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MULTI-LAMELLAR AND MULTI-AXIAL MATURATION OF CELL-SEEDED FIBER-REINFORCED TISSUE ENGINEERED CONSTRUCTS

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INTRODUCTION

The architecture of load-bearing fibrous tissues is optimized to enable a specific set of mechanical functions. This organization arises from a complex process of cell patterning, matrix deposition, and functional maturation [1]. In their mature state, these tissues span multiple length scales, encompassing nanoscale interactions of cells with extracellular matrix to the centimeter length scales of the anatomic tissue volume and shape. Two structures that typify dense fibrous tissues are the meniscus of the knee and the annulus fibrosus (AF) of the intervertebral disc (IVD). The mechanical function of the wedge-shaped knee meniscus is based on its stiff prevailing circumferential collagen architecture that resists tensile deformation [2,3]. Adding to its complexity, radial tie fibers and sheets are interwoven amongst these fibers, increasing stiffness in the transverse direction and binding the tissue together [4]. In the annulus fibrosus, multiple anisotropic lamellae are stacked in concentric rings with their prevailing fiber directions alternating above and below the horizontal axis in adjacent layers [5]. The high circumferential tensile properties of this laminate structure allow it to resist bulging of the nucleus pulposus with compressive loading of the spine. Given their structural properties, unique form, and demanding mechanical environments, the knee meniscus and the AF region of the IVD represent two of the most challenging tissues to consider for functional tissue engineering.

As a first step in the process of engineering these fibrous tissues, we have developed a strategy that takes into account both the overall anisotropy as well as the nanoscale cell-matrix interactions as primary design criteria. In this strategy, we employ fiber-aligned nanofibrous scaffolds, produced by electrospinning, that are tailored to possess both the structural and mechanical anisotropy of the native tissue as well as its non-linear mechanical behavior [6,7]. We have shown that when seeded with mesenchymal stem cells (MSCs), meniscus fibrochondrocytes (MFCs), and AF cells, these scaffolds provide a 3D micro-pattern for directing cell organization and extracellular matrix (ECM) deposition [7,8]. As the next step in our goal of engineering these complex structures, the current study creates multi-lamellar cell-seeded constructs and evaluates their integration strength with time in culture. Further, we characterize the time-dependent evolution of mechanical properties in single-lamella MSC-seeded constructs in both the prevailing and transverse fiber directions with uniaxial and biaxial mechanical testing.

METHODS

Scaffold Fabrication: To carry out these studies, nanofibers were produced from a 14.3% w/v solution of poly(ε-caprolactone) (PCL, 80 kD) dissolved in 1:1 THF:DMF. Electrospinning was carried out at 13 kV with deposition focused onto a 1" mandrel rotating at 8000 rpm (~10 m/s) [6]. Fibers were collected for 8 hours over a spinneret-to-mandrel distance of 20 cm to form ~1mm thick fiber-aligned meshes. **Cell Culture:** To isolate MFCs, calf menisci were diced into 1-2 mm^3 pieces and placed in tissue culture dishes containing basal medium (DMEM containing 1XPSF and 10%FBS). MFCs emerged from these pieces over a 1-2 week period, and were expanded through passage 2. Bovine MSCs were harvested from the tibial trabecular bone marrow of the same donors as in [8] and expanded through passage 2. Scaffolds were prepared for seeding as described previously [9]. **Multi-Lamellar Studies:** Scaffolds were cut to 5mm wide by 25mm long strips and seeded with $1x10^6$ MSCs or MFCs. After two weeks of pre-culture in chondrogenic medium [8] changed twice weekly, scaffolds were placed into apposition and sandwiched between two porous polypropylene supports secured with a sterile foil cinch (**Fig 1**). After a further two weeks, the polypropylene supports were removed and the constructs were cultured an additional

four weeks. At bi-weekly intervals, constructs were removed from culture and calibrated images of each side were taken. Construct interface testing was performed at 0.1%/sec of the overlap length until failure. Interface strength was determined from the maximum force normalized to the overlap area. **Multi-Axial Studies:** To assess maturation with respect to the prevailing fiber direction, aligned meshes were cut into six 25 by 25mm samples. Three were seeded with $5x10^6$ MSCs and cultured in chondrogenic medium changed twice weekly for 12 weeks. Three additional samples served as unseeded controls maintained similarly (in PBS). At 12 weeks, samples were cut to yield a 16mm square for biaxial testing (BIAX) and two 4mm wide by 20mm long strips for uniaxial testing, with one sample cut in the fiber (FIB) direction and the other cut in the transverse (TRANS) direction. Uniaxial testing was carried out with elongation at a rate of 0.1%/s and modulus determined from the force-elongation curve and sample geometry. Biaxial testing was carried using a custom device with speckled samples imaged during elongation (0.1%/s) applied in the fiber direction with the transverse boundary fixed. Vic2D software was used to determine local strains and the apparent modulus was calculated from the stress-strain curve. **Histology:** Constructs were fixed in 4% paraformaldehyde and 8μm thick cross-sections were stained with Hematoxylin and Eosin (H&E) or Picrosirius Red (PSR) to visualize cells and collagen. **Statistical Analysis:** ANOVA with Fisher's LSD posthoc tests was used to make comparisons between groups, with significance set at $p<0.05$.

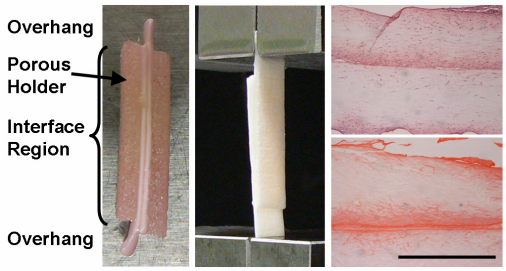

Figure 1: Culture (left) and mechanical testing (middle) of conjoined laminates. H&E (right, top) and PSR (right, bottom) staining of laminate MSC-seeded construct (Day 42). Scale: 1mm.

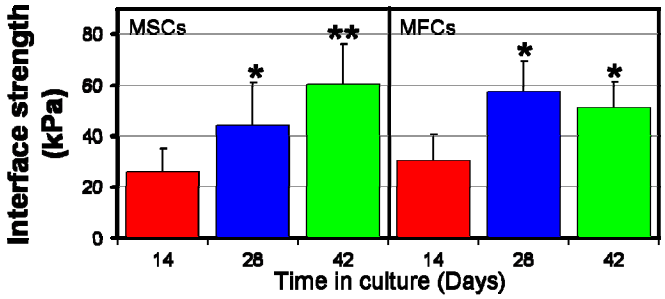

Figure 2: Interface strength of laminates (MSCs: n=11-12/time point, MFCs: n=5/time point). *: p<0.05 vs. day 14, **: p<0.01 vs. day 14 and 28.

RESULTS

Cell-seeded (MFC or MSC) bi-lamellar constructs formed a stable biologic union with time in culture. Within 2 weeks of conjoined culture (**Fig 1**), interface strength developed such that composites could be handled and the porous supports removed. Over the next four weeks, interface strength increased for both cell types ($p<0.05$, **Fig 2**). Histological sections showed high cellularity and fibrocartilaginous matrix deposition at the interface with some infiltration into the scaffold (**Fig 1**). To determine how multi-directional mechanics change with maturation, single-lamella samples were cultured for 84 days with and without seeding with MSCs. Testing in the FIB and TRANS directions showed that both fiber direction and cell seeding had an effect on scaffold modulus on day 84 ($p<0.001$, **Fig 3**). Seeding with MSCs increased modulus in both testing directions ($p<0.05$, FIB: 23%, TRANS: 703%, **Fig 3**). Apparent moduli of samples in biaxial loading increase with seeding ($p<0.001$) and were 40% higher for acellular and 68% higher for MSC-seeded scaffolds compared to fiber-aligned uniaxial values ($p<0.001$), indicating the contributions of developing transverse properties to the overall construct mechanical response.

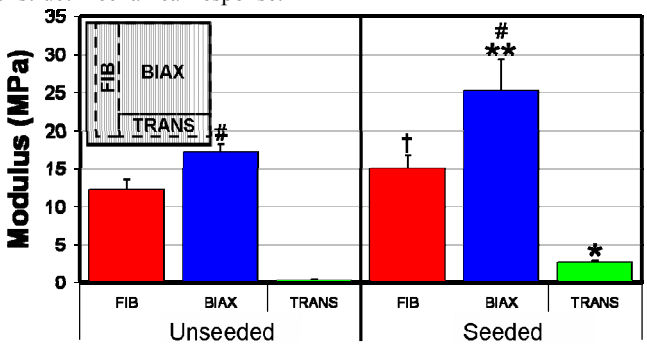

Figure 3: Properties of unseeded and seeded constructs on day 84 (FIB, TRANS: n=6, BIAX: n=3). *: p<0.05, **: p<0.001, and †: p<0.01 vs. unseeded scaffolds in same direction, #: p<0.001 vs. FIB.

DISCUSSION

The specialized function of fiber-reinforced tissues is reflected in their structural properties and architecture. However, these same features that enable function (density, fiber-alignment, hypocellularity) are also a hindrance to self-repair. To address this issue, we propose a tissue engineering approach based on fiber-aligned nanofibrous scaffolds. While these anisotropic scaffolds seeded with cells increase in properties over time, little information is available regarding their transverse and biaxial maturation. In this study, seeding with MSCs and culture in chondrogenic medium for 84 days increased construct properties in both the fiber and the transverse directions. Similarly, the apparent moduli of biaxial test samples were higher with cell seeding. These findings suggest that with culture, cells deposit a functional matrix that improves mechanical properties in multiple directions. In addition to these single-lamella studies, we also demonstrated that, when cultured in apposition, scaffolds form a functional biologic union. This finding will allow for 3D stacking approaches to provide constructs that recapitulate anatomic form. For example, for the meniscus, a multi-lamellar construct may be fabricated that contains both circumferential and radial 'tie' fibers. For the AF, concentric wrapping will recapitulate the lamellar structure of the native tissue (with alternating fiber angles). Such approaches hold great promise for the creation of tissue-engineered fiber-reinforced replacement structures for poorly healing tissue of the musculoskeletal system that are critical for locomotion.

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