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Scaffold architecture and fibrin gels promote meniscal cell proliferation

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Stability of the knee relies on the meniscus, a complex connective tissue with poor healing ability. Current meniscal tissue engineering is inadequate, as the signals for increasing meniscal cell proliferation have not been established. In this study, collagen scaffold structure, isotropic or aligned, and fibrin gel addition were tested. Metabolic activity was promoted by fibrin addition. Cellular proliferation, however, was significantly increased by both aligned architectures and fibrin addition. None of the constructs impaired collagen type I production or triggered adverse inflammatory responses. It was demonstrated that both fibrin gel addition and optimized scaffold architecture effectively promote meniscal cell proliferation. [http://dx.doi.org/10.1063/1.4900885]

The meniscus is critical for joint movement, acting to distribute loads, stabilize the knee joint, and absorb shock.[1] However, the adult meniscus has a poor healing potential, requiring interventions to ensure a favorable outcome after traumatic injury. These interventions include the addition of collagen scaffolds or fibrin gel to the wound site.

With significant improvement in pre-operative function and pain, and arresting cartilage degeneration, collagen type I scaffolds have improved meniscal healing within the clinic.[2,3] However, even after 5 yr post-operation, the regenerated tissue is not as organized as native tissue and is limited to areas of the meniscus with a blood supply.[2,3] Fibrin gels, which occur naturally as part of the clotting system, have been demonstrated to promote extra-cellular matrix (ECM) production and improve wound healing *in vivo*.[4–6] It is hypothesized that the fibrin clot contains cells and growth factors which allows repair to take place, even in a vascular regions.[6] When fibrin clots were tested in meniscal lesions in goats, it was found that adding fibrin did not improve the repair rate obtained by sutures alone, although the organization of the repair tissue improved.[7]

Clearly, current meniscal repair strategies need improvement, which requires an expanded understanding of meniscal cell response to environmental cues within tissue engineering constructs. Cellular in-growth and proliferation can be encouraged by mimicking the composition and mechanical properties of native tissue within tissue engineering constructs.[8–10] It has also been proposed that architectural cues are important cellular signals and it has been demonstrated that architecture can alter meniscal cell morphology on aligned nano-fiber eletrospun mats and within aligned porous silk scaffolds.[11,12] In addition, the combination of a scaffold and fibrin gel has been shown to be beneficial to cellular proliferation in other musculoskeletal tissues, such as tendon.[13]

The key goal in regenerative medicine of the meniscus is to stimulate damaged tissue to regain the function and properties of healthy tissue. While both fibrin gels and scaffolds have been used in the past as tissue engineering constructs, no comparative study has been undertaken, to our knowledge, to assess the relative influence of scaffold architecture and addition of fibrin gel on meniscal cell

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proliferation and metabolic activity. Although proliferation and metabolic activity are important for evaluating tissue engineering constructs, it is also necessary to ensure there are no negative biological effects due to the constructs, such as inflammation or reduced matrix production.

Given the complex architecture of native meniscus, it was hypothesized that scaffold architectural cues would influence the biological activity of meniscal cells both with and without fibrin gel addition. Within this study, meniscal cells were cultured on isotropic scaffolds (without a preferred pore orientation) and on aligned scaffolds (composed of parallel pores). The effect of fibrin gel within both scaffold architectures was assessed and compared to a fibrin gel control in the absence of a scaffold. The constructs were evaluated based on meniscal cell proliferation and metabolic activity. In addition, the expression of gelatinases, which can indicate inflammation, and the production of type I collagen, the major structural protein in the meniscus, were also examined.

Collagen scaffolds were made by hydrating 1 wt. % insoluble bovine Achilles tendon, type I collagen in 0.05 M acetic acid at pH 2. Slurries were homogenized and frozen at −30 °C, for 90 min, with a set cooling rate of 0.9 °C/min. The scaffold was lyophilized using a Virtis freeze drier at 0 °C for 20 h under a vacuum of 80 mTorr. Scaffold architecture was altered via mold design, using principles described previously.[14,15] Molds for aligned scaffolds, those with parallel pores, were created from perspex molds with a copper base and isotropic scaffolds, those without a preferred orientation, were made in stainless steel molds, detailed in Davidenko *et al.*[16] Scaffolds were cross-linked for 4 h in 70% ethanol containing a 5:2:1 ratio of N-(3-Dimethylamino propyl)-N'-ethylcarbodiimide hydrochloride (EDC):N-Hydroxysuccinimide (NHS):collagen COOH groups.[17]

Throughout the study, adult ovine primary meniscal-derived cells were used. They were harvested by digesting minced tissues for 2 h at 37 °C in 0.2% collagenase in complete media containing 10% fetal bovine serum and antibiotics. Prior to seeding cells, cylindrical, aligned scaffolds (diameter 6 mm) and isotropic scaffolds (cross-section 5 × 5 mm), with a length of 10 mm, were prepared. Tissue culture polystyrene (TCPS) plates were coated in SYLGARD (type 184 silicone elastomer, Dow Chemical) as previously described.[18] Scaffolds were pinned to the SYLGARD with minutiens insect pins (0.1 mm diameter, Fine Science Tools GmbH), sterilized in 70% ethanol and washed in 1× phosphate buffered saline (PBS). Scaffolds were blotted with sterilized filter paper (3MM, Whatman) and seeded with 1×10^6 meniscal cells in 75 μl of media or fibrin gel (1% fibrin and 0.15 U/ml aprotinin in PBS). If seeded with fibrin, scaffolds were gelled in 300 μl of 0.4 U/ml thrombin for 10 min before thrombin was replaced with media. Otherwise, scaffolds were incubated (5% CO_2, 37 °C) for 2 h before adding media. Empty, control scaffolds were treated as other scaffolds, without the addition of cells. The fibrin control, adapted from Kapacee *et al.*, consisted of gelling 75 μl of fibrin, containing 1×10^6 cells, across a 10 mm gap between minutiens pins with 75 μl of 0.4 U/ml thrombin.[18] The study was conducted over 3 weeks, which was sufficient time to ascertain if cell proliferation would take place and for the cells to form a characteristic ECM which could be assessed.

Scaffold structure was evaluated using both scanning electron microscopy (JEOL 820), operated at 10 kV, and micro-computed tomography scans, (Skyscan 1072, 25 kV, 132 μA), reconstructed using ImageJ and NRecon, part of the Skyscan system. Aligned scaffolds were composed of planes of collagen in the longitudinal direction, with no predominant directionality in the transverse direction, Figure 1. Isotropic scaffolds consisted of purely equiaxed pores without preferred orientation, Figure 1. To image cell attachment after 24 h, scaffolds were fixed for 1 h in 4% paraformaldehyde, then dried in ethanol dilutions (25%, 30%, 50%, 70%, 80%, 90%, and 100%), with a final immersion in Hexamethyldi-silazane before air drying.[19] On all scaffold structures, meniscal cells were well spread. However, on aligned scaffolds, cells were more elongated in the direction of the pores, Figure 1, which is in agreement with previous literature.[11,12] Alignment in cellular morphology has been shown to influence the organization of the extra-cellular matrix produced by meniscal cells, leading to enhanced mechanical properties which are closer to native tissue.[12]

Metabolic activity is related to both the production of matrix proteins and cellular proliferation within tissue engineering constructs. Cell metabolism was measured using an alamarBlue® assay (Invitrogen). Samples were incubated in media with 10% alamarBlue for 4 h at 37 °C, after which fluorescence of the cell supernatant was read using a Fluorostar Optima plate reader (BMG

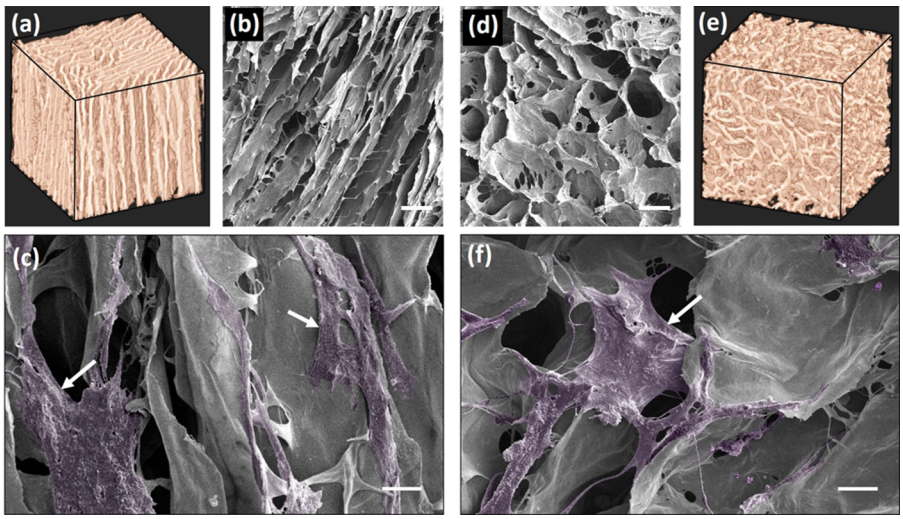

FIG. 1. Scaffold structure and meniscal cell morphology. (a)-(c) Aligned scaffolds consisted of parallel pores and (d)-(f) isotropic scaffolds had equiaxed pores with no preferred orientation. (a) and (e) Three-dimensional micro-computed tomography reconstructions (1 mm^3). (b), (c), (d), and (f) Electron micrographs, (b), and (d) scale bar 100 μm, (c), (f) scale bar is 20 μm. Arrows indicate meniscal cells.

Labtech). Controls confirmed that neither the scaffolds nor fibrin alone interfered with the assay. Cell metabolic activity increased significantly over a three week period for all tissue engineering constructs, except the fibrin control, Figure 2(a). While the fibrin control only had significantly decreased metabolic activity after three weeks, the addition of fibrin to scaffolds significantly

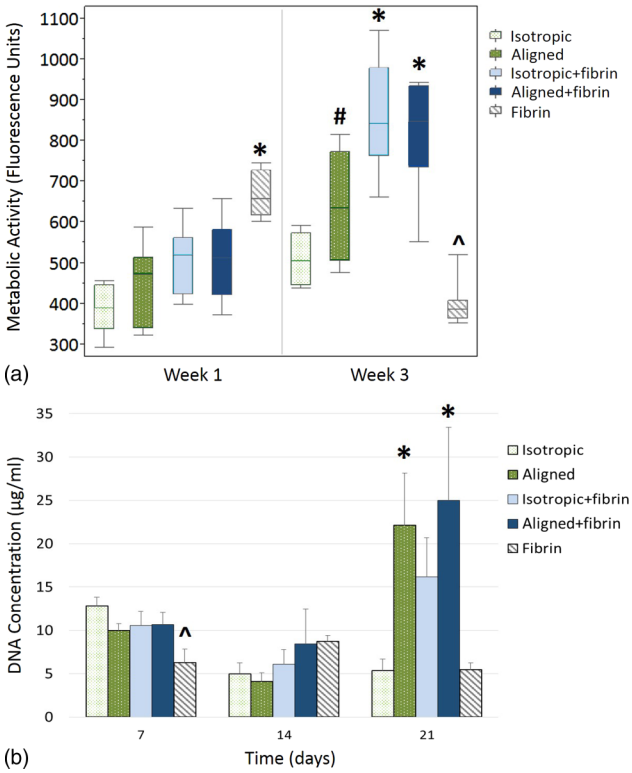

FIG. 2. The influence of scaffold architecture on meniscal cell (a) metabolic activity measured via alamarBlue and (b) cell proliferation, observed from DNA concentration. *Significantly greater than all groups without an asterisk ($p < 0.05$). #Significantly greater than isotropic scaffolds ($p < 0.05$).^Significantly lower than all other groups ($p < 0.05$).

up-regulated metabolic activity, regardless of architectural cues. However, in the absence of fibrin gel, an aligned architecture still promoted greater metabolic activity than an isotropic scaffold, Figure 2(a).

Cellular proliferation was determined via DNA concentration, measured by the Hoechst assay. Cells and scaffolds were digested in papain buffer at 60 °C overnight.[20] The papain digest was diluted 1:10 in 1× Trizma-Sodium Chloride-EDTA buffer (TNE). Afterwards, 0.2 μg/ml Hoechst dye (Bisbenzimide H, Sigma B2883), was added, yielding a final dye concentration of 0.1 μg/ml, and fluorescence was measured (360 nm Ex, 460 nm Em).[21] Without fibrin addition, the cell number doubled within aligned scaffolds over the course of the study, while remaining constant on isotropic scaffolds. Architectural cues stimulated cells independently of fibrin addition, and there was no significant difference between the proliferation within aligned scaffolds with or without fibrin gel addition. The addition of fibrin to scaffolds did increase the proliferation of meniscal cells in isotropic scaffolds 1.5 fold, Figure 2(b). In fibrin controls, there was no significant change in DNA concentration, which was significantly lower than in scaffolds with fibrin, Figure 2(b).

Within the literature, fibrin gels have been shown to encourage matrix production and allow cellular alignment of connective tissue cells to occur *in vitro*.[4,22] However, within the current study, fibrin gels alone did not encourage either high proliferation or metabolic activity over three weeks. This is probably due to the early dissolution of the gel before the meniscal cells could build up a supportive ECM to replace it, which is a known drawback of fibrin gels.[4] When fibrin gel was used in combination with a scaffold, the resulting construct created a supportive environment for cell growth and proliferation, which has been noted in other tissue engineering systems, such as tendon and blood vessel formation.[13,23]

While meniscal cell proliferation and metabolic activity are important for evaluating the regenerative capacity of tissue engineering constructs, it is also necessary to ensure there are no negative biological effects, such as inflammation or reduced matrix production. In response to pro-inflammatory factors, meniscal cells increase the expression of gelatinases (MMP-2 and MMP-9), which degrade the ECM.[24] Thus, the expression of gelatinases was examined after 21 days via gelatin zymography.[25] Cell supernatants were mixed with sodium dodecyl-sulphate (SDS) buffer and separated on a Novex 1 mm gelatin zymography gel (Invitrogen). The gel was renatured for 60 min at room temperature and incubated overnight (37 °C) before staining with Coomassie Blue R. Gels were dried and quantified using densitometry. It was found that meniscal cells expressed gelatinases, both the pro-form of MMP-2 and 9, at 72 and 92 kDa, and the active form of MMP-2 at 62 kDa.[26,27] There was no evidence of abnormal gelatinase activity within any of the tissue culture constructs, Figure 3(a), suggesting no up-regulation of inflammation.

The production of ECM protein within all constructs was also evaluated after 21 days, to ensure that matrix expression was not repressed, using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting. Scaffolds were incubated overnight at 4 °C in cell lysis buffer (Invitrogen) and mixed with 4× SDS sample buffer for a final concentration of 1×, before incubation at 60 °C for 20 min. Standards of native ovine meniscus were prepared by freezing tissue in liquid nitrogen, milling using a dismembrator (B Braun Biotech International GmbH) and suspending in cell lysis buffer (100 mg/ml, Invitrogen). Proteins were separated on 4%–20% Tris-glycine gels (Invitrogen) and transferred to a membrane (Immobilon PVDF, Millipore) for Western blotting. Primary antibodies for fibronectin (SC-9068, Santa Cruz Biotech) and collagen type I (Rockland) were used (1:1000 dilution). Membranes were developed using ECL plus (Amersham) and assessed with a densitometer (BioRad GS-800). Within each sub-figure, all lanes are from the same gel, where lanes have been moved, it has been clearly marked. Data were normalized by DNA concentration, due to interference of serum proteins with protein normalization (data not shown), and reported as the fold change from the aligned scaffold without fibrin. Quantification of the other two major constituents of meniscal ECM, decorin, and proteoglycan, were attempted but the results were masked by the excess proteoglycan associated with the raw collagen material used to make the scaffolds (data not shown).

Fibronectin expression was examined as a general matrix molecule for cellular adhesion. It is widely distributed within the body and is often secreted into the matrix of connective tissue.[28] Bands were detected at both 220 kDa (monomer) and 440 kDa (dimer), as described in literature, and seen

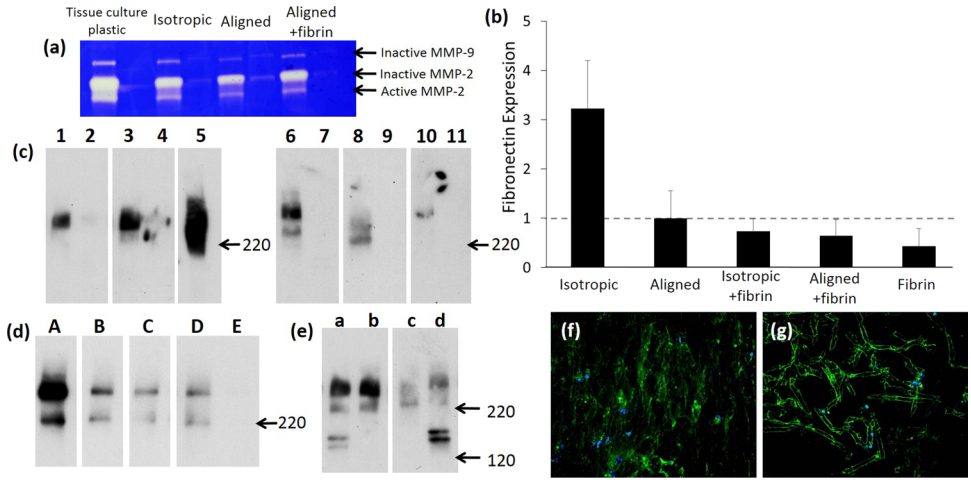

FIG. 3. Meniscal cell behavior on tissue engineering constructs. (a) Zymography of supernatant with and without cells. (b) Densitometry of fibronectin expression (fold change from aligned scaffold ± standard deviation). (c) Only constructs with cells expressed fibronectin. 1: *Isotropic*; 2: *empty isotropic*; 3: *aligned*; 4: *empty aligned*; 5: *meniscal tissue*; 6: *isotropic + fibrin*; 7: *empty isotropic + fibrin*; 8: *aligned + fibrin*; 9: *empty aligned + fibrin*; 10: *fibrin*; 11: *empty fibrin*. (d) Protein expression within constructs. A: *Isotropic*; B: *aligned*; C: *isotropic + fibrin*; D: *aligned + fibrin*; E: *fibrin*. (e) Fibronectin fragments were found in some samples. a: *Isotropic fragments*; b: *isotropic*; c: *aligned + fibrin*; d: *aligned + fibrin fragments*. Immunohistochemistry revealed fibronectin along scaffold pore walls in (f) aligned and (g) isotropic scaffolds. Molecular weights in kDa.

in the meniscal tissue control, Figure 3(c), lane 5.[28] Fibronectin was specific to cellular activity; empty scaffolds, without cells had no signal, Figure 3(c), confirming that the protein was a good marker for cellular activity and matrix production. The addition of fibrin to scaffolds decreased fibronectin production, possibly due to a reduced need for adhesive proteins within constructs. Fibronectin expression was higher on isotropic scaffolds, Figure 3(b), which may be due to the spacing of the scaffold pore struts, which could affect the ease of migration within the structure.

Fibronectin fragments were also observed, appearing as a doublet between 220 and 120 kDa, Figure 3(e). The appearance of fragments was not confined to a single sample type. In total, 2 out of 27 samples produced fragments: an isotropic scaffold and an aligned scaffold with fibrin. Fibronectin fragments are generally found in pathological states.[29] These proteolytic fragments have been documented to cause inflammation and decrease proteoglycan concentration in osteoarthritis.[30] The expression of fragments was not caused by a specific architecture or fibrin addition, so might possibly be linked to the meniscal cells themselves rather than a signal in the tissue engineering constructs.

To assess where fibronectin was localized, constructs were frozen in OCT™ Compound (Tissue Tek) and sectioned to 10 μm with a microtome (Bright, UK). Sections were fixed in 1:1 acetone-methanol, incubated with the primary antibody for fibronectin (1:200) for 4 h, and then 1 h in FITC-conjugated secondary (1:500). Sections were mounted with Vectashield + DAPI (Vector Labs). Fibronectin appeared as long fibrils predominantly located along pore walls, Figures 3(f) and 3(g).

The matrix of meniscus is a complex structure, made up primarily of collagen type I.[31] To stimulate cells to reproduce a functional matrix, constructs should encourage the production of a functional ECM of collagen type I. Meniscal cells expressed collagen type I in all tissue engineering constructs, Figure 4(a), in bands corresponding to the alpha 1(I) and alpha 2(I) chains of collagen, at 139 kDa and 129 kDa, respectively.[26] However, the bands above 220 kDa, multimers of the alpha chains, produced a stronger signal, Figure 4(b). After normalization of protein expression by DNA content, it was shown that the collagen type I expression was unaffected by scaffold architecture or fibrin gel addition, Figure 4(c), which has been noted previously.[12] Instead collagen I expression is linked much more strongly to cell type and culture time.[12]

During the current study, isotropic and aligned architectures were assessed with and without fibrin gel addition to understand the cues which influence meniscal cell proliferation and metabolic

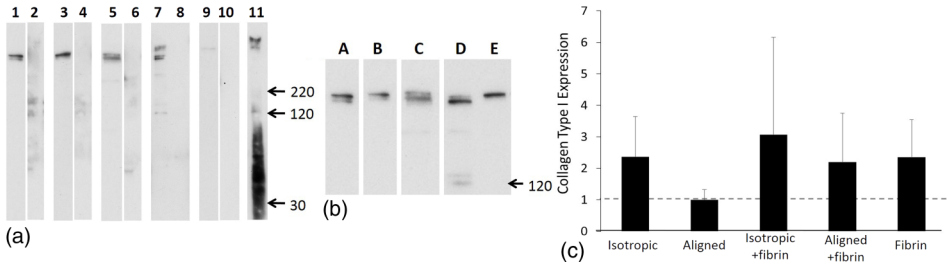

FIG. 4. Collagen type I expression of meniscal cells, visualized using (a) and (b) western blotting. (a) Only constructs with cells had a collagen signal. 1: *Isotropic*; 2: *empty isotropic*; 3: *aligned*; 4: *empty aligned*; 5: *isotropic + fibrin*; 6: *empty isotropic + fibrin*; 7: *aligned + fibrin*; 8: *empty aligned + fibrin*; 9: *fibrin*; 10: *empty fibrin*; 11: *meniscal tissue*. (b) Protein expression within constructs. A: *Isotropic*; B: *aligned*; C: *isotropic + fibrin*; D: *aligned + fibrin*; E: *fibrin*. (c) Densitometry of protein expression (fold change from aligned scaffold ± standard deviation). Molecular weights in kDa.

activity. It was found that both collagen scaffold architecture and the addition of a fibrin gel alter meniscal cell response *in vitro*. As noted in literature, and seen throughout the study, scaffold architecture played an important role on cell morphology.[11,12] Aligned scaffolds maintained meniscal cells in an elongated shape. Over three week in culture, metabolic activity was significantly increased by fibrin addition, regardless of scaffold architecture. None of the constructs tested triggered an abnormal inflammation response in the cells. Importantly, all of the constructs promoted the expression of adhesion proteins and collagen type I, the main structural component of meniscal tissue. As both scaffold architecture and fibrin gel addition can stimulate meniscal cell response, it is recommended that future regenerative medicine should incorporate these tissue engineering strategies.

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