Published in final edited form as:

Biomaterials. 2013 July ; 34(23): 5766–5775. doi:10.1016/j.biomaterials.2013.04.023.

Mechanical Modulation of Nascent Stem Cell Lineage Commitment in Tissue Engineering Scaffolds

Min Jae Song¹, David Dean², and Melissa L. Knothe Tate^{1,3}

¹Department of Biomedical Engineering, Case Western Reserve University, 2071 Martin Luther King Jr. Drive, Cleveland, OH 44106, USA

²Department of Neurological Surgery, Case Western Reserve University, 10900 Euclid Avenue, Cleveland, OH 44106, USA

³Department of Mechanical & Aerospace Engineering, Case Western Reserve University, 2071 Martin Luther King Jr. Drive, Cleveland, OH 44106-7207, USA

Abstract

Taking inspiration from tissue morphogenesis *in utero*, this study tests the concept of using tissue engineering scaffolds as delivery devices to modulate emergent structure-function relationships at early stages of tissue genesis. We report on the use of a combined computational fluid dynamics (CFD) modeling, advanced manufacturing methods, and experimental fluid mechanics (micro-piv and strain mapping) for the prospective design of tissue engineering scaffold geometries that deliver spatially resolved mechanical cues to cells seeded within. When subjected to a constant magnitude global flow regime, the local scaffold geometry dictates the magnitudes of mechanical stresses and strains experienced by a given cell, and in a spatially resolved fashion, similar to patterning during morphogenesis. In addition, early markers of mesenchymal stem cell lineage commitment relate significantly to the local mechanical environment of the cell. Finally, by plotting the range of stress-strain states for all data corresponding to nascent cell lineage commitment (95% CI), we begin to “map the mechanome”, defining stress-strain states most conducive to targeted cell fates. In sum, we provide a library of reference mechanical cues that can be delivered to cells seeded on tissue engineering scaffolds to guide target tissue phenotypes in a temporally and spatially resolved manner. Knowledge of these effects allows for prospective scaffold design optimization using virtual models prior to prototyping and clinical implementation. Finally, this approach enables the development of next generation scaffolds *cum*

© 2013 Elsevier Ltd. All rights reserved.

Address for correspondence: Melissa L. Knothe Tate - Department of Biomedical Engineering, Case Western Reserve University, 512A Wickenden Building, 2071 Martin Luther King Jr. Drive, Cleveland, OH 44106-7207, USA Phone: (216)-368-5884 Fax: (216)-368-4969 knothetate@case.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

CONFLICT OF INTEREST AND DISCLOSURE STATEMENT

One of the authors (DD) and Case Western Reserve University have submitted three patent applications related to the additive manufacturing technology used to create the scaffold tested here.

delivery devices for genesis of complex tissues with heterogenous properties, *e.g.*, organs, joints or interface tissues such as growth plates.

INTRODUCTION

Like tissue *anlagen* or templates formed during the process of prenatal tissue morphogenesis, tissue engineering scaffolds are designed to facilitate the process of postnatal tissue repair and/or to enable nascent generation of tissue in critical sized defects that are too large to be bridged through the natural healing process. Contemporary tissue engineering scaffold design seeks to optimize delivery of mechanical and chemical cues to stem cells seeded within. Scaffolds provide an environment for the *de novo* generation of tissues by cells that themselves produce and lay down, *e.g.*, extracellular matrix comprising structural proteins and inductive factors in spatial patterns optimizing structure-function relationships and building tissues, from the inside out [1]. The future of tissue engineering lies in development of routine, repeatable manufacturing methods and products for translation to the clinic. Currently, there is an acute need for rational scaffold design strategies, to develop scaffold geometries and compositions that modulate the spatial and temporal patterning of nascent tissue formation. Additive manufacturing methods provide a means to achieve patterning through spatial presentation of biological (cells), chemical (*e.g.* growth factors) and biophysical (*e.g.* stress, strain) factors [2-4]. As noted by Hollister and others, “the art of [rational] scaffold [design lies in]... where to put the holes and the biofactors...” [5]. Together with the outer boundary conditions defining the inner and outer surfaces of scaffolds, the spatial distribution of holes within the scaffold not only defines its architecture and the local delivery of biochemical cues but also the delivery of local mechanical and mechanically modulated cues such as molecular gradients via globally defined flow regimes [3].

Recently, the mechanical modulation of stem cell shape and fate has been shown in model embryonic mesenchymal stem cells exposed to small magnitude mechanical stresses, which in turn induce normal and shear strains on cell surfaces and significantly modulate early markers of lineage commitment [6-11]. This approach is of keen interest for application within tissue engineering scaffolds, as a new means to modulate lineage commitment in a spatially and temporally resolved manner. Hence, while previous approaches emphasized the spatial distribution of scaffold structure and composition to achieve mass transport, appropriate cell seeding densities, as well paths to guide cell migration and tissue ingrowth, new approaches will incorporate the delivery of mechanical as well as biochemical cues through the globally modulated local environment of the cell.

Nano-micro fluid dynamics lends itself well for the prediction and optimization of scaffold performance parameters, including fluid flow and permeability, prior to scaffold manufacture [3,6,9,12-19]. Based on Navier-Stokes equations for steady flow induced by a pressure gradient, computational fluid dynamics (CFD) is particularly useful to predict fluid flow through a given scaffold as well as the mechanobiological milieu of cells seeded within. A cell attached to the channel wall within a scaffold experiences normal and shear stresses at its boundaries. Wall shear stress is calculated from the laminar viscosity and the

wall strain rate, determined through the solutions of Navier - Stokes equations within the scaffold,

$$\tau_w = \mu \gamma$$

where τ_w is the shear stress at the wall, μ is the fluid viscosity, and γ is the strain rate determined from the second invariant of the stress tensor.

In the current study, we test the concept of delivering biophysical, including mechanical, cues as a means to control cell behavior at the earliest time points of emergent cell fate. We first tested the feasibility of predicting and delivering mechanical cues to model mesenchymal stem cells (MSCs) seeded on rapid prototyped scaffolds. Then we measured strains on surfaces of live MSCs *in situ* while delivering controlled mechanical cues. Finally, we measured and correlated genetic markers of nascent cell lineage commitment to applied stresses and imbued strains, creating a reference library for mechanical cues correlating significantly to nascent cell lineage commitment to osteogenic, chondrogenic, adipogenic, vasculogenic and hematopoietic cell fate. Ultimately, we aim to guide functional tissue generation through anisotropic extracellular matrix molecule secretion and posttranslational modification [3,20,21].

MATERIALS AND METHODS

1. Manufacture of scaffolds

Two scaffold geometries, modular sections exemplifying dominant architectural features (axial, transverse) of the complete transverse isotropic cylindrical geometry, were modeled and tested (Fig. 1). The scaffolds used in this study were prepared via Continuous-Digital Light Processing (cDLP) Additive Manufacturing (AM) technology (Perfactory® Mini Multi Lens, envisionTEC, Ferndale, MI), configured for $21 \mu\text{m} \times 21 \mu\text{m} \times 50 \mu\text{m}$ (xyz) resolution. Scaffolds were produced from a light polymerizable polymer, poly(propylene fumarate) (PPF), with which our group has significant experience [43].

2. Computational Fluid Dynamics (CFD) prediction of flow through scaffolds without MSC seeding

CFD models were built to predict flow regimes in the near vicinity of cells seeded on scaffolds, based on original CAD design and μ -CT scanned images of manufactured scaffolds (Fig. 3, S2) (Microphotonics Inc. Allentown, PA; CFD-ACE, SOLVER, GEOM, and VIEW by ESI group). Micro-CT images were analyzed using Amira (Visage Imaging, Inc., San Diego, CA). Flow was calculated from the continuity (1) and Navier-Stokes equations (2) using a 2nd order upwind-discretization scheme in three dimensions (3D). Wall shear stress was calculated from the shear rate (3).

$$\nabla \cdot \mathbf{v} = 0 \quad (1)$$

$$\rho (\mathbf{v} \cdot \nabla \mathbf{v}) = \mu \nabla^2 \mathbf{v} - \nabla P \quad (2)$$

$$\tau_{\text{wall}} = \mu \left. \frac{\partial \mathbf{v}}{\partial \mathbf{x}} \right|_{\text{wall}} \quad (3)$$

We assumed that the flow medium is incompressible and that flow is laminar at physiological rates of interest, which is appropriate for a flow medium similar to 0.9% saline, a Newtonian fluid with density comprising 996 kg/m³, at body temperature (310K), and laminar viscosity (0.001 kg/ms). The Navier-Stokes equation (2) was applied, assuming that body forces are negligible and that flow is steady in 3D, also appropriate assumptions for the length and time scale as well as the flow velocity studied [3,9].

Flow is induced using an input pressure gradient of 8.3 Pa and 13.3 Pa for the respective modular axial and transverse geometry scaffolds. These pressure gradients were selected in order to achieve a target shear stress of 1 dyn/cm² (0.1 N/m²) for the actual respective manufactured geometries, and resulting in an order of magnitude difference in shear rates between the high and low shear rate regions. This magnitude shear stress was shown previously to result in significant changes in gene expression (over baseline) associated with early stages of cell lineage commitment of MSCs during mesenchymal condensation [3,6,11].

3. μ -PIV for validation of CFD and analysis of flow regimes at initial time points

To achieve a target shear stress as 0.2 dyn/cm² for CFD validation, flow is induced using input pressure gradients of 1 Pa and 1.6 Pa for the scaffolds representing the axial and transverse modular scaffold geometries, respectively (Fig. 1). The corresponding flow rate was predicted using CFD to deliver a global target shear stress of 0.2 dyn/cm² shear stress to the apical surface of cells seeded within the flow chamber (Fig. 4A, global flow conduit geometry: 1 cm (width) $\times$ 2.3 cm (length) $\times$ 250 μ m (height) [45]). Predicted flow regimes were validated using μ -PIV methods on manufactured scaffolds in both in the presence as well as in the absence of cells. One μ m fluorescent microspheres were diluted (0.5 mL of Tetraspeck (Invitrogen) microparticles suspended in 50 mL of Basal Medium Eagle (BME) supplemented 1% L-Glutamine, and 1% Pen/Strep) to allow for the observation of fluid displacements in time and space, with respect to cells that were labeled fluorescently with Syto9 (Invitrogen). Flow was controlled using a syringe pump (Harvard Apparatus, Holliston, MA). Confocal images were acquired (40 $\times$ objective, SP2 laser scanning confocal microscope, Leica Microsystems, Mannheim, Germany) at high shear rate regions and low shear rate regions within each scaffold, at 0 - 12 μ m distance from the substrate, and 256 $\times$ 256 resolution over 990 ms (Fig. S3). Microsphere displacement was tracked and measured in space and time using Image J (National Institutes of Health, Bethesda, MD). 4D (x,y,z,t) images (Fig. S3, Animation 1) were reconstructed by Volocity (PerkinElmer, Inc., Waltham, MA).

4. Cell preparation and seeding on scaffolds

C3H/10T1/2 cells, a model mesenchymal stem cell (MSC) line derived from the mesenchyme of the murine embryo (CCL-226; ATCC, Manassas, VA) were passaged until P5 or P6 in 10 ml of Basal Medium Eagle (BME) supplemented with 10% fetal bovine

serum, 1% L-Glutamine, and 1% Pen/Strep (Invitrogen, Carlsbad, CA) in T-75 Flasks (Corning, Inc., Corning, NY). These cells do not exhibit phenotypic drift such as that observed previously in primary murine cells derived from the mesoderm at the time of condensation [46]. Furthermore, these cells exhibit multipotency, *i.e.* the capacity to differentiate along osteogenic, chondrogenic, adipogenic, hematopoietic [47-51], and angiogenic lineages [52-55]. Using a hemocytometer, we counted the number of cells in each of four grid regions and averaged the four numbers to determine the total number of cells. Before cell seeding, we applied radiofrequency glow discharge (RFGD) to the scaffolds and soaked them into FBS for 24 h. We used 1×10^6 cells of 0.5 ml suspension for each of three scaffolds (n=3) in 24 ultra low attachment well plates (Corning, Inc., Corning, NY). The cell culture medium was changed two hours after cell seeding, and the seeded cells were incubated for 24 hours prior for flow visualization studies.

5. Strain mapping on cell surfaces under shear flow

One μm diameter microbeads were coated with Concanavalin A (Con A, Polysciences, Inc., Warrington, PA), a lectin and carbohydrate-binding protein, *per* our previously published protocols [6]. Then a solution containing the coated microbeads (4.55×10^7 microbeads) was introduced to the cell culture medium (50 μl microbead solution per well); the Con A on the bead surface bound to the naturally occurring glycoproteins on the surface of the seeded stem cells within scaffolds. During application of controlled flow fields, we imaged displacements of these microbeads in the XY plane during exposure of the cell surface to flow-induced drag forces. Confocal images were taken every 10 minutes during exposure to the target shear stress (1 dyn/cm^2 , 0.76 ml/min for axial geometry, 1.23 ml/min for transverse geometry) (Fig. S3, Animation 2). Linear elastic theory (4) was applied to calculate cell scale stresses and strains, where E is an elastic modulus, and σ , ε indicate stress and strain, respectively.

$$\sigma = E \cdot \varepsilon \quad (4)$$

The elastic modulus was based on a previously validated parametric analysis and estimation from our lab (1.78 Pa for shear stress-strain and 4.83 Pa for normal stress-strain) [6].

6. RNA isolation and real-time PCR

Transcriptional profiles for early markers of mesenchyme condensation and cell commitment to osteogenic, chondrogenic, and adipogenic lineages were measured using quantitative real-time PCR [6,10,11]. Cells were removed using a cell scraper in the high and low shear rate regions of the respective axial and transverse modular scaffolds. Total RNA was isolated from the samples using our previously published protocols [6,10,11] (Qias shredder, Qiagen, Valencia, CA; RNeasy Mini Kit, Qiagen). Quantitative real-time PCR analysis (including cDNA preparation) was performed at the Gene Expression and Genotyping Facility (shared resource of the Comprehensive Cancer Center at Case Western Reserve University, Cleveland, OH) using TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA) and an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems). Sixteen genetic markers (in addition to TBP used as the endogenous control) were examined in each of the samples: Col1a1 (type I collagen), Col2a (type II

collagen), Runx2, Sox9, Msx2, AGC1, Cdh2 (N-cadherins), HAS3, CD44, peroxisome proliferation activated receptor γ 2 (PPAR γ 2), BGLAP (Osc or Osteocalcin), IBSP (BSP), VEGF, TIE2, Runx1, and CD38. The markers chosen are key to mesenchymal condensation and subsequent differentiation (Fig. 6A) [46, 56]. Prior to condensation, cells produce primarily a mesenchymal matrix comprising type I and III collagens; during and after condensation, the cells produce a cartilaginous matrix comprising types II, IX, and XI collagens. Upregulation of Sox9 controls the production of Col2a1 [11, 46]. Hence, activity of markers including Col1, Sox9, Col2, HAS3, CD44, and Cdh2 were assessed to determine the relative stage of mesenchymal condensation for each of the groups studied. Furthermore, to assess differentiation state, early and late markers of osteogenic (Col1, Runx2, BGLAP, and IBSP), chondrogenic (AGC1 and Col2), adipogenic (PPARG2), angiogenic (VEGF and TIE2), and hematopoietic (Runx1 and CD38) lineage commitment were assessed.

7. Statistical analysis

For stress - strain analyses, first, mean microbead displacements were measured for each image of a given image stack. Then, mean shear and normal strains were calculated for each layer at each region from three samples. JMP was used for ANOVA and Tukey-Kramer tests ($p < 0.05$) for comparing shear stresses of each pair such as high versus low shear rate regions for each scaffold geometry (Fig. 4).

RESULTS

1. Prediction and delivery of mechanical cues to MSCs seeded on rapid prototyped scaffolds

CFD models predict orders of magnitude different local stresses on scaffold surfaces exposed to identical global flow regimes (Fig. 1-3,S1). Mechanical stresses at scaffold-fluid interfaces are significantly dependent on local geometry of the scaffold design; like narrowings in a stream, smaller caliber flow geometries result in increased shear rates and thus higher shear stresses at fluid-surface interfaces (Fig. S1). Although sensitive to manufacturing accuracy, no significant difference in shear stress magnitude is observed between target and actual manufactured geometries of a rapid prototyped test scaffold design exhibiting transverse isotropic architecture (Fig. 2, S2). In the manufactured test scaffold, areas of high and low shear rate exhibit more than one order difference in magnitude delivery of shear stress within both the transverse plane and along the longitudinal axis (Fig. 2,3,S2).

Seeding of MSCs on the test scaffold surface significantly attenuates local shear stresses at the cell-fluid interface in areas of high but not in areas of low shear rate, where shear rate is determined by local scaffold geometry (Fig. 2). To measure the relative effects of local geometry as well as the presence of cells *per se* on delivery of mechanical cues, we exposed specific regions of the scaffold to a global flow regime designed to deliver a target “bulk” 0.2 dyn/cm² shear stress (Fig. S1A). Given the transverse isotropic geometry of the scaffold, we used micro-piv to measure flow in scaffolds embodying geometrical features of the axial geometry with those exhibiting the transverse geometry. In unseeded scaffolds exhibiting axial geometry, the mean local shear stresses delivered in high and low shear rate regions

were 0.115 dyn/cm^2 and 0.024 dyn/cm^2 , respectively (Fig. 2, S3). Upon seeding, shear stresses at the fluid interface in high shear rate regions of the axial geometry were reduced significantly, to $0.08 \pm 0.003 \text{ dyn/cm}^2$ (a $25 \pm 2.6\%$ decrease). Shear stresses in low shear rate regions did not differ significantly between seeded and unseeded scaffolds (Fig. 2, S3). Similarly, in unseeded scaffolds exhibiting transverse geometry, the mean local shear stresses delivered at high and low shear rate regions were 0.118 dyn/cm^2 and 0.029 dyn/cm^2 , respectively (Fig. 2, S3). Upon seeding, shear stresses delivered at high shear rate regions decreased to $0.07 \pm 0.002 \text{ dyn/cm}^2$ (Fig. 2, S3). Hence, the presence of MSCs in the transverse geometry scaffolds resulted in a $40 \pm 1.7\%$ ($p < 0.05$) decrease in shear stress delivered in high shear rate regions and no significant decrease in low shear rate regions (Fig. 2).

2. Measuring cell strain on MSCs during exposure to controlled stresses

Once we demonstrated the feasibility to deliver spatially resolved mechanical stresses to MSCs seeded within scaffolds, we then aimed to measure actual strains on cells during exposure to controlled stresses. Due to experimental constraints associated with the strain mapping technique, in this experiment we used a global flow regime to deliver a target “bulk” 0.1 dyn/cm^2 stress. We calculated shear and normal components of stress and measured strain, akin to mechanical testing of living, seeded cells *in situ* (Fig. 4, S4). In scaffolds embodying geometric features of the axial geometry, normal as well as shear stresses and strains were significantly higher in the high shear rate regions than in the low shear rate regions (Fig 4A). Interestingly, compressive stresses and strains were observed in the low shear rate regions of the scaffolds exhibiting axial geometries. In scaffolds embodying geometric features of the transverse geometry, no statistically significant differences were observed in components of stress and strain between the low and high shear rate regions.

3. Modulation of stem cell fate using mechanical cues inherent to local geometry and global flow field

After showing that cells in spatially varying geometrical locations and exposed to significantly different local normal and shear stresses and show significant differences in normal and shear strains, we wanted to determine whether geometry-based spatial delivery of mechanical cues could be used to modulate nascent stem cell fate decisions. At early stages in lineage commitment, transcription of specific gene markers serve as a functional measure of unfolding phenotype, like a timestamp in the developmental process (Fig. 6A); to this end, we used real time PCR to measure change in expression of pre- (Msx2 and HAS3) and peri-mesenchymal condensation markers (Col1, Sox9, CD44, N-cadherin [Cdh2], and Col2) as well as markers of cell lineage commitment, including osteogenic (Runx2, BGLAP [Osc], IBSP [bone sialoprotein]), chondrogenic (AGC, Col2), adipogenic (Pparg), angiogenic (VEGF, TIE2) and hematopoietic (Runx1, CD38) markers after one hour exposure to spatially controlled mechanical stresses in the same scaffolds used previously. Thus, cells were seeded on scaffolds with axial and transverse geometries (Fig. 5,6A) and were then exposed to the global flow field engendering 1 dyn/cm^2 shear stress.

In axial scaffolds, one hour exposure to flow and its associated shear/normal stresses and strains resulted in a significantly upregulated expression of genes including *Msx2*, *Col2*, *Cdh2*, and *HAS3* compared to static (nonflowed) baseline controls. In contrast, the same cells exhibited significant downregulation in expression of genes for *CD44*, *IBSP*, *Runx1*, and *CD38*. A significant, two-fold upregulation in *Col2* was observed in cells within high shear rate regions but no observable change in low shear rate regions (Fig. 5,6A). *AGC1* expression varied widely and did not exhibit statistically significant differences in expression over baseline controls (Fig. 5) in axial scaffolds.

In transverse scaffolds, exposure to flow was associated with a significant upregulation in *Msx2*, *AGC1*, *PPARG*, *Cdh2*, *HAS3*, *BGLAP*, *IBSP*, *Runx1*, and *CD38*, while *Col1*, *Runx2*, *Col2*, *Sox9*, and *CD44* were significantly downregulated compared to the baseline control. Among the upregulated markers, *AGC1* showed the largest increase in expression over the baseline control. Furthermore, high shear rate regions exhibited significantly higher expression of *AGC*, with a twelve-fold increase over baseline gene expression; in low shear rate regions, a nine-fold increase over baseline gene expression level (Fig. 5) was observed. Interestingly, in the low shear rate region, expression of *BGLAP* (also referred to as *Osc*, Osteocalcin) was significantly higher than its expression in the high shear rate region.

4. Delineating the library of mechanical cues conducive to guiding cell fate in scaffolds, “mapping the mechanome”

To create a reference library of mechanical cues conducive to guiding nascent lineage commitment in tissue engineering scaffolds, we then “mapped the mechanome”, plotting 95 *per cent* confidence intervals for lineage commitment as a function of stress and strain. Accounting for scaffold geometry (transverse and axial) of the test scaffold, areas maximizing the probability of commitment to target lineage can be defined. For example, for the transverse geometry, two areas representing distinct distributions of stress-strain states at low and high shear rates, respectively, define the 95% confidence interval (CI) for achieving chondrogenesis (Fig. 6, yellow areas). Similarly, for the axial region, a narrow swath of stress and strain distributions in high shear rate regions describe the 95% confidence interval for either achieving chondrogenesis or supporting hematopoiesis, while the low shear rate regions result in an intersecting but unique state distribution (area) defining the 95% CI for achieving chondrogenic, hematopoietic and osteogenic fates.

Interestingly, when all data are pooled (normal and shear stress data from four groups including the axial and transverse, high and low shear rate regions, respectively, and 3 samples each) and correlated with corresponding fold changes in gene expression data, exposure to tensile normal stresses correlates significantly and positively with transcription of *Msx2*, *Col1*, *Runx2* and *Col2*, all markers of pre- and peri-mesenchymal condensation as well as early stages of osteo- and chondrogenesis (Table 1). In contrast, tensile normal stresses correlate significantly and negatively with transcription of *Ppar-gamma* (a marker for adipogenesis), *BSP* and *Osc* (late markers of osteogenesis), as well as *Runx1* and *CD38* (markers of hematopoiesis) (Table 1). Although areas exposed to compressive normal stresses (axial scaffold geometry, low strain rate) exhibit significant upregulation in pre-condensation markers including *Msx2* and *HA3* and downregulation in markers of lineage

commitment further downstream, no cross correlation could be carried out using this factor due to inadequate numbers of samples exposed to compressive stresses. Finally, in pooled data used for the cross correlation analysis, no significant correlation was observed with shear stress and change in regulation of gene transcription.

DISCUSSION

Based on these studies, a combined computational fluid dynamics (CFD) modeling, rapid prototype manufacturing, and experimental fluid mechanics (micro-piv and strain mapping) validation approach allows for prospective design of tissue engineering scaffold geometries to deliver spatially resolved mechanical cues, differing by an order of magnitude or more, to cells within. Local geometry dictates mechanical stresses and strains delivered to cells seeded within tissue engineering scaffolds and exposed to identical global flow fields. Furthermore, nascent lineage commitment of multipotent cells relates significantly to the local mechanical environment of the cell. By plotting the range of stress-strain states for all data corresponding to nascent cell lineage commitment (95% CI) in model embryonic mesenchymal stem cells, we have begun to “map the mechanome”, defining stress-strain states most conducive to targeted cell fates. In so doing, we provide a library of reference mechanical cues that can be delivered to cells seeded on tissue engineering scaffolds to guide target tissue phenotypes in a temporally and spatially resolved manner.

The current study addresses one prototypical tissue engineering scaffold as a test case for the use of a combined computational and experimental approach to engineer complex tissues, and demonstrates the feasibility and potential of the approach. A number of different manufacturing methods are currently in use to produce scaffolds [22-43]. While many meet rigorous technical performance specifications [22-44], even the best manufacturing methods are not perfect and include defined design tolerances for optimal performance. Based on the results of this study, manufacturing can be honed in advance by accounting for these discrepancies in the design process or by accounting for geometrical discrepancies when defining flow regimes for delivery of mechanical cues. For this study demonstrates that there is no significant difference in flow regimes predicted using CFD and those measured using micro-piv; hence, CFD provides a valid means to predict shear stresses in actual manufactured geometries. Interestingly, based on the micro-piv data from this study, the presence of cells *per se* significantly attenuates shear stress magnitudes in high but not in low shear rate regions of the scaffold; the effects of cells on flow can be accounted for when prospectively designing scaffold geometries to deliver specific mechanical cues.

In both the modular axial and transverse scaffold geometries, shear stress in the near vicinity of stem cells is significantly dependent on local flow conditions (Fig. 2,3). Interestingly, the current study data indicate that local mechanical strains (shear, normal) experienced by seeded MSCs differ significantly within and between axial and transverse scaffold geometries as well (Fig. 4). The significant, order of magnitude difference in cellular strain observed in the axial modular scaffold derives from differences between high and low shear rate regions, respectively high and low shear stresses, induced by flow (Fig. 4A).

Emergent fate decisions measured by fold change in gene transcription of markers typical for pre, peri- and post-mesenchymal condensation, as well as hematopoiesis and angiogenesis, correlate significantly to the local stress and strain state of the cell (Fig. 4-6). In general, axial geometry scaffolds exhibit significantly higher mechanical strains at cell-fluid interfaces in regions of high compared to those of low shear rate; further, in areas of high shear rate (and increased stress and strain), Col2, a post-condensation marker for chondrogenic fate, is significantly upregulated (Fig. 5). Expression of late osteogenic markers, including BGLAP and IBSP, as well as hematopoietic markers (Runx1 and CD38) differ significantly between the axial transverse scaffold geometries and depends significantly on the local mechanical state of the cell (Fig. 5,6B). In general, the transcription of these late osteogenic and hematopoietic markers is significantly downregulated by compressive strains on cell surfaces (Fig. 5,6B). Further, their transcription is sensitive to stress magnitude, where highest normal and shear stresses do not significantly alter expression of these genes over baseline, but mid-range magnitude stresses comprising *circa* 0.5-0.8 Pa of shear stress and 0.1-0.2 Pa of normal stress do (Fig. 5,6B). In transverse geometry scaffolds, it appears that subtle change in shear stress within this same range significantly up- or downregulate osteogenic and hematopoietic markers, providing further evidence for stem cells' exquisite mechanosensitivity, which has been noted in a number of recent studies [6-11]. Although AGC1, a late marker of chondrogenic differentiation, does not show statistically significant differences in expression between the high and low shear rate regions, the fold change in expression level is the highest in axial geometry (Fig. 5). A significant upregulation in AGC1 occurs in transverse geometry scaffolds, comparing flowed and baseline control scaffolds. Hence, this mechanical *milieu* is conducive to chondrogenic (axial geometry and transverse geometry) as well as osteogenic and hematopoietic (transverse geometry) lineage commitment (Fig. 6B).

Mechanome maps define stress-strain states most conducive to targeted lineage commitment and provide valuable reference points not only for honing scaffold geometries but also for defining relevant flow regimes for spatiotemporally resolved target tissue development. In addition, mechanical cues delivered directly via flow through scaffolds may act synergistically with other biophysical and biochemical cues to direct nascent cell fate decisions [7,46]. Taken as a whole, the demonstrated stochastic approach to define most probable stress and strain states conducive to achieving target lineage commitments can be further tuned using combinations of biochemical and biophysical cues delivered via dynamic scaffold surfaces and/or bioreactor media.

CONCLUSIONS

The approach demonstrated here should be broadly applicable to the prospective design of scaffold geometries for the cultivation of tissues in bioreactors under defined fluid flow regimes, and potentially in the future for the cultivation of tissues *in vivo* using scaffolds that harness prevailing *in situ* flow regimes. Further, this approach may allow for development of scaffolds exhibiting complex architectures designed to drive fate in a spatial and temporal manner appropriate for the engineering of complex tissues or interfaces between tissues, enabling increased sophistication in tissue engineering to allow for creation of tissue templates that grow and adapt with time.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This research was supported through grants from the NIH (NIDCR, R01-DE013740), NSF (CMMI-0826435), Alexander von Humboldt Foundation, and the Research Foundation of the Department of Neurological Surgery, Case Western Reserve University, Cleveland, OH. In addition, we would like to acknowledge J. Wallace for his significant contribution in manufacturing the scaffolds described in this manuscript.

REFERENCES

1. Knothe Tate ML, Shvartsman S, Friedman A. Cell and tissue engineering--taking cues from nature's engineering paradigm for developing, growing, and repairing tissues. *Tissue Eng Part A*. 2008; 14(9):1459–60. [PubMed: 18774909]
2. Knothe Tate ML, Chang H, Moore SR, Knothe UR. Surgical membranes as directional delivery devices to generate tissue: testing in an ovine critical sized defect model. *PloS One*. 2011; 6(12):e28702. [PubMed: 22174873]
3. Anderson EJ, Knothe Tate ML. Design of tissue engineering scaffolds as delivery devices for mechanical and mechanically modulated signals. *Tissue Eng*. 2007; 13(10):2525–38. [PubMed: 17822359]
4. Hutmacher DW, Sittinger M, Risbud MV. Scaffold-based tissue engineering: rationale for computer-aided design and solid free-form fabrication systems. *Trends Biotechnol*. 2004; 22(7):354–62. [PubMed: 15245908]
5. Hollister SJ, Lin CY, Saito E, Lin CY, Schek RD, Taboas JM, et al. Engineering craniofacial scaffolds. *Orthod Craniofac Res*. 2005; 8(3):162–73. [PubMed: 16022718]
6. Song MJ, Brady-Kalnay SM, McBride SH, Phillips-Mason P, Dean D, Knothe Tate ML. Mapping the mechanome of live stem cells using a novel method to measure local strain fields in situ at the fluid-cell interface. *PloS One*. 2012; 7(9):e43601. [PubMed: 22970134]
7. Zimmermann JA, Knothe Tate ML. Structure-function relationships in the stem cell's mechanical world A: seeding protocols as a means to control shape and fate of live stem cells. *Mol Cell Biomech*. 2011; 8(4):275–96. [PubMed: 22338707]
8. Chang H, Knothe Tate ML. Structure-function relationships in the stem cell's mechanical world B: emergent anisotropy of the cytoskeleton correlates to volume and shape changing stress exposure. *Mol Cell Biomech*. 2011; 8(4):297–318. [PubMed: 22338708]
9. Song MJ, Dean D, Knothe Tate ML. In situ spatiotemporal mapping of flow fields around seeded stem cells at the subcellular length scale. *PloS One*. 2010; 5(9):e12796. [PubMed: 20862249]
10. McBride SH, Knothe Tate ML. Modulation of stem cell shape and fate A: The role of density and seeding protocol on nucleus shape and gene expression. *Tissue Eng Part A*. 2008; 14(9):1561–72. [PubMed: 18774910]
11. McBride SH, Falls T, Knothe Tate ML. Modulation of stem cell shape and fate B: Mechanical modulation of cell shape and gene expression. *Tissue Eng Part A*. 2008; 14(9):1573–80. [PubMed: 18774911]
12. Anderson EJ, Kaliyamoorthy S, Alexander JID, Knothe Tate ML. Nano-microscale models of periosteocytic flow show differences in stresses imparted to cell body and processes. *Ann Biomed Eng*. 2005; 33(1):52–62. [PubMed: 15709705]
13. Gutierrez RA, Crumpler ET. Potential effect of geometry on wall shear stress distribution across scaffold surfaces. *Ann Biomed Eng*. 2008; 36(1):77–85. [PubMed: 17963042]
14. Hidalgo-Bastida LA, Thirunavukkarasu S, Griffiths S, Cartmell SH, Naire S. Modeling and design of optimal flow perfusion bioreactors for tissue engineering applications. *Biotechnol Bioeng*. 2012; 109(4):1095–9. [PubMed: 22068720]

15. Milan JL, Planell JA, Lacroix D. Computational modelling of the mechanical environment of osteogenesis within a polylactic acid-calcium phosphate glass scaffold. *Biomaterials*. 2009; 30(25):4219–26. [PubMed: 19477510]
16. Singh H, Teoh SH, Low HT, Huttmacher DW. Flow modelling within a scaffold under the influence of uni-axial and bi-axial bioreactor rotation. *J Biotechnol*. 2005; 119(2):181–96. [PubMed: 16081181]
17. Truscetto S, Kerckhofs G, Van Bael S, Pyka G, Schrooten J, Van Oosterwyck H. Prediction of permeability of regular scaffolds for skeletal tissue engineering: a combined computational and experimental study. *Acta Biomater*. 2012; 8(4):1648–1658. [PubMed: 22210520]
18. Truscetto S, Schrooten J, Van Oosterwyck H. A computational tool for the upscaling of regular scaffolds during in vitro perfusion culture. *Tissue Eng Part C*. 2011; 17(6):619–30.
19. Vossenbergh P, Higuera GA, van Straten G, van Blitterswijk CA, van Boxtel AJ. Darcian permeability constant as indicator for shear stresses in regular scaffold systems for tissue engineering. *Biomech Model Mechanobiol*. 2009; 8(6):499–507. [PubMed: 19360445]
20. Knothe Tate ML, Ritzman TF, Schneider E, Knothe UR. Testing of a new one-stage bone-transport surgical procedure exploiting the periosteum for the repair of long-bone defects. *J Bone Joint Surg Am*. 2007; 89(2):307–16. [PubMed: 17272445]
21. Curtis A, Riehle M. Tissue engineering: the biophysical background. *Phys Med Biol*. 2001; 46(4):R47–65. [PubMed: 11324976]
22. Beskardes IG, Demirtas TT, Durukan MD, Gumusderelioglu M. Microwave-assisted fabrication of chitosan-hydroxyapatite superporous hydrogel composites as bone scaffolds. *J Tissue Eng Regen Med*. 2012 doi: 10.1002/term.1677.
23. Seol YJ, Park DY, Park JY, Kim SW, Park SJ, Cho DW. A new method of fabricating robust freeform 3D ceramic scaffolds for bone tissue regeneration. *Biotechnol Bioeng*. 2012 doi: 10.1002/bit.24794.
24. Saito E, Suarez-Gonzalez D, Rao RR, Stegemann JP, Murphy W, Hollister S. Use of micro-computed tomography to non-destructively characterize biomineral coatings on solid freeform fabricated poly (L-lactic acid) and poly (epsilon-caprolactone) scaffolds in vitro and in vivo. *Tissue Eng Part C*. 2012 PMID: 23134479.
25. Li W, Lu K, Walz JY. Fabrication of porous nanocomposites with controllable specific surface area and strength via suspension infiltration. *Langmuir*. 2012; 28(47):16423–9. [PubMed: 23113584]
26. Chuang TH, Wilson RE, Love JM, Fisher JP, Shah SB. A novel internal fixator device for peripheral nerve regeneration. *Tissue Eng Part C*. 2012 PMID: 23102114.
27. Lin H, Zhang D, Alexander PG, Yang G, Tan J, Cheng AW, Tuan RS. Application of visible light-based projection stereolithography for live cell-scaffold fabrication with designed architecture. *Biomaterials*. 2013; 34(2):331–9. [PubMed: 23092861]
28. Chantarapanich N, Puttawibul P, Sucharitpwatskul S, Jeamwattananachai P, Ingiam S, Sithiseripratip K. Scaffold library for tissue engineering: a geometric evaluation. *Comput Math Methods Med*. 2012:407805. [PubMed: 23056147]
29. Tseng H, Cuchiara ML, Durst CA, Cuchiara MP, Lin CJ, West JL, et al. fabrication and mechanical evaluation of anatomically-inspired quasilaminate hydrogel structures with layer-specific formulations. *Ann Biomed Eng*. 2013; 41(2):398–407. [PubMed: 23053300]
30. De Nardo L, Bertoldi S, Cigada A, Tanzi MC, Haugen HJ, Farè S. Preparation and characterization of shape memory polymer scaffolds via solvent casting/particulate leaching. *J Appl Biomater Function Mater*. 2012; 10(2):e119–26.
31. Tan JY, Chua CK, Leong KF. Fabrication of channeled scaffolds with ordered array of micropores through microsphere leaching and indirect Rapid Prototyping technique. *Biomedical Microdevices*. 2013; 15(1):83–96. [PubMed: 22923215]
32. Vaquette C, Cooper-White J. A simple method for fabricating 3-D multilayered composite scaffolds. *Acta Biomater*. 2013; 9(1):4599–4608. [PubMed: 22902817]
33. Nseir N, Regev O, Kaully T, Blumenthal J, Levenberg S, Zussman E. Biodegradable scaffold fabricated of electrospun albumin fibers: mechanical and biological characterization. *Tissue Eng Part C*. 2013 PMID: 22881713.

34. Yoo D. New paradigms in internal architecture design and freeform fabrication of tissue engineering porous scaffolds. *Med Eng Phys.* 2012; 34(6):762–776. [PubMed: 22721938]
35. Sharma Y, Tiwari A, Hattori S, Terada D, Sharma AK, Ramalingam M, et al. Fabrication of conducting electrospun nanofibers scaffold for three-dimensional cells culture. *Int J Biol Macromol.* 2012; 51(4):627–31. [PubMed: 22721872]
36. Ovsianikov A, Chichkov BN. Three-dimensional microfabrication by two-photon polymerization technique. *Methods Mol Biol.* 2012; 868:311–25. [PubMed: 22692619]
37. Lu L, Zhang Q, Wootton D, Chiou R, Li D, Lu B, et al. Biocompatibility and biodegradation studies of PCL/beta-TCP bone tissue scaffold fabricated by structural porogen method. *J Mater Sci Mater Med.* 2012; 23(9):2217–26. [PubMed: 22669285]
38. Andric T, Wright LD, Taylor BL, Freeman JW. Fabrication and characterization of three-dimensional electrospun scaffolds for bone tissue engineering. *J Biomed Mater Res A.* 2012; 100(8):2097–105. [PubMed: 22615093]
39. Alge DL, Bennett J, Treasure T, Voytik-Harbin S, Goebel WS, Chu TM. Poly(propylene fumarate) reinforced dicalcium phosphate dihydrate cement composites for bone tissue engineering. *J Biomed Mater Res A.* 2012; 100(7):1792–802. [PubMed: 22489012]
40. Kang HW, Cho DW. Development of an indirect stereolithography technology for scaffold fabrication with a wide range of biomaterial selectivity. *Tissue Eng Part C.* 2012; 18(9):719–29.
41. Wang H, Feng Y, An B, Zhang W, Sun M, Fang Z, et al. Fabrication of PU/PEGMA crosslinked hybrid scaffolds by in situ UV photopolymerization favoring human endothelial cells growth for vascular tissue engineering. *J Mater Sci Mater Med.* 2012; 23(6):1499–1510. [PubMed: 22430593]
42. Shalumon KT, Chennazhi KP, Tamura H, Kawahara K, Nair SV, Jayakumar R. Fabrication of three-dimensional nano, micro and micro/nano scaffolds of porous poly(lactic acid) by electrospinning and comparison of cell infiltration by Z-stacking/three-dimensional projection technique. *IET Nanobiotechnol.* 2012; 6(1):16–25. [PubMed: 22423866]
43. Dean D, Wallace J, Sibiani A, Wang MO, Kim K, Mikos AG, et al. Continuous Digital Light Processing (cDLP): Highly accurate additive manufacturing of tissue engineered bone scaffolds. *Virtual Phys Prototyp.* 2012; 7(1):13–24. [PubMed: 23066427]
44. Kim K, Dean D, Wallace J, Breithaupt R, Mikos AG, Fisher JP. The influence of stereolithographic scaffold architecture and composition on osteogenic signal expression with rat bone marrow stromal cells. *Biomaterials.* 2011; 32(15):3750–63. [PubMed: 21396709]
45. Anderson EJ, Knothe Tate ML. Open access to novel dual flow chamber technology for in vitro cell mechanotransduction, toxicity and pharmacokinetic studies. *Biomed Eng Online.* 2007; 6:46. [PubMed: 18053207]
46. Knothe Tate ML, Falls TD, McBride SH, Atit R, Knothe UR. Mechanical modulation of osteochondroprogenitor cell fate. *Int J Biochem Cell Biol.* 2008; 40(12):2720–38. [PubMed: 18620888]
47. Lancrin C, Mazan M, Stefanska M, Patel R, Lichtinger M, Costa G, et al. GFI1 and GFI1B control the loss of endothelial identity of hemogenic endothelium during hematopoietic commitment. *Blood.* 2012; 120(2):314–22. [PubMed: 22668850]
48. Ross K, Sedello AK, Todd GP, Paszkowski-Rogacz M, Bird AW, Ding L, et al. Polycomb group ring finger 1 cooperates with Runx1 in regulating differentiation and self-renewal of hematopoietic cells. *Blood.* 2012; 119(18):4152–161. [PubMed: 22411870]
49. Swiers G, de Bruijn M, Speck NA. Hematopoietic stem cell emergence in the conceptus and the role of Runx1. *Int J Dev Biol.* 2010; 54(6-7):1151–63. [PubMed: 20711992]
50. Miraglia S, Godfrey W, Yin AH, Atkins K, Warnke R, Holden JT, et al. A novel five-transmembrane hematopoietic stem cell antigen: isolation, characterization, and molecular cloning. *Blood.* 1997; 90(12):5013–21. [PubMed: 9389721]
51. Dagher RN, Hiatt K, Traycoff C, Srour EF, Yoder MC. c-Kit and CD38 are expressed by long-term reconstituting hematopoietic cells present in the murine yolk sac. *Biol Blood Marrow Transplant.* 1998; 4(2):69–74. [PubMed: 9763109]
52. Carmeliet P, Ferreira V, Breier G, Pollefeys S, Kieckens L, Gertsenstein M, et al. Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. *Nature.* 1996; 380(6573):435–439. [PubMed: 8602241]

53. Ema M, Yokomizo T, Wakamatsu A, Terunuma T, Yamamoto M, Takahashi S. Primitive erythropoiesis from mesodermal precursors expressing VE-cadherin, PECAM-1, Tie2, endoglin, and CD34 in the mouse embryo. *Blood*. 2006; 108(13):4018–4024. [PubMed: 16926294]
54. Asahara T, Murohara T, Sullivan A, Silver M, van der Zee R, Li T, Witzenbichler B, et al. Isolation of putative progenitor endothelial cells for angiogenesis. *Science*. 1997; 275(5302):964–967. [PubMed: 9020076]
55. Risau W. Mechanisms of angiogenesis. *Nature*. 1997; 386(6626):671–674. [PubMed: 9109485]
56. Evans SF, Docheva D, Bernecker A, Colnot C, Richter RP, Knothe Tate ML. Solid-supported lipid bilayers to drive stem cell fate and tissue architecture using periosteum derived progenitor cells. *Biomaterials*. 2013; 34(8):1878–87. [PubMed: 23237517]

Figure 1.

Prototypic tissue engineering scaffold design tested in the current study. (A,B) This scaffold shares design features common to many scaffolds, including longitudinal canals through the length of the scaffold for efficient molecular transport and smaller caliber, and transverse channels to aid in seeding and cultivation of cells. Using modern imaging methods, such modular scaffolds can be customized to accommodate patient-specific tissue defect geometries.(3) Scaffolds exemplifying modular sections of the axial (A,C,E) and transverse (B,D,F) cylindrical scaffold geometry were tested in the current study. (C,D) Idealized and (E,F) manufactured geometries of respective axial and transverse scaffold sections including measurements at high and low shear rate regions.

Figure 2.

Shear stresses in the near vicinity (near vicinity areas shown as black and red square outlines in Fig. 3) of stem cells seeded within the tissue engineering scaffolds. Error bar shows SE (n=3), where * indicates statistically significant differences ($p < 0.05$).

Figure 3. Prediction and optimization of flow regimes by CFD to achieve target shear stress of 1 dyn/cm^2 in manufactured scaffolds (A,B). Red and black boxes indicate high shear rate and low shear rate regions, respectively, in axial (A) and transverse (B) modular scaffolds.

Figure 4.

Shear and normal stresses and strains on the surface of stem cells seeded within the modular tissue engineering scaffolds. (A) Statistical analysis of shear and normal strain at high and low shear rate regions for both scaffold geometries. (B,C) Shear (B) and normal (C) stress and strain distributions depending on geometry of scaffolds and local regions. Error bar shows SE (n=3), where * indicates statistically significant differences ($p < 0.05$).

Figure 5.

Fold changes of genetic markers indicative of lineage commitment (*cf.* Fig. 7A) normalized to baseline gene expression level (without flow). The fold changes of gene expression studied in axial (A) and transverse (B) scaffold geometries. *(flow *versus* control), † (low *versus* high shear rate) indicate statistical significance ($p < 0.05$).

Figure 6.

Unfolding of stem cell fate as a function of the cell's local mechanical *milieu* and mapping the mechanome. (A) Fate map for mesenchymal stem cells. After mesenchymal condensation (blue dotted box, E11.5 in the mouse), the first step in skeletogenesis, lineage commitment progresses toward chondrogenic (orange), osteogenic (blue), and adipogenic (green) fates. Transcription levels for specific proteins (red and blue font) give a “chronological fingerprint” for each stage of development over time (46, 56). (B) Each color-coded region depicts a 95% confidence area for lineage commitments corresponding to shear and normal stress states of the cells seeded within regions of high and low shear rate, respectively. The two orange shaded, oval areas depict distinct regions (95% confidence area) corresponding to chondrogenic lineage commitment in high (upper right) and low shear rate (lower left) regions, respectively, of the modular axial geometry scaffold. The purple area includes the 95% CI for cell data corresponding to nascent chondrogenic and support of hematopoietic lineage commitment. The red area includes the 95% CI for cell data corresponding to nascent chondrogenic and osteogenic, as well as support of hematopoietic lineage commitment.

Table 1

Correlation of gene expression to shear and normal stress exposure. Bolded (+/-) text indicates a respective significant positive/negative correlation.

Stress	Col1a1	Runx2	Msx2	Col2a1	SOX9	AGC1	PPARG	CD44	Cdh2	HAS3	BGLAP	VEGF	TIE2	Runx1	CD38	IPSP
shear	0.1723	0.173	0.367	0.454	0.108	0.346	-0.076	0.381	0.173	0.249	0.151	0.173	0.173	0.108	0.0864	0.0648
normal (tension)	0.738	0.738	0.896	0.843	0.580	0.000	-0.824	-0.346	-0.158	-0.344	-0.896	-0.527	-0.609	-0.843	-0.896	-0.948