Cyclic tension promotes fibroblastic differentiation of human MSCs cultured on collagen-fibre scaffolds

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Abstract

Mesenchymal stem cells (MSCs) have been suggested as a potential cell source for tendon/ligament tissue engineering. Extrinsic cues, such as the chemical and physical properties of scaffolds, as well as external forces, play an important role in fibroblastic differentiation of these cells. In this study, we employed a collagen-fibre scaffold that mimics the chemical and fibrous structure and mechanical properties of tendon/ligament, and studied how imparting cyclic tension to these fibrous collagen scaffolds affects tendon/ligament fibroblastic differentiation of MSCs. Human MSCs attached and spread on the surface of the scaffolds, and appeared aligned along the fibres 24 h after seeding. Cyclic tension was then applied to cell-laden scaffolds over a period of 14 days (10% strain, 1 Hz, 3 h on/3 h off). Real time RT–PCR analysis indicated that scleraxis, a transcription factor associated with the tendon fibroblast phenotype, was found to be significantly upregulated only under cyclic tension. Immunohistochemical staining demonstrated that MSCs cultured under cyclic tension after 14 days secreted more extracellular matrix, including collagen I, collagen III and tenascin-C, compared to constructs in static culture, after 14 days in vitro. Our data indicate that cyclic tension can promote fibroblastic differentiation of MSCs in these fibrous collagen-based scaffolds, which may have significant applications in the development of tissue-engineered graft alternatives for tendon and ligament injuries. Copyright © 2014 John Wiley & Sons, Ltd.

Keywords mesenchymal stem cells; tendon/ligament; tissue engineering; tensile strain; fibroblast; fibrous scaffold

1. Introduction

Tendon/ligament injuries caused from trauma or continued overuse are a common clinical problem. An overall annual incidence of 106.2 tendon and ligament injuries/100 000 people has been reported in the USA (Clayton and Court-Brown, 2008). After rupture, many of these patients require tendon/ligament replacements to recover normal activity levels. Although autografts, allografts and prosthetic devices have been used for tendon/ligament surgeries, inevitable shortcomings, such as donor site morbidity (autografts), risk of disease transmission (allografts) and high long-term failure rate (synthetic prostheses), have promoted exploration for alternative strategies (James et al., 2008). Tissue-engineered implants, which combine autologous or allogeneic cells with biocompatible scaffolds, hold great potential as alternative tissue grafts for these applications.

Previous studies in tendon/ligament tissue engineering have focused on identifying ideal scaffold materials and cell sources, as well as examining how external cues could direct and maintain the fibroblast-like phenotype of the cells, thus promoting long-term tissue production in the constructs. Cells that reside in the tendon/ligament tissue have been considered as a source (Kryger et al., 2007). However, the availability of these tendon/ligament fibroblasts is limited, since tendons and ligaments are relatively acellular, and the cells may lose their phenotype during in vitro expansion (Yao et al., 2006). In contrast,
adult mesenchymal stem cells (MSCs) can be efficiently isolated and expanded in vitro (Caplan and Bruder, 2001) before being directed to differentiate toward a tendon/ligament fibroblast phenotype (Chen et al., 2008; Doroski et al., 2010; Kuo and Tuan, 2008; Yang et al., 2012). Because of these advantages, adult human MSCs were chosen for this study.

Scaffolds play an important role in tendon/ligament tissue engineering, since they not only support MSC attachment and growth but also provide important cues for fibroblastic differentiation of MSCs. Previous studies have employed a variety of scaffolds, including poly(glycolic acid) (PGA) (Cao et al., 2006; Deng et al., 2009), poly(lactic acid) (PLA) (Freeman et al., 2007), PLGA (Moffat et al., 2009), silk fibroin (Chen et al., 2010), fibrin (Paxton et al., 2010) and collagen (Butler et al., 2010; Garvin et al., 2003; Juncosa-Melvin et al., 2006; Kuo and Tuan, 2008; Shearn et al., 2007). However, for tendon/ligament tissue engineering, successful differentiation of MSCs into tendon/ligament fibroblast-like cells in vitro using synthetic materials only, such as PGA, PLA and PLGA, has not yet been achieved. Therefore, the biochemistry of the extracellular matrix (ECM) of tendons/ligaments may also play a crucial role in directed differentiation of MSCs into tendon/ligament fibroblasts. Since the ECM of tendons/ligaments is composed primarily of collagen type I (James et al., 2008), type I collagen-based scaffolds are being widely utilized in fibrous tissue engineering. Collagen scaffolds are commonly fabricated as a gel (Garvin et al., 2003; Kuo and Tuan, 2008; Young et al., 1998) or a combination of gel and sponge (Butler et al., 2010; Juncosa-Melvin et al., 2006). Although such materials have demonstrated the ability to help improve tissue mechanical properties in a rabbit model of patellar tendon injury (Butler et al., 2010; Juncosa-Melvin et al., 2006; Shearn et al., 2007), the maximum tensile modulus of such gels remains well below 1 MPa (Roeder et al., 2002), which is inferior to that of tendon/ligament tissues (~200–800 MPa) (Staubli et al., 1999). Clinical studies of commercial soft scaffolds for tendon repairs have indicated that ideal implants would require good initial mechanical properties to prevent gap formation or implant failure, and that suture retention of scaffolds or cell–scaffold constructs may be a key factor in determining their efficacy (Aurora et al., 2007). Therefore, application of collagen scaffolds with high mechanical and suture-retention properties may increase the possibility for clinical success.

With these findings in mind, techniques such as fibre extrusion (Kato et al., 1989), in vitro fibrillogenesis (Caves et al., 2010) and electrospinning (Matthews et al., 2002; Yang et al., 2008) have been applied to reconstruct the aligned and fibrous structure of collagen existing in tendon/ligament tissues. At the same time, other efforts have centred on improving the mechanical properties of collagen scaffolds via chemical crosslinking of collagen fibrils (Kew et al., 2011). As a part of these experiments, a novel collagen-fibre scaffold was developed by wet spinning sub-millimetre fibres from purified, soluble, bovine type I collagen and crosslinking these fibres with nordihydroguaiaretic acid (NDGA). The fibres were then braided at a low picks/inch (five to six) into scaffolds (Koob and Hernandez, 2002; Koob et al., 2001a, 2001b). This scaffold has not only been shown to be cytocompatible (Koob et al., 2001a, 2001b), but also mimics both the chemical and fibrous nature of tendon/ligament (Koob, 2002; Koob and Hernandez, 2002). In addition, the mechanical strength of the scaffolds can be easily tailored by scaling the number of braided fibres to reach comparable properties to those of native tendon (Koob and Hernandez, 2002). The improved mechanical properties of this fibrous scaffold could not only provide better initial mechanical properties to potentially prevent gap formation and failure during postoperative healing, but also allow early loading of the graft material and make it easier to suture in a surgical field for anchoring onto bone or muscle, thus facilitating clinical application for repair of fibrous tissues.

Besides the choice of material substrate, fibroblastic differentiation of MSCs can be influenced by other exogenous cues. Many previous studies have focused on the effects of external forces on the fibroblastic differentiation of stem cells and demonstrated that tensile loading is a key element to encourage differentiation during in vitro culture (Butler et al., 2010; Garvin et al., 2003; Kuo and Tuan, 2008). However, prior experiments using collagen-based scaffolds were carried out by encapsulating MSCs in soft collagen gels (Garvin et al., 2003; Kuo and Tuan, 2008; Young et al., 1998). Thus, the goal of this study was to examine the effects of external cyclic tension on MSCs differentiation on collagen-fibre-based scaffolds that possess significantly higher strength/stiffness than collagen gels and a fibrous structure inspired by native tendon/ligament tissue. To accomplish this, human MSCs were cultured either statically or at 10% cyclic tension on collagen fibre scaffolds for up to 14 days, using a custom-designed bioreactor. At 1, 7 and 14 days, the constructs were assayed for DNA content, real-time RT–PCR was performed for the tendon fibroblast markers scleraxis, collagen I, collagen III and tenascin-C, and immunohistochemistry (IHC) was performed to visualize the presence of any ECM molecules produced.

2. Materials and methods

2.1. Preparation of scaffolds and culture system assembly

The NDGA-crosslinked collagen fibre scaffolds were provided by Mimedx Group Inc. The scaffolds employed in this study were braided from four crosslinked collagen fibres, each of which had a diameter of about 100 μm. Using a custom-made template (Figure 1), two loops were created at the ends of each scaffold to anchor them into our custom tensile culture bioreactor. The length of the scaffold between the two loops was fabricated to be 13 mm. With the current configuration, up to 10 scaffolds
can be mounted onto the tensile culture system at the same time, and cultured under identical loading conditions.

### 2.2. Expansion of MSCs

Cryopreserved human MSCs at passage 2 (a combination of three individual donors, Texas A&M; cell source is human adult bone marrow: http://medicine.tamhsc.edu/irm/msc-distribution.html) were seeded into tissue culture flasks at 3000 cells/cm² and grown in a medium with α-MEM containing 10% fetal bovine serum (FBS), 4 mM L-glutamine (Mediatech) and 1% antibiotic/antimycotic (Mediatech) (note: the FBS lot used was prescreened for MSC growth, while maintaining collagen I, collagen III and tenascin-C gene expression). The MSCs were trypsinized with 0.05% trypsin (VWR) while reaching ~80% confluence, and used at passage 3.

### 2.3. Tensile culture

Before tensile culture, the scaffolds were sterilized by immersion in 70% ethanol for ~1 h, followed by three washes with phosphate-buffered saline (PBS). To homogeneously seed the MSCs on the scaffolds, a custom-made device of poly(tetrafluoroethylene) (PTFE) was utilized (Figure 1). This device has a groove with a length similar to the length of scaffold between the two loops, allowing the scaffold to be held inside the groove. During cell seeding, 80 000 MSCs suspended in 40 μl medium were placed into the groove containing the scaffold and remained in this area, due to the high hydrophobicity of the PTFE. After 1 h of culture at 37 °C, the remaining medium was removed from the groove, the scaffold was rotated 180° along the long axis of the scaffold and the seeding was repeated with 60 000 cells in 30 μl medium. After the second round of cell seeding (total cell number...
was 140 000 cells/70 μl, the cell-laden scaffolds were cultured in six-well plates for 24 h. A portion of scaffolds (referred to as day 0 samples) were harvested after this 24 h preulture and served as negative controls.

After 24 h of preulture, the constructs were loaded into our custom tensile culture system and cultured for 2 weeks. The scaffolds were maintained under a 10% sinusoidal cyclic strain (5% offset, 5% amplitude) at 1 Hz for 3 h, followed by 3 h without strain. This cycle was repeated continuously throughout the culture period, resulting in 12 h/day of total loading. Constructs cultured in the bioreactor over 2 weeks, but subjected to no strain, were used as a static control. The culture medium (same as the expansion medium described above) was changed every 3 days and the samples in both culture conditions were collected at days 7 and 14 for further analysis.

2.4. Determination of cell number

At days 0, 7 and 14, samples (n = 3-4/treatment/time point) were washed three times with PBS and treated with 0.25% trypsin for 5 min in 1.7 ml microcentrifuge tubes (VWR). To completely detach the cells from the scaffold, strong tapping was applied to the tubes several times until all the cells were suspended into the 0.25% trypsin solution. Cell culture medium was then added to neutralize the enzyme activity of trypsin, and the cells were collected by centrifugation, washed with PBS and resuspended in 250 μl Milli-Q water. Three cycles of freezing at –80 °C for 1 h, thawing at room temperature for 30 min and sonicating for 30 min were applied to extract DNA. DNA content, which can be correlated to cell number, was determined by assaying the resulting supernatant via PicoGreen (Invitrogen), using standard protocols (Doroski et al., 2010).

2.5. Observation of cell morphology

To study the morphology of MSCs post-seeding, day 0 samples (n = 3) were washed three times with PBS, followed by fixation in 10% buffered formalin (Fisher) for 1 h. The fixed samples were then stained with 1 μg/ml Hoechst dye (Sigma) for 5 min to visualize the cell nuclei. The nuclei of adherent MSCs were visualized under fluorescent microscopy with a DAPI filter (blue, 435–485 nm). Since the collagen-fibre scaffolds are autofluorescent, images were also taken with TRITC (red, 590 nm) and FITC (green, 520–560 nm) filters to visualize the fibres. Images from all three channels were merged using Adobe Photoshop.

At days 0, 7 and 14, samples from both cyclic tensile and static culture (n = 4–5/treatment/time point) were collected and washed with PBS, followed by RNA isolation using a QiaShredder column (Qiagen) and purification with an RNeasy Minikit (Qiagen). cDNA was then synthesized using Superscript III RT (Invitrogen) and a nucleotide mix (Promega). Amplification of cDNA was performed in the presence of custom-designed primers (Doroski et al., 2010) and SYBR Green (Applied Biosystems), using the eStepOnePlus™ Real-time PCR System (Applied Biosystems). Expression of tendon/ligament-related genes, including collagen I, collagen III, tenasin-C and scleraxis, were measured. α-Smooth muscle actin (α-SMA) expression, which is associated with a myofibroblastic phenotype (Hinz, 2007), and alkaline phosphatase (ALP), runt-related transcription factor 2 (Runx2) and osteocalcin (OCN) expression, which are markers of an osteoblastic phenotype, were also examined.

In this study, we examined three common housekeeping genes, GAPDH, 18S and β-actin. Although both GAPDH and 18S had a constant expression over the 2 week period of culture, β-actin expression increased significantly over time (data not shown). The expression of other genes was therefore normalized to the average of GAPDH and 18S only. Real time RT–PCR data were first analysed with LinRegPCR (v 12.11; http://www.hartfaalcentrum.nl; Ruijter et al., 2009) to determine the baselines and amplifying efficiencies of individual samples, from which the starting concentration of cDNA was calculated. The fold changes of the genes of interest were then calculated by first normalizing to the geometric mean of the two housekeeping genes and then to the average gene expression of day 0 samples.

2.6. Gene expression analysis

At days 0, 7 and 14, samples from both cyclic tensile and static culture (n = 4–5/treatment/time point) were collected and washed with PBS, followed by RNA isolation using a QiaShredder column (Qiagen) and purification with an RNeasy Minikit (Qiagen). cDNA was then synthesized using Superscript III RT (Invitrogen) and a nucleotide mix (Promega). Amplification of cDNA was performed in the presence of custom-designed primers (Doroski et al., 2010) and SYBR Green (Applied Biosystems), using the eStepOnePlus™ Real-time PCR System (Applied Biosystems). Expression of tendon/ligament-related genes, including collagen I, collagen III, tenasin-C and scleraxis, were measured. α-Smooth muscle actin (α-SMA) expression, which is associated with a myofibroblastic phenotype (Hinz, 2007), and alkaline phosphatase (ALP), runt-related transcription factor 2 (Runx2) and osteocalcin (OCN) expression, which are markers of an osteoblastic phenotype, were also examined.

To examine the location of ECM molecules associated with the tendon/ligament fibroblast phenotype, samples (n = 3) were removed from culture, washed with PBS and fixed with 10% buffered formalin after either 0, 7 or 14 days. To cryosection the samples into 30 μm slices, they were first embedded into Histogel (VWR), and then flash-frozen into optimal cutting temperature (OCT) compound (VWR) with liquid nitrogen. The cross-sections obtained were perpendicular to the long axis of the scaffolds. Before staining, the samples were treated with 20 μg/ml proteinase K (Sigma) solution for 20 min at 37 °C in a humidified chamber for antigen retrieval. The samples were collected at days 7 and 14 for further analysis.
were then blocked with 2% goat serum and incubated with primary antibody (1:500, monoclonal mouse anti-human collagen I, collagen III or tenascin-C; Abcam) overnight at 4°C, and then secondary antibody (1:200, goat anti-mouse Alexa 488-conjugated; Invitrogen) was applied for 30 min at room temperature. Nuclei were visualized by staining with 1 μg/ml Hoescht dye for 5 min at room temperature, and the slides were mounted with FluoroGel (Fisher). The ECM protein of interest and the nuclei were imaged with FITC (green, 520–560 nm) and DAPI (blue, 435–485 nm) filters under fluorescent microscopy (n=3). The autofluorescence of the collagen fibres was also imaged using a TRITC filter (red, 590 nm). Images from all three channels were then merged using Adobe Photoshop.

2.8. Statistics

Results are reported as mean ± standard deviation (SD). Before statistical analysis, data were transformed with Box-Cox to obtain a normal distribution. Linear regression was then used to remove outliers. A two-way ANOVA was applied, using Systat, to determine the statistical significance of groups, and Tukey’s post hoc test was used with significance set at p ≤ 0.05 to indicate significance between individual samples.

3. Results

3.1. Cell seeding and morphology

The custom-made grooved device greatly facilitated homogeneous MSC seeding on the surfaces of the scaffolds. The appropriate seeding density for this study was determined by using different cell concentrations (70 000 cells/70 μl, 140 000 cells/70 μl, 210 000 cells/70 μl and 280 000 cells/70 μl, which are equivalent to 1, 2, 3 and 4 million/ml) in the cell suspension placed on the scaffold (data not shown). After seeding at 210 000 or 280 000 cells/70 μl, small aggregates were observed on the surface of the scaffolds via light microscopy, which may produce unwanted variation in local seeding density. However, no cell aggregation was found on the surface of scaffolds when the MSCs were seeded at 70 000 cells/70 μl or 140 000 cells/70 μl. In addition, it appeared that cells seeded at 140 000 cells/70 μl attached well to the surface and grew to almost confluence after the 24 h preculture (Figure 2A). Thus, a cell density of 140 000 cells/70 μl (2 million/ml) was utilized for the remainder of this study.

The morphology of adherent MSCs was visualized via confocal microscopy after staining for actin (Figure 2B, C, flattened Z-stack confocal images). After 24 h of preculture, the cells appeared to spread very well on the surfaces of the scaffolds, as indicated by the evident actin staining (Figure 2B). Interestingly, the orientation of actin fibres appeared relatively aligned along the long axis of the collagen-fibre scaffolds even after only 24 h of attachment with no applied strain. Moreover, this alignment seemed to be maintained in both culture conditions over 14 days (Figure 2B, C).

3.2. Cell proliferation

To better understand how the MSCs proliferated under the various culture conditions, we assayed the cell number at each time point via the PicoGreen assay. Our results indicated that about 10 000 cells adhered to each scaffold, which is only about 7% of the number of cells seeded (140 000 cells/70 μl) (Figure 3). Although there was no significant increase in cell number in either static or cyclic samples at day 7 compared to day 0, MSC number did significantly increase in both the static and cyclic tensile cultures in the second week of culture to ~2.5–3.5 times that found on day 0. However, cyclic tension did not influence MSCs proliferation in this experiment, since there was no significant difference between cell number in the two culture conditions at days 7 and 14.

3.3. Gene expression

After culture we found that, for these specimens, the expression of the tendon-related transcription factor...
scleraxis was highly loading-dependent (Figure 4). Compared to the scleraxis expression in static culture, which remained relatively constant over the 14 days of culture, cyclic tension significantly upregulated scleraxis expression on day 7 and this upregulation was maintained through to day 14. However, our data indicated that gene expression of collagen I increased over time (increase by ~2.5–4 times at both days 7 and 14), regardless of the presence of loading during culture. Similar to scleraxis, both culture time and culture condition played a role in the regulation of collagen III and tenascin-C expression. Collagen III expression increased by day 7 in both culture conditions, but there was no effect of tensile loading observed at this time point. However, cyclic tension increased collagen III gene expression by 14 days, so that there was significantly greater expression than the static control by the last time point. Following a comparable pattern, although there was no difference in tenascin-C expression on day 7 between static and cyclic tensile culture, cyclic tensile culture promoted significantly more tenascin-C expression compared to static culture on day 14 (Figure 4). To examine alternative differentiation pathways, we also analysed the expression of α-SMA, a commonly used myofibroblast marker (Hinz, 2007), which is also reported to be responsive to cyclic loading (Kobayashi et al., 2004). It was demonstrated that α-SMA levels remained low at all time points for both culture conditions (data not shown). Similarly, analysis of the expression of the osteoblast markers ALP, Runx2 and OCN also suggested that osteogenic differentiation of MSCs was minimal in our system, since ALP levels remained low at all time points, and Runx2 and OCN levels only reached a maximum of two-fold upregulation after 14 days, regardless of culture condition (data not shown).

3.4. Histology and immunostaining for ECM molecules

The effects of tensile loading on tendon/ligament ECM production were further confirmed by IHC. Since the collagenous scaffold did not degrade during the 2 week culture period, its fibrous structure enabled the differentiation of the scaffold from collagen production by MSCs. In addition, primary anti-collagen antibodies did not cross-react with the collagen scaffold, so that the difference in fluorescence intensity made the boundary between scaffold and ECM easily observable. As shown in Figure 5, extracellular collagen I was barely detected in statically cultured samples. With cyclic tensile culture, extracellular collagen I...
collagen I was only primarily observable at day 14, but not at day 7 (Figure 5). Similarly, static culture resulted in only low levels of collagen III and tenascin-C visible after 14 days (Figures 6, 7). In contrast, cyclic tension resulted in obvious extracellular tenascin-C and collagen III deposition at day 14 (but not day 7). Taken together, cyclic tension upregulated extracellular collagen I, collagen III and tenascin-C production after 14 days of culture.

4. Discussion

For tissue-engineering applications, fibroblastic differentiation of MSCs can be guided by various external cues, such as the chemical structure and topography of the scaffold employed, or external mechanical forces (Liu et al., 2008; Lui et al., 2011). Although previous studies have explored the roles of combinations of two of these factors, such as chemical structure with cyclic force (Garvin et al., 2003; Kuo and Tuan, 2008), or geometry with external force (Chen et al., 2010), the effects on cellular differentiation of combination of all three of these cues in a single system have yet to be explored. Therefore, in this study, we examined how MSC differentiation can be influenced by cyclic tension after cells have been seeded on fibrous collagen scaffolds that mimic both the chemical composition and the general fibrous structure of tendon/ligament.

The unique collagen type I-based fibrous scaffolds utilized here were inspired by the microstructure of tendon/ligament tissues. Type I collagen is the most abundant tendon component, which constitutes about 60% of the dry mass of the tendon and about 95% of the total weight of collagen (Wang, 2006). Collagen is assembled extracellularly into collagen fibrils, and further into aligned collagen fibre bundles with a diameter in the range 1–300 μm by intermolecular crosslinking the collagen fibrils (Silver et al., 2003). The novel scaffolds employed in these studies are formed from type I collagen fibres, which have a diameter of ~100 μm, which is similar to the diameter of fibre bundles. These collagen fibres were pre-crosslinked via NDGA treatment to increase the mechanical properties of each fibre (Koob, 2002; Koob and Hernandez, 2002; Koob et al., 2001a, 2001b). Although the final scaffold is braided from four collagen fibres, and therefore does not fully recapitulate the structure of aligned fibres found natively, its braided structure increases the mechanical properties of the scaffolds and facilitates cell seeding. Previous studies have indicated

Figure 5. MSCs produced collagen I over time in both static and cyclic culture, but cyclic culture resulted in much more apparent collagen I deposition after 14 days, as indicated by immunohistochemistry. Arrows in the day 0 images indicate fibres of the original scaffold; scale bar = 50 μm. Nuclei, blue; collagen I, green; collagen fibres, merged from blue, green and red. Negative control: samples under cyclic culture after 14 days were stained with secondary antibody only as negative controls. Similar results were seen for day 0 samples and negative controls for all antibodies, but are not shown in the following figures due to space constraints.
that these crosslinked collagen fibres have mechanical properties comparable to those of native collagen fibres, and the resulting scaffolds possess similar stiffness to that of many tendons (Koob, 2002; Koob and Hernandez, 2002). Therefore, these NDGA-crosslinked collagen fibres with superior mechanical properties not only mimic the mechanical cues provided by the individual fibres in tendon/ligament tissues, but also may act as an ideal tissue-engineered scaffold for mechanical substitution or augmentation for tendon/ligament repair (Kew et al., 2012). Moreover, another distinct advantage of this scaffold is that NDGA is a potent anti-oxidant and inhibitor of cyclooxygenases and lypoxygenases involved in pro-inflammatory responses (Kim et al., 2008). NDGA itself has been shown to be an effective anti-inflammatory agent and NDGA-crosslinked collagen does not produce an inflammatory or immune response, as demonstrated in animal models (Ju et al., 2010; Koob, 2002), making this material easily clinically translatable for the repair of tendon and ligament injuries.

In these scaffolds, we found that MSCs were seeded with relatively low efficiency (~7% seeded cells attached after 1 day), possibly due to the low total surface area. While a good first-pass estimate, in the future, it may be
possible to further improve our seeding density calculations by also assaying the medium supernatant immediately after cell seeding. Regardless, at the chosen seeding density for these studies, the cells were relatively homogeneously seeded on the scaffolds at near confluence after 1 day of attachment (Figure 2A). As indicated by confocal microscopy and immunostaining, the MSCs proliferated and formed several layers of cells on the surface by day 14, regardless of the presence of loading during culture, resulting in an increase in total cell number for both sample types by the last time point (Figure 3). The relatively high cellularity after 2 weeks of culture compared to the native tendon/ligament tissue is consistent with the finding that the cellularity of embryonic tendon or immature tendon (Stanley et al., 2007) is comparatively higher than that of mature tendon, and that there is increased cellularity in the proliferative phase of tendon healing after injury (Molloy et al., 2003), and therefore may suggest that these constructs have not reached full maturity over this time period.

Similar to previous studies demonstrating that cells could, to some degree, align along the long axis of microfibres with a diameter of 10–242 μm (Hwang et al., 2009) as early as 24 h after seeding, the MSCs appeared aligned along the scaffold fibres (Figure 2B). Cyclic tension seemed to have no effect on alignment, since no apparent morphological differences between static and cyclic culture were observed via confocal microscopy after 14 days. The cyclic culture parameters employed in this study were based on previous work with this tensile culture apparatus (Doroski et al., 2010; Yang et al., 2012), which demonstrated upregulation of gene markers for fibroblastic differentiation when human MSCs were embedded in hydrogels and subjected to this tensile culture regimen, and fall within the range of parameters used for a wide variety of previous tensile culture studies (Breen, 2000; Kim et al., 2002; Lee et al., 2005). Moreover, because the motor in this bioreactor is displacement-controlled, the stiffness of the scaffold is not expected to have a large impact on the level of strain imparted to the samples. In the future, a wider range of loading parameters can be explored to more fully understand how MSCs respond to a range of strain amplitudes and frequencies on these scaffolds.

Although the cyclic tension did not cause differences in MSC alignment or proliferation (Figure 3), clear effects of tensile strain on fibroblastic differentiation of MSCs in these scaffolds were seen at both gene and protein levels. The transcription factor scleraxis is considered to be a specific marker of the tendon/ligament lineage (Murchison et al., 2007; Schweitzer et al., 2001; Shukunami et al., 2006). It is induced in the earliest stage of fibroblastic differentiation, and is maintained throughout tendon development (Schweitzer et al., 2001). Previous studies have indicated that 3D culture by encapsulating MSCs in collagen I gels can upregulate scleraxis expression at the beginning of culture, compared to when MSCs were cultured on a 2D collagen-coated surface, and cyclic stretching was required to maintain scleraxis expression over time (Kuo and Tuan, 2008). In our system, MSCs were seeded on the surface of collagen scaffolds, which may be considered more like a 2D surface than a 3D gel. However, similar to the previous study within collagen gels, scleraxis gene upregulation was observed, and was seen to be tensile culture-dependent in our system. Its expression was increased by ~ five-fold at day 7 compared to day 0 samples only under cyclic culture, and it was maintained through day 14, suggesting early fibroblastic differentiation of cells in these constructions (Figure 4). It should be noted that, in other work in our laboratory (Doroski et al., 2010; Yang et al., 2012), while other fibroblast-related factors were upregulated in response to cyclic culture, scleraxis was not, suggesting that this combination of fibrous scaffold and tensile culture is particularly efficient at promoting MSC differentiation toward a tendon/fibroblast phenotype, as compared to the synthetic gels previously explored in our laboratory using the same culture system.

The ECM molecules collagen I, collagen III and tenasin-C are also important markers of tendon/ligament fibroblast differentiation (Chiquet, 1999). Although tendons/ligaments are composed predominantly of collagen I, and collagen III is mainly present in the endotenon and epitenon, collagen III is highly expressed in the early stages of development, and is distributed throughout small, immature fibrils (Birk and Mayne, 1997; Silver et al., 2003). Our data indicated that MSCs in both culture conditions demonstrate increased mRNA levels of collagen I over time (Figure 4). In contrast, for collagen III, cyclic tension resulted in an increase in gene expression by ~ five-fold at day 7 and ~ seven-fold at day 14, i.e. significantly higher than in static culture (Figure 4). Most importantly, immunostaining results for each of these molecules indicated that production of collagen I and III was only found in the scaffolds undergoing cyclic tension at day 14 (Figures 5, 6). This suggests that cyclic tension promoted the production of collagen I and III, as the gene expression indicated, but the ECM accumulated and became detectable only at day 14. Furthermore, high collagen III expression indicates that, even at day 14 under cyclic culture, the MSCs are likely still in an early stage of fibroblastic differentiation. Moreover, the low gene expression levels of α-smooth muscle actin support the concept that these cells are not becoming myofibroblastic during the culture period (another potential cause of collagen III production) (Volk et al., 2011).

Tenasin-C is a large ECM glycoprotein, highly regulated by tensile stress (Chiquet-Ehrismann and Tucker, 2004; Chiquet et al., 2009). In development, tenasin-C expression often appears in tissues with high tensile strength (Chiquet and Fambrough, 1984), and muscle immobilization suppresses tenasin-C accumulation in developing tendons (Mikic et al., 2000). Similarly, in our study, increased tenasin-C mRNA was detected at day 14 in both culture conditions. However, cyclic tension promoted tenasin-C expression compared to static culture (Figure 4). Furthermore, the accumulation of tenasin-C became observable via immunostaining only on scaffolds.
undergoing cyclic tensile culture at day 14 (Figure 7). It is noted that, although cyclic culture promoted the gene expression of scleraxis, collagen I, collagen III and tenascin-C compared to static culture, the gene expression of collagen I, collagen III and tenascin-C were also upregulated in static culture over time. However, the upregulation of gene expression in static culture did not translate into significant, observable ECM production, which may be due to inefficient post-translational modifications that may also be mechanically regulated (Kuo and Tuan, 2008).

Taken together, these data demonstrate that these collagen-fibre-based scaffolds with high tensile strength, combined with cyclic tensile culture, promoted fibroblastic differentiation of MSCs as well as substantial ECM production, and therefore represent a promising approach to fabrication of tissue-engineered tendon and ligament grafts. It is possible that these scaffolds provided the appropriate combination of chemical and topographical cues, so that when MSCs were seeded on them in the presence of tensile loading, relatively rapid differentiation was promoted. However, it should be noted that this study was performed only over a period of 2 weeks and at a single combination of strain parameters. From gene expression and immunostaining, as well as overall cellularity measurements, it appears that the resulting tissue was still in a relatively early stage of differentiation. Therefore, in the future, it may be beneficial to characterize when differentiating MSCs begin the transition to maturity, how the cellularity of the construct changes, and the type and amount of ECM deposited over longer times (or under different strain conditions), and how this may affect the mechanical forces imparted to the cells in vitro. These further studies would greatly aid in identifying optimal in vitro culture parameters to provide clinically useful alternative grafts for tendon/ligament repair, keeping in mind both the required mechanical properties of the graft and also how to best encourage integration with surrounding tissues after transplantation.

5. Conclusions

In this study, we examined how cyclic tension, in combination with a scaffold that mimics the chemical, architectural and mechanical properties of native tendon/ligament tissues, can regulate fibroblastic differentiation of MSCs. Strikingly, scleraxis expression in MSCs was significantly upregulated under cyclic tension as early as day 7, and this upregulation was maintained through the rest of the culture period. By day 14, collagen III and tenascin-C gene expression was significantly upregulated in cyclic vs static constructs, and collagen I, collagen III and tenascin-C protein production were obvious only in samples undergoing cyclic culture, suggesting that tensile culture on these scaffolds promotes fibroblastic differentiation of human MSCs over 2 weeks of in vitro culture. These exciting results indicate that fibrous, braided NDGA–collagen scaffolds are excellent candidates for use with a cyclic culture bioreactor, both to prime MSCs for cell-based therapies in tendon and ligament repair and for production of tissue-engineered alternatives to current tendon/ligament tissue grafts.

Conflict of interest

Y.Q., J.L. and J.S.T. disclose that they have no financial interest in the MiMedx Group. T.J.K. is the Chief Scientific Officer of MiMedx and inventor of the collagen fibre technology.

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References

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Yang PJ, Levenston ME, Temenoff JS. 2012; Modulation of mesenchymal stem cell shape in enzyme-sensitive hydrogels is decoupled from upregulation of fibroblast markers under cyclic tension. Tissue Eng A 18: 2365–2375.
