TISSUE ENGINEERING: Part A
Volume 20, Numbers 19 and 20, 2014

DOI: 10.1089/ten.tea.2013.0457

Three-Dimensional Culture and Transforming Growth Factor Beta3 Synergistically Promote Tenogenic Differentiation of Equine Embryo-Derived Stem Cells

Tom Barsby, BSc, Emma P. Bavin, BSc, and Debbie J. Guest, BSc, PhD

The natural reparative mechanisms triggered by tendon damage often lead to the formation of biomechanically inferior scar tissue that is prone to re-injury. Before the efficient application of stem cell-based regenerative therapies, the processes regulating tenocyte differentiation should first be better understood. Three-dimensional (3D) growth environments under strain and the exogenous addition of transforming growth factor beta3 (TGF-β3) have separately been shown to promote tendon differentiation. The aim of this study was to determine the ability of both of these factors to induce tendon differentiation of equine embryo-derived stem cells (ESCs). ESCs seeded into 3D collagen constructs can contract the matrix to a similar degree to that of tenocyte-seeded constructs and histologically appear nearly identical, with no areas of cartilage or bone tissue deposition. Tendon-associated genes and proteins Tenascin-C, Collagen Type I, and COMP are significantly up-regulated in the 3D ESC constructs compared with tenogenic induction in monolayer ESC cultures. The addition of TGF-β3 to the 3D cultures further up-regulates the expression of these genes and also induces the expression of mature tenocyte markers Tenomodulin and Thrombospondin-4. Our results show that when ESCs are exposed to the intrinsic forces exerted by a 3D culture environment, they express tendon-associated genes and proteins which are indicative of tenocyte lineage differentiation and that this effect is synergistically enhanced and accelerated by the addition of TGF-β3.

Introduction

Tendon damage is a common injury in racing Thoroughbreds and other competition horses. As with many other species; this damage leads to the formation of biomechanically inferior scar tissue, resulting in a high probability of re-injury.[1]

For a successful regenerative therapy to be clinically applied and modulated, the molecular pathways regulating and initiating tendon development should be better understood. Transforming growth factor betas (TGF-βs) are a family of cytokines whose isoforms have multiple roles in cell proliferation and tissue morphogenesis.[2] Developmental studies in chickens have demonstrated that the TGF-β3 isoform is present throughout the morphogenesis of tendon tissue.[3] Exogenous addition of TGF-β3 triggers an up-regulation of the tendon progenitor marker scleraxis (*Scx*) in developing limb bud mesenchyme, and null mutants for TGF-β2/3 cause a loss of normal tendon phenotype.[4] Multiple studies have shown that TGF-β is up-regulated after tendon injury[5–7] and after their injection into the injured tendon, pluripotent equine embryo-derived stem cells (ESCs) express SCX.[5] The addition of TGF-β3 induces tenogenic differentiation of *in vitro* cultures of mesenchymal stem cells (MSCs),[8] micromass cultures of undifferentiated limb mesenchyme,[9] and equine ESCs.[5] Taken together, this suggests that TGF-β signaling plays a key role in triggering the initiation of tenocyte differentiation from stem cells and tendon progenitors.

The aim of this study was to develop an *in vitro* model system to better understand and monitor tenocyte differentiation from equine ESCs in a three-dimensional (3D) growth environment. Biomechanical forces can promote cell differentiation during a two-dimensional (2D) culture;[10,11] however 3D culture systems offer many advantages compared with a monolayer culture. Cell migration and alignment can be much more closely observed, organization and re-organization of the extra-cellular matrix (ECM) can be detected over a time course, and the spatiotemporal expression and deposition of matrix proteins can be readily identified in a system which much more closely resembles that of the true *in vivo* environment.

Three-dimensional cell culture systems have been shown to elicit strong effects on the phenotypes and expression patterns of the contained cells. Three-dimensional constructs have also been utilized to manipulate and direct certain pathways of differentiation in stem cells, including those of

Animal Health Trust, Centre for Preventive Medicine, Newmarket, Suffolk, United Kingdom.

tenocyte lineages from MSCs. These include growth under ''static tension'' (produced by anchoring the gel),[8,12–14] ''uniaxial applied tension'' (an externally applied constant force),[15] ''cyclical tension'' (exposure to externally applied and released forces),[12,16] and seeding cells on electrochemically aligned collagen.[17]

When cells are cultured in anchored gels, they are under tension in what has been described as a ''static'' culture system.[12] This tension has also been termed ''uniaxial''[8] to indicate that the tension is applied in one direction between the anchors. However, during the culture and contraction of the matrix, the actual force applied to the cells will change and, until a construct reaches its maximum contraction, the direction of force will be variable. We will, therefore, define the force produced in an anchored gel as ''intrinsic'' in this article to mean that the force is applied by the re-arrangement of the matrix by the cells themselves, no additional applied forces are used, and there is no release from the force during the culture.

The *in vivo* differentiation of ESCs into tenocytes after an injection into tendon lesions[5] may indeed be, in part, due to the exposure of the pluripotent cells to mechanical strain within the healing tendon. We, therefore, hypothesized that ESCs cultured in an anchored 3D environment with an intrinsic force and also treated with TGF-β3 would have enhanced tenogenic differentiation compared with cells cultured in a 2D environment and that by using this model, we could define the expression pattern of tendon-associated genes and proteins which occurs during early development.

Materials and Methods

Two-dimensional cell culture

Three lines (i.e., derived from three different individuals) of previously characterized ESCs[18,19] were used in this study. ESCs were cultured on mitotically inactivated mouse embryonic fibroblasts at 37.5°C, 5% CO_2 in ESC medium [DMEM/F12 containing 15% fetal bovine serum, 2 mM L-glutamine, 1% nonessential amino acids, 1 mM sodium pyruvate, 0.1 mM 2-mercaptoethanol (all from Invitrogen), and 1000 units/mL leukemia inhibitory factor (LIF) (Sigma)]. ESCs were passaged mechanically every 5–7 days in the presence of Effectine (PAA Laboratories).

Equine tenocytes were isolated from healthy equine tendon tissue at postmortem with institutional ethical approval from the Animal Health Trust Research Ethics Committee. Briefly, the tissue was cut into small pieces and then incubated overnight in 1 mg/mL collagenase (Sigma) solution in fibroblast culture media [DMEM high glucose, 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin (all from Invitrogen)]. The cells were pelleted by centrifugation and washed thrice in cell culture media before culture. Equine tenocytes were passaged using 0.25% trypsin-EDTA (Sigma) every 3–4 days just before reaching confluency.

Two-dimensional gene expression studies were performed as detailed.[5] Briefly, ESCs (passage 12–18) were induced to differentiate by passaging into conditions without feeder cells and in ESC media lacking LIF. Cells were allowed to attach for 5 days before the addition of 20 ng/mL TGF-β3 (PeproTech). Cells were then harvested for RNA after 7 or 14 days as detailed next.

Three-dimensional cell culture

0.2 mm-diameter minutien pins were embedded in silicone-coated six-well plates (Sylgard 184 Silicone elastomer; Dow Corning) in pairs that were 15 mm apart.

Mechanically passaged ESCs (passage 12–24) were isolated, and an aliquot dissociated with TrypLE™ Express (Invitrogen) was used to perform representative cell counts. Colonies of ESCs were suspended in a chilled mixture of two parts of ESC medium (without LIF), eight parts PureCol (Bovine collagen type I; Advanced Biomatrix) (with the pH adjusted to 7.2 to 7.6) to give a final suspension of 4×10^5 cells/mL. 200 μL of collagen-ESC suspension was used per construct between each pair of minutien pins. The six-well plates were sealed with parafilm and kept at 37.5°C for 60–90 min to allow the constructs to set. Finally, 3 mL of ESC medium (without LIF) was added to each well, with or without 20 ng/mL TGF-β3. This concentration of TGF-β was previously used to generate a tenogenic response in ESCs[5] and is consistent with working concentrations used to induce scleraxis in mouse mesenchymal progenitor cells.[4] Constructs were cultured for approximately 14 days and during this time, the media was changed every 3–4 days and fresh TGF-β3 was added. Tenocyte-seeded constructs (passage 1–5) were prepared in the same way and with the same cell density, but using fibroblast media instead of ESC media.

Contraction analysis

The 3D constructs were photographed every day until they were harvested. The images were analyzed using ImageJ software (National Institutes of Health). Individual images were calibrated using the well diameter. Measurements were made across the width of each construct and averaged to obtain a contraction value. Contraction data are displayed as a percentage of the Day 0 value. A Student's *t*-test was used to determine statistically significant differences in contraction. For this analysis, three biological repeats were performed using three independent lines of ESCs and adult tenocytes with a minimum of two technical repeats carried out for each cell line. For each technical repeat, a minimum of five constructs were measured at each time point for each condition.

Cell survival assay

Constructs were harvested and digested in 1 mL ESC/fibroblast medium with 1 mg/mL type I collagenase produced by *Clostridium histolyticum* (Sigma) for 1–2 h at 37.5°C. The remaining cells were pelleted by centrifugation, resuspended in 1 mL TrypLE Express (Invitrogen), and also kept at 37.5°C in order to fully dissociate the cells. Cell counts were performed on a hemocytometer, and results are displayed as a percentage of the seeded cell number on Day 0. A Student's *t*-test was used to determine statistically significant differences in cell survival. Cell survival was performed using three independent cell lines of ESCs or adult tenocytes. For each line, a minimum of nine constructs were used to calculate the cell survival at each time point for each condition.

Histology and immunohistochemistry on 3D construct sections

Constructs were embedded in OCT compound (VWR) and snap frozen in liquid nitrogen-cooled isopentane. Longitudinal

sections, 11 μm thick, were cut using a cryostat. The sections were fixed in 100% acetone for 10 min and stored at −20°C until they were used. Histological staining was carried out using Hematoxylin and Eosin (H&E) to view cellular morphology and distribution. Cartilage and bone differentiation were detected using Alcian Blue staining pH1.0 and Alizarin Red S staining, respectively. For these stains, positive controls were carried out using sections of mature equine cartilage and bone tissue. Negative controls were carried out on sections of mature equine tendon tissue.

For immunohistochemistry on 3D construct sections and undifferentiated ESCs: Primary antibody incubations were carried out overnight at 4°C before detection with the fluorescently labeled secondary antibody. Antibodies were used at optimized concentrations in phosphate-buffered saline (PBS). Primary antibodies included rabbit anti-scleraxis 1:100 (Abcam), rabbit anti-tenascin C 1:100 (Abcam), rabbit anti-tenomodulin 1:100 (Santa Cruz), mouse anti-collagen I 1:100 (Abcam), rabbit anti-COMP 1:500 (kindly provided by Professor Roger Smith, Royal Veterinary College, UK), and rabbit anti-thrombospondin 4 1:100 (Santa Cruz). Secondary antibodies included goat anti-mouse FITC 1:200 (Abcam), goat anti-rabbit FITC 1:100 (Sigma), and goat anti-rabbit TR 1:200 (Abcam). Negative controls were carried out using isotype-matched sham primary antibodies and by omitting the primary antibodies. All sections were mounted with vectashield hardset mounting medium containing DAPI (Vector Laboratories).

All histological and immunohistochemical analyses were performed on constructs that were set up using three lines of ESCs and tenocytes.

Immunocytochemistry on undifferentiated ESCs

ESCs were cultured with feeders in ESC media for 5–7 days on gelatin-coated (Sigma) coverslips. The cells were fixed in 3% paraformaldehyde in PBS for 20 min at room temperature and then permeabilized for 1 h with 0.1% triton-X-100 at room temperature before incubation with the primary antibodies using the same conditions as described earlier.

RNA extraction, cDNA synthesis, and quantitative PCR

RNA was extracted using 1 mL Tri-reagent (Sigma) per six to nine constructs and treated with Ambion DNA-free (Life Technologies) according to the manufacturer's instructions. cDNA was made from 1 μg of RNA using Moloney murine leukemia virus reverse transcriptase (Promega) and oligo(dT) and random hexamers as primers (both Promega). Two-microliter aliquots of cDNA were used in a quantitative polymerase chain reaction (qPCR). Primers were designed using primer3 (http://frodo.wi.mit.edu/primer3) and mfold (http://mfold.rna.albany.edu/?q=mfold/DNA-Folding-Form) programs to obtain amplicons with a melting temperature (Tm) of 58–62°C, devoid of a secondary structure at Tm 60°C, and with an amplicon size of 50–150 bp. Primer sequences can be found in Table 1. qPCR was carried out using SYBR Green containing supermix (Bioline) on a Quantica machine (Techne). All PCR reactions were performed in duplicate. PCR cycle parameters were 95°C 10 min, followed by 40 cycles of 95°C for 15 s, 60°C for 15 s, and 72°C for 15 s. At the end of the program, the temperature was reduced to 65°C and then gradually increased by 1°C increments for approximately 95°C to produce a melt curve. Gene expression was normalized to 18s rRNA gene expression levels, which did not change between treatments (data not shown), using the $2^{-\Delta\Delta Ct}$ method.[20] A Student's *t*-test was used to determine statistically significant fold changes in gene expression between the control and treated groups at each time point. qPCR was performed on three lines of ESCs with two technical repeats carried out for each cell line.

Results

ESCs cultured in anchored 3D collagen gels contract the matrix and the process is accelerated by TGF-β3 signaling

When cultured in an anchored collagen matrix and exposed to the intrinsic forces produced, ESCs are able to contract the matrix in a similar manner to adult equine tenocytes (Fig. 1a). Over a period of 14 days, the degree of contraction reached in gel constructs containing ESCs is the same as that produced by constructs containing adult tenocytes (Fig. 1b, c). The contraction rate of tenocyte constructs was unaffected by the presence or absence of TGF-β3 (Fig. 1b). TGF-β3 can promote tenocyte differentiation of ESCs in 2D cultures,[5] and exposure of ESCs in 3D culture to TGF-β3 produced an increase in the initial rate of contraction (Fig. 1c). However, the differences in the degree of contraction are only significant over the first 5 days and by 14 days, the constructs had contracted to the same degree whether or not TGF-β3 was present. The increased rate of contraction was not due to increased ESC survival, which did not significantly alter in the different culture conditions or at the different time points studied (Fig. 2). Despite similar degrees of contraction between constructs containing ESCs and adult tenocytes, the cell survival of tenocytes was significantly lower than that of ESCs after 7 days.

Table 1. Primer Sequences Used in Gene Expression Analysis

Gene	*Forward primer*	*Reverse primer*
Scleraxis	CCCAAACAGATCTGCACCTT	ATCCGCCTCTAACTCCGAAT
Tenascin-C	AACCCGTCCAAAGAGACCTT	GCGTGGGATGGAAGTATCAT
Tenomodulin	GTCCCTCAAGTGAAGGTGGA	CCTCGACGGCAGTAAATACAA
Collagen I	TGCGAAGACACCAAGAACTG	GACTCCTGTGGTTTGGTCGT
Cartilage oligomeric matrix protein	AGAACATCATCTGGGCCAAC	CGCTGGATCTCGTAGTCCTC
Thrombospondin-4	GGGAAATGGGGTTACCTGTT	CGGGTAGCAGGGATGATATT
Sox9	GCTCTGGAGACTTCTGAACGA	GTAATCCGGGTGGTCCTTCT

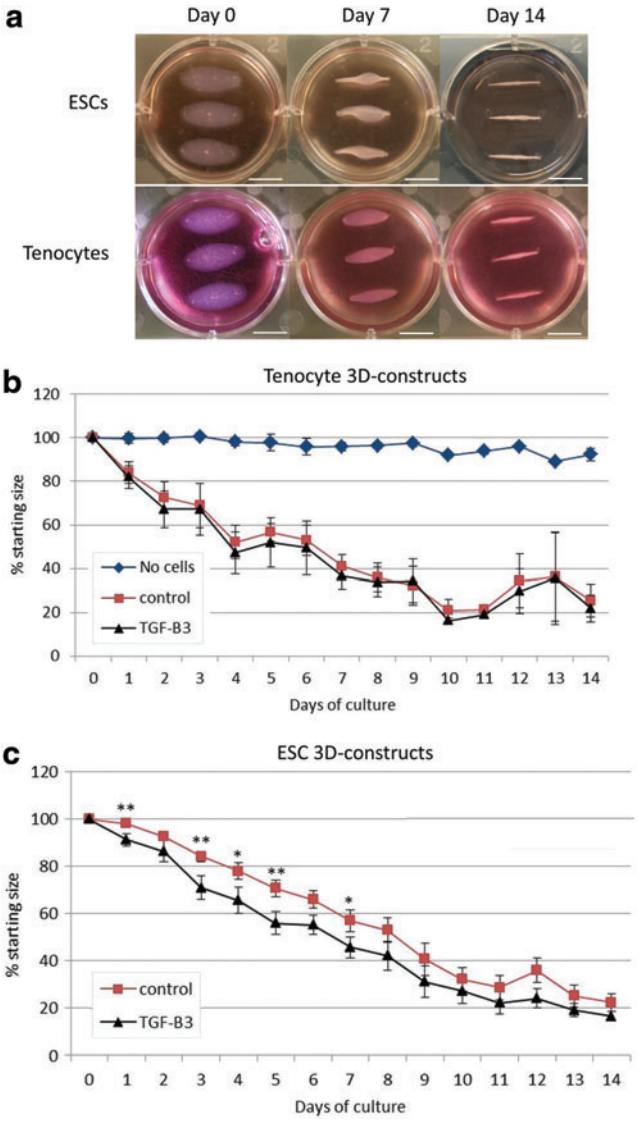

FIG. 1. **(a)** Embryo-derived stem cell (ESC)-seeded constructs in three-dimensional (3D) culture contract the collagen matrix in a comparable manner to tenocyte-seeded constructs over the same time course. Scale bar = 10 mm. **(b)** The contraction rates of tenocyte-seeded constructs are unaffected by the presence or absence of transforming growth factor beta3 (TGF-β3). **(c)** ESC-seeded constructs display enhanced rates of contraction in the presence of TGF-β3, which reach statistical significance over the first 5 days. $^{**}p < 0.05$, $^{*}p < 0.1$. Error bars represent the standard error of the mean. Color images available online at www.liebertpub.com/tea

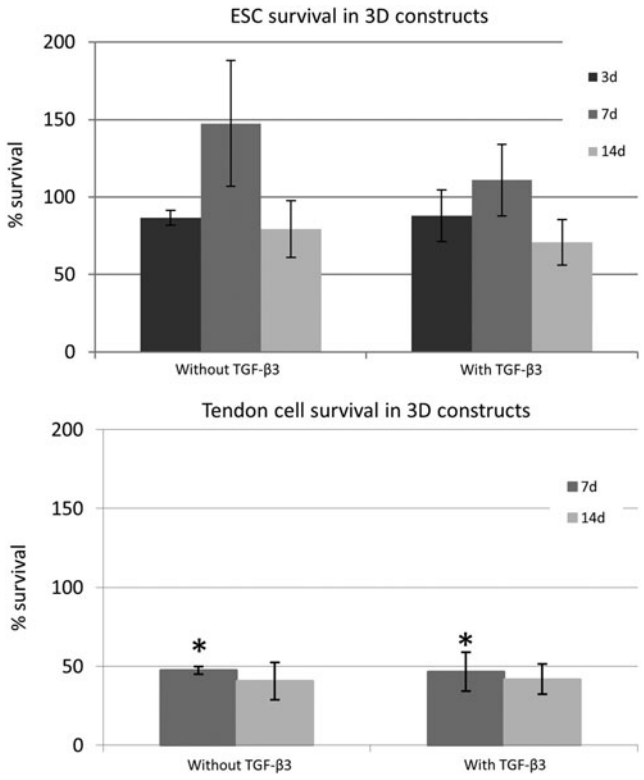

FIG. 2. Cell survival in ESC-seeded constructs is unaffected by the presence of TGF-β3 over the experimental time course. Cell survival of adult tendon cells is significantly lower than that of the ESCs after 7 days of culture $^{*}p < 0.05$. Error bars represent the standard error of the mean.

ESCs organize the collagen construct into a tendon-like matrix without evidence of bone or cartilage formation

After 7 days, gel constructs containing cells had contracted sufficiently to be robust enough to be examined histologically. Before this time point, the constructs were too insubstantial to allow sectioning. H&E staining of ESC-seeded constructs after 7 and 14 days revealed well-organized collagen fibers (Fig. 3). The majority of the ESCs exist as single cells despite being originally suspended in the gels as cell clusters and have taken on a spindle shape aligned in the direction of the static force. There are no qualitative differences in the collagen fiber alignment or cell distribution between ESC-seeded gels cultured in the presence versus the absence of TGF-β3. The constructs formed by the ESCs after 7 and 14 days are strikingly similar to those formed by adult equine tenocytes, and highly resemble artificial tendon matrices from other species described in the literature.[12–14] It should be noted, however, that tenocyte-seeded constructs tended to have a higher proportion of cells throughout the body of the construct than those seeded with ESCs, which showed an almost exclusive cell localization to the periphery of the collagen matrix.

No expression of the cartilage marker *SOX9* was detected in the 7 day cultures, and although expression was detected after 14 days it was not up-regulated in the presence of TGF-β3 compared with the control (Fig. 4a). The level of this later expression of *SOX9* is also much lower than that seen in differentiating 2D ESC cultures (with or without TGF-β3) over the same time course[5] (Fig. 4b). No positive staining for Alcian blue and Alizarin Red S was observed in any constructs formed by ESCs, in the presence or absence of TGF-β3, after 7 or 14 days (Fig. 4c).

ESCs cultured in anchored 3D collagen gels express tendon-associated proteins and genes

Immunohistochemistry demonstrated that the ESCs cultured in the 3D matrix express the tendon progenitor marker SCX (Fig. 5), which is not expressed by undifferentiated

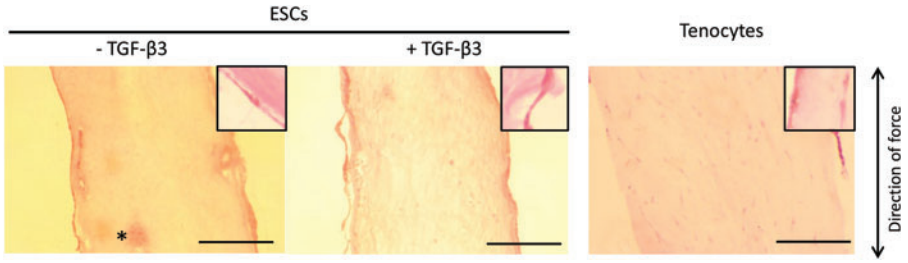

FIG. 3. Hematoxylin and Eosin histological staining of sectioned 3D constructs show a high level of collagen fiber alignment after 14 days of culture. The majority of ES cells have migrated to the periphery of the construct and aligned with the direction of the anchoring tension. Inset boxes show magnified images of spindle cell morphology. No qualitative differences can be seen between ESC-constructs cultured in the presence or absence of TGF-β3. ESC-seeded constructs show a very high degree of similarity to tenocyte-seeded constructs cultured over the same period. *Remnants of initial ESC-seeded cluster. Scale bar = 0.5 mm. Images shown are representative of three biological repeats. Color images available online at www.liebertpub.com/tea

ESCs maintained in 2D cultures[5] (Supplementary Fig. S1; Supplementary Data are available online at www.liebertpub.com/tea). After 7 days of culture, low-intensity staining is only observed in ESCs cultured in the presence of TGF-β3. However, after 14 days, SCX staining is observed in cells cultured in both the presence and absence of TGF-β3. Furthermore, positive staining was observed for the tendon-associated proteins COMP, TNMD, THBS4, and TNC. Although TNMD, THBS4, and TNC staining was mostly observed around the periphery of the constructs where the majority of the cells were located, COMP showed a more diffuse staining across the matrix. Positive staining for these proteins was observed in TGF-β3-treated and -untreated constructs at both time points analyzed. The pattern and intensity of staining was directly comparable to that seen when adult tenocytes were cultured in the same 3D system,

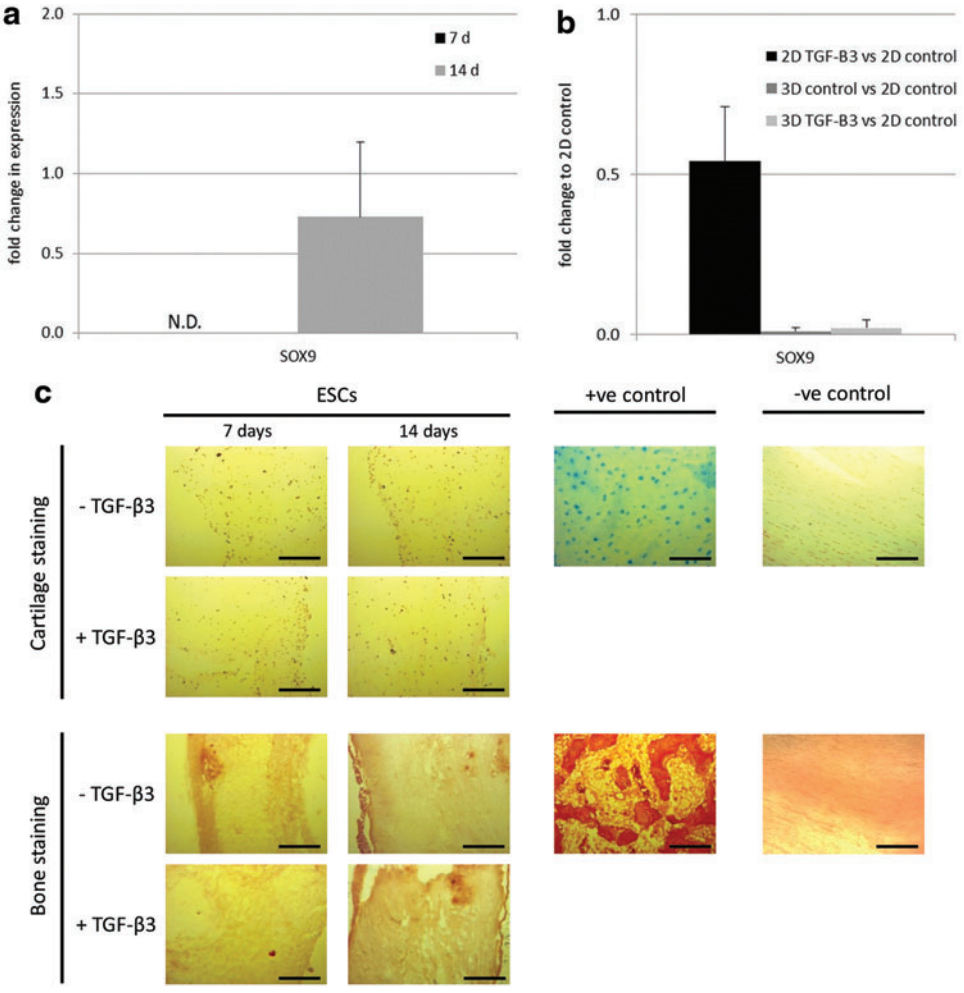

FIG. 4. **(a)** Quantitative PCR demonstrates that *SOX9* expression is only detected in 14 day 3D ESC constructs, and its expression is not up-regulated in the presence of TGF-β3. N.D., expression not detected. **(b)** Relative to spontaneously differentiating two-dimensional (2D) ESC cultures, ESCs grown in the 3D environment (both with and without TGF-β3) for 14 days inhibit the expression of *SOX9*. Error bars represent the standard error of the mean. **(c)** Alcian Blue and Alizarin Red S staining, for cartilage and bone tissue components, respectively, show no positive staining in the ESC-seeded constructs over the 14 day time course, with or without TGF-β3 treatment. Positive controls were carried out with mature equine cartilage and bone sections. Negative controls used mature equine tendon sections. Scale bar = 250 μm. Images shown are representative of three biological repeats. Color images available online at www.liebertpub.com/tea

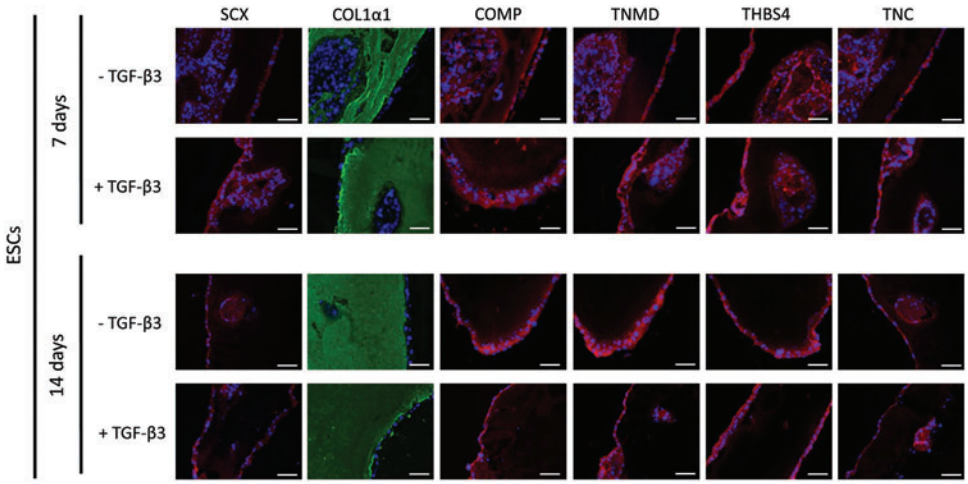

FIG. 5. Immunohistochemistry of longitudinal sections of 3D ESC-constructs shows the presence of tendon-associated proteins at both 7 and 14 days of culture. The intensity of each protein appears unaffected by the presence of TGF-β3, except for SCX, which shows stronger staining at 7 days under TGF-β3 treatment. DAPI staining of the nuclei is shown in blue. Data shown are representative of testing on three cell lines. Scale bar = 40 μm. Images shown are representative of three biological repeats. Color images available online at www.liebertpub.com/tea

which also showed no differences in the presence or absence of TGF-β3 (Supplementary Fig. S1). We were unable to find an antibody to Collagen Type I (COL1α1) that reacted only to the equine protein without cross reacting to the bovine collagen in the construct. The immunohistochemical analysis did not detect any differences in the intensity of COL1α1 staining between TGF-β3-treated and -untreated constructs at any of the time points, but showed a high level of dense collagen fiber alignment in the direction of the intrinsic force within the ESC constructs (Fig. 5).

qPCR revealed that after 3 days of 3D culture, tendon gene expression by the ESCs was often undetectable and varied greatly between replicates (data not shown). *SCX* expression was also undetectable at 7 and 14 days (with or without TGF-β3). However, TGF-β3 affected the expression of the other genes. After 7 days of culture, the presence of TGF-β3 resulted in a two-fold increase in expression of *COL1α1*, although after 14 days there was no significant difference in its expression between cells cultured in the presence versus the absence of TGF-β3 (Fig. 6). *COMP* expression was significantly increased at both 7 and 14 days in the presence of TGF-β3 compared with the untreated controls. The induction of *TNMD* at 7 days and *THSB4* at 7 and 14 days only occurred in constructs treated with TGF-β3. *TNC* expression also showed a trend for up-regulation by TGF-β3 after 7 days, although this did not reach statistical significance. Together, these data support our contraction data and suggest that although TGF-β3 may enhance the initial rate of tenocyte differentiation by ESCs, the intrinsic force of a 3D culture alone is sufficient to drive ESCs to a tenocyte fate.

Three-dimensional culture and TGF-β3 have a synergistic effect on tenocyte differentiation

To further dissect the tenogenic contribution of the 3D intrinsic force, TGF-β3 treatment, and the possible synergistic effect of both, we compared the expression of tendon-associated genes in the presence of tendon-promoting signals (3D culture and/or TGF-β3) relative to their expression in spontaneously differentiating 2D cultures[5] (Fig. 7). From this, we found that, although TGF-β3 promotes tenogenic differentiation to a significant degree in 2D ESC cultures,[5] the level of expression of tenocyte genes is much higher in untreated 3D ESC cultures (with the exception of *SCX*, which is not expressed at the time points studied in the 3D cultures). After 7 days of culture, the expression levels of *TNC*, *COL1α1*, and *COMP* are significantly higher in 3D untreated ESC

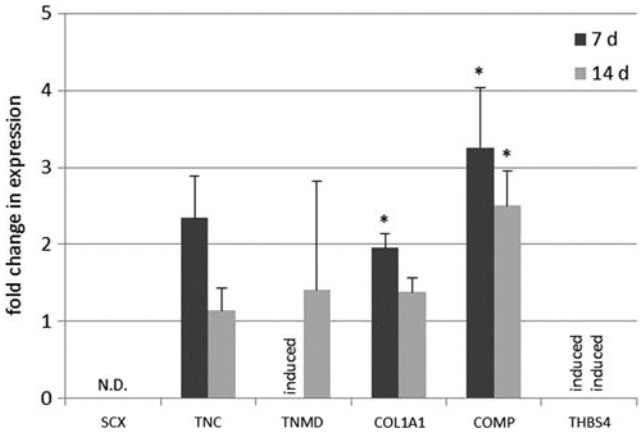

FIG. 6. Quantitative PCR of 3D ESC constructs over 7 and 14 days demonstrates that the tendon-associated genes *COL1α1* and *COMP* are significantly up-regulated at 7 days in the presence of TGF-β3 compared with untreated constructs. *COMP* expression remains significantly higher after 14 days. *TNC* also shows a trend for up-regulation in 3D ESC constructs in the presence of TGF-β3 after 7 days. The induction of *THBS4* at 7 and 14 days, and the induction of *TNMD* at 7 days, only takes place in the TGF-β3-treated constructs. Induced = expression was only detected in the cultures in the presence of TGF-β and not in the control cultures at this time point. N.D., expression not detected. $*p < 0.05$. Error bars represent the standard error of the mean.

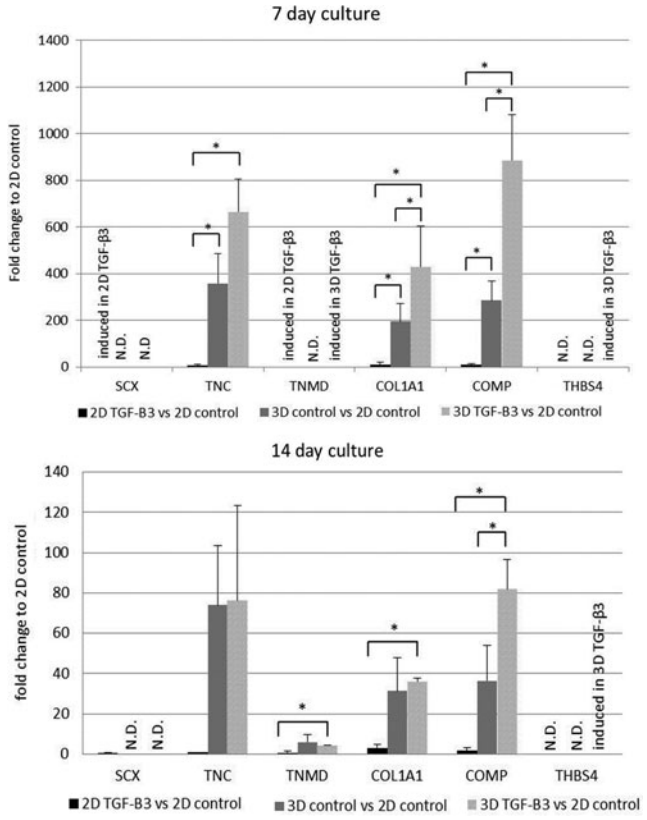

FIG. 7. Quantitative PCR analysis of tendon-associated genes in 3D ESC constructs (with and without TGF-β3) and 2D cultures in the presence of TGF-β3 displayed relative to 2D spontaneously differentiating ESC cultures. *TNC*, *COL1α1*, and *COMP* are significantly up-regulated in untreated 3D cultures compared with TGF-β3-treated 2D cultures. *COL1α1* and *COMP* show further significant up-regulation when TGF-β3 is added to the 3D constructs, whereas *TNC* shows a trend for further up-regulation in 3D culture in the presence of TGF-β3 after 7 days. *TNMD* is only induced in the presence of TGF-β3 at 7 days, whereas *THBS4* is only induced in 3D culture with TGF-β3 treatment. After 14 days, there is still a strong trend for increased tendon gene expression in 3D cultures compared with TGF-β3-treated 2D cultures, and the addition of TGF-β3 to 3D cultures results in a significant increase in the expression of *TNMD*, *COL1α1*, and *COMP*. *THBS4* again is only expressed in 3D cultures in the presence of TGF-β3. Induced = expression was only detected in the cultures in the presence of TGF-β and not in the control cultures at this time point. N.D. = Expression not detected. $*p < 0.05$. Error bars represent the standard error of the mean.

constructs than in 2D TGF-β3-treated ESCs. This increase is in the region of 200- to 400-fold higher than untreated 2D ESCs, in comparison to the 5- to 10-fold expression increase in 2D TGF-β3-treated ESCs. As stated earlier, *THBS4* is only induced in 3D cultures treated with TGF-β3 and *TNMD* is only induced in the presence of TGF-β3. By 14 days, there is still a strong trend of tendon-gene up-regulation in the untreated 3D cultures compared with the 2D TGF-β3-treated ESCs, and although the overall fold changes for *TNC*, *COL1α1*, and *COMP* are lower than those seen after 7 days of culture, they are still ~30- to 70-fold higher than baseline 2D levels.

Overall, the synergistic effect of the 3D culture system and TGF-β3 increases *TNC*, *COL1α1*, and *COMP* expression levels 400- to 900-fold over 7 days relative to untreated 2D ESC cultures, an effect significantly greater than the individual tenogenic inductions of TGF-β3 alone and 3D culture alone. By 14 days of culture, the synergistic effect is less pronounced and expression levels become comparable to the levels seen in the untreated 3D ESC cultures except in the case of *COMP*, which remains significantly higher than the untreated 3D ESC cultures. However, *TNMD*, *COL1α1*, and *COMP* levels are still significantly above those in 2D TGF-β3 cultures and *TNC* expression also shows a very strong trend for up-regulation in 3D cultures. *THBS4* expression is only detected in cultures within the 3D environment with TGF-β3 treatment.

Discussion

The developmental pathways governing tenocyte differentiation are, for the most part, still largely unknown. This is principally due to the lack of tendon-specific gene markers present throughout development and into the adult tissue. For this article, we selected a range of tendon-associated genes that when expressed together are indicative of the presence of tenocytes and, therefore, tenogenic differentiation. The genes were selected on the basis of having been previously shown to be associated with tendon tissue.[21–26] The adult tendon is primarily composed of Collagen Type I,[21] which lacks tissue specificity and is a more general component of multiple ECMs. More robust markers of tenocytes include Scleraxis (SCX), Tenomodulin (TNMD), Tenascin-C (TNC), Cartilage oligomeric matrix protein (COMP), and Thrombospondin-4 (THBS4), which are not only collectively expressed in tendons, but individually also present in other tissue types.[22–26] Developmental studies have also implicated genes such as Six1/2, Eph-A4, Eya1/2, Egr1/2, and Mohawk (Mkx),[25,27–30] although it has not been established whether these genes show temporally restricted expression in tendon tissue.

The aim of this study was to determine the effect of culturing equine ESCs in 3D collagen constructs under an intrinsic force, both with and without exogenous addition of TGF-β3, in order to understand the factors that influence the induction of tenocyte differentiation by monitoring the spatiotemporal expression of tendon-associated genes and proteins. Cultures were performed using a collagen matrix that has previously been shown to promote tendon differentiation of MSCs and inhibit osteogenic differentiation.[11]

The contraction rates of the 3D gel constructs seeded with ESCs displayed a similar pattern of contraction to constructs seeded with adult tenocytes over the 14 day experimental period. This demonstrates that the ESCs are actively migrating and manipulating the surrounding matrix in order to drive this contraction. The addition of TGF-β3, a known tenogenic signal in equine ESCs,[5] significantly increased the rate of contraction over the first 5 days compared with untreated controls. This effect of enhanced contraction by TGF-β3 was not due to differences in cell survival within the gel constructs, which remained constant between TGF-β3-treated and -untreated cultures.

Histological analysis of the constructs was possible after 7 days when they became robust enough to maintain their

form during sectioning. Regrettably, the ability to obtain construct sections during the period of increased contraction with TGF-β3 treatment was not possible due to the insubstantial nature of the gels at the early stages post set up, rendering histological and immunohistochemical analysis impossible. H&E staining of ESC seeded gels after 7–14 days revealed alignment of the collagen fibers, which does not occur in the absence of cells. The ESCs themselves, although seeded in cell clusters, migrate out from the clusters, become independently aligned to the fibers, and take on spindle morphologies, which are typical of tenocytes. This effect was also seen *in vivo* when ESCs were injected into the site of injured tendon.[5,31] Taken together, this suggests that the ESCs are undergoing differentiation within this environment in response to the intrinsic forces, taking on a tenocyte-like morphology and arranging the matrix in regular fibers that align to this force. This mimics the data from constructs seeded with adult tenocytes and, indeed, by the later time point of 14 days; the tenocyte- and ESC-seeded constructs are nearly identical to each other. The only noticeable difference was that tenocyte-seeded constructs tended to have a higher proportion of cells spaced throughout the collagen matrix compared with ESC-seeded constructs, where the majority of the cells localized to the periphery. This may reflect better migratory capacities of ESCs toward the periphery to access the nutrients in the media such as fetal bovine serum, a known stimulator of cell migration.[32] This, in turn, may explain the significantly higher survival of the ESCs than adult tenocytes after 7 days. However, it should be noted that ESCs are routinely cultured in media containing a higher percentage of fetal bovine serum than adult tenocytes, and this may have impacted cell migration and survival. Interestingly, the addition of TGF-β3, although enhancing the contraction rates of the ESC constructs until day 5, has no detectable effect on the architecture of the constructs by the 7 and 14 day time points. This leads to the conclusion that although TGF-β3 may have an early-onset positive effect of forming tendon-like tissue, the intrinsic force of the 3D environment itself elicits a positive tenogenic signal that, given sufficient time, is enough to trigger differentiation of the ESCs and remodel the construct to the same level seen in tenocyte-seeded gel constructs.

No differentiation to bone or cartilage was detected histologically in the ESC-seeded constructs. Therefore TGF-β3 under these experimental parameters does not appear to induce the production of cartilage or bone tissue. SOX9 is a well-established marker of chondrocyte differentiation[33] and although *SOX9* mRNA was detected in 3D ESC cultures, its expression was considerably lower than levels seen in 2D ESC cultures. ESCs cultured within a 3D environment may, therefore, be inhibited from developing into chondrocyte lineages. In this sense, if the intrinsic forces of a 3D environment are considered tenogenic, it may, by some mechanism, impede or even act to block the differentiation of other musculoskeletal tissues within the constructs. This has been previously seen in MSC-seeded 3D constructs, by which the exposure of MSCs to a uniaxial force alone reduced the ''stemness'' of the seeded cells and left them unable to differentiate into osteogenic lineages after retrieval from the construct.[8] The maximum amount of time that the constructs could be maintained for before tearing off the pins was 14 days, as has been previously reported.[34] This limitation makes this model unsuitable for studying the longer-term effects of tissue development, but instead can be used to further analyze early-onset ESC differentiation and the associated molecular pathways.

Immunohistochemical analysis of the constructs found that by 7 days, ESCs cultured in the 3D matrix had detectable SCX protein and that this staining was of a higher intensity in the TGF-β3-treated ESC constructs. By 14 days, however, both treated and untreated constructs showed a comparable intensity of SCX staining. This follows the contraction data showing that TGF-β3 has an early-onset propensity to drive the tenogenic differentiation of ESCs, but this effect is matched over 14 days by 3D culture alone which can also derive a tenogenic response to the same measurable degree. We also found positive staining for COMP, TNMD, THBS4, and TNC proteins in both the TGF-β3-treated and -untreated constructs by day 7 and 14, and the staining intensity showed no differences between the conditions. It is also noteworthy that the staining pattern showed little or no qualitative differences from tenocyte-seeded 3D constructs over the same time course. We, therefore, conclude that the ESCs are undergoing tenocyte differentiation in response to the 3D environment and the presence of TGF-β3.

We utilized qPCR to obtain a quantitative assessment of the expression of tendon-associated genes in the 3D ESC constructs. Interestingly, *SCX* gene expression was not detected after 7 and 14 days, despite detection of the protein. This suggests that the protein has a different rate of degradation from its mRNA.[5,35] SCX is a basic helix-loop-helix transcription factor that can directly regulate tendon-associated genes such as TNMD,[36] Collagen I,[37] and Collagen XIV.[38] It is a specific marker for tendon progenitors,[26] and mice homozygous for an *Scx* null allele have severe tendon development defects.[38] Ectopic expression of *SCX in vitro* has also been shown to induce the differentiation of MSCs into tendon progenitor cells.[39] Therefore, it is likely that *SCX* is expressed early in the differentiation of ESCs into tenocytes. Attempts were made to examine gene expression levels at 3 days, during the period of increased gel contraction in the presence of TGF-β3; however, the expression of all genes was low at this time point with large amounts of variability in their detection, which precluded getting any meaningful data.

Levels of *COL1α1* at 7 days and *COMP* at 7 and 14 days were significantly higher in the presence of TGF-β3 than untreated constructs. There was also a trend for increased *TNC* expression at 7 days. Furthermore, expression of the tenocyte markers *THBS4* and *TNMD* was only detected in the 3D environments treated with TGF-β3 at 7 days. Both of these genes are known markers of adult tendon tissue[23] and, therefore, are indicators of mature tenocytes. If TGF-β3 accelerates the differentiation process, then it follows that over the same time course mature tendon gene markers would be first detected in the TGF-β3-treated constructs.

Analysis of the TGF-β3-untreated and -treated 3D constructs showed increases in tendon gene expression when compared with 2D differentiation. Our previous work has demonstrated that TGF-β3 treatment of ESCs in 2D culture promotes tenocyte differentiation.[5] Comparing gene expression in the 3D cultures with expression in 2D cultures

highlights a striking increase in the level of tendon-associated genes in the 3D collagen environment under a static force alone. Tendon genes are expressed approximately 400-fold higher in the 3D cultures than in differentiating 2D cultures after 7 days, and remain approximately 70-fold higher after 14 days. In comparison, the presence of TGF-β3 in 2D cultures only produces approximately 10-fold increases in tendon gene expression. This shows that the 3D growth environment is a potent inducer of tenogenic lineages from ESCs and elicits a much stronger effect than the TGF-β3-triggered up-regulation of tendon genes in 2D ESC cultures. As described, TGF-β3 results in the up-regulation of tendon genes within the 3D model and by comparing these data back to 2D ESC cultures, we have shown that the synergistic effect of both the 3D environment and the presence of TGF-β3 increases the levels of gene expression approximately 900-fold higher than in 2D cultures without TGF-β3—a level much greater than TGF-β3 or the 3D environment can elicit individually. By 14 days of culture, the synergistic effect is less pronounced and expression levels become comparable to those of 3D culture alone, except in the case of *COMP* whose expression remains significantly higher. Taken altogether, these data show that the synergistic effect of TGF-β3 treatment has the greatest efficacy of accelerating tenogenic differentiation in the earlier stages of the 3D ESC cultures.

Together with the contraction data, we have demonstrated that the 3D culture model can accelerate the differentiation of ESCs into tenocytes far beyond the level of induction in 2D cultures within the same time frame, and that this induction is further strengthened by the addition of TGF-β3, which results in an earlier and stronger expression pattern of tendon-associated genes and proteins. The presence of TGF-β3 is also necessary for the induction of late-onset tenogenic markers, namely *TNMD* and *THBS4*, which were not detected in untreated 3D cultures over the early time course. The ESC 3D constructs did not develop areas of cartilage or bone tissue, and we conclude that the differentiation response is specific to tenocyte lineages. The use of 3D culture in combination with TGF-β3 can, therefore, be used to drive tendon differentiation by equine ESCs to provide a model to ultimately elucidate the molecular pathways underpinning tenocyte differentiation and enable a more targeted and regulated stem cell-based regenerative therapy in horses.

Acknowledgments

The authors would like to thank Professor Roger Smith (Royal Veterinary College) for kindly providing the COMP antibody and the Paul Mellon Foundation for funding.

Disclosure statement

No competing financial interests exist.

References

1. Dyson, S.J. Medical management of superficial digital flexor tendonitis: a comparative study in 219 horses (1992–2000). Equine vet J **36,** 415, 2004.
2. Cox, D.A. Transforming growth factor-beta 3. Cell Biol Int **19,** 357, 1995.
3. Kuo, C.K., Petersen, B.C., and Tuan, R.S. Spatiotemporal protein distribution of TGF-betas, their receptors, and extracellular matrix molecules during embryonic tendon development. Dev Dyn **237,** 1477, 2008.
4. Pryce, B.A., *et al.* Recruitment and maintenance of tendon progenitors by TGF beta signalling are essential for tendon formation. Development **136,** 1351, 2009.
5. Barsby, T., and Guest, D. Transforming growth factor beta3 promotes tendon differentiation of equine embryo-derived stem cells. Tissue Eng Part A **19,** 2156, 2013.
6. Chan, K.-M., *et al.* Expression of transforming growth factor beta isoforms and their roles in tendon healing. Wound Repair Regen **16,** 399, 2008.
7. Chen, C.H., *et al.* Tendon healing *in vivo*: Gene expression and production of multiple growth factors in early tendon healing period. J Hand Surg **33A,** 2008.
8. Kapacee, Z., *et al.* Synthesis of embryonic tendon-like tissue by human marrow stromal/mesenchymal stem cells requires a three-dimensional environment and transforming growth factor β3. Matrix Biol **29,** 668, 2010.
9. Lorda-Diez, C.I., Montero, J.A., Martinez-Cue, C., Garcia-Porrero, J.A., and Hurle, J.M. Transforming growth factors beta (TGF-betas) coordinate cartilage and tendon differentiation in the developing limb mesenchyme. J Biol Chem **284,** 29988, 2009.
10. Engler, A.J., Sen, S., Sweeney, H.L., and Discher, D.E. Matrix elasticity directs stem cell lineage specification. Cell **126,** 677, 2006.
11. Sharma, R.I., and Snedeker, J.G. Biochemical and biomechanical gradients for directed bone marrow stromal cell differentiation toward tendon and bone. Biomaterials **31,** 7695, 2010.
12. Kuo, C.K., and Tuan, R.S. Mechanoactive tenogenic differentiation of human mesenchymal stem cells. Tissue Eng Part A **14,** 1615, 2008.
13. Chen, X., *et al.* Stepwise differentiation of human embryonic stem cells promotes tendon regeneration by secreting fetal tendon matrix and differentiation factors. Stem Cells **27,** 1276, 2009.
14. Chen, X., *et al.* Force and scleraxis synergistically promote commitment of human ES cells derived MSCs to tenocytes. Sci Rep **2,** 977 2012.
15. Yang, G., *et al.* Enhancement of tenogenic differentiation of human adipose stem cells by tendon-derived extracellular matrix. Biomaterials **34,** 9295, 2013.
16. Altman, G.H., *et al.* Cell differentiation by mechanical stress. FASEB J **16,** 270, 2002.
17. Kishore, V., Bullock, W., Sun, X., Van Dyke, W.S., and Akkus, O. Tenogenic differentiation of human MSCs induced by the topography of electrochemically aligned collagen threads. Biomaterials **33,** 2137, 2012.
18. Li, X., Zhou, S.G., Imreh, M.P., Ahrlund-Richter, L., and Allen, W.R. Horse embryonic stem cell-like cell lines from the proliferation of inner cell mass cells. Stem Cells Dev **15,** 523, 2006.
19. Guest, D.J., and Allen, W.R. Expression of cell surface antigens and embryonic stem cell pluripotency genes in equine blastocysts. Stem Cells Dev **16,** 789, 2007.
20. Livak, K.J., and Schmittgen, T.D. Analysis of relative gene expression data using real time quantitative PCR and the $2^{-\text{deltadeltaCT}}$ Method. Methods **25,** 402, 2001.
21. Wang, J.H., Guo, Q., and Li, B. Tendon biomechanics and mechanobiology - a minireview of basic concepts and recent advancements. J Hand Ther **25,** 133, 2012.

22. Docheva, D., Hunziker, E.B., Fassler, R., and Brandau, O. Tenomodulin is necessary for tenocyte proliferation and tendon maturation. Mol Cell Biol **25,** 699, 2005.
23. Jelinsky, S.A., Archambault, J., Li, L., and Seeherman, H. Tendon-selective genes identified from rat and human musculoskeletal tissues. J Orthop Res **28,** 289, 2010.
24. Fang, C., *et al.* Molecular cloning, sequencing, and tissue and developmental expression of mouse cartilage oligomeric matrix protein (COMP). J Orthop Res **18,** 593, 2000.
25. Edom-Vovard, F., and Duprez, D. Signals regulating tendon formation during chick embryonic development. Dev Dyn **229,** 449, 2004.
26. Schweitzer, R., *et al.* Analysis of the tendon cell fate using Scleraxis, a specific marker for tendons and ligaments. Development **128,** 3855, 2001.
27. D'Souza, D., and Patel, K. Involvement of long- and short-range signalling during early tendon development. Anat Embryol **200,** 367, 1999.
28. Lejard, V., *et al.* EGR1 and EGR2 involvement in vertebrate tendon differentiation. J Biol Chem **286,** 5855, 2011.
29. Ito, Y., *et al.* The Mohawk homeobox gene is a critical regulator of tendon differentiation. Proc Natl Acad Sci U S A **107,** 10538, 2010.
30. Berthet, E., *et al.* Smad3 binds Scleraxis and Mohawk and regulates tendon matrix organization. J Orthop Res **31,** 1475, 2013.
31. Guest, D.J., Smith, M.R.W., and Allen, W.R. Equine embryonic stem-like cells and mesenchymal stromal cells have different survival rates and migration patterns following their injection into damaged superficial digital flexor tendons. Equine Vet J **42,** 636, 2010.
32. Haas, V.R., Santos, A.R., Jr. and Wada, M.L. Behaviour of fibroblastic cells cultured in collagen I using the sandwich technique. Cytobios **106 Suppl 2,** 255, 2001.
33. Akiyama, H., and Lefebvre, V. Unraveling the transcriptional regulatory machinery in chondrogenesis. J Bone Miner Metab **29,** 390, 2011.
34. Nirmalanandhan, V.S., *et al.* Combined effects of scaffold stiffening and mechanical preconditioning cycles on construct biomechanics, gene expression, and tendon repair biomechanics. Tissue Eng **15,** 2103, 2009.
35. Guo, Y., *et al.* How is mRNA expression predictive for protein expression? A correlation study on human circulating monocytes. Acta Biochem Biophys Sin **40,** 426, 2008.
36. Shukunami, C., Takimoto, A., Oro, M., and Hiraki, Y. *Scleraxis* positively regulated the expression of *tenomodulin*, a differentiation marker of tenocytes. Dev Biol **298,** 234, 2006.
37. Lejard, V., *et al.* Scleraxis and NFATc regulate the expression of the Pro-alpha1 (I) collagen gene in tendon fibroblasts. J Biol Chem **282,** 17665, 2007.
38. Murchison, N.D., *et al.* Regulation of tendon differentiation by scleraxis distinguishes force-transmitting tendons from muscle-anchoring tendons. Development **134,** 2697, 2007.
39. Alberton, P., *et al.* Conversion of human bone-marrow derived mesenchymal stem cells into tendon progenitor cells by ectopic expression of scleraxis. Stem Cells Dev **21,** 846, 2012.

Address correspondence to:
Debbie J. Guest, BSc, PhD
Animal Health Trust
Centre for Preventive Medicine
Lanwades Park, Kentford
Newmarket, Suffolk CB8 7UU
United Kingdom

E-mail: debbie.guest@aht.org.uk

Received: July 26, 2013
Accepted: March 10, 2014
Online Publication Date: April 21, 2014