a macromolecular scaffold composed of various collagen types (e.g., types IV & V), large glycoproteins (e.g., fibronectin, laminin), and proteoglycans (heparan sulfate, hyaluronic acid) that mediates cell attachment and holds cells together in all solid tissues in vivo. Moreover, the ECM in these soft tissues is under tension or "prestressed" because all of the surrounding cells generate tension within their contractile cytoskeleton (i.e., the internal supporting framework of the cell) and exert tractional forces on their ECM adhesions. This is why the edges of a surgical incision pull away from one another, and why wounds must be sutured. This is also why whole tissues and organs exhibit residual strains, as demonstrated by Professor Fung [Fung and Liu (1989); Omens and Fung (1990)].

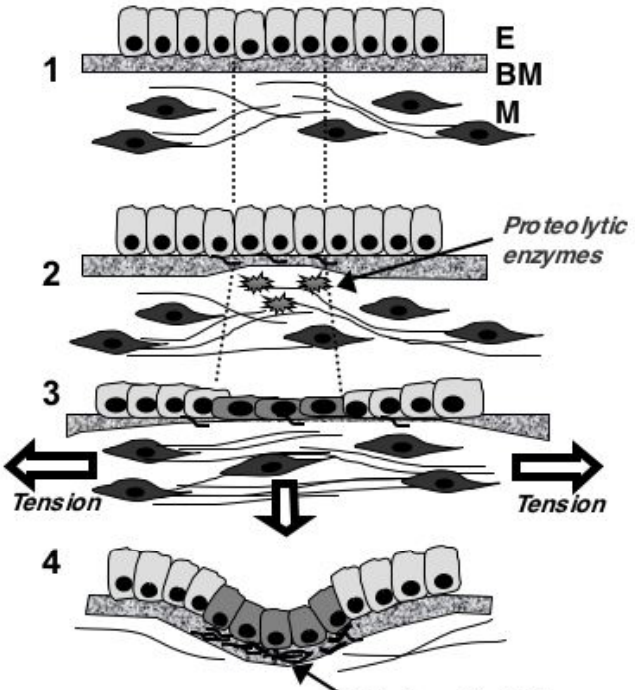

Figure 1 : Mechanical control of tissue patterning. 1) Diagram of a small region of embryonic epithelium (E), mesenchyme (M) and intervening basement membrane (BM). 2) Localized secretion of proteolytic enzymes increases the degradation and compliance of the BM locally. 3) Increased BM flexibility results in BM extension due to residual stresses. 4) Cell distortion supports local growth, BM deposition and bud formation.

If the tissue is mechanically tensed, then a thinned region of the ECM (e.g., basement membrane) that becomes more compliant due to removal of materials through high turnover (increased protein degradation) will stretch out more than the surrounding regions of the same ECM, much like a run in a woman's stocking (Fig. 1). This would change the balance of mechanical forces that are transferred across cell surface receptors, known as "integrins", that mediate cell adhesion to the distorted ECM and physically couple to the internal molecular framework of the cell – the "cytoskeleton".

In short, the concept was that this shift in mechani-

cal forces across the ECM would promote cell and cytoskeletal distortion, and thereby alter the activities of chemical signaling and regulatory molecules that are known to physically associate with the insoluble cytoskeletal lattice [rev. in Ingber (1993a)]. In this manner, distorted cells would activate "solid-state" signaling activities on the cytoskeleton that support cell proliferation in growth factor-stimulated cells and movement in cells stimulated with motogens, whereas neighboring cells that fail to experience the same mechanical distortion would activate intracellular signaling events that promote quiescence in the same chemical microenvironment. The result would be the localized growth and motility that drive pattern formation in all developing tissues. In the remainder of this article, experimental results will be reviewed that provide direct experimental support for this hypothesis. Advances we have made in terms of elucidating the molecular basis of cellular mechanotransduction also will be described, as well as recent work which is beginning to provide insight into how structural networks impact information processing networks in living cells.

2 Cell fate switching through cell shape distortion

A key tenet of the mechanochemical control hypothesis is that cell growth and function may be controlled in the presence of soluble mitogens through physical distortion of the cell and cytoskeleton. To unequivocally demonstrate this point, it was necessary to develop an experimental tool to make cell shape distortion an "independent variable" such that the degree of cell spreading can be altered independently of changes in the density of soluble growth factors (e.g., FGF, EGF) or insoluble ECM adhesive molecules. We accomplished this by microfabricating adhesive islands coated with a saturating density of ECM molecules (e.g., fibronectin, laminin) that were on the same micrometer size scale as individual cells; the islands were surrounded by non-adhesive (polyethylene glycol-treated) regions so that cell spreading was limited to the area of the adhesive island [Singhvi et al. (1994); Chen et al. (1997); Chen et al. (2000)]. These substrates were created by adapting a novel microtechnology (microcontact printing) based on soft lithography and molecular self-assembly of alkanethiols that was first developed by George Whitesides' Laboratory (Harvard U.) as an alternative method for creating microchips for the computer industry [Prime and Whitesides, 1991].

When mammalian cells were plated on these planar substrates, they spread and flattened, eventually taking on the precise size and shape of the islands. Capillary endothelial cells, liver epithelial cells, fibroblasts, smooth muscle cells, and skeletal muscle cells that are normally highly pleiotropic in form in standard culture, appeared perfectly round on circular adhesive islands, and exhibited ninety degree corners on square islands [Singhvi et al. (1994); Chen et al. (1997); Parker et a. (2002)]. Most importantly, we found that in the presence of a constant amount of soluble mitogen (e.g., FGF, EGF), cells that physically distorted (spread) to the greatest degree exhibited the highest growth rates [Singhvi et al. (1994); Chen et al. (1997)], whereas cells that were fully retracted but still adherent to the same ECM underwent apoptosis in the same growth medium [Chen et al. (1997)]. Moreover, cells that were cultured on intermediate size islands that neither promoted growth nor apoptosis, underwent differentiation: hepatocytes secreted liver-specific proteins and capillary endothelial cells organized into hollow capillary tubes [Singhvi et al. (1994); Dike et al. (1999)]. It may therefore be useful to integrate micropatterned surfaces, such as these, in artificial substrates that are used for tissue engineering or for creating cellular microchips in the future. Interestingly, when we stimulated various cells on square islands with motility factors, the cells extended new migratory membrane processes (lamellipodia, filopodia, and microspikes) preferentially from their corners (Fig. 2) [Parker et al. (2002)]. Subsequent studies with cells on similar sized islands of different polygonal shapes revealed that these processes preferentially form at acute, rather than obtuse, angles along the cell periphery [Brock et al. (2003)].

Specific intracellular signaling pathways (e.g., activation of the small GTPase, Rac) control the formation of new membrane processes in motile cells. Thus, our results show that anisotropic distortion of the cell due to the geometry of the ECM substrate (or physical distortion in vivo) is somehow able to dictate *where* these signaling activities are manifested, and hence, the direction in which the cell will move. In addition, when more than one cell was placed on a single large island, pattern formation spontaneously emerged: the two cells began to migrate in the same direction, either clockwise or counter-clockwise [Brangwynne et al. (2000)]. Given that cells normally migrate in a random walk on similar ECM substrates that are not geometrically constrained

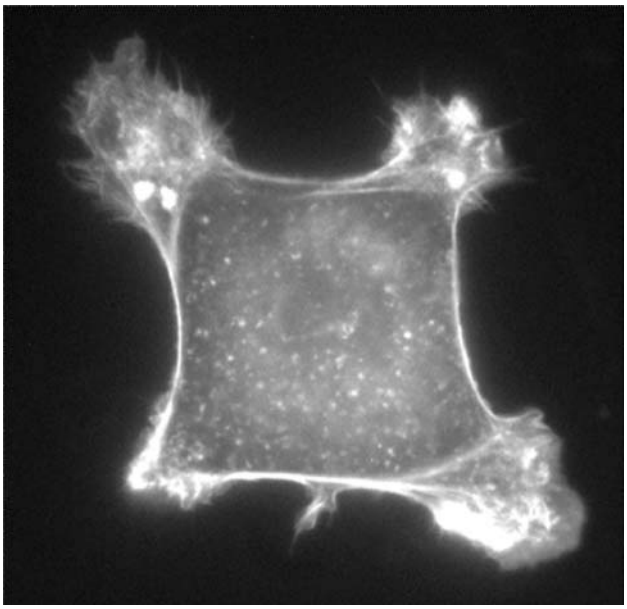

Figure 2 : An endothelial cell adherent to a square fibronectin-coated adhesive island (40 x 40 μm) preferentially extends lamellipodia (stained with fluorescent-phalloidin) from its corners when stimulated with FGF.

(i.e., unpatterned), the emergence of this pattern is a clear example of "symmetry breaking" behavior in a mammalian cell system. It also demonstrates how complex tissue (multicellular) patterns can emerge through interactions among components that are individually governed by simple rules. This is a key feature of complex adaptive systems, and understanding this process by which complex biological behaviors and patterns emerge through multi-component interactions will remain a central challenge in all fields of biology for many years to come.

3 Cellular tensegrity and mechanotransduction

The finding that cells can be mechanically switched between different phenotypes is important because it supports the concept that local changes in ECM mechanics that alter cell shape or structure may lead to the establishment of local differentials in cell growth and function that are key to how fractal-like tissue patterns are generated during morphogenesis [Huang and Ingber, 1999]. But how can physically distorting a cell influence its behavior? Given the classic view of the cell as an elastic membrane surrounding a viscous cytosol, it was originally assumed that mechanotransduction – the process by which cells convert a mechanical signal into a biochemical response - results from generalized membrane deformation. This belief was further strengthened by studies done by engineers who often model the cell as a mechanical continuum composed of an elastic cortex surrounding a viscous or viscoelastic core.

In contrast, we proposed an alternative model that suggested cells respond mechanically like "discrete network" structures; these are systems that are composed of an interconnected series of discrete elements separated by spaces (i.e., areas free of load-bearing components), rather than a mechanical continuum. This concept was based on work in the 1970s which revealed that all cells (i.e., not only muscle cells) contain a cytoskeleton, and that this porous molecular framework contributes to cell shape control. Others had recognized that the cytoskeleton was important for cell mechanics, however, they focused on the gel behavior of these networks, or the biophysical properties of the individual molecular components.

Our approach was different: we took an architectural perspective. Specifically, we proposed that the mechanical properties of the cytoskeleton, and hence the cell, are based on the use of a particular form of architecture known as "tensegrity" that comes from Buckminster Fuller world of geodesic architecture [Ingber et al. (1981); Ingber and Jamieson (1982, 1985); Ingber (1993b, 1998, 2003a)]. These are structures that gain their stability from continuous tension, rather than continuous compression. In their simplest embodiment, tensegrity structures are composed of a series of tensed cables that pull towards the center; however, these elements are stabilized in space because they are balanced by a subset of elements that resist being compressed. Thus, the stability of tensegrity structures depends on maintenance of a prestress (i.e., isometric tension) just like the stability of my arm depends on maintenance of contractile tone in my muscles. Importantly, all cells are known to generate active tension within their contractile cytoskeleton, and to exert tractional forces on their substrate adhesions. Isometric tension can not be "seen" in cells on rigid substrates, however, these underlying forces can be revealed by plating cells on flexible substrata (e.g., silicon rubber) which they crinkle and pull up into "compression wrinkles" between their localized

adhesion sites at the cell-substrate interface [Harris et al. (1980)].

My group and others have shown that different types of molecular struts and filaments interact in a similar manner within the cytoskeleton to stabilize the shape and architecture of living cells [rev. in Ingber (1993b, 1998, 2003a)]. Contractile microfilaments generate tension, and with intermediate filaments they distribute tension throughout the entire cell. These tensional forces are resisted externally by localized regions of the ECM substrate that separate the cell's isolated "focal adhesions" (i.e., spot weld-like anchoring structures that contain clustered integrin receptors as well as cytoskeletal linker proteins) and resist being compressed. Cytoskeletal tension is also balanced internally by microtubules and cross-linked bundles of actin (e.g., within filopodia) that similarly resist being shortened.

The simplest way to visualize the concept of cellular tensegrity is to think of the cell as a tent. One way to stabilize the shape of the canvas membrane is to tense or "prestress" it by pushing upward from within using tent poles (analogous to internal microtubules) while simultaneously anchoring the same membrane at the base using tent pegs (analogous to focal adhesions). However, once the roof is raised, it also may be anchored by being tied off to an overlying tree branch (analogous to a cell-cell adhesion, or another focal adhesion). In this situation, the tent pole would be "decompressed", and thus, it would be possible to then shift the pole to a different position (e.g., to extend a new region of the surface membrane).

Now if you were to break the tent poles in this type of structure, you would transfer the compressive load they normally carry onto the tent's external adhesive tethers. This would result in increased traction on the tent pegs and overlying tree branch. In contrast, if all of the elements of the tent, including the tent poles, were under tension, then disruption of the tensed pole would actually decrease the level of traction exerted on the surrounding adhesions.

Importantly, with our collaborator Ning Wang (Harvard School of Public Health), we have carried out traction microscopy studies with living cells cultured on flexible substrates containing small fluorescent beads that allow us to quantitate bead movement and hence changes in tractional forces that cells exert on their adhesive tethers when different cytoskeletal elements are disrupted. These studies clearly demonstrate that disruption of cytoskeletal microfilaments decreases cell tractional forces, confirming that these contractile elements are tensed. In contrast, disruption of microtubules increases the level of traction cells exert on their adhesive tethers, a result consistent with their ability to resist compression as predicted by the tensegrity model [Wang et al. (2001). Microtubule disruption also can increase traction biochemically by activating myosin light chain kinase, however, we obtained similar results under conditions in which myosin light chain phosphorylation and intracellular calcium levels (another potential regulator of cell contractility) remained unchanged. Finally, time-lapse microscopy of cells expressing green fluorescent protein (GFP)-labeled microtubules clearly showed that at least a subset of microtubules experience end-on compressive loading within living cells [Wang et al. (2001)]. Given that the cell is globally tensed (e.g., cutting the cell anywhere with a micropipette results in cytoskeletal tension-dependent retraction of the cut edges)[Pourati et al. (1998)], these combined results clearly show that cells experience continuous tension and local compression, the key tenets of tensegrity architecture.

Working with Dimitrije Stamenovic (Boston U.), we also developed a theoretical tensegrity model starting from first mechanistic principles [Stamenovic et al. (1996). This formulation of tensegrity and subsequent improved versions of the model have been shown to qualitatively and quantitatively predict many static and dynamic mechanical behaviors of living mammalian cells [rev. in Stamenovic and Ingber (2002); Ingber (2003a); Sultan et al. (2004)]. Dynamic computer simulations of multi-modular tensegrities also exhibit complex behaviors (e.g., undulating movements, integrated retraction throughout the material when its anchors are released) that are displayed by living cells and tissues [Ingber (2003a)]. Interestingly, even the simplest tensegrity configuration embodies many of the key features that are exhibited by more complex tensegrity arrays. In particular, these include a dependence of both elastic and frictional moduli on prestress, as well as the fact that a local stress can result in global structural rearrangements (changes in position and orientation) throughout large regions of the structure (Fig. 3) and at different size scales. Thus, this computational model may provide a handle with which to attack issues relating to the structural complexity and hierarchical features of living cells and tissues in the future.

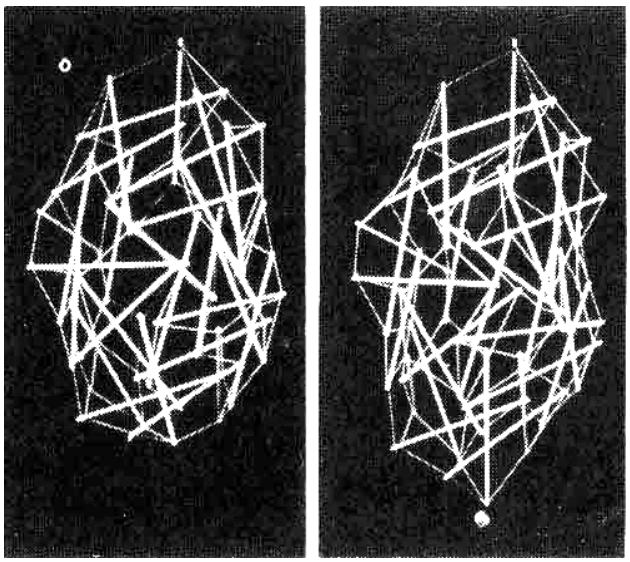

Figure 3 : The discrete elements of a prestressed tensegrity structure composed of sticks and elastic strings undergo global rearrangements when a stress (metal weight) is applied to the lower vertex, as shown on the right.

3.1 Integrins as mechanoreceptors

The important point about tensegrity in the present context of mechanotransduction is that it predicts that transmembrane adhesion receptors that mediate mechanical coupling between ECM and the cytoskeleton, such as integrins (the pegs in the tent analogy), will provided preferred paths for mechanical signal transfer across the cell surface. In contrast, other transmembrane receptors in more flexible regions of the membrane would dissipate this stress locally.

To test this hypothesis, we developed a method whereby we can apply controlled mechanical stresses to specific receptors on the surface membrane of living cultured cells using ligand-coated magnetic microbeads (1 to 10 μm in diameter) in conjunction with applied magnetic fields. Our first technique – magnetic twisting cytometry – utilized ferromagnetic microbeads coated with molecular ligands for integrin receptors (e.g., synthetic RGD peptides from the cell binding site of fibronectin, specific anti-integrin antibodies) or for control receptors (e.g., metabolic receptors, growth factor receptors, histocompatibility antigens) that similarly span the surface membrane but do not mediate anchorage to the internal cytoskeletal lattice [Wang et al. (1993, 1995); Wang and Ingber (1994); Yoshida et al. (1996)]. In this method, cells are allowed to bind to the beads for a few minutes and while they are still bound on the outer surface of the cell, a brief (10 μs) but strong (1000 Gauss) magnetic pulse is applied in the horizontal direction to magnetize the beads, and align the dipoles. Then a weaker (0 to 60 Gauss) but prolonged (1 min) magnetic field is applied in the vertical direction. The magnetized beads rotate to align with this new applied field; this results in application of a controlled torque or shear stress, but only to the receptors that are bound to the ligand-coated beads. More recently, we developed a "magnetic tweezer" [Alenghat et al. (2000)] and a "magnetic microneedle" [Matthews et al. (2004)] that can be used to apply controlled tensional stresses (i.e., rather than rotational stresses) to similarly coated paramagnetic microbeads bound to cell surface receptors. Force induced bead displacements are quantitated in real-time using optical microscopy.

Using these different methods, we consistently found that when we applied mechanical stresses to transmembrane metabolic receptors or histocompatibility antigens that do not normally mediate cell adhesion, there was minimal resistance to stress. In contrast, when we applied stress to integrin receptors on the surface of the same cells, cell stiffness (elastic modulus) increased in direct proportion as the level of stress was raised. This fundamental relationship between stiffness and applied stress is also exhibited by many living tissues, as reviewed in Professor Fung's classic book on bioengineering [Fung (1981)], although the molecular basis for this behavior remains unknown. Interestingly, we showed that simple stick-and-elastic string tensegrity models exhibited identical linear stiffening behavior, apparently because, as stress is applied, global rearrangements through the structure cause the stiffer compression struts to progressively realign along the applied tension field lines (Fig. 3) [Wang et al. (1993)]. This was also shown to naturally fall out from the computational tensegrity model [rev. in Stamenovic and Ingber (2002); Ingber (2003a)], although there may be other explanations for this particular behavior [Heidemann et al. (2000)].

Subsequent magnetic cytometry studies showed that other adhesion receptors, including cell-cell adhesion molecules (e.g., selectins, cadherins), also mechanically couple to the cytoskeleton, although certain integrins (e.g., integrin β1) appear to be more efficient [Wang et al.

(1993,1995); Yoshida et al. (1995); Potard et al. (1997)]. Focal adhesion proteins, such as vinculin, that link the cytoplasmic portion of integrins to the actin cytoskeleton were found to mediate transmembrane mechanical coupling [Ezzell et al. (1997); Goldmann and Ingber (2002)]. Cells that lacked vinculin were much more compliant when stressed through integrins, and the stiffness of this coupling could be restored by transfecting cells with exogenous vinculin protein. Finally, we found that β1 integrin receptors must be chemically activated, either by ligation of the RGD ligand binding site on the integrin receptor or using specific "activating" integrin antibodies, in order for mechanical coupling to take place [Meyer et al (2000); Matthews et al. (2004)]. Integrin ligation/activation is also required for focal adhesion assembly [Miyamoto et al. (1995)], and we found that efficient transembrane mechanical coupling correlates tightly with focal adhesion formation [Wang et al. (1993); Alenghat et al. (2000)]. Most recently, this was confirmed by analyzing the viscoelasticity of individual bead-associated focal adhesions in cells expressing GFP-labeled actin, vinculin and paxillin [Matthews et al. (2004)]. Beads bound to activated β1 integrins that recruited these focal adhesion proteins were significantly stiffer than those that did not even though they were expressed on the surface of the same cell. Interestingly, although focal adhesion formation significantly increased the elastic stiffness of the integrin-associated adhesion complex, it did not alter its viscous behavior.

Taken together, these results confirmed our hypothesis that transmembrane adhesion receptors that couple extracellular adhesive scaffolds (ECM and other cells) to the cytoskeleton, such as integrins, function as "mechanoreceptors". Integrins are among the first membrane molecules to sense physical forces, and they transfer these mechanical signals across the cell surface via a specific molecular pathway (i.e., through transmission across the molecules that form the cytoskeletal backbone of the focal adhesion).

3.2 Mechanochemical transduction through integrins

The finding that integrins provide a preferred path for mechanical stress transfer across the cell surface was extremely exciting to us as we and others have shown that many of the signaling molecules that are activated by ligation of both integrins and growth factor receptors are immobilized on the same cytoskeletal backbone of the focal adhesion [Plopper et al. (1995); Miyamoto et al. (1995)]. In fact, the entire cytoskeleton is more than a supporting structure, it also plays a key role in the control of cellular biochemistry because it orients many of the enzymes and substrates that mediate critical cell metabolic functions, including glycolysis, protein synthesis, and mRNA transport; DNA replication and transcription are similarly carried out using solid-state biochemistry on nuclear scaffolds (e.g., nuclear matrix)[rev. in Ingber, 1993a)].

Thus, if external mechanical stresses are preferentially transferred to these intracellular cytoskeletal and nucleoskeletal scaffolds via integrins, then force-dependent changes in the structure (three-dimensional configuration) and mechanics (e.g., flexibility) of certain load-bearing elements could mediate mechanochemical conversion. At the molecular biophysical level, changes in molecular shape and physical forces can impact thermodynamic and kinetic parameters. For example, theoretical work has shown that compressing a microtubule filament will increase the critical concentration of tubulin monomer necessary to maintain the total amount of microtubule polymer constant [Hill and Kirschner (1982)]. Interestingly, shifting forces from the ECM onto the cytoskeleton (e.g., by detaching adhesion receptors) alters microtubule polymerization state precisely in this manner [Dennerll et al. (1988); Mooney et al. (1994)], and a model for regulation of microtubule polymerization in whole cells that incorporates this tensegrity force balance has been published [Buxbaum and Heidemann (1988)]. Mechanical distortion of molecules also can influence their kinetics, such as the opening and closing rates of "mechanosensitive" ion channels [Sachs and Morris (1998)]. Force transmission through integrins and associated cytoskeletal linkages within the focal adhesion that contain signaling components could therefore provide a mechanism to link a mechanical stimulus to an intracellular signaling response [Ingber (1991); Ingber (1997); Alenghat and Ingber (2002)].

Importantly, we demonstrated that by applying controlled stresses directly to activated integrin receptors using magnetic twisting cytometry, we could turn on chemical signaling cascades, such as the cAMP pathway, in cells and activate transcription of cAMP-specific genes in a stress-dependent manner [Meyer et al. (2000)]. We also observed stress-dependent assembly of protein synthetic complexes surrounding focal adhesions at the sites

of force application [Chicurel et al. (1998)]. In contrast, application of the same stress to non-adhesion receptors (e.g., metabolic receptors) or to non-activated integrins (using non-activating antibodies) on the surface of the same cells had no effect.

Recent analysis of the mechanism by which mechanical stress activates the cAMP response has revealed that this is mediated by integrin-dependent and stress-dependent activation of heterotrimeric G proteins within the focal adhesion [Alenghat and Ingber, unpublished data)]. Activated integrins recruit heterotrimeric Gα and Gβ proteins to focal adhesions to a greater degree than non-activated integrins, however, these G proteins are only minimally activated when measured by biochemical techniques. When mechanical stresses are applied to integrin receptors, however, there is stress-dependent induction of G protein activation within focal adhesions at the sites of force application. Biochemical analysis of these focal adhesions after their removal from cells using a magnetic isolation technique [Plopper et al. (1995)] confirmed that Gαs, the key Gα protein involved in control of cAMP signaling, is activated to a much greater degree when stress is applied to activated integrins relative to when it is applied to non-activated integrins or to control receptors.

These results clearly demonstrate that the old concept that cells sense mechanical signals through generalized membrane deformation is incorrect. Instead, cells use specific transmembrane receptors, such as integrins, that physically couple to the internal cytoskeleton through specialized adhesion complexes to sense mechanical signals, as well as to convert them into a biochemical response. Various laboratories have now confirmed the central roles that integrins play in mechanotransduction in vivo as well as in vitro [rev. in Ingber (2003c)].

3.3 Cytoskeleton as a global signal integrator

These studies, combined with others from many different laboratories, have led to a view of the focal adhesion as a "mechanochemical signaling machine"[Alenghat and Ingber (2002)]. These sites represent points of convergence for signals from soluble chemicals, insoluble adhesive molecules, and mechanical stresses. Thus, the focal adhesion is perfectly poised to integrate all three types of regulatory signals directly at the cell surface where they converge, and many studies confirm that cells use this form of local information processing. However, the cellular tensegrity model suggests that a local stress applied to the cell surface also may produce distant responses, if it is applied to transmembrane molecules, such as integrins, that span the membrane and link to the internal cytoskeletal lattice. In the tensegrity model, cytoskeletal filaments may transmit forces over long distances in the cytoplasm and even concentrate or focus stresses at distant sites because of the discrete nature of the load-bearing network. In contrast, if the cell were a mechanical continuum, stress-induced displacements would drop off equally in all directions.

In fact, we have been able to demonstrate long-range force transfer across discrete cytoskeletal paths within living cells using a variety of techniques, including applying stresses to cell surface integrins using ECM-coated micropipettes or microbeads. For example, when pulling forces were applied to surface integrins using fibronectin-coated micropipettes in conjunction with a microscope stage micromanipulator, birefringent (i.e., molecularly aligned) stress fiber bundles were observed to turn ninety degrees and realign along the applied tension field lines within less than 1 second after stress application [Maniotis et al. (1997)]. Impressively, stress application also induced birefringence within nucleoli in the center of the nucleus in these living cells. Similar studies carried out with cells labeled with GFP-mitochondria showed that stress application to integrins results in mitochondrial displacements over twenty micrometers from the site of force application, whereas only minimal intracellular displacements were observed when similar forces were applied to beads bound to cell surface metabolic receptors [Wang et al. (2001)]. Most recently, in a collaboration with Ning Wang using an intracellular stress tomography system he developed, we confirmed that mechanical stresses applied to integrins on the apical cell surface using oscillatory magnetic twisting cytometry are transmitted throughout the cell and concentrated at distant sites, including on the nuclear surface and at basal focal adhesions [Hu et al. (2003)]. Moreover, long-range force transfer was inhibited when cytoskeletal prestress was dissipated using various techniques. Taken together, these findings clearly demonstrate that application of a stress to integrins results in prestress-dependent force transfer across tens of micrometers within the cell, and induces realignment of load-bearing elements throughout the cytoplasm and nucleus, a result consistent with the cellular tensegrity model.

Again, these findings are significant because they suggest that mechanical forces may influence cell behavior by acting at many points in the cell (i.e., not just in the focal adhesion). In fact, we found that when we apply controlled magnetic twisting stresses to integrins in round (suspended) versus spread (attached) cells, similar activation of cAMP signaling was obtained [Meyer et al. (2001)]. Yet, as I discussed earlier, spread cells must somehow be able to integrate this signal with other cues conveyed by their general state of distortion such that they proliferate, whereas round cells sense the same mechanical signal in a retracted state and decide to undergo apoptosis [Chen et al. (1997)]. In other words, cells may act locally via integrins and focal adhesions to sense mechanical forces, however, they apparently "think" globally in that they have evolved a mechanism to integrate these physical cues with other cell-wide signals to make a final decision as to how they should behave in a particular microenvironment [Ingber (2003d)].

Experimental analysis of the mechanism by which cell shape distortion governs the final cell behavioral response once again revealed that the cytoskeleton is the global signal integrator. For example, cell cycle progression can be inhibited and apoptosis induced in spread cells by disrupting the actin cytoskeleton using pharmacological inhibitors or genetic manipulation techniques [Chen et al. (1997); Flusberg et al. (2001); Numaguchi et al. (2003)]. Dissipation of cytoskeletal tension generation (and hence decreasing cell prestress) alone, in the absence of a cell shape change, is sufficient to induce partial growth inhibition [Huang et al. (1998); Numaguchi et al. (2003)]. Importantly, in human microvascular endothelial cells, the actin network is only required for growth during a particular three hour window in mid to late G1 phase of the cell cycle, prior to the critical G1/S transition [Huang and Ingber (2002)]. Disruption of actin filaments after this cytoskeleton-sensitive restriction point has no effect on cell cycle progression, thus confirming that growth inhibition is not due to some non-specific effect of generalized cytoskeletal disruption. Moreover, recent studies have revealed that the signaling associated with a stable actin cytoskeleton in a spread (distorted) cell is conveyed by a specific signaling pathway involving the small GTPase RhoA, and a series of downstream effectors including mDia1, Rho-associated kinase (ROCK), Skp2, and the critical cell cycle inhibitor, $p27^{kip}$ [Mammoto et al., unpublished data]. Induction of apoptosis by cell retraction or disruption of cytoskeletal filaments (microfilaments or microtubules) is also mediated by cytoskeleton-dependent activation of a known apoptosis signaling pathway involving Akt, bcl2 and various caspase enzymes [Flusberg et al. (2001)].

The mechanism by which distorted cells decide the direction in which to move is similarly governed by the cytoskeleton and mechanical forces. Cells on square adhesive islands apply the greatest tractional stresses in their corners where they also organize their focal adhesions [Parker et al. (2002); Wang et al. (2003)]. New process extension can be prevented by dissipating cell prestress using chemical inhibitors of cytoskeletal tension generation. Morover, cells that lack vinculin and fail to efficiently transmit tractional forces across focal adhesions also fail to extend lamellipodia [Goldmann and Ingber (2003)]. This mechanical coupling may be critical for local control of this response as vinculin-deficient cells still fail to extend lamellipodia even when microinjected with constitutively active Rac. Recent studies reveal that local activation of Rac in the corners of square cells (near focal adhesions) may be critical for the observed directional response as cells proteofected with constitutively active Rac extend lamellipodia equally well from sides as well as corners [Brock et al., unpublished data]. Thus, although cells may sense and respond locally to forces applied to integrins, somehow the cell is able to integrate these cues with information conveyed by the overall state of the global cytoskeleton in order to decide on a particular behavioral response.

3.4 Cytoskeletal Control of Organ Patterning

Taken together, these in vitro studies suggest that the major governor of whether cells will grow, move, or die when stimulated by soluble factors is the cytoskeleton, and the degree to which it is physically distorted or prestressed. This is certainly consistent with the mechanochemical model of tissue morphogenesis that first led us to initiate these studies. But are these findings relevant in vivo?

All of our studies were initiated based on a model in which tissue morphogenesis is controlled mechanically through local alterations in mechanical forces that cells balance between their cytoskeleton and their ECM. To explore this mechanism more directly, we recently studied the effects of modulating this force balance in whole lung rudiments explanted from embryonic mice on day

12 of development. We found that epithelial budding morphogenesis in the lung could be inhibited by dissipating cytoskeletal prestress using various inhibitors of cytoskeletal tension generation (e.g., Y27632 to inhibit Rho kinase; BDM to inhibit myosin ATPase; ML9 to inhibit myosin light chain kinase) or cytoskeletal disrupting agents (e.g., cytochalasin D)[Moore et al. (2001)]. Inhibition of cell tension generation also resulted in a loss of basement membrane thinning in regions where new buds would normally form [Moore et al., unpublished data]. Furthermore, all of these effects were fully reversible upon removal of drug.

In addition, we could actually accelerate budding morphogenesis (i.e., increase the number of buds and branches) by increasing cytoskeletal tension through activation of the Rho pathway using the chemical activator, CNF-1[Moore et al. (2001)]. Biochemical studies revealed that these results correlated more closely with the effects of CNF-1 and the other cytoskeletal modulators on myosin light chain phosphorylation (and hence tension generation) than on Rho activity. Quantitation of effects on cell proliferation also suggested that changes in tension influenced these morphogenetic changes by altering the spatial distribution of cell growth, and not by producing general growth suppression [Moore et al., unpublished data].

These results are consistent with our mechanochemical model in which the cellular balance of forces transmitted between the cytoskeleton, integrins and the ECM plays a key role in the establishment of the local differentials of cell growth and function that drive pattern formation during tissue morphogenesis. These findings also validate the physiological relevance of the past results we have obtained using simplified two-dimensional model systems with cultured cells. We therefore believe that the cytoskeleton serves as a critical control element, as well as the major cell-wide signal integrator, during tissue development.

4 Cellular information processing

Our work has demonstrated that cellular control lies in the balance of forces between the cytoskeleton and the ECM across integrins. However, at the same time, we found that relevant physiological functions of the cell – growth, differentiation, motility, apoptosis – are controlled through distortion of the *whole* cell. How does this work, and where is the specificity?

We gained some insight into this mechanism through the work of my associate, Sui Huang, who noted that the way in which continuously varying cell shape produced discrete cell fate transitions (either apoptosis or differentiation or growth) was in the widest sense a "biological phase transition". This effect was analogous to a phase transition in physical systems [Huang and Ingber (2000); Huang (2002)] in that the continuous variation of one control parameter produced sudden, qualitative changes in the entire system. For example, similar abrupt switches in behavior are observed as the temperature of water is raised, and it sequentially expresses the different material properties of solid, liquid and gas.

Physicists studying complex systems recognize that system-wide ("macroscopic") features in simple inorganic materials are *emergent* properties in that they are not properties of any individual component. However, biologists still tend to focus on the importance of individual genes or signaling components and assume that their properties directly map to system properties. More recent approaches from the burgeoning field of "Systems Biology" attempt to explain system-wide properties by comprehensive characterization of all the component parts of a system and developing quantitative models of their interactions. Yet they still tend to focus on isolated signaling modules whose properties they assume directly map into system properties. Cell regulatory pathways appear to be organized within modules, however, all of these pathways are connected within a single genome-wide network covering almost the entire genome ("giant component") [Jeong et al. (2001); Salgado et al. (2001); Lee et al. (2002)]. Given that the stable cell fates (e.g., growth, differentiation, apoptosis) which are regulated by thousands of different genes across the genome are mutually-exclusive, this suggests that cell behavioral control must be exerted at the level of the cell-wide (genome-wide) regulatory network so that when one state is turned on, all of the others are switched off.

Importantly, computational models of generic networks carried out by scientists studying complex systems have revealed that because of dynamic constraints imposed by the regulatory interactions [Kauffman (1969,1993)], stable states, known as "attractors", will spontaneously emerge in large interconnected networks that exhibit a particular class of network architecture. In fact, recent data provided by genomics has revealed that biomolecular networks exhibit a structure that belongs to this

class of architecture [Glass and Hill (1998); Fox and Hill (2001); Jeong et al. (2001)]. Thus, we have explored the possibility that the few stable cell fates that mammalian cells express (e.g., growth, differentiation, motility, apoptosis) represent attractor states in their genome-wide regulatory network.

A simple way to conceptually visualize attractors is to imagine a landscape containing multiple hills and valleys. If a droplet of rain were to land on this terrain, it would roll down the hillsides within one of the same set of possible valleys or "basins of attraction", eventually coming to rest at a stable low point (attractor) in one of these valleys. In the cell analogy, the position of the droplet at any time would represent the internal state of the cell which, when activated by some stimulus, would roll through gene state space (hillsides) always falling into one of the same set of possible cell fates (valleys)(Fig. 4).

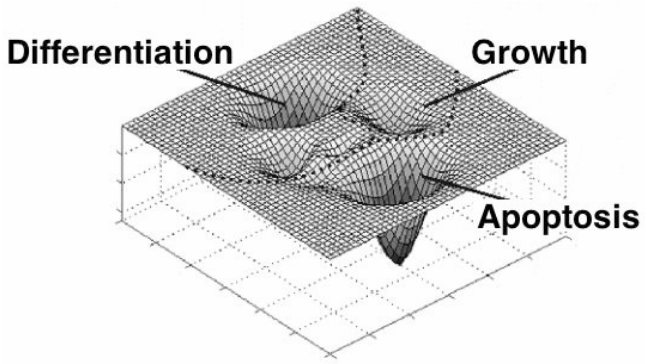

Figure 4 : A landscape of the state space of the genome-wide gene regulatory network with stable cell phenotypes (growth, differentiation and apoptosis) as stable attractor states which appear as valleys in this schematic diagram.

First, we used Boolean network models that incorporated experimental data relating to the activities of known growth signaling molecules (e.g., p27, cyclin D1, Rb) that we measured in studies in which we varied cell shape, ECM adhesion and growth factors independently. Computational models based on these simple networks lead to emergence of a cycling attractor state that corresponded to the mammalian cell cycle, as well as two other, distinct attractors that closely mimicked the different resting "G0" states induced by serum-starvation versus ECM detachment [Huang and Ingber (2000)]. The results of these modeling studies showed that even with a coarse model in which proteins are represented by binary switches, the network of interactions itself is the essential ingredient for producing ordered, system-level behavior. Specifically, they supported the concept that stable cell fates represent "default" states (attractors) in the cell regulatory network; however, direct experimental evidence at the genome scale in support of this hypothesis was lacking.

To further pursue this hypothesis, we needed to be able to simultaneously analyze changes in genome-wide signaling activities over time in response to multiple experimental perturbations. If attractors exist, such analysis would reveal that cells can visit entirely different internal network states, yet end up in the same final state, like the rain droplets converging to the bottom of the valleys. To accomplish this, we developed an entirely new bioinformatics visualization tool – the gene expression dynamics inspector (GEDI) – that identifies gene expression patterns and presents their changes as a movie [Eichler et al. (2003)]. When published gene expression profile data were analyzed, this tool clearly revealed the genome-wide coordination of changes in gene activity that underly various cell behavioral responses. For example, analysis of gene array data from studies of Dictyostelium development revealed that the entire gene profile underwent an abrupt change in pattern exactly at the time when the cells switched from an amoeboid to a stalk phenotype [Eichler et al. (2003)]. When a similar analysis of gene array data collected at multiple time points during the mammalian cell cycle were viewed as a movie, the entire genome cycled in a coordinated manner across thousands of genes [movies available at: www.chip.org/∼ge/samples.htm]. In other words, this form of inspection revealed genome-wide regulation and distributed information processing – the prerequisite for attractors in the genome-wide network, rather than changes in a dominant signaling pathway or module.

More recently, we have obtained experimental evidence in direct support of the attractor hypothesis by analyzing time-dependent behavior of genome-wide gene profiles during induction of a cell fate switch in human HL60 cells. These are promyelocytic precursor cells (HL60) that can be induced to differentiate into neutrophils by treatment with either all trans-retinoic acid (atRA) or dimethylsulfoxide (DMSO) [Huang et al., unpublished

data]. The possibility that the neutrophil differentiation represents a default attractor state is already supported by the fact that the same fate switch can be triggered by both a specific hormone (atRA) and a non-specific solvent (DMSO). Most importantly, genome-wide gene expression profiling over the time course of both differentiation processes revealed that differentiation of human promyelocytes into neutrophils triggered by either atRA or DMSO occurs along two distinct gene expression state space trajectories that first diverge, but then converge as cells transition into the common phenotype. In other words, cells in both treatment groups visit different sites within the same valley, or basin of attraction, but then converge toward the same attractor.

These results provide direct support for the existence of high dimensional attractors in gene expression state space in human cells, whereas these data cannot be plausibly explained by any other mechanism known at the present time. The discovery that the state space contains attractors and distinct trajectories is also consistent with Waddington's "epigenetic landscape" that was proposed almost 60 years ago as an intuitive metaphor to capture the typical features of cell fate dynamics during embryological development [Waddington (1940)]. Our work suggests the relative position of a cell in this landscape may determine the developmental potential of the cell, rather than activation of a particular "instructive" pathway or series of specific genes.

The existence of attractors in the genome-wide regulatory network that confer stability with respect to thousands of dimensions (gene expression levels) is important because it helps to explain how a non-specific stimulus like cell shape distortion could have been harnessed by evolution to impact the same biochemical machinery responsible for distinct cell fate switches that is actuated by growth factors which bind with high specificity to their own cell surface receptors [Ingber (2003b)]. It also explains how cells can simultaneously sense multiple chemical, adhesive and mechanical inputs and yet only switch on one of a limited number of specific and reproducible behavioral responses (e.g., growth or differentiation or apoptosis). Finally, a feature of the attractor model is that multiple regulatory elements (e.g., genes, signaling proteins) must change in order to produce an attractor switch. Given that cell shape distortion likely impacts many cytoskeletal-associated signaling molecules simultaneously, this may explain how global changes in shape are able to control cell fate switching [Ingber (2003b)].

5 Conclusions

The current focus of biology and medicine on molecular genetics ignores the physical basis of disease, even though many of the clinical symptoms that cause patients to visit the doctor's office result from changes in tissue structure or altered mechanics. In fact, a wide range of diseases included within virtually all fields of medicine and surgery share a common feature: their etiology and clinical presentation result from abnormal cell and tissue responses to mechanical stress [Ingber (2003c)]. The overall goal of the studies we have carried out over the past twenty years, and that were briefly reviewed here, was therefore to help integrate biophysics and mechanics into our understanding of the molecular basis of development and disease.

The contributions of Professor Fung which inspired this Symposium introduced the tools and thinking of mechanical engineering into tissue physiology. The work presented here shows how application of similar engineering approaches at the cell and subcellular levels can provide new insights into how Nature builds. Specifically, we discovered that cells respond mechanically like prestressed network structures that are stabilized using tensegrity architecture, and not like a bulk material or mechanical continuum. Because of the use of discrete networks, mechanical stresses are transmitted across the surface membrane of the cell over specific transmembrane molecules, such as integrins, that anchor internal cytoskeletal scaffolds to extracellular support scaffolds (i.e., ECM and other cells). Moreover, these same integrin receptors mediate mechanochemical transduction by recruiting signal transducing molecules to the cytoskeletal backbone of the focal adhesion complexes that form at sites of cell-ECM attachment. Signaling molecules that form part of the load-bearing network of the cell experience mechanical stresses that are transmitted over these receptors, whereas neighboring soluble molecules within the viscous cytosol that permeates the cytoskeleton do not. Mechanical distortion of these molecules can impact biochemistry through changes in molecular biophysical parameters (e.g., kinetics, thermodynamics). Based on this form of solid-state biochemistry, mechanical forces govern the pattern in which load-bearing elements assemble and orient, as well as what signaling pathways

are activated inside the cell. The architecture of the cell, combined with the level of tension or prestress in the cytoskeleton, then governs the cellular response to subsequent mechanical distortion. In essence, the cell is entirely mechano-chemical; this is the key to all living systems.

The challenge for the future is to understand how forces applied to the cytoskeleton through adhesion receptors impact cellular information processing. The mechanotransduction field is now focused on the site of adhesion, with many studies analyzing how stress application to integrins modulates focal adhesion assembly and signaling. However, mechanical forces can be transmitted over long distances and focused on distant sites in the cell because of tensegrity. Thus, to meet this challenge, we need to develop new experimental approaches and theoretical models to attack the question of how the whole cell processes mechanical signals. Our venture in the area of cellular information processing has revealed the existence of stable default states (attractors) in gene state space due to regulatory constraints within the genome-wide gene regulatory network. To switch cells between different stable phenotypes would require multiple genes or other regulatory elements to simultaneously switch their activity status. The cytoskeleton, with its multiple associated signaling components, is perfectly positioned to provide this multiplexed switching activity. In fact, our work shows that the cytoskeleton plays a fundamental role in distortion-dependent control of cell cycle progression, movement, and contractility, as well as apoptosis.

In conclusion, the "take home message" is that we must go back to structure to understand function. But neither the molecular cell biologist, the engineer, nor the computer scientist alone will be able to fully explain how complex biological structures form and function. Success in this area will require training of a new class of investigators with all three of these skills. This requires more than recruiting biologists into engineering departments, although that is a great beginning. It also requires the openness of programs in graduate education. Each discipline, whether in engineering or biology, has its own specialized vocabulary, techniques, and principles of operation. These boundaries need to be broken down when young investigators are still in training. Newly formed bioengineering departments have been especially receptive to this idea, with many graduate students gaining hands-on experience in molecular cell biology. On the other hand, biology programs rarely offer electives in biomechanics or engineering, let alone state these as requirements. If we can instill this inherent need for scientific integration in all training programs, and support collaborations between established engineers and biologists that frame biological questions which are physiologically relevant, then we will undoubtedly catalyze the melding of these different disciplines. In the process, we will gain new insights into the mechanical basis of tissue regulation which may lead to development of improved medical devices, engineered tissues, and biologically-inspired materials for tissue repair and reconstruction.

Acknowledgement: I would like to thank all of the present and past members of my laboratory, as well as all of my collaborators, without whom this work would not be possible. This work was supported by grants from NIH, NASA, DARPA, DoD and ACS.

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