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Advanced Bioreactor with Controlled Application of Multi-Dimensional Strain For Tissue Engineering

Advanced bioreactors are essential for meeting the complex requirements of in vitro engineering functional skeletal tissues. To address this need, we have developed a computer controlled bench-top bioreactor system with capability to apply complex concurrent mechanical strains to three-dimensional matrices independently housed in 24 reactor vessels, in conjunction with enhanced environmental and fluidic control. We demonstrate the potential of this new system to address needs in tissue engineering, specifically toward the development of a tissue engineered anterior cruciate ligament from human bone-marrow stromal cells (hBMSC), where complex mechanical and biochemical environment control is essential to tissue function. Well-controlled mechanical strains (resolution of $<0.1\ \mu$m *for translational and* $<0.1°$ *for rotational strain) and dissolved oxygen tension (between 0%–95%* $\pm 1\%$*) could be applied to the developing tissue, while maintaining temperature at* $37+/-0.2$°C *about developing tissue over prolonged periods of operation. A total of 48 reactor vessels containing cell culture medium and silk fiber matrices were run for up to 21 days under 90° rotational and 2 mm translational deformations at 0.0167 Hz with only one succumbing to contamination due to a leak at an medium outlet port. Twenty-four silk fiber matrices seeded with human bone marrow stromal cells (hBMSCs) housed within reactor vessels were maintained at constant temperature* $(37+/-0.2$°C*), pH* $(7.4+/-0.02)$*, and* pO_2 $(20+/-0.5\%)$ *over 14 days in culture. The system supported cell spreading and growth on the silk fiber matrices based on SEM characterization, as well as the differentiation of the cells into ligament-like cells and tissue (Altman et al., 2001).* [DOI: 10.1115/1.1519280]

Introduction

Tissue engineering can potentially address tissue and organ failure by providing functional tissue constructs grown *in vitro* that have a capacity to continue to develop *in vivo* and integrate with the host tissues. In addition, engineered tissues can serve as physiologically relevant models for quantitative *in vitro* studies of biological mechanisms inherent in tissue development. The clinical and scientific utility of tissue engineering critically depends on our abilities to (a) direct cells to form specialized tissues, (b) provide and characterize the specific regulatory signals (biochemical, physical) that are known to modulate cell function and tissue development, and (c) precisely control the environmental conditions (e.g. pH, temperature) and mass transport of chemical species (e.g. nutrients, oxygen, metabolites, growth factors) to and from the cells. To meet these requirements and advance the utility of tissue engineering, improved bioreactor systems are needed.

Bioreactors are a key component of tissue engineering, providing a controlled environment to direct cellular responses toward specific tissue structures. Ideally, a tissue engineering bioreactor must provide: (1) an appropriate environment (e.g., temperature, humidity), the maintenance of biochemical conditions (e.g., pH, pO2, concentrations of nutrients and growth factors) to support cell proliferation and/or differentiation into functional tissues, (2) sufficient metabolite transport to and from the developing tissue, and (3) structurally defined support for cell attachment and tissue formation.

Mechanical stress plays a significant role in tissue formation and repair *in vivo*. Recently, more focus has been given to the utilization of mechanical signals *in vitro*, either in the form of shear stress generated by fluid flow, hydrodynamic pressure or as direct mechanical stress applied to the scaffold upon which cells were growing. Constant strain applied to fibroblasts seeded on collagen gels, induced fibroblast elongation and alignment of the cells and enhanced the functional assembly of the extracellular matrix (ECM) [1–3]. 2D cyclic mechanical strain promoted

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smooth muscle cell proliferation, and ECM synthesis [4] and collagen I synthesis by anterior cruciate ligament (ACL) fibroblasts [5]. Uniaxial cyclic loading via a piston was used to generate a cyclic traction reactor for the long-term culture of fibroblasts in collagen gels [6]. 3D dynamic shear and compression bioreactors have been used to stimulate explants of cartilage or bone [7]. 3D reactors with pulsatile flow have been used to induce smooth muscle cell alignment for blood vessel engineering [8]. Dynamically fluctuating hydrodynamic shear and pressure during bioreactor cultivation of engineered cartilage [9–13], cardiac muscle [14–16], and blood vessels [17] resulted in improved structural and functional properties of the engineered tissues. However, the existing *in vitro* systems utilizing mechanical strain are, at best, only crude approximations of the complex *in vivo* environments. This gap between *in vitro* and *in vivo* conditions was the impetus for the development of a new bioreactor that would better simulate some of the physiological conditions *in vitro* and permit the systematic study of cell growth and differentiation into functional tissue structures.

The ACL was selected as a model tissue due to the complexity in mechanical forces applied under normal physiological conditions [18] as well as the diverse biochemical environment of the knee joint [19]. Thus, the ACL provides a suitable target against which the efficacy of the new bioreactor design can be judged. In addition, there is a clinical need for improved ACL tissue replacements due to donor site morbidity, lengthy rehabilitation periods, and increased risk of tendonitis.

A number of studies used ligament fibroblasts in bioreactors to generate ligament-like tissues [2,5]. Recent findings of the potential of adult stem cells to lead to a variety of differentiated cell types [20,21] suggest that this cell type will provide important new options to forming biologically and functionally relevant ligament tissues *in vitro*. However, this goal can only be achieved if suitable environmental signals, both mechanical and biochemical, can be provided to the cells to direct their differentiation path. Importantly, none of the known biochemical regulatory factors has been shown to promote adult stem cell differentiation into ligament-like cells *in vitro*. Previously, we successfully demonstrated that human bone marrow stromal cells (hBMSCs), in the absence of specific ligament growth and regulatory factors, could differentiate into ligament-like cells through the application of physiologically relevant cyclic multi-dimensional mechanical strain [22]. Based on those findings, a novel bioreactor system was developed that could impart sophisticated mechanical signaling, designed to mimic some aspects of the actual *in vivo* environment, to the growing tissue under well-controlled environmental conditions. In this paper we describe the design and validation of a bench-top bioreactor system that provides enhanced control of the biochemical environment and complex mechanical forces applied to the cell-seeded scaffold for ligament tissue engineering. The system has been characterized and its utility demonstrated in experiments using hBMSCs grown on silk fiber matrices.

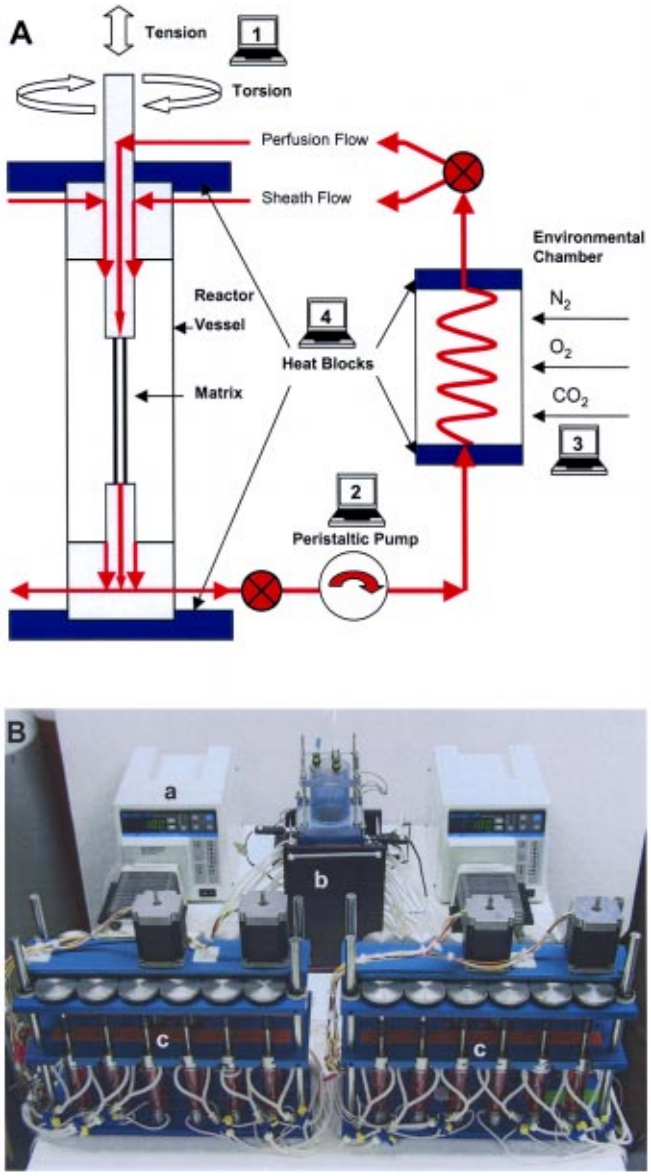

Fig. 1 A. Schematic illustrating the bioreactor system. B. Functioning bioreactor system including (*a*) peristaltic pump, (*b*) environmental gas chamber and (*c*) the two bioreactors containing 24 vessels.

System Overview

The system encompasses two independently controlled bioreactors that share a common environmental control chamber. The system, shown in Fig. 1 A&B, is comprised of the following components/subsystems: (a) the reactor vessels in which the matrix is resident, (b) an environmental chamber to control gas exchange via the growth medium, (c) a multi-channel peristaltic pump to recirculate the growth medium, (d) a motion control subsystem to subject the matrix to mechanical stimuli and (e) a thermal control subsystem to maintain appropriate tissue growth temperatures. Each reactor vessel has its own independent loop to avoid cross contamination and allow change in biochemical factors from one vessel to another.

Reactor Vessel

The bioreactor provides independent but concurrent control over translational and rotational strains imparted to the growing tissue housed within the reactor vessel. The co-axial reactor vessels are designed to provide two degrees of freedom for mechanical deformation of the growing ligaments. Each vessel is comprised of top and bottom mounts, a vessel tube, and two anchor-shafts (Fig. 2A). Custom-designed parts are machined from 304 stainless-steel (S.S.). Off of the shelf items are: 18-8 S.S. set screws, polycarbonate tubing, nylon or kynar luer lock connections, bronze hubs and rubber Buna-O O-rings. The polycarbonate tubing, which comprises the vessel wall, is optically clear allowing real time observation. The inside diameter of the tube is fixed at 1.91 cm (0.75″) while the length of the tube is sized to accommodate the length of the matrix or tissue desired. Vessels are designed to allow for quick assembly/disassembly and all parts can be steam or gas sterilized.

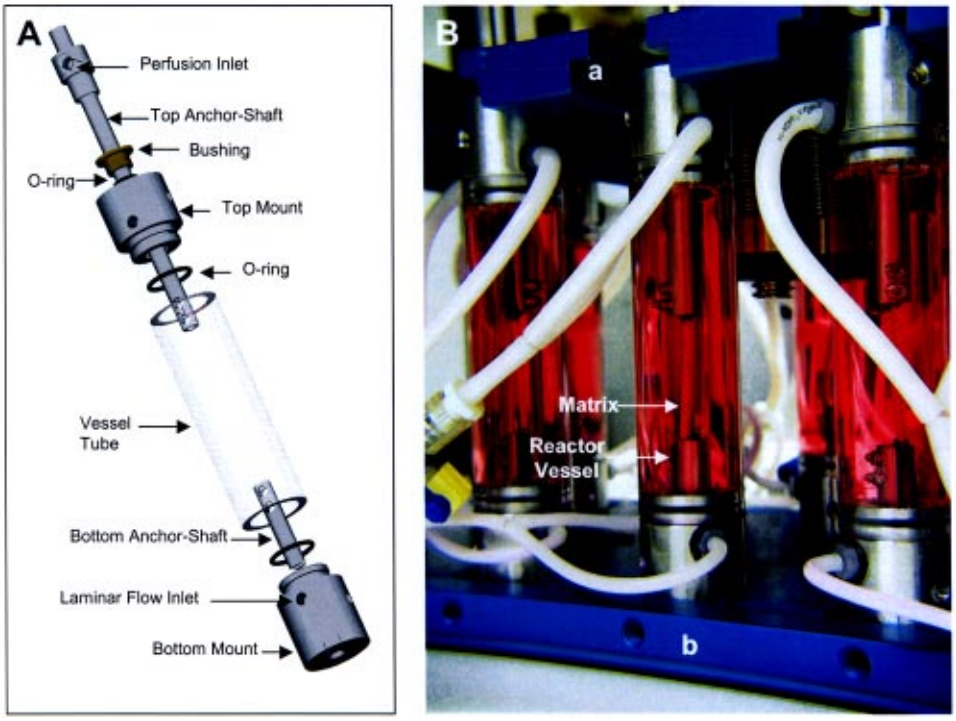

Fig. 2 A. Exploded-view of the reactor vessel assembly. B. Reactor vessel containing an anchored silk fiber matrix during culture with seeded hBMSCs. Vessel 'seats' (*a*) within the fixed-position plates and vessel locking bar (*b*) are shown.

Vessel Mounts. The top and bottom mounts provide two diametrically opposed radial inlets (or outlets). These feed 1.27 cm (0.5″) long annuli in the mounts. The annuli (of outer diameter of 0.95 cm (0.38″)) allow a radially invariant sheath flow around the 0.64 cm (0.25″) diameter shaft and into the main volume of the vessel. The annuli dimensions were calculated on the basis of the flow rates provided by the peristaltic pump (0.25–25 ml/min). When used to culture ligaments, laminar flow can be achieved around the surface of the growing tissue by varying the flow rate. Both mounts employ O-ring grooves for sealing of the polycarbonate tube to the mounts (Fig. 2A). All inlet and outlet connections were made with nylon or kynar plastic luer lock fittings.

The bottom mount (Fig. 2A) contains a co-axial blind hole into which the anchor-shaft can be affixed via set-screws. The bottom mount also contains two blind threaded holes on the exterior which do not protrude into the reactor vessel. One of the holes is radial while the other is coaxial. The radial hole is used to attach a stabilizing arm for use during vessel disassembly and while the axial hole exists for vessel loading into an Instron or a base-fixture.

The top mount (Fig. 2A), designed with a 6.38 mm (0.25″) through hole and a counterbore at the top of the mount for an O-ring and bronze bushing, allows the top anchor-shaft to protrude from the interior of the vessel, through the O-ring and bushing, to the exterior of the vessel where it is coupled to the bioreactor for mechanical manipulation. The depth of the bushing into the counterbore controls the degree of compression of the O-ring against the top tissue anchor shaft, providing a barrier against contamination while allowing the shaft to move freely with two degrees of freedom, an axial motion and a rotational motion about that axis.

Tissue Anchor-Shafts. The anchor-shafts (Fig. 2A) were machined from 304 S.S. tubing, (6.35 mm OD, 3.175 mm ID). The bottom and top anchor-shafts, 2.54 cm and 10.16 cm long, respectively, allow for fluid flow through their centers. The top anchor-shaft serves two functions, an external fluid inlet into the shaft system for perfusion flow through the tissue and coupling of the tissue/matrix to the mechanical manipulation. Two internally located 8–32 threaded holes and two clearance holes oriented diametrically opposed were provided to allow for a variety of tissue anchoring options (Fig. 2B). Fluidic control (0.25 ml/min to 25 ml/min) and pathways for perfusion through and/or sheath flow about the tissue were designed to increase possible cell seeding methodologies and support tissue growth and differentiation (Fig. 1A & 2B).

Bioreactor Motion Control Subsystem

Each bioreactor houses two banks of six reactor vessels supporting the growth of up to 12 independent engineered ligaments (Fig. 3A). Each bioreactor contains two fixed-position aluminum plates, a traveling plate with mounted rotational and translational gear trains and 2 high-torque stepper motors. The fixed position plates provide a number of functions: (1) 12 ''seats'' or slots (Fig. 2B) for 12 reactor vessels that allow easy removal/replacement of individual bioreactor vessels without disturbing the adjacent vessels, (2) anchors for the bioreactor vessels preventing their translation when tension is applied to the top anchor-shaft, (3) the bottom plate connected via a locking bar (Fig. 2B) and set-screw to prevent rotation of the reactor vessel as torsion is applied to the top anchor-shaft, (4) two plates to provide heat to the vessel mounts and medium as it is recirculated through the system maintaining internal vessel temperature, and (5) the top fixed plate fitted with two anti-back lash nuts against which the lead screws from the traveling plate act. The distance between plates is easily adjustable to accommodate a wide range of reactor vessel lengths.

Traveler. A traveling plate (Fig. 3A) situated above the top fixed position plate allows mounting of the two independent gear trains and provides one degree of freedom (axial) for the translation of the gear trains and the motors while constraining the other five degrees of freedom. The traveler is fitted with (1) two inde-

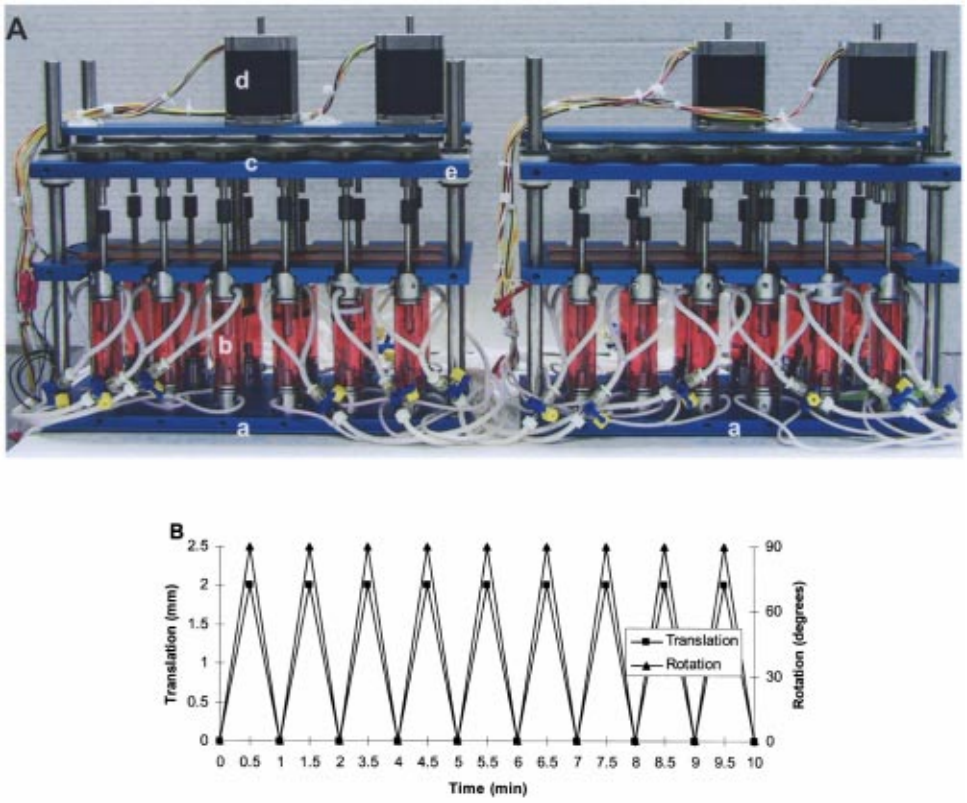

Fig. 3 A. System bioreactors (*a*) with 24 loaded reactor vessels (*b*). The traveler (*c*) housing both rotational (in view) and translational gear trains, the high torque stepper motors (*d*), and the linear bearing (*e*) are shown. B. Chart illustrating empirical translational and rotational displacement data for a programmed 10 cycle 2 mm and 90° regime at 0.0167 Hz.

pendent gear trains, (2) 4 self-aligning recirculating ball linear bearings for traveler translation, and (3) a motor mount plate allowing both stepper motors to engage their specific gear trains.

Drive Mechanisms. The gear trains, when driven by their specific stepper motors, independently control rotational and translational deformation. The rotational gear train includes 12 $\times$5.08 cm diameter spur gears (72 pitch) in a 1:4 ratio with the stepper motor. Each gear is aligned co-axially with the reactor vessel seated below and sits on top of a bronze thrust bearing and attaches to a 6.35 cm (2.5″) long precision ground 0.635 cm (0.250″) 316 S.S. rod. The rod extends from the gear, through the bearing in the traveler, and protrudes 1″ from the bottom surface of the traveler towards the reactor vessels. The rod is held in place via a 6.35 mm (0.250″) long collar pressed against the bottom of the thrust bearing. The remaining 1.91 cm (0.75″) of the rod is used to attach the anchor-shaft extending up from the reactor vessel via a coupling. The coupling provides an independent way of attaching/detaching the reactor vessel from the system during the course of an experiment

Translational deformation is produced via two precision 1.27 cm (0.5″) diameter 2.54 mm (0.1″) pitch stainless steel lead screws reacting against the anti-backlash nuts affixed to the top fixed-position plate. The lead screws are driven through a 1:3 reduction by a stepper motor. The stepper is capable of microstepping (51,200 microsteps/revolution). The combination of the motor's resolution and gear reduction results in sub-micron axial translation. When actuated, the entire mass of the traveler including the gear trains for both rotation and translation as well as the motors and the associated hardware (bearings, bushings, etc.) move relative to the top fixed plate and, hence, relative to the bioreactor vessels' bottom tissue anchors producing axial translation of the upper tissue anchor. This method allows for uncoupled axial and rotational displacements.

Motion Control Software. LPT Indexer motion control software, high torque stepper motors (50 lbs-in torque) with accompanying micro-step drivers (51,200 steps/rev) and power supply were purchased from Servo-Systems Co., Montville, NJ. Custom software to engage the LPT Indexer software was written in Visual Basic V. 6.0 to control the stepper motors. The software provides precise independent control over the rotational and linear movements. Rates for linear and rotational movement of the top tissue anchors range from zero to 6.5 inches/sec and 48.8 revolutions/sec. The software allows the user to input the forward and return rotational and linear rates, total excursion (rotational and linear), provides for an intermediate period of rest or static mode at the extreme point, a rest or static mode at the home point, and the number of repetitions. Several different cycles with varying strain regimes can be programmed and run for the duration of a experiment. A common loading regime for the culture of ligaments is shown in Fig. 3B.

Environmental Chamber and Gas Exchange

A multi-component environmental chamber (Fig. 4) was built to provide precise pH and pO_2 control to all 24 reactor vessels by silicone tubing gas exchangers (a coil of 1.83 m tubing, 1.588 mm (1/16″) I.D., 3.175 mm (1/8″) O.D., for each vessel). Environmental chamber components include (1) 12 individual chamber sections designed to house two coils per section, (2) an additional chamber to house two thermocouples to measure chamber wall and internal temperature, (3) a gas mixing plenum with 3 gas

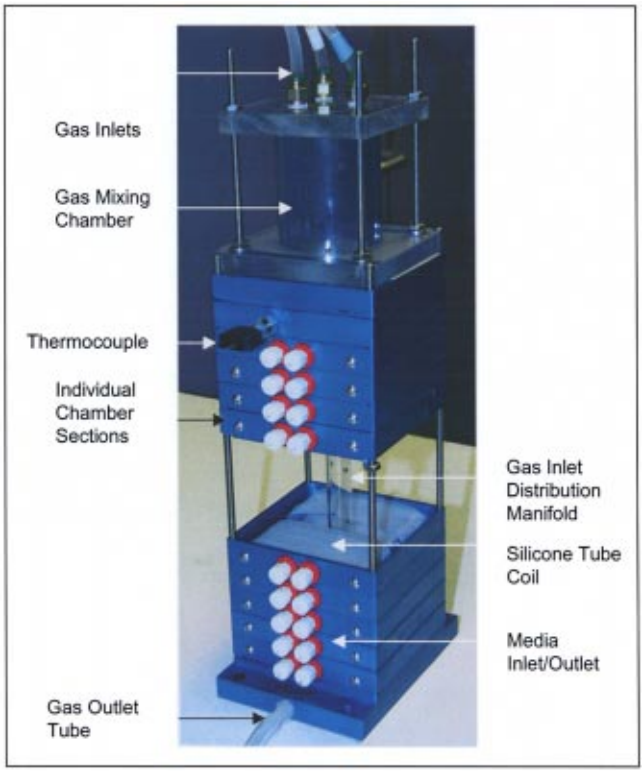

Fig. 4 Environmental chamber prior to closure to show the internal silicone hose coils and gas inlet distribution manifold

inlets, (4) a gas distribution manifold and (5) a chamber outlet port. Each chamber section provides, on opposite faces, an inlet and outlet port for recirculating vessel medium. The chamber sections were designed as independent components to provide flexibility to the system. Chamber sections are stackable and removable for quick turn-around, sterilization without entire system shutdown, and easy clean up in the case of leaks. The entire chamber or individual sections, including inlet and outlet ports, can be steam sterilized. Hose dimensions and length were chosen to achieve vessel medium equilibrium with the gas environment of the environmental chamber over the range of possible medium flow rates provided by the pump system. Vessel medium is recirculated via a multi-drive microbore peristaltic pump (12 channels per pump) (Masterflex, Cole-Parmer, Vernon Hills, IL) through the silicone gas permeable hose at 1.7 ml/min to achieve equilibrium with chamber atmosphere; therefore, control over inlet gas flow rates can be used to adjust the dissolved gas concentrations of the reactor vessels.

Independent mass flow controllers (MKS, MA) individually control the flow rates of the three gasses into the mixing plenum. The ratiometrically correct mixture is then distributed into the chamber by the orifices of the gas inlet distribution manifold that have been sized for even distribution of the gas into the chamber taking into account the mass flow rate and the associated drag of the gas mixture. The gas permeable platinum-cured silicone hose (Cole-Parmer, IL) of the coils allows diffusion of the gases into the medium. Thus, pH and dissolved pO_2 are maintained at the same levels in all reactor vessels. The chamber gas outlet tube remains opened to allow gas flow through the chamber maintaining steady state. Low inlet gas flow rates were maintained such that inexpensive commercially available CO_2, O_2, and N_2 tanks will last for approximately 3 weeks.

Temperature Control System

Vessel medium temperature is maintained by heating both fixed plates of each bioreactor and the walls and inlet gas in the environmental chamber. Four PID type CN-9000 programmable temperature control units (Omega, Stamford, CT) via temperature feedback through J-type thermocouples were used to maintain the top mounts of each reactor vessel and the wall and the inlet gas of the environmental chamber temperatures such that recirculating medium about the developing tissue was maintained at 37°C. Four 60-watt silicone heating strips (2.54 cm×30.48 cm) were used per bioreactor (2 per fixed position plate) to maintain the vessel mounts at 37°C thus heating the medium as it enters and exits the vessel.

Experimental Methods

Biocompatible Matrix. Several materials including collagen type I gels and *Bombyx mori* silkworm silk fiber matrices have been anchored, seeded with adult stem cells (hBMSCs) and grown in the system for up to 21 days. For example, acid soluble collagen type I (Sigma Type III) was neutralized, mixed with hBMSCs and gelled within the reactor vessel between anchors [22]. Silk fiber matrices were either embedded in biocompatible epoxy adhesive (3M DP-100, McMaster-Carr, New Brunswick, NJ) or sutured using a ''whip-stitch'' for attachment into the anchor-shafts. Matrices were 3 cm long between anchors. Direct clamping of the silk matrix's ends via set-screws in the anchor-shafts provided stable anchoring that can withstand $\geqslant$200 N of force before the matrix would pull-out.

Cells. Human bone marrow stromal cells (hBMSC) were obtained from bone marrow aspirates from consented donors $\leqslant$25 years of age (Clonetics-Poietics, Walkersville, MD). Twenty-five ml aspirates were resuspended in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 0.1 mM nonessential amino acids, 100 U/ml penicillin, 100 mg/L streptomycin (P/S), and 1 ng/ml basic fibroblast growth factor (bFGF) (Life Technologies, Rockville, MD) and plated at $8-10\ \mu$l aspirate/cm^2 in tissue culture flasks. BMSCs were selected based on their ability to adhere to the tissue culture plastic; non-adherent hematopoietic cells were removed during medium replacement after $\sim$5 days in culture. Medium was changed twice per week thereafter. When primary BMSC became near confluent (12–14 days), they were detached using 0.25% trypsin/1 mM EDTA and replated at 5×10^3 cells/cm^2. First passage (P1) hBMSCs were trypsinized and frozen in 8% DMSO/10% FBS/DMEM for future use.

Silk Matrix Cell Seeding. Frozen P1 hBMSCs were defrosted, replated at 5×10^3 cells/cm^2 (P2), trypsinized when near confluency, and used for matrix seeding. Sterilized (ethylene oxide) silk matrices were seeded with cells in customized seeding chambers (1 ml total volume) machined in Teflon blocks to minimize cell-medium volume and increase cell-matrix contact. Seeded matrices, following a 4 hour incubation period with the cell slurry (3.3×10^6 BMSCs/ml) were transferred into the anchor-shafts and assembled into the reactor vessel. Each vessel was loaded into the bioreactor system and infused with 30 ml of cell culture medium without bFGF.

Tissue Culture Conditions. Reactor vessels containing seeded silk matrices were loaded into a servo-hydraulic Instron 8511 (Instron Corp., Canton, MA), a 5 N tear load under load-control is imparted to the matrix via the top anchor-shaft, and the position of the top anchor shaft is set via set-screws located within the top vessel mount. This sets the initial ''zero'' position of the scaffold before loading into the bioreactor. The reactor vessels containing the pre-stressed matrices were then loaded into the bioreactor, coupled to the rotational gear train and the mechanical regime (dynamic or static) was imparted to the developing cell-

scaffold complex. Reactor vessel medium was changed batch-wise (50%/change) twice a week. pH was maintained at 7.4 and dissolved oxygen at 20% pO_2.

Experimental Analysis

Bioreactor Motion Control Subsystem Characterization. As is the case in a fully utilized bioreactor, reactor vessels occupied all available seats during linear and angular displacement characterization. Translational hysteresis was measured using vernier calipers with resolution of 25.4 μm clamped to the bottom fixed plate of the bioreactor. Caliper jaws were situated between the anchor shafts and displacement between the top and bottom anchor shafts for vessels seated in the third and sixth positions of the bioreactor was measured for a 2 mm cyclic translation at 0.0167 Hz. A spring mounted between the traveling plate and top caliper jaw was used to ensure continuous measurement throughout the cyclic regime by keeping the caliper jaw in constant contact with the top anchor shaft. Initial displacement between shafts was set to 3 cm, the caliper zeroed and displacement at the initial home and extreme positions measured and recorded for 10 cycles. Temporal changes relative to both linear and rotational displacements were measured using a stopwatch to verify programmed cyclic frequency.

Rotational hysteresis was measured on the axis of the bioreactor vessel furthest (along the gear train) from the driving axis of the stepper motor used to control rotational displacement. The bioreactor in this position suffers from the most hysteresis as hysteretical error compiles in a linear (gear train) system. The hysteresis was measured using a mirror and a coherent light source (laser). The central shaft of the reactor vessel seated in the sixth position was machined to allow placement of the reflective plane of the first surface mirror to be collinear with the axis. The laser (HeNe 3mW) was placed such that the plane of the incident and reflected light to/from the mirror remained normal to the reflecting surface of the mirror and intersecting the axis of the bioreactor vessel. Hysteresis was measured by causing, through the motion control software and stepper motor, cyclic rotational displacement. This resulted in a linear translation of the reflected spot on a diffuse reflecting screen (e.g., white wall) approximately 3 meters from the reactor vessel and mirror; the angular measurement system resulted in 0.0268° resolution. The displacement of the reflected spot was marked relative to the initial position (zero displacement). The locations of the spot at the extreme point of rotation and following the return to the initial condition were also recorded and repeated for 9 additional cycles. In order to achieve desired resolution, available room space limited rotational displacement to 30°. Two sets of measurements ($N=10$ cycles per set) were performed for 20° and 30° of rotation and maximum deviations calculated.

Reactor Vessel Medium Temperature Characterization. Extensive testing was performed to evaluate the temperature about the developing tissue in the reactor vessel. Feedback temperature control of the top anchor mount and maintenance of environmental chamber wall temperature at 37°C was used to control medium temperature around the 3 cm long silk fiber matrix over 21 days in culture. A submersible miniature thermocouple (Omega, CT) calibrated at 37°C by the manufacturer (resolution $+/-0.2$°C) was used to measure temperature about the mid-section of the matrix as well as the area where the top and bottom anchor shafts anchor the matrix. Three ports were introduced to the reactor vessel tube wall at desired locations along the length of the matrix for insertion of the thermocouple during the 21-day experiment. Measurements were taken at least twice a day and deviations from 37°C recorded.

pH and Dissolved pO_2 Measurements. Medium pH and dissolved O_2 were measured off-line using a blood gas analyzer (Instrumentation Laboratory 1610, Lexington, MA). Following 50% medium exchange, 15 ml of vessel medium extracted from the reactor vessel into a 20 ml syringe was immediately assayed for pH and pO_2.

Scanning Electron Microscopy. The matrices were harvested at timed intervals, washed with 0.2 M sodium cacodylate buffer, fixed overnight in Karnovsky fixative, dehydrated through an ethanol series and left to dry in Freon overnight. The samples were sputter-coated with Au using a Polaron SC502 Sputter Coater (Fison Instruments), and imaged at 15 keV with a JEOL JXA 840 Scanning Electron Microscope.

Results and Discussion

A new bench-top bioreactor system (Figs. 1A, B) was designed, constructed and utilized to support tissue engineering starting from isolated cells and 3D matrices. The bioreactor system had ability to apply independent multi-dimensional complex and well-controlled mechanical strains (at <0.1 μm for translational and <0.1° for rotational strain) to developing tissue, and to precisely and accurately control the biochemical and fluidic environments. The bench-top system can accommodate up to 24 individual reactor vessels (Fig. 2A) in two independently controlled bioreactors to permit the concurrent study of varied strain rates and percent strain in order to identify optimal parameters for tissue development without the needed for an incubator. Enhanced flexibility has been achieved through the modular bench-top design allowing concurrent but independent operation of up to 24 reactor vessels; individual reactor vessels can be added, replaced or withdrawn at any time during the course of an experiment without disturbing system function. Matrices can be anchored into the reactor vessels to support the culture of developing tissue within a mechanically dynamic environment. Cell seeding options have been improved through enhanced fluidic control (0.25–25 ml/min) utilizing perfusion through and/or sheath flow around the matrix (Fig. 1A).

Reactor vessels, via the top mount O-ring and bushing, provide a barrier to contamination while allowing the tissue cultured within the vessel to be exposed to multi-dimensional strains. Each bioreactor can apply a unique programmed mechanical regime to its 12 housed vessels, i.e., forward and return rotational and linear rates, total excursion (rotational and linear), intermediate static mode at the extreme point and/or the home point, the number of cycle repetitions for a specific regime, and the duration between different regimes can be controlled (Fig. 3B). An unlimited number of regimes could be programmed. The use of anti-backlash nuts and micro-step drivers combined with 1:4 (angular) and 1:3 (translation) gear reductions minimized linear system hysteresis to within the resolution of our measuring equipment (vernier calipers with resolution of 25.4 μm) and technique. Linear hysteresis over 10 cycles was measured to be less than the resolution of the caliper's at less than 25 μm or 1.25% for 2mm of travel (Fig. 3B). Deviations between the third and sixth bioreactor seat positions from the programmed theoretical displacements were not discernable as well. Rotational hysteresis was measured to be less than 0.2° (0.36%) for a angular displacement of 30° (Fig. 3B) and was less than 0.1° for a 20° displacement over 10 cycles. It is reasonable to expect when extrapolating to 90° of rotation, hysteresis of substantially less than 1% of total displacement will result. The accuracy of the programmed cyclic frequency derived from the motion control software was verified to within $+/-0.5$ sec or the resolution of the stopwatch and timer.

A total of 48 reactor vessels containing cell culture medium and silk matrices were run for up to 21 days under 90° rotational and 2 mm translational deformations at 0.0167 Hz. Of the 48 vessels, only one became contaminated during culture due to a leak on the outlet port. Feedback temperature control of the top anchor mount to 37°C and the maintenance of wall temperature at 37°C in the environmental chamber resulted in constant temperature within the resolution of the measuring thermocouple ($+/-0.2$°C) about the 3 cm long silk matrix housed in the reactor vessel. A submerged wire thermocouple place in the middle and at the two ends

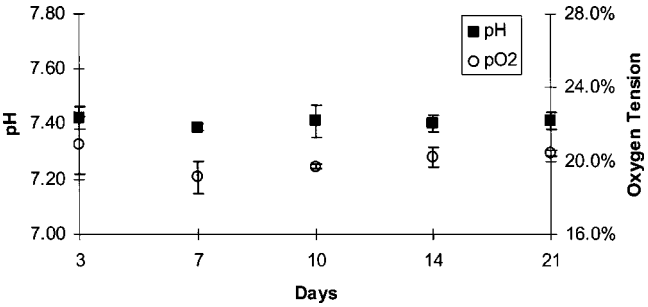

Fig. 5 Experimental data of pH and pO_2 levels measured during a 21 day experiment

of the matrix anchored in the reactor vessel demonstrated that constant temperature was maintained at $37+/-0.2°C$ (mean+/−standard deviation) about the silk matrix over the 21 days of operation.

The system functioned by recirculating (via the peristaltic pumps) cell culture medium through a closed loop consisting of the reactor vessel, C-Flex, and silicone tubing from the reactor vessel through the tubing and environmental chamber and back into the reactor vessel. Fine adjustment and control of medium pH and pO_2 was achieved by controlling the gas flow rates into the environmental chamber (Fig. 4); the environmental chamber provided control over dissolved oxygen tension (between 0%–95% ±1%) by achieving equilibrium with the recirculating medium. Twenty-four reactor vessels containing silk matrices seeded with hBMSCs maintained a constant 7.4 pH and 20% pO_2 over 21 days in culture (Fig. 5). The bioreactor system supported hBMSCs spreading and growth on silk fiber matrices as demonstrated by SEM at a flow rate of 2.5 ml/min, pH of $7.4+/-0.02$ and pO_2 of $20+/-0.5\%$ (Fig. 6).

Mechanical stimulation (cyclic rotation (90°) and translation (2 mm) at 0.0167 Hz without rest at home or extreme positions) applied up to 21 days via the bioreactor induced the elongation of bone marrow stromal cells seeded within a collagen type I 3-dimensional gel and increased cross-sectional cell density ~2-fold as compared to statically grown tissue (Fig. 7). We have recently shown that directed multi-dimensional strains that mimic the physiological environment of the ACL could specifically direct the differentiation of BMSCs towards ligament lineage [22].

The relevance of applying both translational and rotational strains to developing ACL tissue is seen in the structure-function relationship of the ligament. The ACL has a unique helical fiber organization and structure to perform its stabilizing functions. The mode of attachment to bone and the need for the knee joint to rotate ~140° (extension/flexion) results in a 90° twist of the ACL major fiber bundles developing a helical organization. In full extension, individual fibers of the ACL are attached anterior-posterior and posterior-anterior from the tibia to the femur in the sagittal plane of the knee. This helical geometry allows the individual ACL fiber bundles, during knee joint flexing, to develop a flexion axis about which each individual fiber bundle or fascicle twists thereby remaining isometric in length. Fiber bundle isometry allows the ACL to equally distribute load to all fiber bundles, maximizing its strength. It is this unique structure-function relationship that allows the ACL to sustain high loading through all degrees of knee joint extension and flexion. Therefore, it is hypothesized that a tissue engineered prosthesis exposed to physiologically relevant translational and rotational strains will develop a structure suitable for function following implantation *in vivo*. The lack of rotational strains (e.g., only translation) during *in vitro* ligament engineering would fail to support the development of a helical structure capable of effectively distributing load throughout the tissue placing the prosthesis at a higher risk for rupture. While rotational strain alone acts to translate individual fibers organized in a helical geometry, translational strains are needed to control fiber pitch angle and mimic anterior draw loads typically stabilized by the ACL. Thus, the combination of both translational and rotational strains is needed for ACL tissue engineering.

The new reactor system extends the current state-of-the-art by: (a) allowing the application of multidimensional strains to the 3D matrices, (b) provides options to select perfusion and/or sheath flow for cell seeding and/or tissue culture, (c) offers a bench-top modular design for easy interchange of individual reactors and medium exchange, (d) provides enhanced environmental control for oxygen, pH and temperature, and (d) maintains accurate and precise motion control. This combination of features provides options to better mimic *in vitro* the physiological features to be encountered by engineered tissues once placed *in vivo*. The bench-top bioreactor system developed in the present study supported cell and tissue growth on the silk fiber matrices while providing a multitude of biochemical and mechanical parameters for manipulation. Enhanced mechanical, biochemical and fluidic control systems now exist for accurate and precise control of the environment for developing tissue. The bench-top design increases system flexibility as well as the total number of reactor vessels that can be cultured in parallel. Optimal biochemical and mechanical parameters (e.g., oxygen tension, strain, strain rate, medium flow rate, type of flow) to induce ligament-specific differentiation of hBMSCs seeded on silk matrices are currently being identified. Additional capabilities, including a force monitoring system and enhanced fluid handling capabilities (i.e., for medium exchange or the programmed introduction of relevant biochemical factors) are in development.

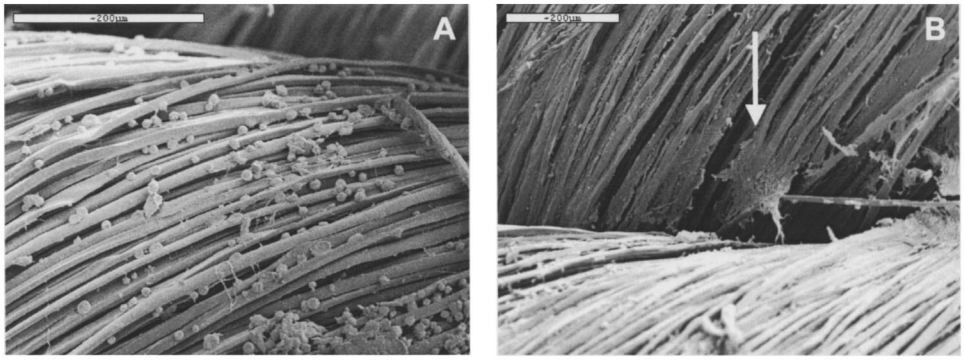

Fig. 6 Scanning electron micrographs of hBMSCs seeded on the silk fiber scaffold (*A*) 1 hr post-seeding prior to loading into the reactor, and (*B*) grown in the bioreactor for 14 days. The arrow indicates cell sheet and possible extra-cellular matrix formation.

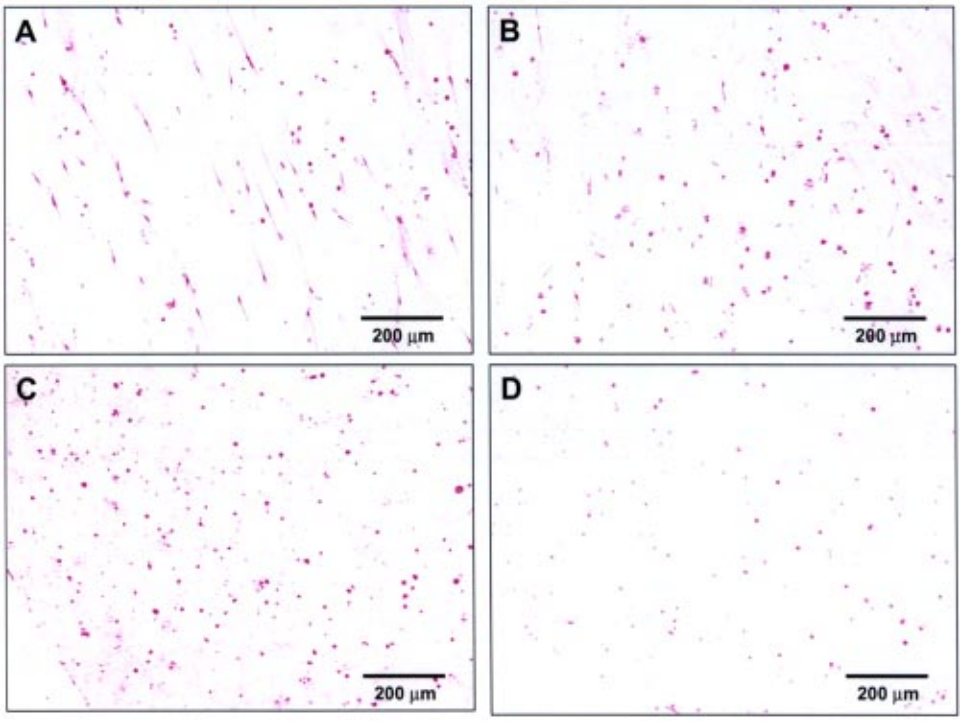

Fig. 7 A. H&E of mechanically stimulated collagen gel longitudinal section after 14 days of culture. B. H&E of longitudinal section of static control. C. H&E of mechanically stimulated gel cross-section. D. H&E of static control cross-section.

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