Review: Roles of Microenvironment and Mechanical Forces in Cell and Tissue Remodeling

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Abstract

This article provides a brief review of current research in cellular and tissue biomechanics, with an emphasis on the roles of the extracellular matrix and external forces on cell and tissue remodeling. In this rapidly developing interdisciplinary field, molecular biology, biochemistry, material science, biomedical engineering, and clinical medicine are integrated to understand intracellular signal transduction, gene regulation, tissue remodeling, and functional restoration or regeneration of injured or degenerated tissues and organs. Analysis of the responses to various types of externally applied force over a range of magnitude scales has helped to elucidate how cells sense external forces and respond to microenvironments from the single cell to tissue levels, from the micro to marco scales, and from two dimensions to three dimensions.

Keywords: External force, Cell biomechanics, Tissue remodeling, Signal transduction

1. Introduction

The human body is continuously exposed to various types of external force, such as the stretching of skeletal muscles, the compression of bones and cartilage, and the shear stress acting on blood vessels. The concept of tissue engineering was articulated by Fung in 1985 [1]. In the first tissue engineering symposium held in 1988, Saklak and Fox stated that as an "integrated interdiscipline of molecular biology, biochemistry, material science, clinical medicine, and biomedical engineering, tissue engineering has been shown to evolve toward a powerful new paradigm of functional restoration or regeneration of lost or degenerated tissues and organs". During the past decade, tissue engineering has indeed evolved toward this new paradigm [2].

The essence of tissue engineering is to use living cells, together with either natural or synthetic extracellular components, in the development of implantable parts or devices for the restoration or replacement of tissue function. The major topics of research in the tissue engineering field include scaffolds, growth factors, cells [3], the extracellular matrix (ECM), and mechanical forces. In recent years, there have been efforts to advance therapeutic strategies in functional tissue engineering by improving the biomechanical properties of regenerated tissue [4,5]. Based on an understanding of tissue formation and regeneration, tissue engineering aims to induce new functional tissues rather than simply implant new spare parts [6]. With advances in knowledge in biology and engineering, tissue engineering has progressed to investigating living systems at the cellular and even molecular levels. Researchers have experimentally explored various innovative cell- and tissue-based therapies in several areas.

Many principles of cell and tissue remodeling were described by Fung in his three-volume classic *Biomechanics: Mechanical Properties of Living Tissue* [7-9]. These textbooks are pioneering works in biomedical engineering [10]. Chen et al. confirmed that changes in the force balance at the cellular level can influence intracellular biochemistry and gene expression in the nucleus and cause cell remodeling [11,12]. The general concept promulgated by Cannon in his book *The Wisdom of the Body* is that the functions of the entire body are coordinated to keep physiological homeostasis in the face of perturbation [13]. This hypothesis was recently extended to the maintenance of cellular homeostasis in response to external forces; i.e., the adaptive processes of endothelial cells (ECs) to an externally imposed perturbation represent a case of the "wisdom of the cell" [14]. This extended hypothesis states that tissue and cell remodeling interacts with mechanical factors, and that the way a cell senses mechanical forces and transduces signals into gene regulation for remodeling is an important issue in regenerative medicine. The major mechanisms of

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cellular mechanics are mechanosensing, mechanotransduction, and mechanoresponse [15,16].

There are several review articles related to cell and tissue remodeling, discussing topics such as nanotechnology for cell-substrate interactions [17], mechanotransduction at cell-matrix and cell-cell contacts [18], biochemistry and biomechanics of cell motility [19], the regulation of cell functions by local force- and geometry-sensing [15], the molecular basis of the effects of shear stress on vascular ECs [20,21], and the molecular basis of the effects of mechanical stretching on vascular smooth muscle cells (VSMCs) [22]. The present review summarizes several new technologies used for cellular biomechanical studies and discusses mechano-transduction and its broader implications in regenerative medicine. The scale of force in cellular biomechanics, the various types of external force, and the microenvironment that can alter cell and tissue remodeling are also introduced.

2. Scales for cellular biomechanics

The measurements of stress and strain are essential in investigations on the biomechanical properties in tissue- and cell-based tissue engineering. Biomechanical research in the past two decades has focused on the large length scale, such as joint movement, torque, or forces experienced during level walking [23,24] or exercise [25,26]. The scales at which the kinematic and kinetic parameters of body segments or joints are measured are different from those required for tissues and cells. Figure 1 illustrates the scales of length relevant to research at the molecular, cellular, and tissue levels. Table 1 compares the length scales between classical biomechanics and cellular biomechanics in SI units (modified from [27]).

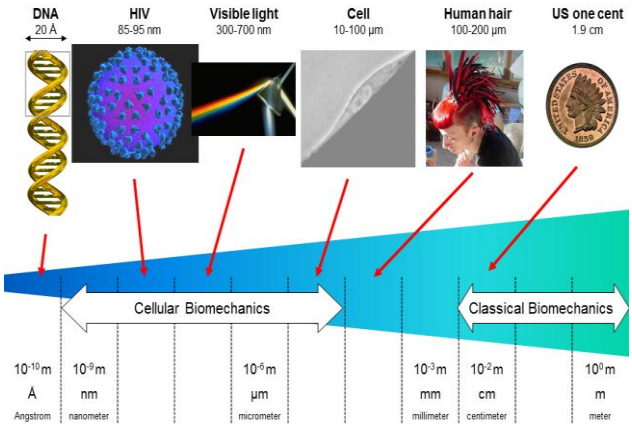


Figure 1. Comparison of length scales.

With traditional mechanical techniques, it is difficult to quantitatively apply external stress to a single cell, which is about 30-50 meter (µm, 10^{-6} meters) in diameter, or to measure its strain (or deformation). Several modern techniques in semi-conductor industries have been developed at resolutions of up to the nanometer (nm, 10^{-9} meter) scale. Nanotechnology is the science and engineering of the design, synthesis, characterization, and application of materials or devices whose smallest functional organization is on the nanometer scale in at least one dimension.

Table 1. Comparison of classical biomechanics in macro SI units and cellular biomechanics in micro SI units

	Classical biomechanics		Cellular biomechanics	
	Units	Application	Units	Application
Distance	cm	Kinematics	µm	All
Force	N	Force bearing during motion	pN (10^{-12} N)	Molecular bonds (soft cells)
			nN (10^{-9} N)	Stiff cells
Pressure (stress)	N/m^2	Foot pressure	pN/µm^2 (Pa)	Soft cells (blood cells)
			nN/µm^2 (kPa)	Stiff cells
Tension	N/m	Ligament force	pN/µm (10^{-3} mN/m)	Cortical elasticity of soft cells
			nN/µm (mN/m)	Elasticity of lipid bilayer

At this scale, it is important to consider individual molecules and interacting groups of molecules in relation to the bulk macroscopic properties of the material or device. Knowledge of the fundamental molecular structure can be used to understand the macroscopic physico-chemical properties. Biomaterials and devices designed for medical applications interact with the body at cellular and molecular scales with a high degree of specificity. Consideration of such specificity helps to translate biomaterials and devices into targeted cellular- and tissue-specific clinical applications designed to obtain maximal therapeutic effects with minimal side effects [28,29]. The understanding of the effects of external force on the human body will facilitate the determination of cell-based biomechanical properties and the development of effective tissue engineering approaches. There is a critical need to develop non-invasive and quantitative systems that can apply external force to manipulate cells and record their deformation at high resolution. The examination of cellular biomechanics in response to externally applied forces and those self-generated by the cell is discussed below.

2.1 Applied force

The remarkable advances in the semiconductor industry and modern optical-/nano-technologies have been applied to study the effects of biomechanical stimulation at cellular or subcellular levels, including gene/protein interactions for tissue regeneration.

To determine the amount of force that cells experience under regular conditions and to apply the appropriate scale of forces, common forces must be considered based on the principles of physics and their application in cellular biomechanics. The techniques used to investigate cell mechanics with controlled forces ranging from the pico-Newton to the micro-Newton include optical tweezers, magnetic tweezers, micropipette-based suction with force transducers [30,31], and an atomic force microscope (AFM) [32,33]. The ranges of forces that can be measured with these instruments are listed in Table 2.

(a) Optical tweezers

Optical tweezers use the radiation pressure of light to trap and manipulate objects such as dielectric spheres [34], sperm cells [35], and DNA [36]. Light carries a momentum of h/λ per photon, where h is Planck's constant and λ is the wavelength of

the light. When light travels through an object and is reflected or refracted, the momentum of the outgoing light is different from that of the incident light. The principle of optical tweezers is based on the force balance of the changed optical momentum as a laser passes through the trapping subject after being focused by the objective lens [34]. Using optical trapping techniques, the binding force of a cell on a fibronectin substrate [37] and the mechanical properties of single collagen molecules [38] can be quantitatively measured.

Table 2. Instruments used in studies on cellular mechanics and their force ranges.

System	Force range	Newtons	Application
AFM cantilever	nN~μN	10^{-9}~10^{-6}	Cell indentation [32] Cell detachment [50] Ligand binding force measurement [131]
Electromagnetic tweezers	10 pN~nN	10^{-11}~10^{-9}	Cell deformation in local area in rigid cell [42,43] 3D orthogonal coordinative force application [44]
Optical tweezers	1~200 pN	10^{-12}~10^{-10}	Cell deformation in local area in soft cell [39] Initial binding force measurement [50] Visualizing the mechanical activation of Src [41] DNA deformation [36] Bio-material deformation [38,132]
Cell aspiration (invasive)	100 pN~μN	10^{-10} ~10^{-6}	Cell stiffness measurement [30,31,133] Cell-cell detachment [134]

Optical tweezers can be used to micro-manipulate cells and quantitatively measure the binding force of a single cell to various types of ECM in pico-Newtons of force [39]. A dual-beam laser can be used to hold and move two free objects to measure their binding force, or to hold one object and apply a bending force to another. In a study on DNA stretching, force was measured directly from the momentum flux of light [40]. The combination of optical tweezers and fluorescence resonance energy transfer (FRET) techniques can be used to monitor mechanotransduction in living cells in response to a local mechanical stimulation [41]. The main limitation of optical tweezers in force applications is that they can exert a force of only up to about 200 pN.

(b) Electromagnetic tweezers

Electromagnetic tweezers and ferromagnetic beads can be used to apply torque to cells and determine their material properties [42]. This technique was used to apply a torque of 6 Pa (pN/μm^2) with a sinusoidal oscillation (0.1 Hz, peak to peak) to the integrin-bound beads on human-airway smooth muscle cells [43]; the storage (elastic) modulus (G') and the loss (frictional) modulus (G") were computed from the resulting bead rotation. A 3D magnetic twisting device was developed to investigate mechanical anisotropic properties using three pairs of orthogonally aligned coils [44]. The orthogonal coordinates were defined, and oscillatory mechanical torque could be applied to any axis desired. Using this system, it was discovered that mechanical anisotropy originates from intrinsic cytoskeletal tension within the stress fibers. Furthermore, the deformation patterns of the cytoskeleton and the nucleolus were sensitive to loading direction, suggesting anisotropic mechanical signaling.

(c) Cytodetachment

Once a cell spreads out to adhere to the substrate, the cell adhesion force is too strong for optical or magnetic tweezers to detach the cell. In the cytodetachment method, a glass probe [45,46], fiber-optic probe [47], or commercial AFM probe [48,49] is used to apply a shear force on a single cell and measure the detachment force from the deformation of the probe, thus allowing the quantification of the adhesion force of the cell.

The combination of optical trapping and cytodetachment techniques can be used to determine the effects of focal adhesion kinase (FAK) on adhesion force during the initial binding (5 s), at the beginning of spreading (30 min), and after spreadout (12 h), as well as growth factor-induced migration [50]. Optical tweezers have been used to investigate the force of interaction between the trapped object and a binding subject by using a suspending cell and a glass coverslide (SC/G), a trapped bead and an initial binding cell (B/IC), or a trapped bead and the apical surface of a long-term spread cell (B/ LC) (Fig. 2).

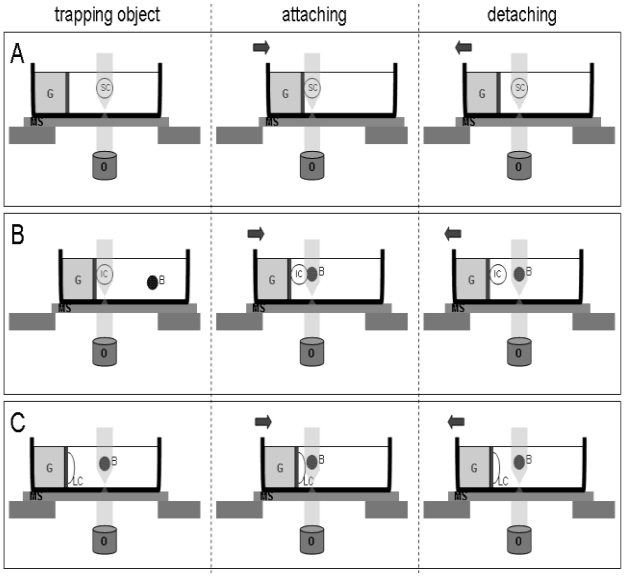


Figure 2. Schematic drawing and microscopic images of (A) SC/G, (B) B/IC, and (C) B/LC groups for measuring the initial binding force by optical trapping. The suspending cell was trapped by optical tweezers and then a collagen-coated glass surface was moved by controlling a motorized stage in the SC/G group, or the trapped bead approaching the opposite side of the cell under initial adhesion or long-term spreadout in the B/IC and B/LC groups, respectively. The initial binding force between the cell and glass surface (A), or between the cell and beads (B and C) was measured from the rupture forces determined by adjusting the laser power. Modified from Wu et al. (2005).O, 100x objective lens; MS, motorized stage; G, glass coverslide coated with collagen I; B, polystyrene bead (10 μm) coated with collagen I; SC, suspending cell; IC, initial binding cell; and LC, long-term spreadout cell.

These strategies allow the investigation of the effect of an adhesion protein such as FAK on the initial binfing force between the two surfaces and the subsequent strengthening of

adhesion in various stages of cell attachment. By integrating cytodetachment equipment with an optical tweezers workstation, the measurement of adhesion force can be extended to the order of nano-Newtons or micro-Newtons for cells with different expression levels of FAK during the spreading, spreadout, and migration phases (Fig. 3). The cytodetachment equipment used in this study was a 50-μm-wide commercial AFM cantilever which can cover the entire cell spreading area during detachment. The advantage of the side-view method is a clear distinction between the cell adhesion interface and the substrate during cell detachment. The disadvantage of this approach is its inability to determine stress distribution and the lack of a high degree of accuracy in measuring the cell adhesion force due to random cell alignment and direction of cell migration.

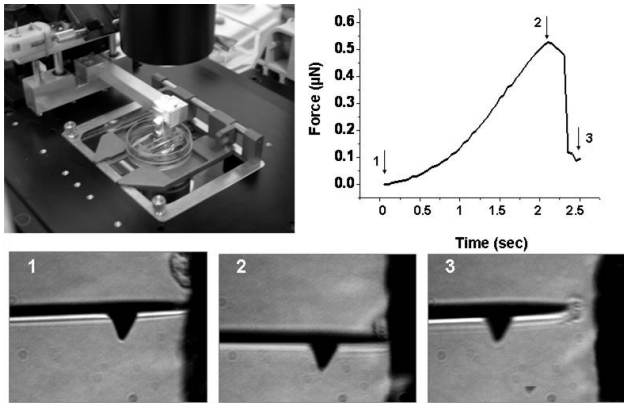


Figure 3. Cytodetachment equipment used for measuring the adhesion force by fixing the AFM cantilever on the holder and moving the seeded cell in combination with an optical tweezers workstation. With the known spring constant of the AFM probe, the cell adhesion force curve was calculated using Hook's law from the deformation of the AFM probe in detachment images. Modified from Wu et al. (2005) [50].

The forces applied by various techniques have many distinct differences. The forces applied on the cell by optical tweezers and a micropipette are normal to the cell surface, whereas the shear force applied by cytodetachment is parallel to the cell surface. In long-term spreadout cells with strong adhesion, it is difficult to detach an extended cell without damaging it, and the adhesion force detection is sensitive to the size of the micropipette's opening end, cell spreading area, cell alignment, direction of migration, and cytoskeleton rearrangement. Therefore, the measured adhesion forces in spreadout cells greatly vary with the type of cell, measurement instrument, and measurement strategy.

2.2 Force measurement

Cells continuously generate force that binds them to the substrate during the stages of cell attachment and adhesion [51]. The attachment phase occurs rapidly and involves physicochemical linkages between cells and the substrate, involving ionic, van der Waals, and other types of force. The adhesion phase occurs over a longer term and involves various biological molecules: ECM proteins, cell membrane proteins, and cytoskeleton proteins, which interact with one another to induce signal transduction, thus promoting the action of transcription factors and consequently regulating gene expression. Several technologies have been developed to measure the forces generated by cells, such as traction force microscopy [52], microcantilevers [53], and microneedles [54].

(a) Traction force microscopy

Traction force microscopy uses polyacrylamide gel embedded with fluorescent beads. The advantage of using polyacrylamide is that its stiffness can be readily adjusted by controlling the monomer and cross-linker concentrations. In brief, the acrylamides are mixed with 0.2-μm fluorescent latex beads and allowed to polymerize. Then, a droplet is immediately placed onto the surface of an activated coverslip, sandwiched by another coverslip, and turned upside down to allow the fluorescent beads to settle near the cell adhesion surface. After the non-activated coverslip is removed and the surface is coated with ECM, such as collagen type I, it is possible to quantitatively measure the traction force exerted by single motile cells with a spatial resolution of about 5 μm and a maximum intensity of about 10^4 pN/μm^2 [52]. In NIH3T3 fibroblasts during steady locomotion, a total propulsive thrust of 2 μN (i.e., 0.2 dyn) is directed to the substratum within 15 μm of the leading edge of the cell. Traction force microscopy can be used to investigate the traction force in H-ras-transformed fibroblasts [55] and the shear enhancement of traction force in migrating endothelial cells [56].

There are some limitations to tracking the displacement of fluorescent beads embedded in the substrate. The discrete markers, which are not equally spaced, do not fully cover the displacement and deformation on the continuous surface. Thus, finding a unique solution of forces using marker displacements can only be achieved by placing constraints on the deformation field or the location of adhesions [57].

(b) Microcantilevers

In this device, a horizontally mounted cantilever is deflected along one axis as individual cells migrate across it [53]. This method circumvents computational and materials problems, but can be used only to measure the force projected along one axis and generated at one location.

(c) Microneedles

Microneedles, also called microfabricated post-array-detectors (mPADs), use micro-electro-mechanical systems (MEMS) technology to create arrays of closely spaced vertical posts of silicone elastomer [54]. Cells are attached and spread across multiple posts, bending the post-like vertical cantilevers as they adhere to or migrate on the array surface. For small deflections, the posts behave as simple springs in that the deflection is directly proportional to the force applied by the attached cell. This behavior occurs for beams composed of a linearly elastic material under pure bending. Changing the post geometry without altering the bulk mechanical properties or the surface chemistry of the material used to fabricate the substrate can cause variation in the post stiffness [58]. This strategy can be used to control the spatial presentation of the mechanical properties across a surface with a micrometer resolution and measure traction forces generated by cells at multiple locations

[59]. An important factor for mPADs is the geometry of the array: posts that are too close or too soft may result in crosstalk, whereas posts that are too far apart may allow the cells to migrate underneath the mPAD surface. The diameter, vertical length, and spacing of the posts must to be optimized for each type of cell.

3. Microenvironments

The regulation of survival and death of cells such as the endothelial cells is critical to tissue development and homeostasis. Perturbations of this balance in the cardiovascular system contribute to various vascular diseases [60]. Several studies have revealed that microenvironment modulations affect cell spreading and adhesion, thus regulating cell growth, differentiation, migration, or apoptosis under static conditions [11].

3.1 Substrate geometry

Microfabrication techniques have been used to pattern ECM proteins, growth factors, and cells to investigate the role of ECM geometry in controlling cell functions [10,44,61,62]. Micropatterning technology, developed by the George Whitesides' Laboratory (Department of Chemistry and Chemical Biology, Harvard University, Boston, Mass), is based on a method used for creating microchips for the computer industry [63]. Advances in modern MEMS and nanotechnologies have made it possible to reach the micrometer or even the nanometer scale, which is at cellular or subcellular levels. Using micropatterning technology, adhesive islands have been fabricated with sizes on the order of micrometers and coated with a saturating density of immobilized ECM molecules. These adhesive islands are surrounded by barrier regions containing polyethylene glycol, which prevents protein adsorption and is therefore non-adhesive. By gradually reducing the size of islands coated with fibronectin to restrict the spreading out of endothelial cells, the cell fate can switch from proliferation to apoptosis (Fig. 4A) [11].

In micropatterning techniques, an elastomeric stamp made of polydimethylsiloxane (PDMS) is used to contact-transfer patterns of protein-adhesive alkanethiols onto gold-coated surfaces (see [64] for a detailed description of the technique). The remaining regions are coated with ethylene-glycol-terminated alkanethiols that resist protein adsorption and cell adhesion. In some studies, cell-adhesive molecules have been printed onto reactive polymer coatings, or vice versa [18]. These stamps are reusable, and many can be cast repeatedly from a single silicon master. Once the master containing the desired pattern has been generated, patterned substrates can be made without the specialized equipment and cleanroom environment that are required for photolithography. A detailed description of the micropatterning technique can be found in [64].

An alternative approach of micropatterning uses a PDMS membrane with holes or a stamp containing channels to physically mask a region on a substrate. A solution is allowed to flow into the holes or channels to functionalize the exposed region [65]. This approach has the advantage of being compatible with many types of surface. To create non-adhesive regions, it is possible to use the physical adsorption of Pluronic surfactants, a series of nontoxic tri-block polymers (polyethylene glycol–polypropylene glycol–polyethylene glycol) that have been used for several in vitro and in vivo applications [66]. The hydrophobic polypropylene segment stabilizes a Pluronic onto a surface via hydrophobic interactions, while the two polyethylene segments extend into the bulk aqueous medium, thus protecting the surface from protein adsorption and cell adhesion [67-69].

An increasing number of studies have utilized micro-patterning techniques to create anisotropic micro- environments. Figure 4 summarizes the concept of studying the interaction of the microenvironment and an external force.

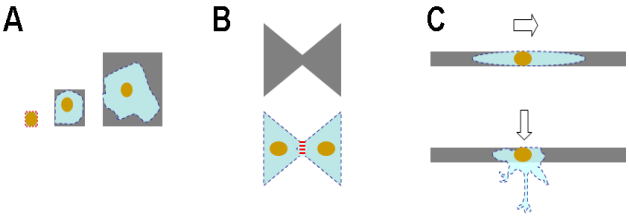


Figure 4. Single cells (blue represents the projection of the cell spreading area and brown represents the cell nucleus) can be resuspended and then seeded on ECM, such as FN (gray), that have been of micropatterned with different geometries to modulate the microenvironment for cell remodeling. (A) Concept from Chen et al. (1999). With a decrease in the size of the island, the cell spreading area decreases and the cell fate changes from proliferation to apoptosis (red dots). (B) Concept from Nelson et al. (2003). Bowtie-shaped ECM pattern (B, upper half) can be used to investigate the cell-cell interaction (B, lower half) regulated by junction proteins such as VE-cadherin or β-catenin (red lines). (C) Modified from Wu et al. (2007). When an external force (arrow) is applied in different directions to the patterned strips of ECM, the resulting orientation of the cell is controlled by the direction of the micropattern and shear flow.

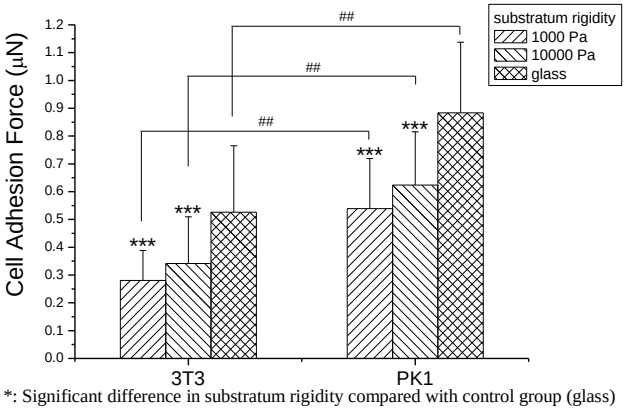
A bowtie-shaped micropattern was created for progressively altering the degree to which cell spreading is inhibited by cell-cell contact (Fig. 4B) [70]. A crossbow micropattern was created for studying the establishment of cell polarity [71]. The results demonstrate that ECM geometry can determine the orientation of the cell polarity axes. An anisotropic distribution of cell adhesions and stress fibers was induced by contact printing various shapes of ECM, such as [frame], [V], [T], and [tripod] [72]. The present authors used microlithography techniques to create anisotropic cell morphology and applied mechanical force with different directionalities to study endothelial cell remodeling and survival (Fig. 4C) [73]. The results indicate that cell apoptosis can be modulated by changing the microenvironment in terms of ECM micropatterning, anisotropic cell morphology, and mechanical forces.

3.2 Substrate rigidity

Polyacrylamide gel can be prepared to provide various levels of substrate rigidity to investigate the effect of this parameter on cell locomotion and focal adhesions. Studies on normal rat kidney epithelial cells and 3T3 fibroblastic cells [74]

showed that cells on flexible substrates had reduced spreading and increased rates of motility or lamellipodial activity. Microinjections of fluorescent vinculin showed that the focal adhesions on flexible substrates were irregularly shaped and highly dynamic. In contrast, adhesions on firm substrates exhibited a normal morphology and were much more stable. Cells on flexible substrates also contained a reduced amount of phosphotyrosine at the adhesion sites. These results demonstrate the ability of cells to sense the mechanical properties of their surrounding environment and suggest the possible involvement of both protein tyrosine phosphorylation and myosin-generated cortical forces in this process. This response to physical parameters likely represents an important mechanism of cellular interaction with the surrounding environment within a complex organism [75]. The rigidity of a cell culture substrate has also been shown to direct the lineage of cell differentiation in human mesenchymal stem cells [76]. A soft substrate that mimics the brain in elastic modulus is neurogenic, a stiff substrate is myogenic, and a rigid substrate that is similar to collagenous bone in rigidity is osteogenic. To understand the effects of substrate rigidity on cell behavior, the present study quantitatively measured the adhesion force between cells and collagen-coated substrates (rigidity values of 1000 and 10000 Pa) and a glass surface (control group) for epithelia cell line LLC-PK1 and NIH-3T3 fibroblasts, respectively (Fig. 5). Substrate rigidity significantly affects the cell adhesion force.

Collagen gel is a natural flexible substrate with various levels of rigidity that correlate to the ages of rat tail tendons (1, 4, and 8 months old) according to the degree of cross-linking. The rigidity of collagen gel is 30~100 Pa as measured by a rheometer and dynamic mechanical analyzer [77]. Three-dimensional (3D) collagen gel has been used as a cell culture vehicle for the study of morphogenesis [78]. Collagen fibrils can transduce signals through integrins and receptor tyrosine kinase (RTK), and recruit several cytoskeletal proteins to form the focal adhesion complex [79]. In 3D cultures of different cell lines on collagen gel, the FAK protein activities are down-regulated and the collagen gel induces the highest level of cell apoptosis in epithelial cells; cell apoptosis was lower in cancer cell lines than in controls [80]. Collagen-gel-induced apoptosis is through the de-regulation of activator protein-1 (AP-1), but not the mitochondrial pathway [81].



*: Significant difference in substratum rigidity compared with control group (glass)
#: Significant difference between 3T3 & PK1

Figure 5. Cell adhesion force between cells and collagen-coated substrates (rigidity of 1000 and 10000 Pa) and a glass surface for the epithelia cell line LLC-PK1 and NIH-3T3 fibroblasts, respectively, was quantitatively measured using the cytodetachment technique. The cell adhesion force is significantly affected by substratum rigidity.

An enzyme and its substrate being activated by their relative movement in position to each other during the rearward transport of actin in response to stretching has been proposed as a rigidity sensing mechanism. When optical tweezers are used to generate local stretching on the ligand, the strength of the ligand linkage between the bead and the cell surface determines how far the cytoskeleton-linked complexes will be displaced in a given time period (as compared to the displacement of beads without restraining by optical tweezers) or whether the force will be sufficient to activate the force sensors [15]. On rigid surfaces, the enzyme and substrate components may be close enough and the linkers are phosphorylated. In contrast, on soft surfaces, the enzyme and substrate components may be separated before the applied force can activate the reaction. However, the detailed mechanism between signal activation and the mechanical properties of these components is still unclear.

4. Types of external force

There are several types of external force that are used to investigate the molecular mechanisms of cellular responses. Table 3 lists some examples of mechanotransduction research that used different types of external force.

Table 3. Examples of mechanotransduction research.

Force type	Force range	System type	Example studies
Shear stress	1~2 Pa (dyn/cm^2)	2D steady (laminar) flow	Mechanosensing [82]
			Mechanotransduction [84]
			Mechanoresponse in gene regulation and cell function [135]
		Oscillatory flow	Bone remodeling [88,104,107]
		Step flow	Create disturbed flow to mimic branch point of vessel [94,95]
		2D co-culture flow chamber	Interaction between EC and VSMC under flow condition [96-99]
Stretching	5~20% deformation	Bi-axial stretching	Mechanotransduction [100,101]
			EC remodeling [14,114]
		Uni-axial stretching	Cytoskeleton remodeling in ECs [111,113]
			Remodeling in VSMCs [22]
		3D biomaterial stretching	Tissue engineering approaches in tendon and ligaments [117,119]
		Ex vivo	Whole skeletal muscle ex vivo culturing and mechanical stimulation [115,116]
Compression	1~20% deformation (Pa~MPa)	Hydrostatic compression	Static hydrostatic pressure [126-128]
			Cyclic hydrostatic pressure [129,130,136]
		Dynamic contact compression	Cartilage remodeling [120]
			Compression-induced remodeling [80,121,122]

4.1 Shear stress

ECs in blood vessels are continuously subjected to shear stress due to their constant exposure to blood flow. Shear stress can convert mechanical stimuli into intracellular signals that affect cellular functions such as proliferation, apoptosis, migration, permeability, and remodeling, as well as gene expression (for review please see [20]). ECs use multiple sensing mechanisms to detect changes in mechanical forces, leading to the activation of signaling networks. For two-dimensional (2D) *in vitro* studies, the most commonly used shear stress is simple laminar flow (with a shear stress of 1~2 Pa to mimic the EC responses in the straight parts of the aorta).

It is still not clear how the cells sense the mechanical stimulations (mechanosensing mechanism) and convert them into biochemical events. Mechanosensing might start from integrin activation [82,83], RTKs [84], ion channels [85], G-proteins and G-protein coupled receptors [86], and intercellular junction proteins [87]. Some studies showed evidence that mechanical-force-induced signal transduction events can be initiated by increasing intracellular calcium concentration [88,89] through the opening of the stretch-activated (SA) channel [90]. This action leads to the release of nitric oxide (NO) [91,92]. The cytoskeleton provides a structural framework for an EC to transmit mechanical forces between its luminal, abluminal, and junctional surfaces and its interior, including the cytoplasm, the nucleus, and focal adhesion sites. In terms of cellular responses, the transient effects of shear stress on ECs are the up-regulation of genes related to cell growth, leukocyte chemoattraction and adhesion, thrombogenesis, and lipid uptake and synthesis; sustained laminar shear stress has the opposite effects.

In addition to steady (laminar) shear, other types of flow have been used for applying shear stress, including pulsatile shear at 1.2 ± 0.4 Pa and reciprocating (oscillatory) shear at 0.05 ± 0.4 Pa [14,93]. Other flow patterns, such as disturbed shear in a step-flow channel, have also been used. In the flow device that creates such a pattern, two silicone gaskets create a vertical step expansion of channel height near the entrance. The top gasket has a longer longitudinal cutout than the lower gasket [94]. The disturbed flow pattern beyond the step consists of a region with flow recirculation in a direction opposite to that of the inflow, and a region of flow reattachment (where the wall shear stress is near zero, but the shear stress gradient is high) [95].

Several cell-cell interaction models have been developed to study the interaction between ECs, VSMCs, and leukocyte adhesion by co-culture under shear stress stimulation [96-99]. ECs and VSMCs are grown on different sides of a thin membrane (e.g., thickness: 10 μm; pore size: 0.4 μm). Exposing the EC side of the co-culture to shear flow attenuated the proliferation of VSMCs [96]. Utilizing the parallel-plate co-culture flow system, other studies elucidated cell-cell interaction in the cardiovascular system for leukocyte attraction [97], subendothelial migration [100], and gene expression in ECs [98]. These results indicate that cell-cell interactions, together with shear stress, play important roles in vascular homeostasis and pathophysiological processes.

Shear flow has also been applied in studies on other types of cell, such as the study of its effects on osteoblast metabolism (for review see [101]). Mechanical stimulations due to fluid flow or matrix strain and loading-induced electric fields have potent effects on bone cell metabolism, including modulations of the bone density, strength, adaptation, and fatigue at the macroscopic level [102]. Effects at the microscopic and sub-microscopic levels include cellular remodeling, molecular signaling, and gene expression [103]. It has been hypothesized that normal blood pressure provides a baseline level of flow in the lacunar and canalicular network in bone structure *in vivo* to maintain bone metabolism [104], and that changes in mechanical loading cause alterations in intraosseous pressure and fluid flow. The mechanical loadings *in vivo* due to daily life activities, e.g., walking, are usually dynamic and oscillatory in nature [105]. An oscillatory flow is usually chosen to mimic the effects of fluid flow loading on osteocytes in the canaliculi [106]. A recent study demonstrated that COL1 (which is important for ECM synthesis) and OPN (which regulates calcium deposition) are up-regulated through the MAPK signal pathway when human osteoblasts are subjected to oscillatory shear stress (0.05 ± 0.4 Pa) [107] and steady shear stress (12 Pa) (Fig. 6).

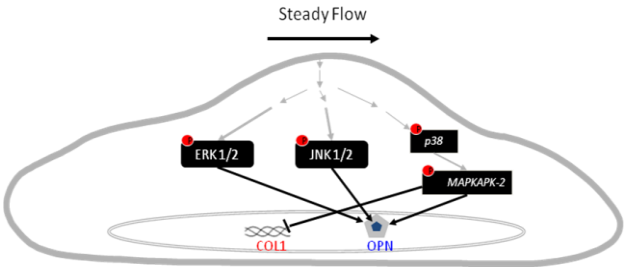


Figure 6. Steady flow caused the down-regulation of COL1 and up-regulation of OPN when human osteoblasts are subjected to steady shear stress (12 Pa). The OPN induction is related to all 3 types of MAPK. The p38 sustain activation is also involved in the down-regulation of COL1.

4.2 Stretching

The responses of cells to stretching exhibit many similarities to those to shear stress, including mechanosensing, intracellular signaling, and mechanoresponses for gene expression and functional modulation. Under physiological conditions, muscle cells are usually subjected to stretch loading, either passive stretching or an actively generated force. In 2D *in vitro* experiments, stretching is usually applied by seeding cells on an elastic membrane. Mechanical devices that cause stretch deformation [108] include a vacuum device (Flexcell International Corporation, Hillsborough, NC, USA), which directly stretches an elastic membrane by two-point holding, and a vertical indenter [109-111].

ECs and VSMCs share many similarities in their mechanisms of mechanosensing of shear stress and stretching. Stretching also involves integrins (whose activation requires specific cognate ECM proteins), RTKs (Flk-1 for EC shearing and PDGFRa and EGFR for VSMC stretching), and ion channels (Ca^{2+} channels for both EC shearing and VSMC stretching and non-selective ion channels for VSMC stretching)

(for review please see [22]). Mechanotransduction for both the shear-induced EC and stretch-induced VSMC occurs through signal pathways such as FAK, Src, small GTPase, MAPK, PI3K/Akt, and PKC. Mechanoresponses to stretching activate VSMC genes related to ECM and vascular remodeling, e.g., MMP-2, fibronectin, collagen, and elastin.

A major difference between shear stress and stretching mechanisms is that the shear stress can act on the apical surface, whereas stretching-induced activation is mainly transduced from the basal surface. The cytoskeleton provides a structural framework for a VSMC to transmit mechanical forces between its luminal, abluminal, and junctional surfaces, as well as its interior, including the focal adhesion sites, the cytoplasm, and the nucleus.

In 2D *in vitro* studies, various types of stretching have been applied to VSMCs [112]. Both uniaxial and biaxial stretching results in an increased number of actin stress fibers in bovine aortic endothelial cells (BAECs), but they have different effects on fiber orientation and the time course of JNK activation [113]. Uniaxial stretching causes an alignment of stress fibers in the direction perpendicular to the stretching force as a function of the magnitude and duration of stretching. Rho GTPase plays a significant role in enhancing stress fiber orientation perpendicular to the direction of stretching; an active mutant of Rho has an effect equivalent to around 3% stretching [114].

The effects of mechanical stimulation on skeletal muscle remodeling and the signal transduction mechanism have been studied using *ex vivo*-prepared mouse hindlimb EDL muscle subjected to 15% intermittent passive stretching [115,116]. It was found that rapamycin (mTOR) signaling is necessary for mechanical-load-induced skeletal muscle growth and that this involves phospholipase D (PLD) and the lipid second messenger phosphatidic acid (PA). Goh et al. studied the repair and regeneration of tendons and ligaments and found that cells incorporated onto a knitted poly-L-lactide (PLLA) scaffold had a stronger failure force [117-119].

4.3 Compression

The function of bones is to provide a skeletal system to support the human body shape. Different cartilage structures provide various degrees of freedom in joint movement and play an important role in bearing force during movement. Therefore, compression-related studies focus primarily on the mechanisms of bone and cartilage cells. Mechanical stimulation is an essential component in the regeneration of cartilage and bone tissues [45]. Compression-related studies generally involve hydrostatic pressure or direct compression. In 2D *in vitro* compression studies, mechanosensing, mechanotransduction, and mechanoresponse mechanisms have rarely been studied. 3D compression tests have been performed on cartilage disks (diameter: 3 mm, thickness: 1 mm) isolated from calves to investigate the biosynthetic responses to compression at frequencies of 0.002 to 0.01 Hz and amplitudes of up to 10% (around 0.5 MPa) [120]. The compression stimulated proteoglycan synthesis in as little as 1 hr and the effect remained unabated 2 days after the release of compression. Charged ECMs, interstitial fluid, and ions have all been proposed as signal transducers in cartilage tissue [105]. Recent studies indicate that compression-induced mechanotransduction involves intracellular calcium and cyclic AMP [121], NO production [122], and MAPK [123]. Compression creates multiple gene expression patterns that indicate cartilage remodeling [121].

Kino-Oka et al. developed a strategy for culturing chondrocytes with a monolayer for cell expansion and subsequent 3D growth. This technique produces cultured cartilage [124]. The authors also proposed a kinetic model of mass expansion and 3D culture with Aterocollagen gel for chondrogenesis under static conditions [125]. The equilibrium, creep, and dynamic behaviors of agarose gels (2.0~14.8%) have also been investigated in confined compression [19]. The results of dynamic stiffness and deformation-dependent permeability behavior provide important insights into biomaterial remodeling under dynamic mechanical loading. Material inhomogeneities can significantly change mechanical and electrochemical events within the ECM, and hence the microenvironments around chondrocytes. Knowing the nature of the mechanical and electrochemical events in the ECM and their variations with time and location during and after loading is essential to understanding the mechanical signal transduction mechanism(s) in chondrocytes and articular cartilage [105].

Hydrostatic pressure can also influence a variety of functions in both mammalian cells and sea organisms. Among mammalian cells, mouse 3T3 cells cultured under extremely high hydrostatic pressure (40~70 MPa) were found to induce the activation of PKC and the production of cytokines, such as interleukin (IL) -6 and -8, and monocyte chemoattractant protein-1 (MCP-1) [126,127]. A stainless steel pressurization vessel and a hydrostatic pressure apparatus were used to create hydrostatic pressure with gradual rates of compression (3~5 MPa/s) and decompression (-20~-40 MPa/s) acting on cells cultured on a glass slide or a flask. The hydrostatic pressurization increased cell proliferation at 5 MPa in 3T3 cells, but disrupted the actin and tubulin filaments at 40 MPa and caused irreversible cell death when the pressure was over 60 MPa [128].

A cyclic pressure system was used to simulate the physiologic blood pressure levels in the vasculature by injecting a pressurized 5% CO_2, 21% O_2,74% N_2 gas mixture into a sealed chamber [129]. The sinusoidal pressures (140/100 mmHg at 1 Hz) were able to reduce the death of human umbilical vein endothelial cells (HUVECs) under low serum/growth factor conditions. Under normal serum/growth conditions, exposure of HUVECs to either 60/20 or 100/60 mmHg cyclic pressures resulted in increased DNA synthesis. In contrast, cyclic pressures of 140/100 mmHg reduced DNA synthesis after 24 hr of stimulation [129]. The cell proliferation was mainly correlated to the induction of the VEGF-C gene under 60/20 mmHg cyclic pressure. The cyclic pressure also affected the transcription of several genes involved in hemostasis (tissue plasminogen activator), cell adhesion (integrin-alpha2), cell signaling (Rho B, cytosolic phospholipase A2), and TGF-beta2 [130].

Recently, the present authors developed a system that combines a compression component with a material testing component (mini Bionix II, MTS) (Fig. 7). In this system, the dynamic force factors can be programmed to provide various types of mechanical stimulation, such as programmed stress or strain. The mechanical properties of the material can also be continuously monitored during the stimulation. Such systems may facilitate the elucidation of feedback control strategies for cartilage regeneration.

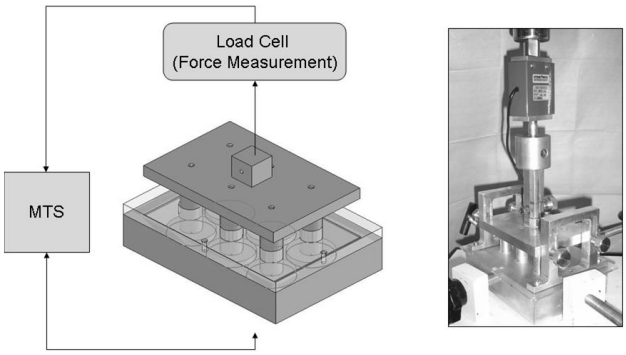


Figure 7. Integrated system for dynamic application of compression force and measurement of the changes of material properties for orthopedic related research.

5. Conclusions

Many researchers have used the bottom-up strategy (from the cell/subcellular level to the organ/tissue level, the application of external force from 2D to 3D, and from individual cell types to co-cultures) to investigate the remodeling mechanisms in the human body from the mechanical behaviors and responses at cellular and subcellular levels. The single-cell approach can be used to answer the most fundamental questions raised at the subcellular level, such as intracellular mechanosensing, how signals transfer from one side of the cell to the other side, and how different cell parts work together dynamically in response to external forces. 2D-monolayer studies can simplify the complications of using 3D tissue to study biomechanical properties in response to mechanical force. The 2D *in vitro* model can simulate many cell remodeling mechanisms based on molecular biology and genomic approaches. Studying the 3D structure and biomechanical stimulation of tissues, however, is essential for elucidating multi-dimensional interactions between force application and cell responses. The knowledge gained from such studies is essential for the regeneration of functional tissues or organs to restore lost functions of the human body, which is the ultimate goal of tissue engineering.

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