Design and Evaluation of a Multi-Phased Scaffold for Functional Ligament Tissue Engineering

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Statement of Purpose: The ACL is the most frequently injured ligament of the knee, with over 100,000 reconstructions performed annually[1]. However, common tendon autografts fail to re-establish the native ACL-bone interfaces, resulting in poor clinical outcomes [2]. Thus there is an unmet need for alternative ACL reconstruction options. The goal of this work is to develop a functional and integrative tissue engineering scaffold for mesenchymal stem cell (MSC)-mediated[3] ACL regeneration. We have developed a five-phased nanofiber-based ACL scaffold (Fig. 1) for simultaneous regeneration of the functional ACL. The objectives of this study are to optimize MSC seeding density *in vitro* and to evaluate the scaffold *in vivo*. We hypothesized that increased seeding density will enable greater matrix formation *in vitro* and the multi-phased scaffold will result in bone, interface and ligament regeneration *in vivo*.

Methods: Each phase of the scaffold was fabricated via electrospinning[4]. The ligament and bone phases consisted of aligned 5:1 poly(ε-caprolactone) (PCL, Sigma-Aldrich):poly(lactide-*co*-glycolide) (PLGA, 85:15, Lakeshore Biomaterials) nanofibers, with 35% (w/w) hydroxyapatite (HA) nanoparticles (Nanocerox, 100-150nm) incorporated for the bone phase. The interface phase was comprised of bi-phasic nanofiber collars with parallel PLGA and PLGA-HA (15% HA) regions which mimic the non-mineralized and mineralized regions of the native ACL-bone interface. *Cell Density Optimization:* Scaffolds were seeded with MSCs (Lewis rat) at $2.5x10^5$, $5x10^5$, or $1x10^6$ cells/scaffold and total DNA, collagen, GAG deposition, and ALP activity were quantified (n=5) for each phase over 7 days. Expression (n=5) of fibroblast (collagen I, III, fibronectin, tenascin-C, scleraxis, tenomodulin), osteoblast (osteocalcin, osteopontin), and interface (collagen II, X, aggrecan) markers was determined by real-time RT-PCR. In Vivo *Evaluation:* ACL reconstruction was performed on Lewis rats[5]. Experimental groups received an acellular or MSC-seeded multi-phased graft. Control groups received a flexor tendon or single-phased (ligament-only) graft. Rats were sacrificed at 4 and 10 weeks and knees were harvested for histological (n=3) and biomechanical (n=10, week 10 only) analysis. *Statistical Analysis:* ANOVA and the Tukey-Kramer post-hoc test were used for all pair-wise comparisons (*$p<0.05$).

Results: *Cell Density Optimization:* Significantly higher GAG content and ALP activity were seen on the interface and bone phases, respectively, in the $1x10^6$ cells/scaffold group at day 7 (Fig. 1). On the interface phase, collagen II and aggrecan were upregulated at day 1 at the highest density (Fig. 1). Thus, a $1x10^6$ cells/scaffold density was determined to be optimal for *in vivo* evaluation. In Vivo *Evaluation:* All synthetic scaffolds remained intact after 10 weeks *in vivo*. At 4 weeks, collagenous tissue surrounding synthetic scaffolds within and at the tunnel exits, and within the mid-substance was evident (Fig. 2). Bone formation within the tunnels appeared to be accelerated with the multi-phased scaffold. Positive GAG staining was observed surrounding the interface phase of the multi-phased graft. The stiffness and failure load of the acellular and MSC-seeded multi-phased scaffolds was similar to the flexor tendon control. Single-phased grafts resulted in a significantly lower failure load, as well as a lack of fibrocartilage formation or osteointegration.

Discussion/Conclusions: In this study, a novel multi-phased ACL scaffold was optimized and evaluated. *In vivo* evaluation showed that the graft was well-tolerated and reduced the duration of the reconstruction procedure. The presence of HA within the bone phase of the scaffold accelerated the formation of mineralized tissue within the scaffold, similar to other studies evaluating the use of HA for bone regeneration and tendon-bone integration[7,8]. Most importantly, the formation of a multi-tissue, structurally-integrated bone-ligament-bone complex was observed *in vivo*, with significantly higher mechanical properties over singled-phased control. Collectively, these findings demonstrate the promise of the multi-phased scaffold for functional and integrative ACL reconstruction.

References: [1]Glickson *et al*., 2004 [2]Tsuda *et al.*, 2001 [3]Pittenger *et al.*, 1999 [4]Moffat *et al*., 2009 [5]Hays *et al*., 2008 [6]Awad *et al*., 2000 [7]Wen *et al*., 2009 [8]Hasegawa *et al*., 2009

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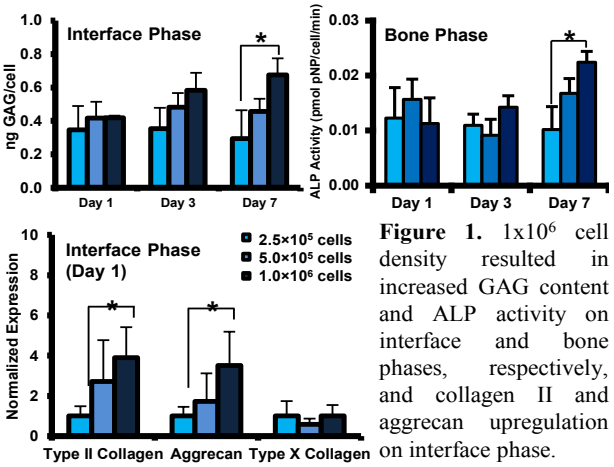

Figure 1. $1x10^6$ cell density resulted in increased GAG content and ALP activity on interface and bone phases, respectively, and collagen II and aggrecan upregulation on interface phase.

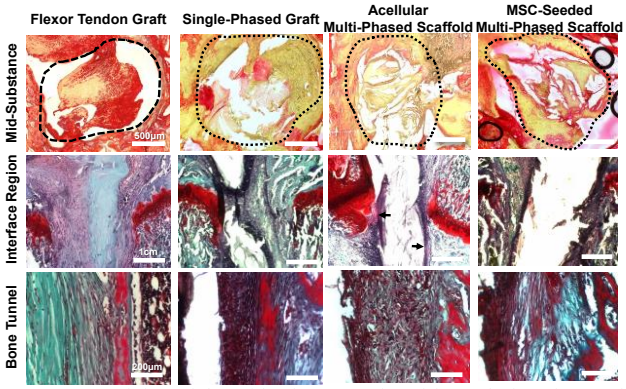

Figure 2. Staining of the mid-substance (picrosirius red), interface region (safranin-O), and bone tunnels (modified Goldner's trichrome), week 4.

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