Using Functional Tissue Engineering and Bioreactors to Mechanically Stimulate Tissue-Engineered Constructs

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Bioreactors precondition tissue-engineered constructs (TECs) to improve integrity and hopefully repair. In this paper, we use functional tissue engineering to suggest criteria for preconditioning TECs. Bioreactors should (1) control environment during mechanical stimulation; (2) stimulate multiple constructs with identical or individual waveforms; (3) deliver precise displacements, including those that mimic *in vivo* activities of daily living (ADLs); and (4) adjust displacement patterns based on reaction loads and biological activity. We apply these criteria to three bioreactors. We have placed a pneumatic stimulator in a conventional incubator and stretched four constructs in each of five silicone dishes. We have also programmed displacement-limited stimuli that replicate frequencies and peak *in vivo* patellar tendon (PT) strains. Cellular activity can be monitored from spent media. However, our design prevents direct TEC force measurement. We have improved TEC stiffness as well as PT repair stiffness and shown correlations between the two. We have also designed an incubator to fit within each of two electromagnetic stimulators. Each incubator provides cell viability like a commercial incubator. Multiple constructs are stimulated with precise displacements that can mimic ADL strain patterns and record individual forces. Future bioreactors could be further improved by controlling and measuring TEC displacements and forces to create more functional tissues for surgeons and their patients.

Introduction

BIOREACTORS ARE DESIGNED to mechanically and chemically stimulate cells and tissue-engineered constructs (TECs) in culture. Spinning flasks and rotating vessels promote perfusion, while other systems deliver hydrodynamic pressure or rotational forces to control stresses and strains.^{1,2} Systems can stretch cells on monolayer and in 3D using flexible membranes^{3–5} or directly compress tissue explants.⁶ Some investigators design and fabricate their own specialized systems to fit within standard incubators.^{7–9}

In 1998, the U.S. National Committee on Biomechanics (USNCB) proposed functional tissue engineering (FTE) to improve repair by changing how TECs are designed.¹⁰ The USNCB cited the need “to increase awareness... about... restoring function in construct designs, to identify critical mechanical requirements needed for tissue engineered constructs, and to encourage tissue engineers to incorporate these functional criteria into design... of tissue engineered constructs.”^{10,11} USNCB emphasized the need to measure

mechanical signals *in vivo* and to use these signals to “precondition” TECs to their future *in vivo* setting.¹⁰ Such mechanical stimulation could enhance protein expression and matrix organization as well as tissue stiffness in culture and shorten fabrication time.

We have applied these principles of FTE in designing bioreactors to deliver more precise and relevant mechanical stimuli to TECs in culture. Applied to tendon repair, we have (1) recorded force transducer voltages in tendons in the goat (patellar tendon, PT) and rabbit (patellar, Achilles, and flexor digitorum profundus tendons) for activities of daily living (ADL), and (2) calibrated the instrumented tendons *in vitro* to determine patterns and peak *in vivo* forces.^{12–15} We have then estimated strains from these recordings using tissue constitutive properties. We have found that *in vivo* forces range between 11% and almost 40% of failure force,^{12–15} that peak strains can reach 2.4%,^{12–15} and that these patterns are more complex than typically delivered by most systems. Others have estimated using human cadaveric tissues that peak joint compressive

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stresses can reach 2–8 MPa on articular cartilage surfaces during moderate to severe ADLs^{16,17} and that menisci transmit 50–85% of these loads.¹⁶ What remains challenging is to design bioreactors to exceed these loading demands while also imposing compressive displacements of less than 0.4 mm^{18,19} and *in vitro*^{20,21} and *in vivo* compressive strains of 3–20%.^{22,23}

To achieve these FTE demands, we contend that a bioreactor should adhere to at least four design principles. Bioreactors should (1) control culture environment during mechanical stimulation; (2) stimulate multiple constructs with either identical or individual waveforms; (3) deliver precise displacements to compliant TECs, including signals that mimic *in vivo* ADLs; and (4) monitor and adjust displacement patterns based on reaction loads and relevant gene and protein expression. We are applying these criteria to bioreactors in our laboratory.

Over the past 10 years, we have developed pneumatic and electromagnetic bioreactor systems that stimulate TECs to improve musculoskeletal soft tissue repair. (1) Both systems permit us to create TECs with adequate cell viability over time in culture. The pneumatic system fits within a standard incubator, while the two electromagnetic systems (ELF 3200; BOSE Corp., Eden Prairie, MN) incorporate small incubators. (2) The pneumatic system stretches five silicone dishes with individual waveform patterns, each dish having wells to accommodate four TECs. Each electromagnetic system imposes a waveform on 6 (tensile stimulator) to 12 (compressive stimulator) constructs. (3) Our electromagnetic systems deliver dynamic, controlled displacement waveforms with micron-level precision. These systems have even delivered tensile strain profiles that mimic tendon patterns for three ADLs. Our pneumatic system has a less precise waveform, being regulated by pressure differential and displacement stops to limit peak strains. (4) Most stations in our electromagnetic systems have load cells that monitor real-time forces as the stimulated TECs mature in culture. The silicone dishes in our pneumatic system cannot monitor construct force.

In this paper, we (1) describe design characteristics for our bioreactors, including validation of culture environment and applied or measured displacements as well as preliminary data on system performance in stimulating 3D constructs; (2) summarize important findings; and (3) propose future systems needed to more effectively and efficiently stimulate TECs for load-bearing applications

Principles for Bioreactor Design

Control culture environment during mechanical stimulation

Tissue engineers seek a culture environment where cells within TECs are capable of appropriate gene and protein expression. Engineers design bioreactors to ensure that 3D TECs receive nutrients (culture medium and gases) as they undergo physical stimulation. Engineers can place the mechanical stimulator within an incubator (our pneumatic system), or they can place the incubator within a mechanical stimulation system (our ELF 3200 systems). We first discuss the design and control of our electromagnetic incubators and then present cell viability and proliferation results for cells in TECs of both systems.

Controlling incubator culture conditions within an electromagnetic system. Our group designed a two-stage incubator to fit within each of the two commercial testing systems (ElectroForce 3200; BOSE Corp.). We describe only the compressive system here. For the main chamber, we adapted an 8" square incubator (Ported Chamber and Saline Bath accessory; BOSE Corp.) to control cell culture environment in the stimulated TECs. We maintained air temperature and relative humidity by heating a 2 L water bath in the bottom of the chamber using an internal heating system with PID control. We then secured a second, custom acrylic antechamber to the main chamber. We pumped CO₂ from an external tank into the antechamber, adjusted a CO₂ controller (Model 3057; ThermoForma, Marietta, OH) in the chamber to regulate flow to the blower, and ported CO₂ through a 2" opening in the common wall of both chambers. A sensor mounted within the main chamber continuously monitored CO₂ levels. The controller then injected CO₂ into the main chamber whenever levels fell below our specified limit (5.0%).

Characterizing environment in the small incubator. Air humidity and temperature were independently recorded by a traceable hygrometer (Model 11-661-18; Fisher Scientific, Pittsburgh, PA), and CO₂ levels were measured using a feedback sensor on the controller and a Fyrite gas analyzer (Model 11-7029; Bacharach, New Kensington, PA). We measured the temperature of 5 mL of culture media within a centrally placed tissue culture dish by a traceable digital thermometer (Model 11-661-9; Fisher Scientific). Environmental conditions were monitored to determine the time needed to reach steady state. The chamber door was then opened for 5 min to simulate dish exchanges, and conditions were again monitored to determine the time required to re-establish steady state.

Our results show that culture environment is well controlled in the small incubator. Media temperature reached 37°C within 100 min of heating the water bath to 39°C. Relative humidity reached 75% in 80 min, comparable that of a standard incubator (70–76%). Similarly, CO₂ levels reached a 5% steady state within 14 min as measured by both sensors. Opening the chamber door for 5 min only reduced media temperature by 1°C, and relative humidity was restored to approximately 70% within 10 min of closing the chamber door. Opening the chamber door did not alter pH levels in the media as evidenced by the fact that phenol red remained red/pink within the physiological range at all times.

Cell viability and proliferation within the small bioreactor. Marrow-derived murine MSCs from three animals were plated at P2 onto 35 mm tissue culture dishes at 100,000 cells per dish. Each dish was covered with Teflon film secured via a Delrin ring to maintain sterility. Control dishes were placed in a standard incubator (Model 370; Thermo Electron Corp., Marietta, OH). Media was changed every 2 days. Cell proliferation and viability were examined after 3, 7, and 14 days of culture using a Student's *t*-test ($p < 0.05$). At each time point, three dishes were removed from both the bioreactor and standard incubator. Cell proliferation was determined using an MTT assay (Vybrant MTT Cell Proliferation Assay Kit; Invitrogen, Carlsbad, CA) where live cells reduce MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to a strongly pigmented formazan product to provide

FIG. 1. Viability of cells cultured in the bioreactor is comparable to that of cells cultured in a standard incubator over 14 days, increasing 61–64% by day 7 before decreasing 6–10% relative to day 7 values ($n=3$ for each time and condition in culture; mean + 1 SEM).

colorimetric indicators of cell viability. Absorbance of formazan was measured at 570 nm in a microplate spectrophotometer (Spectra Max M2; Molecular Devices, Sunnyvale, CA), and absorbance values were plotted on a known standard curve to give cell numbers.

Using a Student's *t*-test, no differences were found in the number of viable, proliferative cells for the small vs. standard incubators up to 14 days of culture ($p > 0.25$; Fig. 1). Cell numbers first increased 61–64% in both systems between 3 and 7 days but then decreased by a total of 6–10% at day 14 relative to the peak values that we measured on day 7.

Cell viability and proliferation in the pneumatic system. TECs were created using MSCs and collagen scaffolds.

Cell isolation and proliferation: MSCs were isolated from the iliac crest of 89 one-year-old, skeletally-mature, female New Zealand white rabbits and passaged to P2 (see Refs.^{24–30}).

TEC fabrication: Four groups of TECs were created by mixing each of two MSC concentrations (0.1×10^6 cells/mL [K] or 1×10^6 cells/mL [M]) from 16 rabbits with each of two bovine collagen gel concentrations (1.3 mg/mL [L] or 2.6 mg/mL [H]; Vitrogen at 2.9 mg/mL, 95–98% type I collagen; Cohesion Technologies, Palo Alto, CA). Each cell-gel mixture was then pipetted into our silicone dishes that permitted contraction around posts (Fig. 2). All constructs remained in an incubator for 2 weeks and were fed high-glucose Dulbecco's modified Eagle's medium (DMEM) with ascorbic acid and 10% fetal bovine serum twice weekly. ANOVA was used to determine significant differences in viability among groups ($p < 0.05$).

Cell separation and viability: After 14 days, cells were retrieved from the gel using collagenase (0.25 mg/mL; Gibco BRL/Life Technologies, Inc., Gaithersburg, MD) and viability was measured using a Trypan blue (Sigma Chemical, St. Louis, MO) exclusion method and hemacytometer (see Ref.²⁴). After 14 days of contraction, cell viabilities in the KH, KL, MH, and ML (cell-gel) constructs averaged 92%, 89%, 79% and 72%, respectively. Only KH versus MH and KH versus ML showed significant differences in viability ($p = 0.001$). No significant differences were found among the

FIG. 2. Each silicone dish consists of four wells into which we pipette cells and collagen gels. Cells contract the gels around two posts (from Ref.²⁵).

other groups ($p = 0.1$). Collagenase digestion times were the same in each case.

Simultaneously stimulate multiple constructs with identical or individual waveforms

We typically use our computer-controlled system of pneumatic actuators to mechanically stimulate our TECs (see Refs.^{24–31}). Five stimulation stations fit within a frame mounted within the incubator (Steri-Cult Model 3033; Forma Scientific, Marietta, OH). Air pressure independently controls pressure differential at each station between adjustable displacement limits, allowing us to manipulate strain amplitude, frequency, and rest period between cycles. Each station can stimulate one silicone dish (Fig. 2) containing up to four TECs. End-to-end dish displacements are monitored by linear variable differential transducers in line with each actuator. The advantages of this system are that it can be placed within an incubator and has high throughput capacity, permitting us to stimulate dishes with different waveforms and to measure multiple responses (e.g., construct stiffness, histology, and gene expression).

Our two Enduratec-Bose ElectroForce 3200 systems can also stimulate multiple TECs in tension or compression. For each system, we designed and manufactured a fixture to simultaneously apply controlled displacement profiles using one actuator. For the compressive bioreactor (Fig. 3a), the fixture consisted of an anodized aluminum top plate mounted to the actuator and a parallel bottom plate attached to an adjustable arm fed through the bottom of the chamber and water bath (Fig. 3b). Each plate slid on low friction bearings along stainless steel rods (12.5 mm diameter) for smooth movement and proper plate alignment. The bottom plate had 12 recessed stations to house 35-mm-diameter tissue culture dishes. Twelve thin aluminum rods extended down from the top plate above the dish locations. The position of these rods could be modified when not in operation by adjusting the threaded portion of the rod near the top plate. Each rod also had a female component of a quick-connect coupler (MC1002; Colder Products, St. Paul, MN) attached to its free end (Fig. 3b).

FIG. 3. (A) Compressive stimulus bioreactor. (B) Environmental chamber and loading apparatus within the compressive bioreactor. (C) Schematic of culture dish with Delrin disk, ring, and film assembly.

A specialized culture dish system was also developed to speed development time so that multiple constructs could be stimulated while ensuring TEC sterility. The male end of the coupler (MC281032; Colder Products) was attached to a polymer disk (8 mm thick $\times$ 15 mm diameter) through a 100- μ m-thick film (4" $\times$ 4"; Teflon FEP 100A; DuPont, Circleville, OH). We stretched and secured the film over the dish using a polymer ring with a small threaded hole for media exchange (Fig. 3c). The film, permeable to O₂ and CO₂, prevented media evaporation and reduced contamination without impeding actuator motion. This coupler allowed each dish to be easily removed from the system, its media exchanged, and specimen groups to be rotated to increase daily throughput. Once assembled, TECs could be mechanically stimulated using the system actuator, which smoothly moved the top plate and pressed the polymer disk onto the specimen dish.

We then adapted a second ELF3200 system to stimulate constructs in tension. As with its compressive counterpart, we designed and manufactured a custom, rigid, stainless steel fixture to apply controlled displacements to multiple constructs from a single actuator shaft. The six-station fixture (Fig. 4) was placed within an environmental chamber like in our compressive system. The system is nearly frictionless

FIG. 4. Environmental chamber and loading apparatus within the tensile bioreactor. Color images available online at www.liebertonline.com/ten.

and includes a quick-release mechanism allowing rapid attachment and removal of construct batches from the fixture.

Deliver precise displacements, including those that mimic in vivo ADLs

Cell-scaffold constructs are usually stimulated in displacement rather than load control due to their weak and compliant nature during early maturation. Stimulating TECs with actual *in vivo* forces would certainly damage them before surgery unless they can be placed in a load-protected environment like a central PT defect (e.g., Refs.^{24–30}). In our pneumatic actuator, we actually measure end-to-end dish displacements using our LVDTs. We then know the corresponding displacements between the posts in the base of each well from prior calibrations. We currently suspend rabbit MSCs (0.14×10^6 cells) in 0.4 mL of high-glucose DMEM media on top of a type I collagen sponge (P1076; Kensey Nash Corporation, Exton, PA) cut to fit over the well posts (Fig. 2).²⁸ After 2 days of incubation and regular feeding (high-glucose DMEM, 5% ascorbic acid, 1% antibiotic/antimycotic, and 10% fetal bovine serum), the dishes containing TECs are typically stretched using a simple trapezoidal stimulus up to 2.4% peak strain, the largest PT strain for inclined hopping.¹² We stimulate once every 5 min for 8 h/day for up to 2 weeks. Stimulated constructs show 2.5 and 4 times the linear stiffness and linear modulus of nonstimulated constructs, respectively (Fig. 5a). While we still do not know if such improvements in gene expression are biologically relevant, it is important to recognize that these observed changes might increase protein expression and assembly and result in improvements in construct stiffness. The exact temporal relationships among gene expression, protein production, and biomechanical improvements must still be elucidated. These preconditioned TECs also significantly improve repair of rabbit PT defect injuries at 12 weeks postsurgery compared to nonstimulated constructs.^{28,30} We are now using the pneumatic system to optimize construct stiffness for different signal components³¹ and scaffold materials.²⁹ However, we also recognize that adjusting pressure differential cannot precisely control all aspects of a displacement waveform (e.g., rise and fall times and complex shape).

We now seek to further enhance construct and repair biomechanics by matching even more components of the strain–time profile. We are using both the electromagnetic devices to stimulate multiple constructs with compressive (Fig. 3) or tensile (Fig. 4) displacements accurate to the micron level and customized for strain profiles in different musculoskeletal tissues.^{13–15} Each ELF 3200 system allows us to control complex wave shapes using an actuator with a stroke length of 12.5 mm and if necessary, a minimum velocity of 0.006 $\mu\text{m/s}$. We can now rapidly examine the effects of ADL-dependent rise and fall times as well as rapid reversals in direction. Such experiments are critical in determining whether the added sophistication of mimicking such complex profiles leads to improved construct stiffness and ultimately repair biomechanics like we see when applying *in vivo* strain levels (Fig. 7).

Monitor/adjust displacement patterns based on reaction loads and biological activity

In our experience, cell lines from different rabbits can exhibit quite different rates and extents of maturation once the cell–scaffold TECs are created. Some cell lines proliferate more quickly than others and reach confluence more quickly, and when suspended in the scaffold, the resulting TECs contract, mature, and stiffen more rapidly between the posts (Fig. 2). Such cell-based differences in stiffness suggest the need to individually monitor construct stiffness (and thus TEC force) in real time. Displacement profiles and chemical stimuli (e.g., growth factors and cytokines) can then be selected or adjusted to (1) improve TEC stiffness without mechanical damage to achieve the maximum benefit from mechanical stimulation, and (2) control cell phenotype and thus the extracellular matrix that the cells synthesize.

Monitoring real-time reaction loads and altering individual TEC displacements

Recording TEC reaction loads allows us to compute real-time stiffness for each maturing construct. Currently, we cannot measure individual construct loads using our pneumatic stimulator because forces are shared among four TECs plus the walls of the silicone dish (Fig. 2). Recording individual loads would require that we instrument one silicone post in each well of the dish (which we have found to be extremely difficult). By contrast, 6 of the 12 stations in the compressive fixture (Fig. 3b) and all 6 of the stations in the tensile fixture (Fig. 4) of the electromagnetic systems contain individual load cells to measure TEC reaction load during maturation and to calculate changes in construct stiffness. In the tensile bioreactor, the moving posts attached to the actuator are each instrumented with a load cell for continuous monitoring (Fig. 4). The compliant constructs also require that we use more sensitive load cells (Model 31, 0.5 lb capacity; Sensotec, Inc., Columbus, OH) that include stops to prevent load cell overload damage during construct handling. As constructs stiffen in culture, we can then increase peak displacement in each station to challenge the construct and accelerate its maturation in culture. In the compressive bioreactor (Fig. 3), we continuously monitor reaction load of each cell–agarose construct using a higher capacity, submersible load cell (Model 31, 25 lb capacity; Honeywell Sensotec, Columbus, OH) that provides load feedback to

detect early changes in TEC stiffness. However, given the cell-dependent variability in TEC stiffness after stimulation in our tensile and compressive bioreactors, we recognize that to speed and customize development, we will likely need to individually adjust displacement profiles delivered to our constructs in culture. We are including these features in our upcoming bioreactor designs.

Monitoring and controlling biological activity during and after stimulation

Like others, we seek to discover how different mechanical stimulation patterns applied to TECs in culture affect cellular gene and protein expression as well as biomechanical stiffness and modulus.

Monitoring spent media. One way to indirectly track a TEC's activity for different mechanical stimuli is to monitor culture media. Once collected, the media could be analyzed using gel electrophoresis. After separation, each gel could be imaged to determine those conditions showing the greatest increase or decrease for each fraction. Fractions exhibiting the largest changes could then be analyzed using mass spectroscopy to identify the proteins from which assays could be developed to determine actual quantity of protein in the media. Such a strategy might be most useful as an earlier indicator of TEC maturation.

Controlling activity in spent media. Tissue engineers have still not identified the timetable for media exchange (time of day, day of the week, etc.) that optimizes maturation of a 3D construct before surgery. In some cases, cells might acclimate to a new environment with fewer media changes, while in others, cellular activity might accelerate nutrient depletion, requiring more frequent exchanges to ensure cell viability and phenotype. Rather than using a set schedule, it might be more prudent to automatically perturb the media schedule to regulate *in vitro* outcome. One could imagine programming exchange to increase rate of cellular differentiation and protein expression and then perturbing the media to reduce the time from cell isolation to surgical implantation. These efforts might be expected to (1) improve the TEC's mechanical integrity, (2) decrease the cost to manufacture, and (3) screen for low cellular activity or even termination of the experiment for that construct or cell line.

Effects of tensile stimulation on *Col1* gene expression in rabbit MSC–collagen sponge constructs. After seeding MSCs (140,000 per construct) from 10 New Zealand white rabbits onto 40 type I collagen sponges, we mechanically stimulated half the constructs (Fig. 2) in our pneumatic stimulator as described above,²⁸ while the other half remained in an incubator without stimulation for 2 weeks. In half the stimulated and nonstimulated constructs, cells were extracted and quantitative real-time RT-PCR performed to determine mRNA expression for collagens 1 and 3, decorin, fibronectin, and glyceraldehyde-3-phosphate dehydrogenase. Remaining constructs were failed in tension to determine TEC stiffness and modulus. The effects of stimulation were determined using a paired Student's *t*-test. Compared to controls, stimulated constructs showed 3–4 times greater *Col1* ($p = 0.0001$) and *Col3* gene expression ($p = 0.001$) as well

as 2.5 times the linear stiffness and 4 times the linear modulus (Fig. 5a). Stimulation did not affect either decorin or fibronectin gene expression ($p=0.2$).²⁷ This study offers one method to assess the potential benefits of stimulation in culture on gene expression before initiating more expensive and time-consuming *in vivo* repair studies. The observed increase in gene expression coupled with improved TEC stiffness suggests corresponding improvements in type I collagen protein, but this cannot be readily measured when using type I collagen sponges as scaffolds.

Effects of compressive stimulation on *Col2* gene expression in murine chondrocyte–agarose constructs. To measure changes in gene and protein expression as well as aggregate modulus due to mechanical stimulation, we cultured chondrocytes from the ribs of six singly transgenic mice³² in media (BGJb + 10% fetal calf serum), and then mixed these passage 1 cells with equal volumes of 4% (w/v) agarose (Sigma Chemical). Half the constructs were cyclically compressed (at 1 Hz to 10% peak strain for 1 h followed by a 1 h rest period for 6 h/day for 7, 14, 21, and 28 days in culture).⁹ Nonstimulated constructs were cultured in the same bioreactor for 0, 7, 14, 21, and 28 days. Media was changed daily. Constructs were mechanically homogenized,

Enhanced Cyan Fluorescent Protein (ECFP) was quantified in Relative Fluorescence Units (RFUs) using a spectrophotometer (Spectra Max M2; Molecular Devices), and mRNA expression was tracked using quantitative real-time RT-PCR. Type II collagen content was also measured at each time interval using ELIZA. Significant differences were examined using two-way ANOVA ($p<0.05$). Stimulated constructs showed 20.2%, 14.5%, and 17.1% higher RFU values at 7, 14, and 28 days, compared to time-matched, nonstimulated controls, respectively (Fig. 5B, $p<0.005$). These stimulated TECs also showed 33.5%, 22.7%, 25.1%, and 31.1% higher *Col2* mRNA values at 7, 14, 21, and 28 days relative to controls, respectively ($p<0.002$). Moreover, stimulated constructs showed 33.3% and 25.1% higher type II collagen content at 21 and 28 days, respectively ($p<0.001$), and an average 41.3% increase in aggregate modulus at day 28 compared to time-matched, nonstimulated controls ($p<0.0001$). When all results are synthesized, stimulated constructs showed earlier increases in *Col2* gene expression, leading to later increases in type II collagen content, and still later increases in aggregate modulus (Fig. 5C). Comparing the magnitude and temporal relationships among these *in vitro* measures due to stimulation combined with the repair outcomes from preclinical studies offers tissue engineers

FIG. 5. (A) Tensile stimulation of rabbit MSC-seeded sponge constructs increases (a) linear stiffness and (b) linear modulus when compared with nonstimulated constructs but also increases (c) *Col1* and *Col3* gene expression.²⁷ Shown are mean + SEM. (B) Compressive stimulation significantly increases ECFP RFU at all time points investigated ($n=6$, mean + SEM). (C) Fold increase from day 0 in stimulated murine chondrocyte–agarose constructs. Note the earlier increase in *Col2* expression, followed by delayed increase in type II collagen content, then followed by an increase TEC construct modulus ($n=6$, mean $\pm$ SEM).

the ability to rapidly assess the fate of TECs in culture and even predict their subsequent outcome after surgery (Fig. 6). Optimizing the stiffness of these constructs in culture (Fig. 7) offers the potential to then increase and control gene and protein expression in culture as well, providing a more complete connection between biological and biomechanical fate in culture and after surgery. Should we also be able to create repairs that are 1000 times stiffer than the TECs before surgery, what may be needed is a more compliant but protected TEC after surgery.

Future Directions

While bioreactors remain important for FTE, future devices may function quite differently.

Environmental chambers

Standard incubators may still accommodate mechanical stimulators, but more stimulators may be needed to speed

FIG. 6. Positive correlation between (A) construct linear stiffness at 14 days in culture and repair linear stiffness at 12 weeks after surgery ($r = 0.6$; $p = 0.02$) and (B) construct linear modulus at 14 days in culture and repair linear modulus at 12 weeks after surgery ($r = 0.7$; $p = 0.004$). CM, construct modulus; CS, construct stiffness; RM, repair modulus; RS, repair stiffness. Solid squares represent stimulated repairs, and open squares represent nonstimulated repairs (adapted from Ref.²⁸).

FIG. 7. This response surface for our TECs containing rabbit MSCs in collagen sponge constructs indicates that delivering a mechanical stimulus with specific components (2.4% peak strain and 3000 cycles/day for 12 days in culture) produces the peak *in vitro* stiffness among conditions examined (from Ref.³⁶). Color images available online at www.liebertonline.com/ten.

development. Alternatively, manual and even automated “change-out” strategies may introduce these TECs to the mechanical stimulator. Should chambers fit within stimulators, designers must be certain that environmental conditions remain stable.

Systems to rapidly and individually stimulate multiple constructs

Regardless of which bioreactors are used, future systems will likely need to individually precondition more and larger TECs. This is important as tissue engineers study repair in larger animals and then create constructs for human implantation. Companies may even change the bioreactor by inserting multiple mechanical stimulators in a single bioreactor room, but only if they can ensure conditions are controlled. This may even lead to patient-specific chambers or lines if autologous cells are needed and contamination is to be avoided.

Systems to deliver complex (even *in vivo*) waveforms in displacement and load control

If investigators show that preconditioning a TEC with *in vivo* signals dramatically improves construct stiffness and shortens maturation time in culture, additional research will be needed to measure these *in vivo* ADL signals for different tissue types.^{10,11} Given these patterns will likely be 3D in nature, future stimulators may need to be precise robots with up to six degrees of freedom to fully duplicate the *in vivo* signals (see below).

Systems to feedback and regulate mechanical and biological state

Tissue engineers may ultimately employ sophisticated bioreactors that not only monitor mechanical and biological states, but can also be programmed to automatically adjust mechanical and chemical stimuli to more effectively mature 3D constructs before surgery. To realize this dream will require that we understand complex interactions among these signals³³ and prioritize which measures we most want to

improve. This strategy will be difficult to establish and likely application and species dependent.³⁴

Controlling cost

One of the greatest challenges facing tissue engineers in translating their research is to be financially competitive with current clinical approaches. The initial boon of tissue engineering companies faltered under the mounting costs of long product development cycles, regulatory hurdles requiring exhaustive *in vitro* and *in vivo* data, and scale-up efforts to meet an expanding market demand.³⁴ To keep these therapies financially viable during development, our field needs to consider how culture environment is established and maintained, and how to keep the cost of custom chambers competitive with standard incubators. Bioreactor capacity will also need to be maximized. A system with limited throughput has little utility as basic research translates into clinical practice. Finally, we must discover how complex a mechanical signal really needs to be and then engineer stimulators specific to that application. Full-scale robots with multiple actuators may be ideal but too expensive and cumbersome unless they can precisely stimulate large numbers of constructs with just enough degrees of freedom to effect the desired outcome.³⁵ Performing such mass stimulation will challenge our goal to deliver individual mechanical signals to different constructs unless certain degrees of freedom are assigned to individual stimulation stations. Controlling bioreactor costs may ultimately dictate how sophisticated our stimulation efforts will be unless research absolutely mandates their inclusion in the development process.

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