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

Dinorath Olvera^{a,b}, Binulal N. Sathy^{a,b}, Simon F. Carroll^{a,b} and Daniel J. Kelly^{*a,b,c,d}

^aTrinity Centre for Bioengineering, Trinity Biomedical Sciences Institute, Trinity College Dublin, Dublin 2, Ireland.

^bDepartment of Mechanical and Manufacturing Engineering, School of Engineering, Trinity College Dublin, Dublin 2, Ireland

^cTissue Engineering Research Group, Department of Anatomy, Royal College of Surgeons in Ireland, Dublin 2, Ireland

^dAdvanced Materials and Bioengineering Research Centre (AMBER), Royal College of Surgeons in Ireland and Trinity College Dublin, Dublin 2, Ireland

*Corresponding author at: Department of Mechanical and Manufacturing Engineering, School of Engineering, Parson's Building, Trinity College Dublin, Dublin 2, Ireland. Tel: +353-1- 8963947, e-mail address: kellyd9@tcd.ie (D.J. Kelly).

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 β 3 (TGF β 3) 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 TGF β 3, 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 motivated 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 TGF β 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 β 3 (TGF β 3) 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 TGF β 3 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 ($\varnothing$ 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 ($n \geq 15$) 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 $\varphi = 1 - \frac{\rho_{scaffold}}{\rho_{PCL}}$; the density of the scaffold ($\rho_{scaffold}$) 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 ($n \geq 6$). The principal axis of fiber orientation was computed using the Directionality plugin of ImageJ based on Fast Fourier Transform (FFT) analysis ($n \geq 10$ 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 μ g/mL) (all from Gibco, Biosciences) and 0.25 μ g/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 μ l/ml β -mercaptoethanol (Sigma-Aldrich) and stored at -80°C ($n=5$ /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 μ l 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.

Gene	Forward	Reverse
GAPDH	TTTAACTCTGGCAAAGTGG	GAACATGTAGACCATGTAGTG

COL1A2	TAGACATGTTTCAGCTTTGTG	GTGGGATGTCTTCTTCTTG
COL2A1	CGACGACATAATCTGTGAAG	TCCTTTGGGTCCTACAATATC
COL3A1	TCATCCCACTGTTATTTTGG	CTCTATCCGCATAGGACTG
COL10A1	GTAGGTGTTTGGTATTGCTC	GAGCAATACCAAACACCTAC
ACTA2	CAAAAGAGGAATCCTGACC	CATTGTAGAAAGAGTGGTG
ACAN	GACCACTTTACTCTTGGTG	TCAGGCTCAGAACTTCTAC
LOX	TGTGAAATTCGCTACACAG	GGGAGTTGCTTTCTAATACG
TNMD	GCTAATTGTCCTATTTTGGGG	GAAAGTGTGTTCCATGTCG
SOX9	CAGACCTTGAGGAGACTTAG	GTTGAGTTGCCTTTAGTG
RUNX2	CCAACAGAGGCATTTAAGG	CCAAAAGAAGTTTGTGAC
ALPL	TTCACTCTTCTTAGTGCTG	CGTTACGGAATGAGGAAAC
BMP2	ATGTGGAGGCTCTTTCAATG	CATGGTCGACCTTTAGGAG

Table 1. Primer sequences used for qPCR

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 Bisbenzimidazole 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 $\pm$ standard deviation, and mean $\pm$ 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 $\sim 300 \mu\text{m}$ 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 $\sim 5 \mu\text{m}$ ($5.2 \pm 0.2 \mu\text{m}$ and $5.1 \pm 0.3 \mu\text{m}$ respectively; $P = 0.39$). The porosity of aligned and randomly-aligned scaffolds was 0.76 ± 0.004 and 0.79 ± 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 TGF β 3 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.

TGF β 3-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 TGF β 3 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 TGF β 3. 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 TGF β 3-stimulated MSCs. As expected, TGF β 3 supplementation was necessary to upregulate *SOX9* (a gene essential for cartilage formation [40]) and *COL2A1*. Chondrogenic gene expression was higher in TGF β 3-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 TGF β 3-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 TGF β 3-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 $\rightarrow$ TGF β 3 and TGF β 3 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 TGF β 3 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 TGF β 3-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 conducive 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 TGF β 3 is dependent on substrate alignment.

Temporal co-stimulation of CTGF and TGF β 3 induced MSC differentiation into cells expressing high levels of both types I and II collagen and aggrecan. Sequential supplementation of CTGF and TGF β 3 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 TGF β 3 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 TGF β 3 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 (TGF β 3 $\rightarrow$ CTGF) and the rest of the groups. Cells stimulated with TGF β 3 followed by CTGF showed a downregulation of *COL10* while upregulating *COL1A1*, *COL2A1* and *ACAN*. These observations suggest that CTGF and TGF β 3 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 TGF β 3. 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.

Acknowledgements

This work was funded by Irish Research Council (IRC), Grant: IRCSET P/G S/Ship.13; and by Science Foundation Ireland (SFI), Grant: 12/US/I2489. The imaging for this project was carried out at the Advanced Microscopy Laboratory (AML) at the AMBER centre, CRANN Institute, Trinity College Dublin, Ireland. AML is an SFI supported imaging and analysis centre.

References

- [1] M. Benjamin, E.J. Evans, L. Copp, The histology of tendon attachments to bone in man, J. Anat. 149 (1986) 89–100.
- [2] C.-H. Chen, Graft healing in anterior cruciate ligament reconstruction, Sports Med. Arthrosc. Rehabil. Ther. Technol. 1 (2009) 21. doi:10.1186/1758-2555-1-21.
- [3] S.A. Rodeo, S.P. Arnoczky, P.A. Torzilli, C. Hidaka, R.F. Warren, Tendon-healing in a bone

- tunnel. A biomechanical and histological study in the dog, *J. Bone Joint Surg. Am.* 75 (1993) 1795–803.
- [4] A. Weiler, R.F.G. Hoffmann, H.J. Bail, O. Rehm, N.P. Südkamp, Tendon healing in a bone tunnel. Part II: Histologic analysis after biodegradable interference fit fixation in a model of anterior cruciate ligament reconstruction in sheep, *Arthrosc. J. Arthrosc. Relat. Surg.* 18 (2002) 124–135. doi:10.1053/jars.2002.30657.
- [5] H.B. Ellis, L.M. Matheny, K.K. Briggs, A.T. Pennock, J.R. Steadman, Outcomes and Revision Rate After Bone–Patellar Tendon–Bone Allograft Versus Autograft Anterior Cruciate Ligament Reconstruction in Patients Aged 18 Years or Younger With Closed Physes, *Arthrosc. J. Arthrosc. Relat. Surg.* 28 (2012) 1819–1825. doi:10.1016/j.arthro.2012.06.016.
- [6] P. Faunø, L. Rahr-Wagner, M. Lind, Risk for Revision After Anterior Cruciate Ligament Reconstruction Is Higher Among Adolescents, *Orthop. J. Sport. Med.* 2 (2014). doi:10.1177/2325967114552405.
- [7] J.M. Deneweth, M.J. Bey, S.G. McLean, T.R. Lock, P.A. Kolowich, S. Tashman, Tibiofemoral Joint Kinematics of the Anterior Cruciate Ligament-Reconstructed Knee During a Single-Legged Hop Landing, *Am. J. Sports Med.* 38 (2010) 1820–1828. doi:10.1177/0363546510365531.
- [8] C.K. Kuo, J.E. Marturano, R.S. Tuan, Novel strategies in tendon and ligament tissue engineering: Advanced biomaterials and regeneration motifs, *BMC Sports Sci. Med. Rehabil.* 2 (2010) 20. doi:10.1186/1758-2555-2-20.
- [9] T. Nau, A. Teuschl, Regeneration of the anterior cruciate ligament: Current strategies in tissue engineering., *World J. Orthop.* 6 (2015) 127–36. doi:10.5312/wjo.v6.i1.127.
- [10] J.E. Phillips, K.L. Burns, J.M. Le Doux, R.E. Guldberg, A.J. García, Engineering graded tissue interfaces, *Proc. Natl. Acad. Sci. U. S. A.* 105 (2008) 12170–5. doi:10.1073/pnas.0801988105.
- [11] S.R. Caliarì, D.W. Weisgerber, W.K. Grier, Z. Mahmassani, M.D. Boppart, B.A.C. Harley, Collagen Scaffolds Incorporating Coincident Gradations of Instructive Structural and

- Biochemical Cues for Osteotendinous Junction Engineering, *Adv. Healthc. Mater.* 4 (2015) 831–837. doi:10.1002/adhm.201400809.
- [12] H. Li, J. Fan, L. Sun, X. Liu, P. Cheng, H. Fan, Functional Regeneration of Ligament-Bone Interface Using a Triphasic Silk-based Graft, *Biomaterials*. (2016). doi:10.1016/j.biomaterials.2016.08.012.
- [13] J.P. Spalazzi, S.B. Doty, K.L. Moffat, W.N. Levine, H.H. Lu, Development of controlled matrix heterogeneity on a triphasic scaffold for orthopedic interface tissue engineering, *Tissue Eng.* 12 (2006) 3497–508. doi:10.1089/ten.2006.12.3497.
- [14] S. Samavedi, S.A. Guelcher, A.S. Goldstein, A.R. Whittington, Response of bone marrow stromal cells to graded co-electrospun scaffolds and its implications for engineering the ligament-bone interface, *Biomaterials*. 33 (2012) 7727–35. doi:10.1016/j.biomaterials.2012.07.008.
- [15] L. Ghasemi-Mobarakeh, M.P. Prabhakaran, M. Morshed, M.H. Nasr-Esfahani, S. Ramakrishna, Bio-functionalized PCL nanofibrous scaffolds for nerve tissue engineering, *Mater. Sci. Eng. C*. 30 (2010) 1129–1136. doi:10.1016/j.msec.2010.06.004.
- [16] B.M. Baker, R.L. Mauck, The effect of nanofiber alignment on the maturation of engineered meniscus constructs, *Biomaterials*. 28 (2007) 1967–77. doi:10.1016/j.biomaterials.2007.01.004.
- [17] N.L. Nerurkar, B.M. Baker, S. Sen, E.E. Wible, D.M. Elliott, R.L. Mauck, Nanofibrous biologic laminates replicate the form and function of the annulus fibrosus, *Nat. Mater.* 8 (2009) 986–92. doi:10.1038/nmat2558.
- [18] J.S. Choi, S.J. Lee, G.J. Christ, A. Atala, J.J. Yoo, The influence of electrospun aligned poly(ϵ -caprolactone)/collagen nanofiber meshes on the formation of self-aligned skeletal muscle myotubes, *Biomaterials*. 29 (2008) 2899–2906. doi:10.1016/j.biomaterials.2008.03.031.
- [19] C.H. Lee, H.J. Shin, I.H. Cho, Y.-M. Kang, I.A. Kim, K.-D. Park, J.-W. Shin, Nanofiber alignment and direction of mechanical strain affect the ECM production of human ACL fibroblast,

- Biomaterials. 26 (2005) 1261–70. doi:10.1016/j.biomaterials.2004.04.037.
- [20] C.A. Bashur, L.A. Dahlgren, A.S. Goldstein, Effect of fiber diameter and orientation on fibroblast morphology and proliferation on electrospun poly(D,L-lactic-co-glycolic acid) meshes, *Biomaterials*. 27 (2006) 5681–8. doi:10.1016/j.biomaterials.2006.07.005.
- [21] G. Criscenti, A. Longoni, A. Di Luca, C. De Maria, C.A. Van Blitterswijk, G. Vozzi, L. Moroni, Triphasic scaffolds for the regeneration of the bone–ligament interface, *Biofabrication*. 8 (2016) 15009. doi:10.1088/1758-5090/8/1/015009.
- [22] H.M. Pauly, B.N. Sathy, D. Olvera, H.O. McCarthy, D.J. Kelly, K.C. Popat, N.J. Dunne, T.L. Haut Donahue, Hierarchically Structured Electrospun Scaffolds with Chemically Conjugated Growth Factor for Ligament Tissue Engineering, *Tissue Eng. Part A*. (2017) ten.tea.2016.0480. doi:10.1089/ten.tea.2016.0480.
- [23] R.D. Cardwell, L. a Dahlgren, A.S. Goldstein, Electrospun fibre diameter, not alignment, affects mesenchymal stem cell differentiation into the tendon/ligament lineage, 2014. doi:10.1002/term.1589.
- [24] Z. Yin, X. Chen, H.-X. Song, J.-J. Hu, Q.-M. Tang, T. Zhu, W.-L. Shen, J.-L. Chen, H. Liu, B.C. Heng, H.-W. Ouyang, Electrospun scaffolds for multiple tissues regeneration in vivo through topography dependent induction of lineage specific differentiation, *Biomaterials*. 44 (2015) 173–85. doi:10.1016/j.biomaterials.2014.12.027.
- [25] S.D. Subramony, B.R. Dargis, M. Castillo, E.U. Azeloglu, M.S. Tracey, A. Su, H.H. Lu, The guidance of stem cell differentiation by substrate alignment and mechanical stimulation, *Biomaterials*. 34 (2013) 1942–53. doi:10.1016/j.biomaterials.2012.11.012.
- [26] N.L. Nerurkar, W. Han, R.L. Mauck, D.M. Elliott, Homologous structure-function relationships between native fibrocartilage and tissue engineered from MSC-seeded nanofibrous scaffolds, *Biomaterials*. 32 (2011) 461–468. doi:10.1016/j.biomaterials.2010.09.015.
- [27] A.C. Bean, R.S. Tuan, Fiber diameter and seeding density influence chondrogenic differentiation of mesenchymal stem cells seeded on electrospun poly(ϵ -caprolactone)

- scaffolds., *Biomed. Mater.* 10 (2015) 15018. doi:10.1088/1748-6041/10/1/015018.
- [28] C. Shukunami, Y. Yoshimoto, A. Takimoto, H. Yamashita, Y. Hiraki, Molecular characterization and function of tenomodulin, a marker of tendons and ligaments that integrate musculoskeletal components, *Jpn. Dent. Sci. Rev.* 52 (2016) 84–92. doi:10.1016/j.jdsr.2016.04.003.
- [29] C.H. Lee, B. Shah, E.K. Moiola, J.J. Mao, CTGF directs fibroblast differentiation from human mesenchymal stem/stromal cells and defines connective tissue healing in a rodent injury model, *J. Clin. Invest.* 120 (2010) 3340–9. doi:10.1172/JCI43230.
- [30] L. Bian, D.Y. Zhai, E. Tous, R. Rai, R.L. Mauck, J.A. Burdick, Enhanced MSC chondrogenesis following delivery of TGF- β 3 from alginate microspheres within hyaluronic acid hydrogels in vitro and in vivo, *Biomaterials.* 32 (2011) 6425–6434. doi:10.1016/j.biomaterials.2011.05.033.
- [31] R. Tuli, S. Tuli, S. Nandi, X. Huang, P.A. Manner, W.J. Hozack, K.G. Danielson, D.J. Hall, R.S. Tuan, Transforming growth factor-beta-mediated chondrogenesis of human mesenchymal progenitor cells involves N-cadherin and mitogen-activated protein kinase and Wnt signaling cross-talk, *J. Biol. Chem.* 278 (2003) 41227–36. doi:10.1074/jbc.M305312200.
- [32] B. Johnstone, T.M. Hering, A.I. Caplan, V.M. Goldberg, J.U. Yoo, In vitro chondrogenesis of bone marrow-derived mesenchymal progenitor cells, *Exp. Cell Res.* 238 (1998) 265–72. doi:10.1006/excr.1997.3858.
- [33] N.S. Binulal, M. Deepthy, N. Selvamurugan, K.T. Shalumon, S. Suja, U. Mony, R. Jayakumar, S. V Nair, Role of nanofibrous poly(caprolactone) scaffolds in human mesenchymal stem cell attachment and spreading for in vitro bone tissue engineering--response to osteogenic regulators, *Tissue Eng. Part A.* 16 (2010) 393–404. doi:10.1089/ten.TEA.2009.0242.
- [34] Q.P. Pham, U. Sharma, A.G. Mikos, Electrospun poly (e-caprolactone) microfiber and multilayer nanofiber/microfiber scaffolds: Characterization of scaffolds and measurement of cellular infiltration, *Biomacromolecules.* 7 (2006) 2796–2805. doi:10.1021/bm060680j.
- [35] S.D. Thorpe, C.T. Buckley, A.J. Steward, D.J. Kelly, European Society of Biomechanics S.M.

- Perren Award 2012: The external mechanical environment can override the influence of local substrate in determining stem cell fate, *J. Biomech.* 45 (2012) 2483–2492.
doi:10.1016/j.jbiomech.2012.07.024.
- [36] B.N. Sathy, U. Mony, D. Menon, V.K. Baskaran, A.G. Mikos, S. Nair, Bone Tissue Engineering with Multilayered Scaffolds—Part I: An Approach for Vascularizing Engineered Constructs *In Vivo*, *Tissue Eng. Part A*. 21 (2015) 2480–2494. doi:10.1089/ten.tea.2015.0098.
- [37] T.D. Schmittgen, K.J. Livak, Analyzing real-time PCR data by the comparative CT method, *Nat. Protoc.* 3 (2008) 1101–1108. doi:10.1038/nprot.2008.73.
- [38] W. Kafienah, T.J. Sims, Biochemical Methods for the Analysis of Tissue-Engineered Cartilage, in: *Biopolym. Methods Tissue Eng.*, Humana Press, New Jersey, 2004: pp. 217–230.
doi:10.1385/1-59259-428-X:217.
- [39] N.Y. Ignat'eva, N.A. Danilov, S. V. Averkiev, M. V. Obrezkova, V. V. Lunin, E.N. Sobol', Determination of hydroxyproline in tissues and the evaluation of the collagen content of the tissues, *J. Anal. Chem.* 62 (2007) 51–57. doi:10.1134/S106193480701011X.
- [40] W. Bi, J.M. Deng, Z. Zhang, R.R. Behringer, B. de Crombrughe, Sox9 is required for cartilage formation., *Nat. Genet.* 22 (1999) 85–9. doi:10.1038/8792.
- [41] N.W. Garrigues, N. William, *Electrospun Scaffolds for Cartilage Tissue Engineering: Methods to Affect Anisotropy, Material and Cellular Infiltration*, Duke University, 2011.
- [42] M.A. Woodruff, D.W. Hutmacher, The return of a forgotten polymer—Polycaprolactone in the 21st century, *Prog. Polym. Sci.* 35 (2010) 1217–1256.
doi:10.1016/j.progpolymsci.2010.04.002.
- [43] F.H. Silver, J.W. Freeman, G.P. Seehra, Collagen self-assembly and the development of tendon mechanical properties, *J. Biomech.* 36 (2003) 1529–1553. doi:10.1016/S0021-9290(03)00135-0.
- [44] C. Shukunami, A. Takimoto, M. Oro, Y. Hiraki, Scleraxis positively regulates the expression of tenomodulin, a differentiation marker of tenocytes, *Dev. Biol.* 298 (2006) 234–247.

doi:10.1016/j.ydbio.2006.06.036.

- [45] V. Kishore, W. Bullock, X. Sun, W.S. Van Dyke, O. Akkus, Tenogenic differentiation of human MSCs induced by the topography of electrochemically aligned collagen threads, *Biomaterials*. 33 (2012) 2137–44. doi:10.1016/j.biomaterials.2011.11.066.
- [46] A. Weiler, F.N. Unterhauser, H.J. Bail, M. Hüning, N.P. Haas, α -Smooth muscle actin is expressed by fibroblastic cells of the ovine anterior cruciate ligament and its free tendon graft during remodeling, *J. Orthop. Res.* 20 (2002) 310–317. doi:10.1016/S0736-0266(01)00109-7.
- [47] M.M. Murray, M. Spector, Fibroblast distribution in the anteromedial bundle of the human anterior cruciate ligament: the presence of alpha-smooth muscle actin-positive cells, *J. Orthop. Res.* 17 (1999) 18–27. doi:10.1002/jor.1100170105.
- [48] O. Adam, K. Theobald, D. Lavall, M. Grube, H.K. Kroemer, S. Ameling, H.-J. Schäfers, M. Böhm, U. Laufs, Increased lysyl oxidase expression and collagen cross-linking during atrial fibrillation, *J. Mol. Cell. Cardiol.* 50 (2011) 678–685. doi:10.1016/j.yjmcc.2010.12.019.
- [49] E. a Makris, D.J. Responde, N.K. Paschos, J.C. Hu, K. a Athanasiou, Developing functional musculoskeletal tissues through hypoxia and lysyl oxidase-induced collagen cross-linking, *Proc. Natl. Acad. Sci. U. S. A.* 111 (2014) E4832-41. doi:10.1073/pnas.1414271111.
- [50] Y. Mori-Akiyama, H. Akiyama, D.H. Rowitch, B. de Crombrughe, Sox9 is required for determination of the chondrogenic cell lineage in the cranial neural crest, *Proc. Natl. Acad. Sci. U. S. A.* 100 (2003) 9360–5. doi:10.1073/pnas.1631288100.
- [51] L. Bian, M. Guvendiren, R.L. Mauck, J.A. Burdick, Hydrogels that mimic developmentally relevant matrix and N-cadherin interactions enhance MSC chondrogenesis, *Proc. Natl. Acad. Sci. U. S. A.* 110 (2013) 10117–22. doi:10.1073/pnas.1214100110.
- [52] S. Shang, F. Yang, X. Cheng, X.F. Walboomers, J.A. Jansen, The effect of electrospun fibre alignment on the behaviour of rat periodontal ligament cells, *Eur. Cell. Mater.* 19 (2010) 180–92.
- [53] Y.M. Kolambkar, M. Bajin, A. Wojtowicz, D.W. Hutmacher, A.J. García, R.E. Guldberg,

- Nanofiber orientation and surface functionalization modulate human mesenchymal stem cell behavior in vitro, *Tissue Eng. Part A*. 20 (2014) 398–409. doi:10.1089/ten.TEA.2012.0426.
- [54] T. Watabe, K. Miyazono, Roles of TGF- β family signaling in stem cell renewal and differentiation, *Cell Res.* 19 (2009) 103–115. doi:10.1038/cr.2008.323.
- [55] S.J. Baek, S.K. Kang, J.C. Ra, In vitro migration capacity of human adipose tissue-derived mesenchymal stem cells reflects their expression of receptors for chemokines and growth factors, *Exp. Mol. Med.* 43 (2011) 596–603. doi:10.3858/emm.2011.43.10.069.
- [56] C.H. Lee, S. a Rodeo, L.A. Fortier, C. Lu, C. Eriskin, J.J. Mao, Protein-releasing polymeric scaffolds induce fibrochondrocytic differentiation of endogenous cells for knee meniscus regeneration in sheep, *Sci. Transl. Med.* 6 (2014) 266ra171. doi:10.1126/scitranslmed.3009696.
- [57] K. Frazier, S. Williams, D. Kothapalli, H. Klapper, G.R. Grotendorst, Stimulation of fibroblast cell growth, matrix production, and granulation tissue formation by connective tissue growth factor, *J. Invest. Dermatol.* 107 (1996) 404–11.
- [58] M.M. Murray, K. Rice, R.J. Wright, M. Spector, The effect of selected growth factors on human anterior cruciate ligament cell interactions with a three-dimensional collagen-GAG scaffold, *J. Orthop. Res.* 21 (2003) 238–244. doi:10.1016/S0736-0266(02)00142-0.
- [59] C.S. Visconti, K. Kavalkovich, J.-J. Wu, C. Niyibizi, Biochemical Analysis of Collagens at the Ligament–Bone Interface Reveals Presence of Cartilage-Specific Collagens, *Arch. Biochem. Biophys.* 328 (1996) 135–142. doi:10.1006/abbi.1996.0153.
- [60] L. Galatz, S. Rothermich, K. VanderPloeg, B. Petersen, L. Sandell, S. Thomopoulos, Development of the supraspinatus tendon-to-bone insertion: localized expression of extracellular matrix and growth factor genes., *J. Orthop. Res.* 25 (2007) 1621–8. doi:10.1002/jor.20441.
- [61] H. Fujioka, G.J. Wang, K. Mizuno, G. Balian, S.R. Hurwitz, Changes in the expression of type-X collagen in the fibrocartilage of rat Achilles tendon attachment during development, *J.*

Figures

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 10⁵ cells, day 10) and high cell seeding densities (5 X 10⁵ 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 mm² 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 $\pm$ 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 picosirius 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 TGF β 3-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: 1.25×10^5 cells/scaffold. **b**, Quantification of clusters area in TGF β 3 groups; $*P < 0.05$. **c**, Number of clusters per mm² in TGF β 3 groups; $n \geq 580$ 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 $\pm$ SEMs of N=5 scaffolds for each condition, * $P < 0.05$. Abbreviation: undet., undetermined.