Modulating microfibrillar alignment and growth factor stimulation to regulate MSC differentiation for interface tissue engineering

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Abstract

The ideal tissue engineering (TE) strategy for ligament regeneration should recapitulate the bone–calcified cartilage–fibrocartilage–soft tissue interface. Aligned electrospun-fibers have been shown to guide the deposition of a highly organized extracellular matrix (ECM) necessary for ligament TE. However, recapitulating the different tissues observed in the bone-ligament interface using such constructs remains a challenge. This study aimed to explore how fiber alignment and growth factor stimulation interact to regulate the chondrogenic and ligamentous differentiation of mesenchymal stem cells (MSCs). Without growth factor stimulation, MSCs on aligned-microfibers showed higher levels of tenomodulin (TNMD) and aggrecan gene expression compared to MSCs on randomly-oriented fibers. MSCs on aligned-microfibers stimulated with transforming growth factor β3 (TGFβ3) formed cellular aggregates and underwent robust chondrogenesis, evidenced by increased type II collagen expression and sulphated glycosaminoglycans (sGAG) synthesis compared to MSCs on randomly-oriented scaffolds. Bone morphogenetic protein 2 (BMP2) and type I collagen gene expression were higher on randomly-oriented scaffolds stimulated with TGFβ3, suggesting this substrate was more supportive of an endochondral phenotype. In the presence of connective tissue growth factor (CTGF), MSCs underwent ligamentous differentiation, with increased TNMD expression on aligned compared to randomly aligned scaffolds. Upon sequential growth factor stimulation, MSCs expressed types I and II collagen and deposited higher overall levels of collagen compared to scaffolds stimulated with either growth factor in isolation. These findings demonstrate that modulating the alignment of microfibrillar scaffolds can be used to promote either an endochondral, chondrogenic, fibro-chondrogenic or ligamentous MSC phenotype upon presentation of appropriate biochemical cues.

Keywords

Electrospinning; growth factors; chondrogenesis; mesenchymal stem cells, interfacial tissue engineering
1 Introduction

Soft tissues such as the anterior cruciate ligament (ACL) attach to bone through a fibrocartilage interface which minimizes stress concentrations and mediates load transfer [1]. Successful surgical reconstruction of such tissues depends on the osteo-integration of an autograft or allograft and the re-establishment of the fibrocartilage interface [2–4], termed the enthesis. However current ACL reconstruction techniques generally fail to regenerate the graded structure of the enthesis, predisposing the tissue at the bone insertion site to high stress concentrations which is believed to contribute to high failure rates [5–7]. This has motived the development of tissue engineering (TE) strategies for ligament regeneration [8,9]. A number of studies have attempted to engineer stable fibrocartilaginous tissue or a mineral gradient to interface between the calcified region and the main body of the engineered ligament [10–14], although the ideal solution has yet to be identified. Electrospinning of polymeric scaffolds is a particularly promising approach for engineering load-bearing musculoskeletal tissues as the mechanical properties of such biomaterials can be tuned to specific applications [15–18]. Furthermore, electrospinning permits the fabrication of aligned fibers that serve as a template for the deposition of a unidirectional organized extracellular matrix (ECM) by resident cells, thereby generating anisotropy for tissues where direction dependence is essential for function. This makes them an ideal biomaterial for ligament tissue engineering [19–22]. However, directing stem cell differentiation within such scaffolds towards the different tissue types that make up the bone-ligament interface remains a challenge.

Scaffold topography is a potent structural cue that regulates the fate of stem cells seeded onto such constructs. For example, aligned electrospun fibers that replicate the anisotropy of the collagen fibers in ligaments and tendons have been shown to promote the expression of key ligament/tendon markers [23,24]. Furthermore, in the absence of aligned structural cues, it has been demonstrated that cells are unable to generate a highly organized ECM architecture, even when mechanically stimulated [24,25]. Both aligned and randomly-oriented electrospun scaffolds have both been used to generate fibrocartilaginous tissues for meniscus and annulus fibrosus regeneration [16,26]. As well
as scaffold fiber alignment, fiber diameter has also been shown to direct cell fate [23,27]. Scaffolds engineered using electrospun micro fibers, as opposed to nano fibers, have larger pores and a higher porosity for cell infiltration and nutrient diffusion, while inducing higher expression of type I collagen, decorin and scleraxis [23] (marker genes of mature ligamentocytes [28]). Further, chondrogenic gene expression, and collagen and sGAG synthesis has been reported to be enhanced on microfibers in comparison to nanofiber scaffolds [27].

MSCs have shown their potential to differentiate towards the tissue types that make up the bone-ligament interface tissues. Appropriate presentation of growth factors is key to regulating the differentiation of MSCs [29,30]. Numerous studies have reported fibroblastic differentiation of MSCs using CTGF [22,29], while TGFβ superfamily members are key mediators of chondrogenesis [31,32]. The hypothesis of this study is that MSCs can differentiate into either ligament or cartilage forming cells within electrospun scaffolds when presented with the appropriate underlying fiber alignment and growth factors. To test this hypothesis, MSCs were cultured on aligned and randomly-aligned microfibers and exposed to either transforming growth factor β3 (TGFβ3) or connective tissue growth factor (CTGF). Changes in cell morphology and cartilage and ligament-specific gene expression were assessed over time. We then investigated how the sequential supplementation of TGFβ3 and CTGF regulated gene expression and matrix synthesis within electrospun microfibrillar scaffolds, demonstrating how the integration of structural and biochemical cues can be used to support either the endochondral, chondrogenic, fibrochondrogenic or ligamentous differentiation of MSCs.

2 Materials and Methods

2.1 Electrospinning of polycaprolactone (PCL) microfiber scaffolds

Polycaprolactone (PCL, $M_w$ 70 – 90 kDa, Sigma-Aldrich) was dissolved at 25% w/v in chloroform. Electrospinning of microfibers was performed in a custom-made electrospinning set-up by modifying previously used parameters [33]. The polymer solution was loaded in a 20-ml syringe and extruded using a syringe pump through a 20G blunt-end needle charged to 15 kV. Continuous polymeric fibers
were collected on a grounded mandrel (Ø 10 cm) with a flow rate of 4 mL/h. The aligned microfibers were collected with the mandrel rotating at 1500 rpm positioned 15 cm from its center to the tip of the needle. The randomly-aligned microfibers were collected with the mandrel rotating at 200 rpm positioned 18 cm from its center to the tip of the needle. Scaffolds were punched from the electrospun sheets into 8 mm diameter discs using dermal biopsy punches (Hibernia Medical).

2.2 Characterization of scaffolds

Samples were sputter coated with a mixture of gold palladium (80:20) to a thickness of ~ 10 nm (Cressington 108) and imaged by scanning electron microscopy (SEM, Zeiss Ultra FE-SEM) at an accelerating voltage of 5 kV. The average fiber diameters were determined by measuring the fibers from SEM micrographs (n≥ 15) using DiameterJ plugin for ImageJ (National Institutes of Health). A total of 1,500 – 3,000 fibers were analyzed for aligned and randomly-aligned scaffolds. Porosity was calculated by gravimetry according to the equation \( \varphi = 1 - \frac{\rho_{\text{scaffold}}}{\rho_{\text{PCL}}} \), the density of the scaffold (\( \rho_{\text{scaffold}} \)) was determined by measuring its mass and volume [34]. The volume of the scaffold was determined using the known diameter (8 mm) and measured thickness using electronic calipers (n ≥ 6). The principal axis of fiber orientation was computed using the Directionality plugin of ImageJ based on Fast Fourier Transform (FFT) analysis (n ≥ 10 images per scaffold).

2.3 Isolation and expansion of bone marrow-derived MSCs

Bone marrow derived porcine MSCs were isolated as previously described [35]. MSCs were expanded in expansion media consisting of high-glucose Dulbecco’s modified Eagle’s medium (DMEM) GlutaMAX supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/mL)-streptomycin (100 μg/mL) (all from Gibco, Biosciences) and 0.25 μg/mL amphotericin B (Sigma-Aldrich) in a humidified atmosphere at 37 °C, 5% CO₂ to passage 2.

2.4 Scaffold cell-seeding and culture conditions

Electrospun scaffolds were sterilized by ethylene oxide (EtO) (Anprolene, Anderson products). Sterile scaffolds were hydrated and conditioned by incubating in expansion media overnight before cell
seeding. At passage 2, MSCs were trypsinized and seeded onto one side of the electrospun scaffolds at a density of 500,000 cells per scaffold or 125,000 cells per scaffold using custom designed scaffold holders as previously reported [36]. Scaffolds retained 82 – 87% of the seeded cells (data not shown). Cell-seeded scaffolds were maintained in expansion medium, ligament induction medium, or chondrogenic medium at 5% CO₂ for 20 days. Ligament induction medium was adapted from previously published studies [29], it consisted of high-glucose DMEM GlutaMAX supplemented with 2% FBS, penicillin (100 U/mL) streptomycin (100 μg/mL), 0.25 μg/mL amphotericin B, 50 μg/mL L-ascorbic acid 2-phosphate and 100 ng/mL recombinant human connective tissue growth factor (CTGF; ProSpec-Tany, TechnoGene Ltd.), hereafter referred to as CTGF treatment. Chondrogenic medium consisted of high-glucose DMEM GlutaMAX, penicillin (100 U/mL)-streptomycin (100 μg/mL), 100 μg/mL sodium pyruvate, 40 μg/mL L-proline, 4.7 μg/mL linoleic acid, 50 μg/mL L-ascorbic acid 2-phosphate, 1.5 mg/mL bovine serum albumin (BSA), 1X insulin-transferrin-selenium, 100 nM dexamethasone, 0.25 μg/mL amphotericin B (all from Sigma-Aldrich), and 10 ng/mL recombinant human transforming growth factor-β3 (TGF-β3; ProSpec-Tany, TechnoGene Ltd.), hereafter referred to as TGFβ3 treatment. Medium was changed twice weekly. MSC-seeded scaffolds were cultured on the scaffold holders suspended above the underlying tissue culture surface.

2.5 Cell viability and fluorescent imaging of cell morphology

Cell viability was assessed by staining cell-seeded scaffolds using the Live/dead assay kit (Invitrogen, Bioscience) with 4 mM calcein-AM and 2 mM ethidium homodimer-1 in phenol-free DMEM for 1 h at 37 °C. Samples were examined under a scanning confocal microscope (Olympus FV1000). F-actin staining was used to evaluate the morphology of MSCs. Glutaraldehyde-fixed specimens were permeabilized in 0.5% Triton-X 100, incubated in 1.5% bovine serum albumin (BSA) to block non-specific labelling and stained with a 1.5% BSA solution containing rhodamine phalloidin (1:40) for 1 h at room temperature (RT). Cell nuclei were stained with 4’,6-diamidino-2-phenylindole (DAPI, 1:100) for 10 min at RT (all from Sigma). Samples were imaged using a scanning confocal microscope (Leica
SP8). Cell-formed aggregates were measured and quantified from confocal images \((n = 4)\) using particle analysis in ImageJ.

2.6 RNA isolation and Quantitative Real-Time PCR

Cells seeded on the scaffolds were analyzed for their gene expression using quantitative real-time PCR at day 10 and 20 of culture. Cells were lysed using RLT lysis buffer (Qiagen) supplemented with 10 μl/ml β-mercaptoethanol (Sigma-Aldrich) and stored at −80°C \((n=5/\text{group/time point})\). At the time of isolation, lysates were thawed and homogenized using QIAshredder columns (Qiagen). Total RNA was isolated and further purified using RNeasy Mini kits (Qiagen) following the manufacturer’s instructions. RNA yield and purity were quantified using a NanoDrop spectrophotometer (Labtech International). 50 ng of RNA per sample was reverse transcribed into cDNA per 20 µl of reaction volumes using High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems) as per manufacturer’s instructions. Quantitative PCR was performed using SyberGreen select master mix (Applied Biosystems) in an ABI 7500 sequence detection system (Applied Biosystems). Expression of collagen type I (COL1A2), II (COL2A1), III (COL3A1), X (COL10A1), tenomodulin (TNMD), α-actin (ACTA2), lysyl oxidase (LOX), aggrecan (ACAN), SRY-Box 9 (SOX9), bone morphogenetic protein 2 (BMP2), runt-related transcription factor 2 (RUNX2), alkaline phosphatase (ALPL), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was evaluated. The porcine specific primer sequences (KiCqStart SYBR Green Primers, Sigma) used for amplification are listed in Table 1. C\(_T\) values were analyzed using the comparative C\(_T\) method with GAPDH as the internal control [37]. Relative expression of the genes is presented as fold change relative to the same population of cells harvested at day 0 that were not seeded on scaffolds (except for COL2A1). COL2A1 was normalized to control group (MSCs on scaffolds cultured in expansion media) as expression of this gene at day 0 was undetected.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
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<tr>
<td>GAPDH</td>
<td>TTTAACTCTGGCAAAGTGG</td>
<td>GAACATGTAGACCATGTAGTG</td>
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Table 1. Primer sequences used for qPCR

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<th>Gene</th>
<th>Primer sequence 1</th>
<th>Primer sequence 2</th>
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<td>COL1A2</td>
<td>TAGACATGTTGCTTCTTCTTG</td>
<td>GTGGGATGTCCTCTTCTTG</td>
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<td>COL2A1</td>
<td>CGACGACATACTGTGAAG</td>
<td>TCTTTGCGTCTAACATATC</td>
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<tr>
<td>COL3A1</td>
<td>TCGACACTGTATTTTGG</td>
<td>CTCTATCCGCTAGGACTG</td>
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<td>ACAN</td>
<td>GACCACCTTACTCTTGGTG</td>
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<tr>
<td>BMP2</td>
<td>ATGTGGAGGCTTCTTCAATG</td>
<td>CATGGTCGACCTTTAGAG</td>
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</tbody>
</table>

2.8 Biochemical analysis

Biochemical content was analyzed from samples at day 0 (24 h after cell seeding) and day 21 (n=4). Samples were digested with 3.88 U/ml papain enzyme in 100 mM sodium phosphate buffer containing 5 mM ethylenediaminetetra-acetic acid (EDTA) and 10 mM L-cysteine hydrochloride (HCl), pH 6.5 (all Sigma-Aldrich) for 18 h at 60 °C under constant rotation. Following digestion of the samples, DNA content was quantified using the Hoescht Bisbenzimide 33258 dye assay (Quant-iT ssDNA assay kit, Biosciences). Sulphated glycosaminoglycan (sGAG) content was quantified using the dimethylmethylene blue (DMMB) dye-binding assay (Blyscan, Biocolor Ltd.). Collagen content was determined by quantifying the hydroxyproline content using the dimethylaminobenzaldehyde and chloramine-T assay [38]. A hydroxyproline-to-collagen ratio of 1:7.69 was used [39].
2.9 Histological analysis

Samples were embedded in optimal cutting temperature (O.C.T.) compound (VWR Chemicals) and flash frozen with liquid nitrogen. Embedded samples were cryo-sectioned longitudinally to 10 μm thickness perpendicular to the fiber plane. Sections were fixed in 4% paraformaldehyde (PFA) for 5 min at RT and stained with Picrosirius Red or Alcian Blue (Sigma-Aldrich) to assess collagen or sGAG content, respectively.

2.10 Statistics

Statistical analyses were performed in GraphPad Prism software (v.6, GraphPad). Statistical differences were determined by analysis of variance (ANOVA) followed by Tukey’s multiple comparison test or Student’s t-test where appropriate. Statistical significance was accepted at $p \leq 0.05$. Data are presented as mean ± standard deviation, and mean ± standard error (SEM) for qPCR. Sample size ($n$) is indicated within the corresponding figure legends.

3 Results

3.1 Fabrication of microfibrillar scaffolds with defined architectures

Polycaprolactone (PCL)-fiber scaffolds consisting of aligned or randomly-aligned fibers with a thickness of ~300 μm were fabricated by electrospinning. Anisotropy of the microfiber-scaffolds was confirmed by scanning electron microscopy (SEM) and FFT analysis (Fig. 1). Aligned fibers were parallel to each other with a small angle of dispersion ($4.48 \pm 0.9^\circ$), while random fibers showed no preferred fiber orientation with nearly equal distributions at all angles (Fig. 1a, b). Our electrospinning parameters produced aligned and randomly-aligned microfibers with diameter ~5 μm ($5.2 \pm 0.2$ μm and $5.1 \pm 0.3$ μm respectively; $P = 0.39$). The porosity of aligned and randomly-aligned scaffolds was $0.76 \pm 0.004$ and $0.79 \pm 0.12$ ($P = 0.0015$) respectively. MSCs remained viable on microfibrillar scaffolds (Fig.1c) with similar numbers of cells adhering to scaffolds 24 hours after seeding (data not shown).
3.2 MSCs adopt distinct morphologies in response to CTGF and TGFβ3 stimulation depending on the alignment of the underlying substrate

To identify the effect of the underlying substrate alignment on MSC morphology, we examined cytoskeletal organization in MSCs cultured on aligned and randomly-aligned microfiber-scaffolds at two seeding densities (1.25 × 10^5 and 5 × 10^5 cells/scaffold). In the absence of growth factor stimulation, cells predominately aligned in the direction of the underlying substrate. Cells on aligned microfibrillar scaffolds elongated and organized parallel to the orientation of the fibers. On randomly-aligned fibers, cells spread and adopted more random orientations (Fig. 2 a).

In the presence of connective tissue growth factor (CTGF), MSCs adopted a more elongated morphology, characteristic of a fibroblastic phenotype, displaying long and thin cell bodies without protrusions on both aligned and non-aligned scaffolds (Fig. 2 b). On both fiber alignments, MSCs appeared to follow the orientation of the individual microfibers to which they attached. As a result, at higher cell-seeding densities CTGF-stimulated MSCs on aligned fibers formed a highly organized cellular structure, while this organization was lost on random fibers. Thus, the aligned fibers serve as a template for the aligned CTGF-stimulated MSCs to organize unidirectionally.

TGFβ3-stimulated cells adopted distinct morphologies depending on the underlying fiber-alignment. MSCs on aligned fibers clustered into large aggregates (these clusters were easily identifiable in the lower cell-density samples) (Fig. 2 c; Supplementary Figure 1). In comparison, cells cultured on random fibers spread and formed fewer and smaller clusters (Fig. 2 d, e). This clustering of MSCs on aligned fibers were observed at different external oxygen tensions (Supplementary Figure 1).

3.3 MSCs display distinct gene expression profiles in response to CTGF and TGFβ3 stimulation that depend on the underlying substrate alignment

We next assessed the gene expression profile of MSCs in response to the combination of fiber alignment and growth factor stimuli (Fig. 3 shows data for 5 × 10^5 cells/scaffold; Supplementary Fig.2 shows data for 1.25 × 10^5 cells/scaffold). The expression profile of cells that were not subjected to
growth factor stimulation (labelled ‘control’) was also assessed to elucidate the inherent effect of fiber alignment on MSC differentiation. In these control conditions, MSCs on aligned microfibrillar scaffolds expressed higher levels of tenomodulin (TNMD) and aggrecan (ACAN) compared to cells on randomly-aligned microfibrillar scaffolds. In general, however, the expression of key markers of MSC differentiation did not dramatically change over baseline levels seen at day 0 in the absence of growth factor stimulation.

The ligament markers – collagen types I (COL1A1) and III (COL3A1), α-actin (ACTA2) and TNMD – were highly upregulated in CTGF-stimulated MSCs compared with controls and with MSCs cultured in the presence of TGFβ3. Consistent with their elongated cell shape on both substrates, the relative expression of these ligamentogenic genes in CTGF-stimulated MSCs was comparable on aligned and randomly-aligned fiber-scaffolds. The exception to this finding was for TNMD expression, which was significantly higher on aligned scaffolds compared to randomly-aligned scaffolds. TNMD and ACTA2 were exclusively upregulated in CTGF-stimulated MSCs. LOX (which plays a key role in cross-linking collagen) expression was also ~6-fold higher in CTGF-stimulated groups compared to other conditions.

The chondrogenic markers – collagen types II (COL2A1) and X (COL10A1), aggrecan (ACAN) and SRY-Box 9 (SOX9) – were highly upregulated in TGFβ3-stimulated MSCs. As expected, TGFβ3 supplementation was necessary to upregulate SOX9 (a gene essential for cartilage formation [40]) and COL2A1. Chondrogenic gene expression was higher in TGFβ3-stimulated cells on aligned fibers, especially COL2A1 expression, which correlates with the formation of larger cell aggregates on this substrate (Fig. 2 c-e).

Bone morphogenetic protein 2 (BMP2) and collagen type I (COL1A1) expression was significantly higher in TGFβ3-stimulated cells on randomly-aligned fibers, suggesting that such scaffolds were supporting progression along the endochondral pathway. The relative gene expression profile was maintained with time and in lower cell-seeding densities (Supplementary Fig. 2). Furthermore, at
lower seeding densities runt-related transcription factor 2 (Runx2), an essential molecular switch for bone formation, was higher in TGFβ3-stimulated cells on randomly-aligned fibers.

### 3.4 Sequential supplementation with CTGF and TGFβ3 supports fibro-cartilaginous differentiation on microfibrillar scaffolds

We next examined whether the sequential supplementation of CTGF and TGFβ3, coupled with the appropriate substrate alignment, could promote MSC specification towards the fibrocartilaginous lineage (cells that express high levels of both types I and II collagen). To evaluate the effects of the sequential supplementation, we analyzed the gene expression of MSCs stimulated with CTGF for 10 days followed by TGFβ3 for a further 10 days (CTGF → TGFβ3) or vice versa (TGFβ3 → CTGF).

MSCs exposed to the temporal growth factor supplementation expressed higher levels of COL1A2 and COL2A1 on both aligned and randomly-aligned substrates compared to control conditions (Fig. 4). COL2A1 gene expression was significantly higher on aligned-fiber scaffolds for all groups stimulated with TGFβ3, transient or continuous. Introducing CTGF supplementation to the TGFβ3-stimulated cells (TGFβ3 → CTGF) on aligned fibers decreased the expression of type II collagen (COL2A1) compared to that of cells continuously stimulated with TGFβ3. In the same way, introducing TGFβ3 to cells stimulated with CTGF (CTGF → TGFβ3) decreased the expression of COL1A2 compared to that of cells exclusively stimulated with CTGF. There was a slight increase in COL1A2 expression in the sequential supplementation groups compared to TGFβ3 only group, but this effect was not statistically significant. MSCs exposed to TGFβ3 → CTGF maintained a gene expression profile more similar to cells exclusively exposed to TGFβ3, compared to cells exposed to the reverse (CTGF → TGFβ3). MSCs on aligned substrates exposed to CTGF → TGFβ3 expressed higher levels of type 10 collagen (COL10A1) compared to those on randomly-aligned scaffolds. In contrast, there was a downregulation of COL10A1 under TGFβ3 → CTGF supplementation on both aligned and randomly-aligned scaffolds.

Consistent with the gene expression data at day 10 (Fig. 3), we observed that TNMD was upregulated exclusively on CTGF-stimulated cells, and COL2A1 upregulation was highest in the aligned
microfibrillar scaffolds. After 20 days in culture, the expression of COL10A1 was lower in TGFβ3-stimulated cells compared to day 10.

3.5 Growth factor stimulation regulates tissue development with MSC-seeded electrospun scaffolds

We next assessed the effects of single growth factor and the temporal growth factor supplementation on collagen and sulphated glycosaminoglycans (sGAGs) synthesis. MSCs seeded on aligned and randomly-aligned scaffolds penetrated the scaffold, proliferated, and deposited collagen and sGAGs on the surface and through the thickness of the scaffold (Fig. 5 a, b). Stronger picrosirius red staining for collagen was prevalent at the surface of the aligned fiber-scaffold for CTGF-stimulated cells. A significant amount of collagen also accumulated in the CTGF→TGFβ3 and TGFβ3 groups. These same groups stained intensely for alcian blue, while low levels of sGAGs deposition were observed in CTGF and control groups.

An increase in DNA content in randomly-aligned substrates indicated cell proliferation at day 21 in the control group. MSCs appeared to undergo higher levels of proliferation on random fibers, but this difference was not significant (Fig. 5 c). MSCs subjected to TGFβ3 stimulation, transient or continuous, produced significantly higher levels of collagen and sGAG compared to other groups (Fig. 5 d, e). Many of the aligned fiber scaffolds – particularly hosting TGFβ3-stimulated cells – folded due to cellular overgrowth and cell-mediated contraction, a phenomenon observed by others [41].

4 Discussion

In this study, we investigated how microfibrillar alignment and growth factor stimulation interact to regulate the chondrogenic, fibrochondrogenic and ligamentous differentiation of MSCs. The electrospun microfibrillar scaffold utilized here has advantageous properties for tissue engineering applications, including a high surface area for cell attachment and matrix deposition and the possibility to functionalize the polymer chains with cell-adhesive peptides, growth factors or natural components of the extracellular matrix (ECM) to further increase cell adhesion and bioactivity [15]. Further, PCL is used in certain FDA approved biomedical devices [42]. Micro-scale as opposed to nano-scale fibers
were used to encourage cell infiltration and ECM deposition through the thickness of the scaffold, to resemble the collagen fiber diameter range in ligaments [43] and to enhance ligament differentiation [23]. Consistent with the literature [24], it was demonstrated that in the absence of biochemical cues, MSCs oriented parallel to fibers on aligned fiber scaffolds, while cells spread in all directions on randomly-aligned fiber scaffolds. This correlated with higher TNMD expression on aligned scaffolds, although no dramatic upregulation in key markers of differentiation were observed over time in the absence of growth factors. When coupled with appropriate growth factor stimulation, it was found that modulating the alignment of microfibrillar scaffolds could be used to promote either an endochondral, chondrogenic, fibro-chondrogenic or ligamentous phenotype. Such control over progenitor/stem cell differentiation is central to successful interface TE strategies.

Our results demonstrate that aligned microfibers and co-stimulation with CTGF preferentially supported ligamentous differentiation of MSCs. In the presence of CTGF, MSCs on aligned microfibrillar scaffolds adopted an elongated fibroblastic phenotype, with cells aligning parallel to the fiber orientation. These environmental conditions promoted an upregulation of COL1A1 and COL3A1 gene expression and the highest expression of TNMD. TNMD, a transmembrane glycoprotein regulated by scleraxis, is a highly specific marker of ligamentocytes and tenocytes [44]. TNMD is implicated in collagen organization, and localized to cells within thick bundles of aligned collagen fibers [44]. Up-regulation of ligamentous genes in MSCs has been reported on highly aligned collagen type I fibers in vitro in the absence of bioinductive molecules [45], suggesting that the alignment of the collagen effectively induces the expression TNMD and other ligamentous factors. In this study, scaffold anisotropy in the absence of CTGF was not sufficient to promote increased TNMD expression over day 0 values, suggesting that further growth factor stimulation is required on PCL scaffolds to induce robust differentiation. ACTA2 was also more strongly expressed in the CTGF-stimulated cells. Previous studies have shown that fibroblasts expressing ACTA2 are involved in the earliest stages of fiber bundle formation in the intact as well as the remodelled human ACL [46,47].
Given the upregulation of fibrillar collagens I and III in CTGF-stimulated cells, we examined whether these environmental conditions also led to an increase of collagen crosslinks, a result that has been demonstrated for cardiac fibroblast and CTGF activation during atrial fibrillation [48]. Thus, we extended our qRT-PCR analysis to examine lysyl oxidase (LOX), a key player for collagen crosslinking [49]. CTGF-stimulated cells showed a ~ 6-fold increase in LOX gene expression compared to controls (MSCs on scaffolds cultured in expansion media). Associated upregulation of LOX mRNA expression likely indicates the crosslinking of fibrillary pro-collagens I and III in CTGF-stimulated cells which will form the final collagen types I and III fibers that are highly resistant against proteolytic enzymes and possess improved tensile strength, critical for ligament TE. Together, these results suggest that substrates promoting both MSC elongation and coordinated alignment in a single direction are required to promote robust ligamentous differentiation following CTGF stimulation.

TGFβ3-stimulated cells on both aligned and randomly-aligned microfiber-scaffolds expressed higher levels of cartilage specific markers and increased the deposition of collagen and sGAGs. On aligned fibers, MSCs formed large condensations which correlated with higher levels of collagen type II expression compared to MSCs seeded onto random fibers. Stem cell condensation is a key initiating step in chondrogenesis [29,38,49,50], with biomaterials that mimic aspects of condensation enhancing chondrogenesis [51]. Condensation was not an effect of increased proliferation, as evidenced by similar DNA values in both random and aligned scaffolds at day 21 under TGFβ3 stimulation, but likely due to increased migration on aligned scaffolds. We hypothesize that aligned fibers provide a substrate more conductive to MSC migration and hence aggregation. In agreement with this concept, aligned fibers have previously been shown to facilitate cell migration [52,53]. The fact that this clustering is exclusively observed in the presence of TGFβ3 is likely due to the known proliferative and chemotactic properties of this growth factor [54,55].

As expected, TGFβ3-stimulated cells expressed SOX9, a transcription factor essential for chondrocyte differentiation and cartilage formation [40], and activation of chondrocyte-specific extracellular
matrix genes: collagen II (COL2A1) and ACAN, all suggestive of robust chondrogenic differentiation. Notably, BMP2 and collagen I expression was upregulated in cells on randomly-aligned fibers, suggesting progression along the endochondral pathway. This finding is in agreement with other studies that have reported osteogenesis on randomly-aligned fibers in the absence of osteogenic media [24]. It should be noted, however, that alkaline phosphatase (ALPL) gene expression was undetectable for all groups and no alizarin red positive staining was present after 20 days of culture (data not shown), suggesting only early progression along the endochondral pathway. These observations, combined with studies showing chondrogenesis and subsequent ossification on randomly-aligned fibers [24], provide further evidence that differentiation of MSCs stimulated with TGFβ3 is dependent on substrate alignment.

Temporal co-stimulation of CTGF and TGFβ3 induced MSC differentiation into cells expressing high levels of both types I and II collagen and aggrecan. Sequential supplementation of CTGF and TGFβ3 has been shown to induce fibrochondrocytic differentiation of human MSCs [56]. Consistently in our electrospun microfibrillar scaffolds, MSCs treated with sequential growth factor supplementation deposited fibrocartilaginous-like tissues that stained positive for picrosirius red (collagen) and alcian blue (sGAGs). In terms of total matrix accumulation, this temporal co-stimulation was found to be more powerful than supplementation with either growth factor alone as the highest collagen/DNA and sGAG/DNA were attained in these groups. The improved matrix synthesis can be explained from the observations of others that CTGF can induce proliferation and ECM synthesis [57], and furthermore that TGFβ3 plays a key role in cell proliferation and matrix production [58]. In the ACL to bone junction, the fibrocartilage interfacial region subdivides into mineralized and non-mineralized fibrocartilage. The mineralized tissue shows expression of collagen X, as well as type II and aggrecan, while the un-mineralized tissue shows expression types I and II collagen and aggrecan [59,60]. Collagen X is expressed at sites of endochondral ossification and in areas of transition from mineralized to soft tissue [61]. Cells stimulated with CTGF followed by TGFβ3 on the aligned scaffold showed upregulation of COL1A1, COL2A1, ACAN compared to day 0, and an increased level of COL10 gene
expression compared to the reverse (TGFβ3 → CTGF) and the rest of the groups. Cells stimulated with TGFβ3 followed by CTGF showed a downregulation of COL10 while upregulating COL1A1, COL2A1 and ACAN. These observations suggest that CTGF and TGFβ3 sequential stimuli to cells on microfibrillar topography can further modulate MSC differentiation.

5 Conclusions

The coupled effects of microfibrillar-scaffold alignment and growth factor stimulation on MSC differentiation were investigated. Aligned microfibrillar scaffolds supported either ligamentogenesis, chondrogenesis or fibrochondrogenesis of MSCs acting when appropriately stimulated with CTGF and/or TGFβ3. Thus, these results have broad implications for regenerating other musculoskeletal interfaces such as articular cartilage-bone, tendon-bone, and meniscus-bone. This study opens the possibility of using aligned microfibrillar scaffolds that are spatially functionalized with specific growth factors to direct MSC differentiation for engineering the bone-ligament interface. Future work will investigate the spatial incorporation of the growth factors along the length of the scaffold to compartmentalize MSC differentiation.

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Figure 1. Microfibrillar scaffolds with specific fibre diameter and alignment. a, Scanning electron micrographs (SEM) of aligned and randomly-aligned microfibers. Scale bars, 20 μm. b, Principal axes of fiber orientation of representative images for aligned and randomly-aligned fibers; plot shows alignment angle (x-axis) and frequency (y-axis; arbitrary units). c, Cell viability on aligned and randomly-aligned microfibres was demonstrated with live/dead staining (green: ‘live’, red: ‘dead’); white arrow indicates fiber orientation on the aligned scaffold. Scale bars, 300 μm.
Figure 2. Fiber alignment and growth factor stimuli influence MSC morphology. F-actin stained of representative MSCs cultured in expansion media (‘Control’) (a), in the presence of CTGF (b) and TGFβ3 (c). MSCs were stained for F-actin (red) and cell nuclei (blue) at low (1.25 X 10^5 cells, day 10) and high cell seeding densities (5 X 10^5 cells, day 20). Aligned and randomly-aligned fibres are shown in green. d, Quantification of cluster area in TGFβ3 group; *P < 0.05. e, Quantification of number of clusters per mm^2 in TGFβ3 group; n ≥ 580 regions of interest (ROI), *P < 0.05.
Figure 3. Gene expression at day 10. Collagen type I (COL1A2), II (COL2A1), III (COL3A1), X (COL10A1), tenomodulin (TNMD), α-actin (ACTA2), lysyl oxidase (LOX), aggrecan (ACAN), SRY-Box 9 (SOX9), bone morphogenetic protein 2 (BMP2), runt-related transcription factor 2 (RUNX2). Data is reported as fold-change relative to the same population of cells harvested at day 0 that were not seeded on scaffolds. COL2A1 was normalized to control group (MSCs on scaffolds cultured in the absence of growth factors) as expression of this gene at day 0 was undetected. The values plotted are means ± SEMs of N=5 scaffolds for each condition, *P < 0.05. Alkaline phosphatase (ALPL) expression was undetected in all groups. Abbreviation: undet., undetermined.
Figure 4. Gene expression at day 20. MSCs exposed to CTGF treatment for 10 days followed by TGFβ3 treatment for 10 days (CTGF → TGFβ3) and vice versa (TGFβ3 → CTGF). MSCs exposed to CTGF for 20 days (CTGF) and those exposed solely to TGFβ3 treatment (TGFβ3). COL2A1 was normalized to Control group (MSCs on scaffolds cultured in the absence of growth factors) as expression of this gene at day 0 was undetected. The values plotted are means ± SEMs of N=5 scaffolds for each condition, *P < 0.05, ^: significantly different from Control group, P < 0.05.
Figure 5. Evaluation of collagen and sGAG content. a, b, Cross-sections of aligned and randomly-aligned scaffolds stained using picrosirius red and alcian blue for collagen and sGAGs, respectively. Stains reveal collagen and sGAG-rich regions through the thickness of the scaffold. CTGF-TGFβ3 and TGFβ3 aligned fiber-scaffold groups folded by the longitudinal axis. c, DNA content; +: $P < 0.05$, significantly different from day 0. d, sGAG content; ^, v: $P < 0.05$, significantly different from aligned and randomly-aligned controls. e, collagen content; <, >: $P < 0.05$, significantly different from aligned and randomly-aligned controls.
Supplementary Figures

Supplementary figure 1: number of clusters and cluster’s area in TGFβ3-stimulated cells on aligned and randomly-aligned scaffolds at 5% O₂. a, Actin cytoskeletal organization of representative MSCs on aligned and random fibres stained for F-actin (red) and cell nuclei (blue) at day 10, initial cell seeding density: 1.25 X 10⁵ cells/scaffold. b, Quantification of clusters area in TGFβ3 groups; *P < 0.05. c, Number of clusters per mm² in TGFβ3 groups; n ≥ 580 regions of interest (ROI), *P < 0.05. The area of the clusters was significantly larger in those on aligned fibers compared to those on randomly-aligned fibers. The number of clusters formed in the aligned fibers was higher than on the random fibers.
Supplementary figure 2: qPCR gene expression at day 10 in lower cell-seeding density groups. ALPL expression was undetected in all groups. The values plotted are means ± SEMs of N=5 scaffolds for each condition, *$P < 0.05$. Abbreviation: undet., undetermined.