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Regulating tension in three-dimensional culture environments

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Abstract

The processes of development, repair, and remodeling of virtually all tissues and organs, are dependent upon mechanical signals including external loading, cell-generated tension, and tissue stiffness. Over the past few decades, much has been learned about mechanotransduction pathways in specialized two-dimensional culture systems; however, it has also become clear that cells behave very differently in two- and three-dimensional (3D) environments. Three-dimensional in vitro models bring the ability to simulate the in vivo matrix environment and the complexity of cell-matrix interactions together. In this review, we describe the role of tension in regulating cell behavior in three-dimensional collagen and fibrin matrices with a focus on the effective use of global boundary conditions to modulate the tension generated by populations of cells acting in concert. The ability to control and measure the tension in these 3D culture systems has the potential to increase our understanding of mechanobiology and facilitate development of new ways to treat diseased tissues and to direct cell fate in regenerative medicine and tissue engineering applications.

Keywords

Mechanobiology; Tension; Stiffness; Extracellular matrix; Collagen gel; Fibrin gel; BioMEMS

Introduction

The processes of development, repair, and remodeling of virtually all tissues and organs are dependent upon tension at the cell level [1]. Genetic and soluble factors strongly regulate growth, but mechanical forces clearly guide the formation of structures [2]. Mechanical signals are also known to contribute to many pathological states including sclerotic diseases and cancer and have been implicated in determining lineage fate for stem cells [3,4] demonstrating our need for a fundamental understanding of mechanobiology for treating and hopefully preventing disease [5].

Tension acts both as a potent stimulus to the cell and a driver of extracellular matrix (ECM) reorganization. Understanding how cells transduce and generate tension has been studied intensely over the past two decades, predominantly in two-dimensional (2D) culture systems. Much has been learned about the importance of specific proteins involved in cell-cell and cell-ECM adhesions [6], the role of cytoskeletal tension [7], and mechano-specific signaling pathways [8]. Three-dimensional (3D) culture systems have been used extensively to study the “dynamic reciprocity” between cells and ECM involved in remodeling, but cell

tension is quantified in only a limited number of studies. In the next section we provide a brief background of the study of mechanobiology in 3D protein matrices, the most utilized in vitro models systems and discuss the concept of “dimensionality.” In these systems, the ECM transmits external forces to cells, and cells exert traction on the ECM [9]. The embedded cells establish a homeostatic tension level [10] with the surrounding matrix stress, and the tension level is, in turn, a critical regulator of cell motility, proliferation, phenotype, and ECM remodeling [11].

In this review, we describe the role of intrinsic and global stiffness in the regulation of cell-generated tension and cell behavior in 3D collagen and fibrin matrices. We highlight recent advances in the modulation and quantification of cell tension which facilitate more refined study of the effects of tension on cell behavior in these 3D systems. The focus of the review is limited to mesenchymal cells cultured within statically constrained gel systems; for cell and matrix responses to cyclic stretch please see the review by Elson and Genin in this volume; for endothelial cell network formation in protein matrices, please see the review by Morin and Tranquillo in this volume.

In protein gel systems, the tension level is regulated by a variety of factors including the intrinsic stiffness of the matrix and local ligand density as discussed in the third section, and whether the cell-populated gel is cultured rigidly attached or free-floating as discussed in the fourth section. In the fifth section we provide a detailed discussion of methods for graded control of tension (level and direction) which involve more refined manipulation of the global boundary conditions. We also highlight the interaction between soluble and mechanical cues in the regulation of cell tension. In the last section we draw general conclusions and look to the future of mechanobiological studies in 3D gels.

Background of mechanobiology in 3D

In the 1980s, soft wrinkling culture surfaces [12], tunable stiffness substrates [13], and dynamic stretching devices [14] were developed which allow systematic study of how cells apply traction to their surroundings and how external loads affect cell behavior [15]. However, the importance of tension on cell behavior was actually highlighted in 3D matrix culture much earlier. Cell behavior has been studied in 3D matrices of clotted plasma and lymph since the early 1900s (see review by Grinnell and Petroll [11]). Over 50 years ago Weiss [16] demonstrated that cells locally reorganize fibers in a fibrin gel clot model between cell explants indicating long-range effects of traction forces. In the early 1970s, Elsdale and Bard [17] cultured cells on and in reconstituted collagen gels and found that they retain in vivo-like bipolar spindle morphology indicating lines of tension, and Bell [18] systematically studied the effect of free-floating and rigidly anchored boundaries on the structure of collagen gels compacted by fibroblasts of different proliferative potential. In the next decade, Stopak and Harris [19] found that fibroblast traction in 3D collagen matrices is sufficient to form patterns of tension, compression, and fiber alignment with similarities to wrinkling caused by cell traction on thin polymer membranes [12].

These biopolymer culture models provide a tissue-like spatial arrangement missing in 2D culture, and reveal many differences in cell behavior on flat surfaces and within a fibrous matrix. Most notably, cell morphology is markedly different in gels than on 2D surfaces [20-22]. Cell growth [17], motility [21,23], differentiation [24], tumorigenicity [25], and response to soluble factors are also altered when cells are extracted from 3D tissues and cultured on 2D substrates [26]. Reasons for discrepancies in cell behavior between 2D and 3D environments are an active topic of debate in the literature [27]. “Dimensionality” itself is not an independent stimulus, rather a complex set of factors that must be decoupled to better understand the critical determinants of cell behavior (see reviews [28] and [29]). Cell-

generated tension in 3D matrices, the focus of the present review, is affected by many factors including cell morphology, adhesion, soluble factors, and the resistance of the cells surroundings to deformation (effective stiffness), all of which differ between 2D and 3D systems. Cell morphological states and migration are restricted by the fibrous meshwork such that cells spread and align along fibers and cells need to squeeze through (ameboid-like) or enzymatically degrade the proteins if the fiber mesh is dense and/or cross-linked [11]. Further, specialized cell–matrix adhesions [30] are formed in 3D systems with more symmetric adhesions over the surface of the cell which alters the concentration of ligands available for binding [31]. Diffusion of nutrients and growth factors is also limited in 3D gels in a protein density-dependent manner, and the matrix can act as a repository of factors which can be enzymatically released by the cell in a tension-dependent manner [32].

Combinations of ECM components and biological hydrogels (e.g., collagen and fibrin [33], collagen and agarose [34], etc.) are utilized as 3D models of varied complexity to mimic specific tissues and to facilitate particular cell–matrix interactions; however, single protein matrices of collagen or fibrin remain the most widely utilized model systems for the study of mechanobiology in 3D (as reviewed by Pedersen and Swartz [28]). Type I collagen is the most abundant protein found in interstitial tissue and is widely used in tissue engineering applications. Collagen monomers can be obtained as small aggregates by acidic digestion of connective tissues (e.g., rat tail tendon, bovine cartilage, and skin) and by pepsin extraction [20]. Collagen monomers form in vitro by self-assembly when the pH is brought to physiologic level, a process accelerated with increasing temperature (generally 4–37 °C). Although the reconstituted collagen gel has much weaker mechanical properties than collagen structures in native tissues, it has proved useful both for fundamental cell–matrix studies and for clinically available engineered tissues (e.g., Apli-graf®, Organogenesis Inc.). Fibrin is the major component of the provisional matrix during wound healing and is a widely utilized model matrix as reviewed by Janmey et al. [35]. Fibrin matrices are formed by polymerization of fibrinogen (obtained from blood) with thrombin in the presence of calcium. Fibrin matrices actively bind growth factors, heparin, and different integrin types making them suitable tools for researching fibroplasia [36], stem cell differentiation [37], tumor angiogenesis, and tissue engineering [38,39]. The cell tension within these compliant gels can be modulated locally by modifying the intrinsic properties and globally by modifying the boundary conditions, as reviewed in the following sections.

Modulation of intrinsic matrix stiffness

Changing the mechanical properties of the matrix within which cells are cultured is a common means for investigating mechanobiology in 3D matrices. The intrinsic stiffness of protein gels can be modulated by changing the protein concentration, altering the polymerization conditions, or chemically cross-linking the fibers as described below.

Increasing collagen concentration increases the stiffness of collagen gels in a dose-dependent manner [33,40–42] and influences the behavior of the resident cells [33,43–47]. Typical initial collagen concentrations range from 0.3 to 5 mg/ml [44,48–50], and the initial matrix stiffness varies over a very wide range depending upon polymerization conditions and method of characterization (e.g., 0.3–50 Pa for shear storage modulus and 500–33,000 Pa for uniaxial tensile modulus at low strain) as reviewed in [20]. Harley et al. [44] found that increasing collagen concentration promotes cell proliferation and metalloproteinase activities, and Grinnell et al. [51] report increased cell spreading, more organized actin stress fibers, and a decrease in ability of the cells to translocate local collagen fibrils with increased collagen concentration. Grinnell and colleagues postulate that changes in collagen translocation are primarily due to altered matrix stiffness, and they attribute changes in cell spreading and migration to collagen matrix porosity. However, stiffness, porosity, and the

number of sites available for cell adhesion are all dependent upon collagen concentration, which further confounds analysis.

Polymerization conditions also have significant effects on the physical properties of collagen and fibrin gels. Matrix stiffness, fiber diameter, fiber density and pore size are altered by changing polymerization temperature and pH [40-42,52], yet there is a very limited range over which these factors can be altered while keeping the embedded cells viable. With judicious choice of polymerization conditions, culture media components, and gel thickness, the fiber microarchitecture and bulk concentration may be modulated independently in collagen gels [53]. It remains unclear if the stiffness of the matrix correlates more strongly with the collagen fiber structure or concentration under these conditions.

Since fibrinogen is cross-linked with thrombin and calcium (Ca^{2+}) which are not cytotoxic even at relatively high concentrations, it is possible to tune fibrin gel mechanical properties over a larger range than collagen gels [54-56]. Increased fibrinogen concentration causes a roughly linear increase in initial Young's modulus [56] and storage modulus [55] of the fibrin gel and decreases proliferation and spreading [56,68]. Increases in Ca^{2+} and thrombin concentration result in bimodal changes in fibrin gel stiffness [55,56]. Increasing fibrinogen or thrombin concentration decreases fiber diameter and increases fiber density, while increasing Ca^{2+} has an opposite effect on fiber density and diameter [55].

The matrix concentration, and thus intrinsic stiffness, can also be increased mechanically. Brown et al. [57] developed a simple method to rapidly increase collagen cell stiffness up to 40 fold by plastically compressing cell-laden collagen gels. This method results in less than 15% cell death and shows promise for quickly generating cell-containing materials for tissue engineering applications; it has also been utilized for studying the effect of stiffness on cell-generated tension and migration [43].

The intrinsic stiffness of a collagen gel can be directly altered with biochemical additives including ribose glycation [58-60], genipin fixation [61,62], photo-crosslinking [63], and non-enzymatic nitrite modification [64]. Glutaraldehyde has been utilized to assess stiffness effects for cells cultured on top of pre-cross-linked 3D matrices [51], but this treatment cannot be used to treat gels with embedded cells due to its cytotoxic effects. Similar to collagen gels, fibrin gel stiffness and strength can also be increased many-fold by exogenous cross-linking e.g., by Ruthenium-catalyzed photo-cross-linking [66] or genipin [67]. Most often, these treatments are utilized to modify the macroscopic properties of the material for biomaterial and tissue engineering applications. In a limited number of studies, the effects on cell behavior have been investigated, although cross-linking may obstruct or alter cell binding sites on the proteins and potentially modulate the cell behavior as well. Conflicting effects of "stiffness" on cell behavior in 3D gels have been reported from studies utilizing different methods for modulating the matrix properties. For example, increasing collagen gel stiffness by adding ribose prior to polymerization [58] or by increasing collagen concentration [44] promotes cell spreading and proliferation, whereas glycation has been reported to decrease cell adhesion, spreading [60,65], and viability [59,60].

One reason for the conflicting results between the aforementioned studies may be that the concentration of the gel changes over time due to compaction of the protein by the cells [18], even for relatively low initial cell density (0.01 million cells/ml) [18]. The extent of compaction generally increases with initial cell density [18,44,48,49,69] and decreases with initial collagen density [33,44,48,50,70]. However, even if the final collagen concentration is measured, the final gel modulus cannot be predicted from this value due to non-uniform compaction around the cells [48]. The existence of elevated density in the pericellular region has recently been quantitatively modeled and measured by Stevenson et al. [70]; the authors

report that the final collagen concentration increases with initial concentration when the compaction is limited (e.g., <45% decrease in volume) in agreement with their prediction. In contrast, for high initial cell concentration and/or low initial collagen concentration, an inverse relationship between initial and final collagen concentration has been reported [48,50,71]. The final concentration also depends upon how the gel is constrained during culture (as discussed in detail in the next section); for example, fibroblasts in collagen gels (initially 1.5 mg/ml) reach 22 mg/ml in attached gels and 55 mg/ml in floating gels after 4 days in culture [72]. In attached gels, active cell-generated tension is greater when initial collagen concentration is higher; while the stress is lower due to a larger final cross-sectional area in the less-compacted tissue [73,74].

As reviewed above, many modifications to the standard collagen and fibrin gel model systems have been shown to be successful in creating 3D matrices with tunable stiffness without significantly affecting cell viability; however, they lead to concomitant changes in fibrous structure (e.g., pore size) and biochemistry which alter cell-matrix interactions and make it difficult to independently assess the effect of these factors on cell tension. Further, quantifying the traction an individual cell exerts within 3D fibrous matrices is a highly challenging technical problem which is only now being tackled effectively, as reviewed in this volume by Wu and colleagues. Altering the mechanical conditions at the outer boundaries of cell-seeded gels offers a way to modulate the resistance to cell deformation without directly changing the physical properties and structure of the matrix as described in the following two sections.

Free-floating, rigidly anchored and released gels

Most commonly, cell-populated gels are either anchored to a rigid culture surface or cultured freely floating in the media following polymerization. When cells are cultured at sufficiently high density (typically 0.1–3 million cells/ml) within protein gels for extended culture duration (hours), they act collectively and the forces they generate are transmitted through the matrix to other cells and to the boundaries of the tissue [10,18,75]. As the population of cells compact the matrix globally, how the edges of the tissue are constrained (or released from constraint) determines the overall resistance of matrix deformation i.e., effective stiffness. This resistance regulates the traction forces that the cells are able to generate and sustain within the matrix.

The resulting cell morphology, migration, contractility and protein secretion are markedly different under “free” (floating) and “fixed” (rigidly anchored) boundary conditions, despite identical *initial* protein and cell density [9]. The matrix can also be released from a rigid boundary which leads to a decrease in tension within gel and further changes in cell behavior (Fig. 1). The tension that populations of cells generate against stiff boundaries are many fold greater than against soft boundaries [26,73], and tension in free gels, although not directly measurable, is likely to be negligible in the center of a floating gel compared to an anchored gel. For this reason, anchored matrices are termed “mechanically loaded,” “high tension,” or “stressed” gels, and free floating matrices are termed “unloaded,” “low tension,” or “unstressed” gels. Differences in the ability of the cells to generate tension against these boundary conditions also lead to substantial variations in the resulting microstructure of the gels which may affect cell behavior. Differences between the volume of floating and anchored gels [76,77] result in dramatic variances in the ECM density, pore size, diffusional coefficients and distances between cells [46,78,79] (although actual gel volume and structural parameters are seldom measured experimentally). The behavioral differences under the different boundary conditions have also been attributed to the relatively high intrinsic stiffness of anchored gels compared to floating gels due to greater anisotropic compaction [76,80]. Further, the organization of the fibers is quite different between these

two cases due to the altered direction of compaction [26]. These confounding factors hinder efforts to decouple the effects of tension from the architecture and properties of protein gels and have led to extensive development of synthetic polymer systems for studying mechanobiology in 3D [34,81]. Regardless of these limitations, cell behavior and fate in free floating, rigidly anchored, and released collagen gels have been studied extensively and have added greatly to our understanding of cell mechanics and motility (as reviewed by Grinnell and Petroll [11]).

In freely floating gels (zero radial force at outer boundary), cells contract the 3D gels by two mechanisms: initial cell elongation and spreading and cell tractional forces due to cell locomotion [82]. Cells are round in shape when they are first trypsinized and re-suspended in gel solution. Within hours they elongate and branch and apply traction forces to the surrounding material. Following elongation and spreading, they apply traction to the matrix while they migrate. In free gels, fibroblasts have a dendritic morphology similar to those in native interstitial tissues rather than the pancake-like shape observed in 2D monolayers [9,83,84] and they do not express stress fibers [85-87] or require fibronectin for stress generation or compaction [83,88,89]. Instead, the cells generate force by α - and β -actinin constituting the cortical cytoskeleton [21,90]. Since there is low resistance to collagen translocation, cells are not able to generate significant tension and thus are not exposed to tension generated by other cells. Due to the low tension in the center of the gel (generally the area analyzed), fibroblasts do not differentiate into myofibroblasts, a highly contractile and synthetic phenotype, and they do not express organized α -smooth muscle actin (α -SMA) rich stress fiber structures, even in the presence of transforming growth factor- β (TGF- β) [86,88]. At the edges of free-floating fibroblast-populated collagen gels, the cells and collagen are aligned circumferentially suggesting anisotropic stresses in these areas and a portion of the cells are α -SMA-positive indicating heightened tension, as predicted by analytical analyses [91,92].

Inside anchored matrices (zero radial displacement at the outer boundary), cells initially appear similar to cells in free floating matrices and migratory forces dominate contractile forces for short culture duration [93]. With cooperative cell remodeling of the matrix over many hours, the cells become extended and stellate or bipolar [10,86], and the tension in the gel rises rapidly over 24 h to an equilibrium “homeostatic” tension [94]. Under these conditions, cells can generate sufficient tension against the fixed boundary to differentiate into phenotypes observed on stiff substrates; however, the means by which they achieve the high tension differ in 2D and 3D systems. In soft protein gels, as in wound granulation tissue [95], the cells generate tension between each other via the matrix which must be restrained at the boundaries. On 2D substrates, single cells can generate high levels of tension against the stiff material, as occurs in stiff fibrotic tissues. In both systems, fibroblasts initially form α -SMA-negative stress fibers, generate contractile forces via Rho kinase (contractile remodeling), and have a stellate-like morphology; these cells are termed proto-myofibroblasts by Tomasek et al. [85]. In the presence of TGF- β , proto-fibroblasts become differentiated myofibroblasts as defined by expression of α -SMA-positive stress fibers and by generation of increased contractile forces [10,76,85,96]. The actions of growth factors such as TGF- β are strongly regulated by the tension that the cell can generate within the ECM. For example, in the low tension environment of floating matrices, TGF- β stimulates fibroblast contraction directly as an agonist; in anchored matrices TGF- β stimulates differentiation into the myofibroblast phenotype and increases generation of residual stress in the matrix [97].

To decrease the tension in cell-populated gels, an anchored gel may be released after compacting for a period of time (generally 3–5 days) (Fig. 1) [98]. This experimental condition, often termed stress relaxation but more aptly called release, is thought to

represent an accelerated transition between granulation tissue and late-stage wound healing where the cells are shielded from extrinsic stress [88,95,99]. Following release from rigid constraints, the tissue rapidly contracts (retracts) due to a combination of passive residual stress [100] and active cell contraction involving a stress-fiber dependent smooth muscle-like mechanism [98] (Fig. 2). The rate and magnitude of retraction are often utilized as metrics of the cells ability to generate contractile force [101]; however, these metrics should be interpreted cautiously as the immediate retraction is due to residual stress stored in the matrix, not active muscle-like contraction. Further, the ensuing change in shape depends both on the stress that the cells can actively generate and on the compressive stiffness of the matrix against which they contract, thus even if the cells are able to generate substantial force, little retraction will be observed if the matrix is very stiff due to extensive remodeling. Methods for measuring tension (described in the following section) are more reliable means of determining the contractile ability of the cell population. Upon retraction, the cell morphology changes profoundly, cell proliferation and collagen synthesis decrease [102], cells switch from an active remodeling to a quiescent phenotype [102], and myofibroblasts dedifferentiate and/or apoptose [103,104]. The magnitude and rate of the drop of tension required for phenotypic switching and other biological sequelae following release are not currently known as experimental systems have not been developed for this purpose.

In the majority of studies utilizing collagen and fibrin gels, a circular well is utilized with uniform radial boundary conditions. Mixed boundary conditions (alternating free and anchored regions) and different shapes (square, rectangular, annular, cruciform, tubular, etc.) allow researchers to alter the patterns of tension in the samples as the cells compact protein matrices and to correlate the stress field with cell behaviors such as matrix deposition. The cells and collagen fibers in rectangular and annular gels become highly aligned parallel to the free surfaces, indicating the lines of tension [106]. In cruciform gels, *de novo* collagen fibrils in cell-laden fibrin gels align parallel to the free edges in the direction of highest tension [107], and the degree of alignment can be controlled by the relative width of the arms [108]. When cells are embedded in fibrin gels in a cross-shaped mold with different arm widths, collagen production is higher in narrower arms where the stress is higher (as computed by multi-scale models [107]).

Nested collagen matrices are another innovative means for investigating cell–matrix interactions with mixed boundary conditions [109,110] (Fig. 3a). In this system, a dermal equivalent, obtained by the compaction of a free-floating collagen gel by fibroblasts, is embedded in an acellular collagen gel. Cells migrate into the outer acellular matrix and collagen is pulled by the cells towards the dense dermal equivalent. In free-floating nested gels, the collagen flow from the outer region to the cell-populated inner region is more pronounced than for attached nested gels, while cell migration is lower (Fig. 3 b and c). These results highlight the fact that cell migration and collagen translocation in a collagen gel depend on the overall resistance of the matrix to traction forces applied by cells [110], i.e., the effective stiffness. Similar cell–matrix interactions are observed when the resistance of the matrix near a cell in a collagen gel is altered by applying compressive local strain. When collagen fibers are pushed towards the front of a cell, the tension the cell can generate decreases and the cell temporarily shortens, then the cell extends again by reprotrusion and pulls the collagen fibrils inward to reestablish a homeostatic tension level [11].

Comparison of the cell–matrix interactions in floating and anchored nested gels illustrates that proximity to the lower boundary, not just lateral boundaries, also affects the ability of cells to generate tension within a protein gel. Feng et al. [111] recently reported on a microfluidic system created to investigate the effects of gel thickness and fiber alignment in thin stacked gels. The authors found that cell morphology and motility of cells in 3D gels were affected by both fiber alignment and substrate thickness and attributed the changes to

an increase in effective stiffness of the thin gels relative to the thick gels. By culturing cells on the surface of anchored gels of varied thickness, we have also observed a progressive increase in cell spreading with proximity to the stiff underlying boundary. Enhanced spreading is observed even when the boundary is over 100 μm from the cell, indicating long-range tethering of the fibers which limits collagen translocation and facilitates the generation of cell traction (M. Rudnicki, H. Cirka, M. Aghvami, E. Sander, Q. Wen, and K. Billiar, Biophys J.).

Refined control and measurement of tension: adjustable boundary conditions

As discussed in the previous section, free-floating and rigidly anchored boundary conditions result in very different states of tension within cell-populated collagen and fibrin gels and strongly affect cell behavior. The rate and extent of gel initial compaction and retraction upon release [100,102] provide relative measures of cell activity in these systems, yet these are indirect measures of motility and contractility. For refined mechanobiological investigations, methods for quantitatively measuring and controlling tension within cell-populated collagen and fibrin gels have been developed as described below.

To measure forces generated by cells in cell-populated collagen gels, Lapiere et al. [112] and Kolodney and Wysolmerski [113] developed culture force monitors (CFMs) in the early 1990s. A CFM consists of a cell-populated collagen gel suspended in media between a highly sensitive isometric force transducer and a rigidly fixed anchor (Fig. 4). The cell-generated tension is propagated hundreds of microns through the fibrous matrix between cells and to the attached boundaries [90] and can be measured externally if the cell density is sufficiently high and if the matrix is compliant enough to avoid stress shielding [10]. Initially, when cells are resuspended in the gels, the force generated increases rapidly due to traction applied to collagen fibrils by cells during migration [114,115]. After approximately 24 h, the tension reaches a plateau as the compaction phase ends and the remodeling phase commences. In the remodeling phase, the cells cross-link the collagen in the new compacted arrangement and lock in residual stresses [116]. To further investigate the effects of the boundaries on the cell tension, Brown, Eastwood and colleagues [10] modified the CFM such that one side could be actuated, and they found that cells quickly respond to extension and retraction of the rigid boundary to maintain a level of tensional homeostasis [10]. The particular homeostatic tension level developed by a population of cells is dependent upon soluble and mechanical factors such as growth factors, initial protein concentration, and boundary conditions [26,73,74]. To determine the contractile potential of the cells, the cells can be stimulated by depolarization (e.g., with potassium chloride) or by vasoactive agents (lysophosphatidic acid, thrombin, etc.). The basal tension can be eliminated by agents which disrupt the cytoskeleton (e.g., Cytochalasin-D) to determine the residual stress that is built up in the matrix a result of remodeling [26,73].

Whether comparing cell types, the effects of soluble factors, or cell–matrix interactions, the most important metric from culture force measurements is the force generated per cell. The force per cell is generally calculated by dividing the total measured tension by the number of cells in the gel [112,114]. Using this calculation, dermal and cardiac fibroblasts generate 0.1–10 mN per million cells (i.e., 0.1–10 nN per cell) in both uniaxial [112,114] and biaxial [26,117] systems. Although straightforward, this calculation assumes that all cells act in parallel, which is clearly an over-simplification (see schematic, Fig. 5a). On the other extreme, if we assume that all of the cells act in series, the total force measured would be equal to the force generated by a single cell, which is clearly an overestimate (Fig. 5b). Since the cells are distributed approximately uniformly throughout a cell-populated collagen gel, cells act in parallel with some cells and in series with others. In practice, systems which

utilize a high aspect ratio (Fig. 4b) [112] yield a lower force per cell than those using a low aspect ratio (Fig. 4a) [113] for the same cell density and contractile activity and produce different levels of cell and matrix alignment [93]. As an improvement to simple parallel and series models, Kolodney and Wysolmerski [113] divide the measured uniaxial force by the cell cross-sectional area measured from histological cross-sections to calculate cell stress (they term tension). Alternatively, calculating the stress in the tissue can be used to normalize the total force to the cross-sectional area of both cells and gel. This method is appropriate for comparing the cell contractility of cells within gels with similar cell density; however, if the cell density is markedly different, changes in cell activity may be misinterpreted. For example, as shown schematically in Fig. 5c, for two tissues with identical cross-sectional area and measured force, if one tissue has twice the force per cell but only half of the cell density, the calculated stresses would be identical; thus the stress measure would mask the difference in individual cell contractile force. As a relatively straight-forward solution that does not require histological analysis, a representative volume element (RVE) containing one cell and the surrounding volume of ECM (equal to the total volume divided by the total cell number) (Fig. 5d) can be used where total force measured is equal to the sum of forces for all RVEs in a perpendicular cross-section. Assuming all cells are aligned in the direction of the measured force, the force per cell, F_C , is simply equal to the total force, F_T , divided by the number of RVEs in parallel (n_E). Using this method for a biaxial configuration (where the total force is divided by two to account for random cell orientation [26]), dermal fibroblasts generate 17–100 nN per cell for low and high stiffness boundary conditions, respectively, after three days of culture in standard media.

Another approach to study the effects of tension on cell behavior is to apply a force boundary condition which is resisted by cell-generated forces [117]. This method has the benefit of being able to prescribe the total force generated by the population of cells, including anisotropic loading (different weights on each axis); however, there is a very limited range of weights that can be applied. If the load is too low, the cells will compact the gel similar to a free gel, and if too high for the cells to generate an equal and opposite force, the cell-populated gel will stretch out until the anchors hit the rigid stops [117]. Costa and colleagues [118] utilized this method to study the effect of altering the axis of tension and found, for the first time, that cell realignment precedes collagen fiber realignment indicating that a change in global loading can overcome local contact guidance.

The tension within a cell-populated protein matrix may be modulated more subtly by anchoring the boundaries with compliant beams which are able to bend with cell-generated forces. This method also provides the ability to monitor the tension by measuring beam deflection which is proportional to applied force. Flexible beams were first used to measure the force generated by fibroblasts seeded in collagen–glycosaminoglycan sponges [119], and Freyman et al. report force per cell values of ~1 nN regardless of overall stiffness of the system (intrinsic matrix stiffness plus beam stiffness). We developed an analogous system utilizing thin stainless steel wires as compliant anchors to modulate the boundary stiffness of suspended collagen gels in a biaxial configuration [26]. In this system, the resistance to deformation can be tuned from negligible stiffness (free-floating) to infinite stiffness (rigidly anchored) boundary conditions (Fig. 6) [26]. In contrast to the aforementioned study in collagen-GAG sponges [120], we found that increased boundary stiffness (0.048–0.64 mN/mm) elicits enhanced basal tension and potassium-stimulated active contractile force from fibroblasts. Remodeling of the collagen matrix also increases the intrinsic matrix stiffness as a function of boundary stiffness indicating stiffness-dependent phenotypic regulation of the cells which is synergistically enhanced by growth factors (e.g., TGF- β 1). An analogous compliant-boundary system has been developed utilizing cantilevered silicone posts for the development and characterization of uniaxial millimeter-scale engineered cardiac tissues. Cell-populated fibrin [121] and collagen gels [122] are suspended between 3–3.5 mm high

poly(dimethylsiloxane) (PDMS) posts, and the beating force generated by neonatal rat cardiomyocytes has been measured by dynamically tracking the post deflections.

Utilizing the same principle of controlled-stiffness boundaries but on a smaller scale, Chen and colleagues [73] developed an array of silicone elastomer-based microwells (<1 mm in length) containing PDMS cantilevers (termed micro-tissue gauges, μ TUGs) to culture cell-populated collagen gels between compliant anchors in a more high-throughput manner (Fig. 7). The small size of the samples offers the advantage of low material costs, reduced diffusion limitations, and ability to utilize powerful optical microscopy methods. Using the μ TUG system, NIH 3T3 fibroblasts and rat cardiomyocytes have been shown to generate greater force per cell against stiff anchors than soft anchors [73,74]. Higher initial collagen density (which increases intrinsic stiffness) results in higher total force but lower tissue stress due to decreased compaction [73,74]. The same system has also been used to measure changes in tension as a response to chemical [123], electrical [74], and optical [124] stimuli in airway smooth muscle cell-, cardiomyocyte-, and myoblast-populated collagen gels, respectively. The intrinsic stiffness of the microtissues can be measured by attaching magnetic beads to one of the cantilevers in each well and pulling the tissues by magnetic force [125]. Combining this technique with treatments to eliminate cell contraction, the authors determined that the tissue stiffness is not affected significantly by the active cell tension. They also note that residual tension remains in the tissue following cell deactivation, indicating substantial collagen remodeling during the culture period [125].

We have utilized the μ TUG system to systematically examine the mechanobiology of aortic valvular interstitial cells (VICs) within fibrin gels. In our preliminary studies, we have begun to quantify the relationship between tension (modulated by boundary stiffness) and growth factor sensitivity (e.g., TGF- β 1) (M. Kural and K. Billiar, submitted). With soft cantilevers, the force comes to an equilibrium level after ~24 h regardless of TGF- β 1 concentration in serum-free medium (Fig. 8a). Under stiff boundaries and in the presence of higher than 0.5 ng/ml TGF- β 1, tensional homeostasis is delayed significantly in agreement with the findings of Marenzana et al. [115]. Fig. 8b shows the force response surface as a function of cantilever stiffness (0.17, 0.56 and 1.01 nN/nm) and TGF- β 1 concentration (0, 0.5 and 5 ng/mL) after seven days of culture demonstrating synergy between cantilever stiffness and TGF- β 1 in stimulating cell force generation. In the absence of TGF- β 1, the boundary stiffness does not have a significant effect on the forces generated by the VICs, and 0.5 ng/mL TGF- β 1 appears to be a threshold concentration for triggering an increase in cell-generated force in this cell type. We have also investigated the modulation of collagen production by tension. VICs embedded in fibrin gels were cultured in the μ TUG system for five days either with either 0 or 5 ng/mL TGF- β 1, and either 0 or 5 μ M Blebbistatin. Our preliminary results indicate that reduced tissue stress results in a decrease in *de novo* collagen accumulation in the neotissues (Fig. 9). These data, and the variety of other quantitative data obtained with compliant boundary culture systems in just the past four years since their development, clearly demonstrate the versatility and power of these devices for the study of mechanobiology.

Conclusions

For over half of a century, studies utilizing collagen and fibrin gels have provided great insight into the regulation of cell tension in a tissue-like environment. These 3D systems have facilitated the study of the effects of cell-generated tension on cell morphology, motility, synthesis, proliferation, and ECM remodeling [9,11]. Continued refinements in polymerization conditions, controlled boundary conditions, imaging, and mathematical modeling are providing ever more quantitative knowledge of the mechano-regulation of cell behavior and fate in 3D.

For single-cell analysis, studies combining the ability to independently alter protein density, structure, and stiffness [53] and measure traction force in 3D are likely to reveal detailed quantitative relationships between local matrix parameters and cell-generated forces (see the review by Hall et al. in this volume). Advanced imaging and mathematical models are also beginning to shed light on the mechanisms of long-range mechanical signaling through the fibrous structure to overcome cell–cell and cell-boundary interactions which confound analysis of single-cell behavior in these gels.

For cell population studies, innovative nesting of cellular and acellular gels are offering innovative ways to study how gel boundaries modulate the balance between migration and contraction and regulate ECM reorganization [109,126]. More refined control and measurement of cell-generated tension is also being facilitated by improvements in controlling and monitoring the global boundaries of densely populated gels [26,73,107]. It is becoming clear that analysis of both the local and global mechanical environments is needed for a more complete understanding of tension regulation in 3D gels since, due to collective cell behavior, the ability of cells to generate tension is regulated by the overall resistance to deformation of the matrix.

Future studies utilizing collagen and fibrin gel systems will undoubtedly provide a more complete picture of mechanobiology in 3D tissues. Further work is needed to determine mechanisms of long-distance force transfer to and from cells to separate the effects of local mechanical properties and cell–cell forces on cell behavior. Continued effort is also required to deconvolve mechanical, chemical, and other changes which occur with compaction concomitant with increased cell-generated tension. Although it is clearly important to isolate the individual effects of these stimuli, more extensive combinatorial studies are also essential for quantifying synergistic (and inhibitory) relationships between stimuli which never act alone in vivo; these studies must utilize graded levels of stimuli to assess nonlinear interactions between the variables. Further, 4D culture systems need to be developed to enable control and measurement of mechanical and chemical signals within the gels over time, e.g., changing global or local stiffness on the fly, expanded use of reporter constructs, and new methods for dynamically masking/presenting ligands within the gels. Such studies will add to our knowledge of the regulation of cell-generated tension by soluble, material, and mechanical cues within 3D matrices, and aid in our understanding of how the state of a cell's internal tension modulates its sensitivity to external stimuli. The quantitative knowledge gained from these studies will facilitate development of new ways to treat diseased tissues and to direct cell behavior and fate in regenerative medicine and tissue engineering applications.

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Fig. 1.

Fibroblasts in free floating gels resemble the quiescent cells in interstitial tissues, while cells in anchored gels are activated and differentiate into myofibroblasts (in the presence of TGF- β) as observed in active wound healing. Releasing anchored gels simulates the accelerated completion of wound healing.

Fig. 2.

(a) Photographs of a fibroblast-populated fibrin gel floating within a 24 mm diameter culture well 40 s and 7 min post release showing the decrease in area from the constrained size (indicated by dotted circle) due to retraction. (b) Total retraction in the tissue, $[1 - (A/A_0)] \times 100\%$, where A and A_0 are final and initial areas of the gel, increases roughly exponentially with time to an equilibrium value with a time constant of approximately eight minutes. To determine the active cell component (R_A) of the observed total retraction (R_T), the immediate passive retraction (R_0) can be subtracted from R_T . (c) Growth factors differentially affect active cell retraction. From [105] (a and b) and [100] (c).

Fig. 3. (a) Nested collagen matrices contain contracted floating collagen matrices re-embedded in cell-free matrices which can be anchored to the culture surface or floating as shown in (b). (b and c) Cell migration into the acellular region is more extensive into anchored nested gels. From [110].

Fig. 4. Culture force monitors with (a) low aspect ratio and (b) high aspect ratio. Modified from [113] (a) and [112] (b).

Fig. 5.

Schematic representing idealized arrangements of cells in a collagen gels in (a) parallel and (b) series formations for the purpose of estimating the force per cell, F_C , from total measured force, F_T , from the total number of cells, n_C , assuming uniaxial restraint. (c) Hypothetical volume elements with identical cross-sectional area the upper element has twice the cell density of the lower element but each cell generates half of the force; in the case of equal cross-sectional area, A , the calculated stress would be the same for both elements. This calculation would not reflect the higher contractile activity of the cells in the lower relative to the upper element demonstrating the flaw of tissue stress calculations for the estimation of cell contractility. (d) Micrograph of a uniaxially constrained cell-populated fibrin gel showing a representative volume element (RVE) containing one cell within its associated volume which is equal to the tissue volume in the central region divided by the cell number in the central region. n_E = number of RVEs in parallel in a given cross section. Note that F_C is lowest in (a), highest in (b), and in between in (d).

Fig. 6.

(a) Schematic view of a compliant-boundary system. (b) Bottom view of compacted collagen gel after culturing for three days (scale bar 10 mm). (c) Finite analysis of distribution of stresses generated by cells compacting the gel against four stiff boundaries (simulated by decreasing the temperature of the linear elastic material in the FE software). Adapted from [26].

Fig. 7.

(a) Arrays of microtissues are simultaneously generated in a PDMS substrate. (b) Cross-section view of a single micro-tissue gauge well. (c) Representative images depicting the time-course of a contracting microtissue. (Scale bars: *A*, 800 μm ; *B* and *C*, 100 μm). From [73].

Fig. 8.

(a) Valvular interstitial cell-generated forces reach an equilibrium after one day with soft boundaries, but increase for over five days under stiff boundaries with 0.5 ng/mL or more exogenous TGF- β 1 when cultured within fibrin gels. (b) Preliminary response surface of cell-generated forces in fibrin gels for combinations of three boundary stiffness levels and three TGF- β concentrations after seven days in culture.

Fig. 9.

Preliminary data indicating that *de novo* collagen deposition (measured by image analysis of fluorescence) by valvular interstitial cells (VICs) within fibrin gels is tension-dependent. VIC-populated fibrin gels were cultured for 5 days anchored by flexible but relatively stiff boundaries ($k=1.1$ nN/nm) with either 0 or 5 ng/mL TGF- β 1 and either 0 or 5 μ M Blebbistatin. (a) Top: collagen staining (red) is shown alone for clarity. Bottom: composite image of collagen (red), actin (green), and nuclei (blue); scale bar: 100 μ m. (b) Collagen staining intensity as a function of calculated tissue stress.