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Tenogenic differentiation of human embryonic stem cells

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Abstract

Tendon healing is complex to manage because of the limited regeneration capacity of tendon tissue; stem cell-based tissue engineering approaches may provide alternative healing strategies. We sought to determine whether human embryonic stem cells (hESC) could be induced to differentiate into tendon-like cells by the addition of exogenous bone morphogenetic protein (BMP)12 (growth differentiation factor(GDF)7) and BMP13 (GDF6). hESC (SHEF-1) were maintained with or without BMP12/13 supplementation, or supplemented with BMP12/13 and the SMAD signalling cascade blocking agent, dorsomorphin. Primary rat tenocytes were included as a positive control in immunocytochemistry analysis. A tenocyte-like elongated morphology was observed in hESC after 40-days continuous supplementation with BMP12/13 and ascorbic acid. These cells displayed a tenomodulin expression pattern and morphology consistent with that of the primary tenocyte control. Analysis of tendon-linked gene transcription in BMP12/13 supplemented hESC demonstrated consistent expression of *COL1A2*, *COL3A1*, *DCN*, *TNC*, *THBS4*, and *TNMD* levels. Conversely, when hESCs were cultured in the presence of BMP12/13 and dorsomorphin *COL3A1*, *DCN*, and *TNC* gene expression and tendon matrix formation were inhibited. Taken together, we have demonstrated that hESCs are responsive to tenogenic induction via BMP12/13 in the presence of ascorbic acid. The directed *in vitro* generation of tenocytes from pluripotent stem cells may facilitate the development of novel repair approaches for this difficult to heal tissue.

Introduction

Tendon is a major component of the musculoskeletal system (1) playing a vital role in force transmission between bone and muscle and enhancing joint stability (2). Acute trauma, overuse and ageing can lead to tendon injuries (3,4). Current treatments have limited capacity to achieve successful tendon healing since the tissue is poorly vascularized, and scar tissue or fibrous adhesions often develop during the healing process (5). Treatment can involve many different types of surgical intervention, such as xenograft or allograft to treat large tendon defects, but potential problems with this method (such as foreign body reaction) can occur (3). A lack of adequate strategies for tendon repair has led to the development of engineered replacement tendon tissue for use in surgical implantation (4). Stem cell based intervention may provide new strategies for tendon repair. Embryonic stem cells (hESCs) are derived from human blastocysts and due to telomerase activity self-renew indefinitely. Effectively the cells are immortal in culture, a property unique amongst the cell types with potential in regenerative medicine applications, providing cells in unlimited numbers. Furthermore, they are pluripotent and accordingly can differentiate into cells of all three embryonic germ layers, namely mesoderm, ectoderm and endoderm (6) conferring upon them potential across the whole field of regenerative medicine. Consequently,they are favoured for tissue engineering in therapeutic applications both *in vitro* and *in vivo* (7–9).

hESC are considered a valuable resource due to their intrinsic plasticity in differentiation capacity. However, there is a surprising paucity of research describing *in vitro* directed tenogenic differentiation of hESC. To date reports have favoured the engineering and rolling of

cell sheets derived from connective tissue growth factor (CTGF)-supplemented hESC-derived mesenchymal stem cells (hMSC) (10,11). These sheets then progress to display tendon-like morphological appearances, the expression of genes including *SCX*, *COL3A1*, and *DCN*, but not *TNMD* (12).

Bone morphogenic proteins 12 and 13 (BMP12/13, also known as GDF7/6) are members of the TGF-β superfamily, and have individually been shown to play important roles in chemotaxis, proliferation, matrix synthesis, and cell differentiation (13–17). BMP12 and/or BMP13 promote tendon repair in rats and sheep (13,18,19). In addition, BMP12 has been reported to induce the *in vitro* and *in vivo* tenogenesis of MSCs (derived from a wide variety of sources including bone marrow, synovial fluid, adipose tissue) in dog, mouse, rat, rhesus monkey, human, horse and chicken (13,20–28). There are also reports describing a role for BMP12 in tenogenic differentiation of tendon stem cells derived from rat (29). However, to our knowledge, there are no descriptions of the use of BMP12 and/or BMP13 to direct differentiation of hESCs into tendon-like cells. In this study, we investigated whether hESCs could differentiate into tenocyte-like cells when supplemented with BMP12/13 and ascorbic acid (AA). Further, we sought to determine whether SMAD signalling was implicated in BMP12/13 induced changes via inhibition of the SMAD pathway, or whether other signalling cascades were involved in the hESC tenogenic process.

Materials and Methods

Culture of Primary Rat tenocytes and hESCs

Primary tenocytes were isolated from 8 week old Sprague-Dawley rats. The Achilles tendon was isolated, extracted, placed into a dry petri dish and allowed to adhere for 3 hours. Media was added, and the explant cultured for 7 days allowing for tenocyte migration and expansion in ambient oxygen (21% O_2)/5% CO_2 in high glucose Dulbecco's Modified Eagle Medium (DMEM, Lonza) supplemented with 10% fetal bovine serum (FBS, Lonza), 1% non-essential amino acids (NEAA, Lonza), 1% L-glutamine (Lonza) and 1% penicillin, streptomycin and amphotericin B (PSA, Lonza). After 7 days, the rat tenocytes were washed twice with phosphate buffered saline (PBS, Lonza), trypsinised (1% Trypsin/EDTA (Lonza)/PBS solution), centrifuged for 3 minutes (200g), re-seeded into two T-25 culture flasks, and cultured until 70% confluent. Once 70% confluent, the tenocytes were again trypsinised, and re-seeded at 0.5 x10^3 cells/cm^2 onto 6 well plates and cultured for a further 48 hours before being fixed for immunocytochemistry.

hESC were cultured according to the Matrigel substrate and mouse embryonic fibroblast (MEF)-conditioned media (CM) protocol (30). CM was obtained by placing Knockout (KO)-DMEM (Gibco), 20% Serum Replacent (SR) (Gibco), 1% NEAA, 1% L-Glutamine, 4ng/ml bFGF (Peprotech) and 50mM β-mercaptoethanol (Gibco) for 24 hours on 50-60% confluent MEFs. After 24 hours, the culture medium was removed, and 4ng/ml bFGF added prior to filtration. SHEF-1 hESCs were cultured in either ambient (21% O_2) or physiological, low-oxygen (2% O_2) conditions. Differentiation was performed in the 2% O_2 condition only.

Tenogenic differentiation of hESCs

SHEF-1 cells were seeded into 6-well plates at (2×10^3 cells/cm^2) in CM. After 24 hours CM was removed and replaced with differentiation media which consisted of KO-DMEM, 10% FBS, 1% NEAA, 1% L-glutamine, 50mM β-mercaptoethanol and 10mM AA (Sigma) with or without BMP12 (R & D Systems) and BMP13 (Peprotech) both at 10ng/ml. To evaluate the role of SMAD signalling in tenogenic differentiation, hESC were seeded and differentiated as above, with the exception that during differentiation hESC were further supplemented with 1μM dorsomorphin (31,32) (Sigma).

Reverse transcription PCR (RT-PCR)

RNA was collected from undifferentiated hESC at Day 0, and subsequently at Days 5, 10 and 20 in the presence of differentiation media in both 2% O_2 and 21% O_2. In addition to above RNA was collected from hESC in differentiation media supplemented with either BMP12/13 or BMP12/13/dorsomorphin supplementation at days 5, 10, 20 and 40. RNA was collected by first washing with PBS followed by the addition of cell lysis buffer (Qiagen), scraping, collection, and homogenisation with a QIAshredder spin column (Qiagen). RNA extraction was performed with the RNeasy Mini kit (Qiagen) following manufacturer's instructions. RT-PCR was performed with Superscript III One-Step HiFi RT-PCR kit (Invitrogen) again following manufacturer's instructions. The genes analysed were representative of a tenocyte-like phenotype and were *COL1A2*, *COL3A1*, *DCN*, *TNC*, *TNMD* and *THBS4* (33), primers used are shown in Table 1. *GAPDH* level was used as an internal control. Electrophoresis was

performed on 2% agarose gel (Gibco) at 100V for 1 hour. Gels were imaged on the Syngene Gel UV illuminator.

Immunocytochemical analysis

Cells were fixed in 95% methanol for 15 minutes before being washed with PBS. Cells were permeabilised with 0.5% Triton-X for 5 minutes, washed with PBS, and incubated in a 3% albumin solution (Sigma) for 1 hour at room temperature. Primary tenomodulin antibody (C-terminus) (SC98875, Santa Cruz Biotechnologies, Germany, 1:500 dilution in PBS) was then added to each well followed by a 30 minute incubation at 37^{o}C and PBS washes. Secondary antibody (SC2090 Santa Cruz Biotechnologies, Germany, 1:500 dilution in PBS) was then added to the appropriate wells followed by a further incubation at 37^{o}C for 30 minutes, PBS washes, and DAPI (1:500 dilution, Sigma) counterstaining. Images were captured via appropriate filter sets on Nikon Eclipse T1 microscope using a Nikon DSi 1 camera.

Histological analysis

Alcian Blue staining

Cells were fixed at Days 0, 2, 5, 10, 20 and 40 using 95% methanol for 15 minutes followed by PBS washes. Cells were then stained with Alcian blue (A3157-10G, Sigma Aldrich, UK) for 24 hours at room temperature on an R100 Rotateck shaker (Luckham). After 24 hours, the Alcian blue solution was aspirated, and each well washed with sterile double filtered dH_2O. Once all the excess Alcian blue stain had been removed, the plates were dried at room temperature before imaging on Nikon Eclipse TD100 inverted microscope.

153 *Masson's Trichrome Staining*

154 Wells were fixed at days 0, 2, 5, 10, 20 and 40 using 95% methanol for 15 minutes and washed
155 twice with PBS. PBS was aspirated from the wells and Bouin's Solution (Sigma) added to
156 completely cover the well base before being placed on the R100 Rotateck shaker (Luckham) for
157 24 hours. After 24 hours Bouin's solution was aspirated and each well washed with double
158 filtered H_2O to remove residual Bouin's solution. The wells were then counterstained with
159 Haematoxylin (Sigma) for 5 minutes before washing as before and applying Biebrich
160 Scarlet-Acid Fuschin solution (Sigma) for 5 minutes, washing again, and incubating in fresh
161 phosphotungstic/phosphomolybdic acid solution (PT/PMA) (Sigma, 25% ($^{v}/_{v}$) PT, 25% PMA
162 and 50% dH_2O) for 5 minutes at room temperature. Following incubation in the PT/PMA
163 solution and its removal aniline blue solution (Sigma) was added, and the samples incubated at
164 room temperature for 5 mins before removal and incubation in 1% acetic acid at room
165 temperature for a further for 2 minutes before washing again and air drying for 24 hours.
166 Images were collected on a Nikon Eclipse TD100 inverted microscope with a. Canon EOS
167 400D camera.

168 *Quantification*

169 Staining intesity of Alcian blue and Masson's Trichrome stained images was semi-quantified
170 using ImageJ (34)[31]. All images were acquired at low magnification with identical
171 microscope and camera settings and were acquired from the centre of stained wells to avoid
172 user bias. To ensure that only regions positively stained with Alcian blue were considered,
173 RGB images were first colour separated using the ImageJ colour deconvolution algorithm
174 reveloped by Ruifrok and Johnston, with colour vectors determined by region of interest as

previously described (35)[32]. For Masson's trichrome staining, total image intensity was determined.

Statistical Analysis

The significance of difference between groups (n=6 per group) was determined by one-way ANOVA single factor one-tailed comparison analysis. A p value less than 0.05 was considered to indicate statistical significance. Data are presented as mean ± standard deviation (SD). All statistical analysis was performed using Minitab® 16 (Minitab Inc., Pennsylvania, USA).

Results

BMP12/13 stimulated the expression of tendon-linked gene expression in hESCs

SHEF-1 cells cultured in a 21% O_2 environment in differentiation media without BMP supplementation showed continued expression of *GAPDH*, *COL1A2*, and *TNC* over 20 days (Figure 1, Left panel). *COL3A1* and *DCN* expression was apparent by Day 10 and thereafter whereas *THBS4* displayed sequential downregulation over the 20 day timecourse. *TNMD* expression was not detected. Similarly, SHEF-1 cells cultured in 2% O_2 environment in differentiation media without BMP supplementation again showed continued expression of *GAPDH*, *COL1A2* and *TNC* over 20 days (Figure 1, Right panel). *TNMD* expression was noted on Day 5 only and *COL3A1* and *DCN* on Day 10. In contrast to the observed expression pattern in 21% O_2, *THBS4* underwent sequential upregulation of expression in 2% O_2.

SHEF-1 treated with BMP12/13 over 40 days in 2% O_2 resulted in continuous expression of *GAPDH*, *COL1A2*, *COL3A1*, *TNC*, and *THBS4*. *DCN* underwent an apparent upregulation over the first 20 days while *TNMD* expression was maintained to Day 20 and reduced thereafter (Figure 2A Left panel). Differentiation media supplemented with both BMP12/13 and dorsomorphin showed several distinct differences when compared to BMP12/13 supplemented differentiation media (Figure 2A). *COL3A1*, *DCN*, and *TNC* all underwent substantial downregulation of expression, whereas *COL1A2*, *THBS4*, and *TNMD* transcripts displayed sustained expression throughout the timecourse.

BMP-12/13 induced tenomodulin expression in hESCs

SHEF-1 cells cultured in BMP12/13 supplemented differentiation media displayed little or no tenomodulin protein expression over the first 20 days (Figure 2C). However, by Day 40 differentiated cells displayed a distinct tenomodulin staining pattern (Figure 2C), consistent with the synapsing observed with the rat tenocyte positive control (Figure 2B). The addition of dorsomorphin to BMP12/13 supplemented differentiation media resulted in an absence of observable tenomodulin staining over the timecourse (Figure 2C).

Histological staining and colorimetric quantification

Alcian blue

Tendon matrix is comprised primarily of collagen alongside a number of other matrix molecules including glycosaminoglycans (GAGs) (33). We next sought to determine the role of BMP12/13 in altering matrix composition towards a tendon-like GAG-rich composition. The histological stain Alcian blue revealed strong staining after 40 days differentiation vs. control cultures (Figure 3A). Visually Alcian blue positive regions appeared to associate into long, string-like, condensations which appeared to connect with each other. Primary rat tenocyte cultures (images included for observation) did not display histologically detectable GAG deposition in controls or in response to BMP12/13 supplementation and were therefore not quantified. Over 40 days untreated control hESC displayed an approximate 21% increase in positive labelling whereas samples incubated with BMP12/13 registered a 35% increase (Figure 4A). This indicated a spontaneous deposition of GAGs in control hESC cultures that was significantly augmented by BMP12/13 supplementation. Dorsomorphin addition to control

hESC cultures resulted in a complete blockage of GAG deposition, while its addition in the presence of BMP12/13 resulted in a 26% increase in GAG deposition over 40 days which was comparable to untreated control cells (Figure 4A).

Masson's Trichrome

The primary component of tendon matrix is collagen. Masson's trichrome is a convenient stain in the identification of collagen deposition. Differentiated hESCs revealed distinct cord-like patterning of collagen deposition after 40 days supplementation with BMP12/13 (Figure 3B). In contrast, control unsupplemented cultures displayed a diffuse faint relatively ubiquitous patterning. Primary rat tenocytes, again included for observation, displayed evidence of a distinct pattern of collagen deposition when compared to differentiated hESC. Over 40 days BMP12/13 supplementation resulted in a 51% increase in collagen deposition vs. 23% for control cultures (Figure 4B). Conversely dorsomorphin supplementation of control cells resulted in a complete block on collagen deposition which was only marginally improved to a 10% increase in the presence of BMP12/13.

Discussion

In vitro tenogenesis is challenging, and the development of simple protocols for its induction will improve our understanding of tendon biology and the development of future therapies for tendon treatment. This study demonstrated, for the first time, that a growth supplement cocktail containing BMP12, BMP13 and AA can induce hESC *in vitro* tenogenic differentiation under physiologically normoxic (2% O_2) conditions. Stable transcription of tendon-linked and specific genes was observed alongside deposition of a tendon-like matrix and elongated, synapsing, cells with concurrent tenomodulin expression. This represents a forward step in tenogenesis studies and will facilitate the generation of enhanced *in vitro* studies.

The definition of a tenocyte is surprisingly complex. The most basic measure is cell phenotype where the tenocyte is reported frequently as being a long elongated cell which forms cell-cell connections via synapsing (36,37). Frequently panels of gene expression are used as an indicative phenotype measure. These can include the α-chains of collagens type I and III, *DCN*, *TNC*, and *SCX*, amongst others (33). In this study, we adopted *TNMD* and *THBS4*, alongside some of the above, following from the findings of Jelinsky et al (12). In their microarray based studies, they identified *TNMD* and *THBS4* expression as best fitting the definition of tendon tissue specific in both human and rat tissue. Similar to their study no expression of *SCX* was noted. We also adopted cellular expression of the tenomodulin protein in association with synaptic linkage between cells as a measure of tenogenesis (38,39). A combined definition, drawn from previous publications, of a tenocyte could therefore be an elongated, synaptic, cell which expresses the genes *TNMD* and *THBS4* and displays positive labelling for the

tenomodulin protein in association with cell-cell synapses. In agreement with this, the combined addition of BMP12, BMP13, and AA, to a basic hESC differentiation media resulted in a controlled hESC differentiation towards a tenogenic lineage.

There are relatively few reports of supplement-directed tenogenic differentiation which allow comparisons to be made. *SCX* expressing, endogenous or ectopic, hESC-MSCs or hESC-Connective Tissue Progenitors (CTPs) were allowed to be become confluent before being rolled into a sheet and mechanically conditioned for application in an *in vivo* repair model (10,11,40,41). Histology and mechanical properties of *in vitro* and *in vivo* tissue was consistent with tendon, but tendon-linked marker gene expression was either lost after 2 weeks *in situ* or not explored post-transplant and suboptimal regeneration was frequently observed.

BMP12 and BMP13 signalling are transduced by the BMP Type Ia receptor via receptor-regulated SMADS (SMAD1, 5 and 8) association with the common mediator, SMAD4, followed by complex translocation into the nucleus to activate gene transcription (20,29,42–45). BMP signaling has been suggested to be inhibitory to tendon development by decreasing the pool of available tendon progenitor cells and restricting tendon-linked gene expression (40). In this instance, and in agreement with studies documenting an association of BMP12 and/or 13 with tenogenesis (13,18,19,21–29,44), we noted that BMP12 and BMP13 supplementation was required for maintenance of tendon-specific gene expression including *TNMD* and *THBS4*. Berasi *et al* similarly found sustained expression of *THSB4* in response to BMP12/13 supplementation in ectopic tissue in a rat model and a mouse mesenchymal cell

line with no evidence of SMAD1, 5 and 8 activation (46). We noted that dorsomorphin, an inhibitor of BMP signaling, did not inhibit transcription of *TNMD* or *THBS4*, but did inhibit *COL3A1*, *DCN*, and *TNC* to some extent. This is suggestive of an alternative, BMP-signalling independent, control of tendon-specific gene expression. It is also notable that although *TNMD* gene expression was maintained in the presence of dorsomorphin the protein was virtually undetectable via immunofluorescence, indicating the likelihood of a BMP-signalling driven translational machinery or key factor in post-translational stability. A deficiency in extracellular structure or matrix composition was also apparent following on from dorsomorphin treatment, with significant reduction in matrix-associated GAG, collagen and elastin. Taken together, these data indicate a complex scenario of BMP signaling requirements in the development and maintenance of tendon gene expression and tendon tissue.

In this study, we have demonstrated that hESCs are responsive to tenogenic induction via BMP12/13 and ascorbic acid supplementation at 2% O_2. However, the mechanisms by which BMP12/13 maintain tendon-linked and tendon-specific gene expression and histology remain unclear, but appear to dissociate into BMP-dependent (*COL3A1*, *DCN*, *TNC*, tenomodulin immunofluorescence, tendon-like matrix) and BMP-independent (*TNMD* and *THBS4*). These results will help provide greater insight into BMP12/13 driven tenogenesis of hESC and new directions of exploration in the design of hESC based treatments for tendon healing.

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Author Disclosure Statement

No competing financial interests exist.

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454

455 Table 1. Tendon-linked gene expression panel.

456

Gene		Primer (5'-3')	Annealing Temp (^{o}C)	Amplicon Size (bp)
COL1A2	F	GACTTTGTTGCTGCTTGC	50	242
	R	CAAGTCCAACTCCTTTTCC		
COL3A1	F	AAGGACACAGAGGCTTCG	51	210
	R	CTGGTTGACCATCAATGC		
TNMD	F	GCACTGATGAAACATTGG	47	274
	R	ATCCAATACATGGTCAGG		
THBS4	F	CCCCAGGTCTTTGACCTTCTCCC	59	245
	R	ACCTTCCCATCGTTCTTCAGGT		
TNC	F	AAGAGCATTCCTGTCAGC	50	217
	R	CAGTTTGCCGGTAAGAGG		
DCN	F	CTGCTTGCACAAGTTTCC	48	372
	R	TTCCAACTTCACCAAAGG		
GAPDH	F	GAGTCAACGGATTTGGTCGT	55	225
	R	GATCTCGCTCCTGGAAGATG		

457 Gene names, primer pair sequences, annealing temperatures, and expected amplicon sizes are

458 shown.

459

460

Figure 1

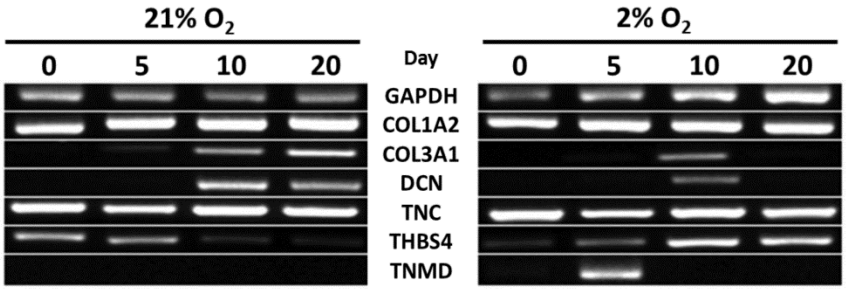
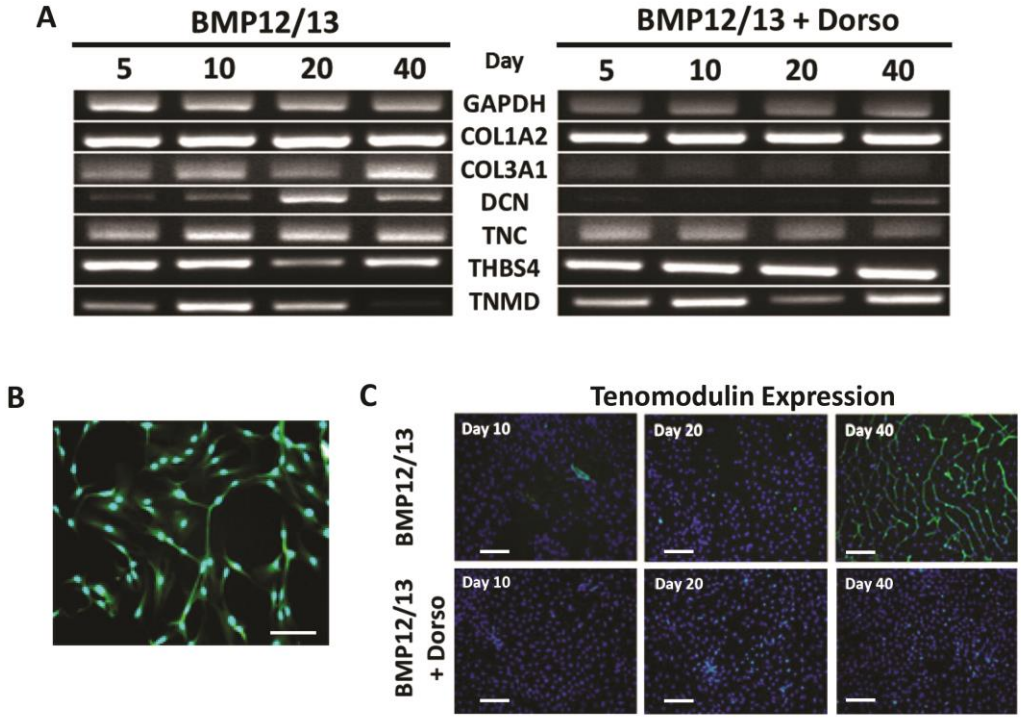


Figure 1. Tendon-linked gene expression in spontaneously differentiated hESC. Expression of RT-PCR amplified tendon-linked genes including *COL1A2*, *COL3A1*, *DCN*, *TNC*, *THBS4*, and *TNMD* is shown. *GAPDH* is included as an internal control. Primer sequences used are described in Table 1. The left-hand and right-hand panels indicate hESC spontaneously differentiating in 21% O_2 and 2% O_2, respectively, over days 0, 5, 10 and 20.

Figure 2



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468

469 Figure 2. BMP12/13 supplementation and 2% O_2 culture promotes stable tenomodulin
470 expression. A) RT-PCR amplification of the tendon-linked genes described in Figure 1. The
471 left-hand and right-hand panels indicate hESC differentiating in 2% O2 with media
472 supplemented with BMP12/13 or BMP12/13 plus dorsomorphin, respectively at days 5, 10, 20,
473 and 40. B) Immunofluorescence detection of characteristic tenomodulin protein expression in
474 primary rat tenocytes. Tenomodulin is green, DAPI (nuclei) is blue. C) Immunofluorescence of
475 fixed samples paired to A). Colours as described in B). Scale bar indicates 100µm.
476

Figure 3

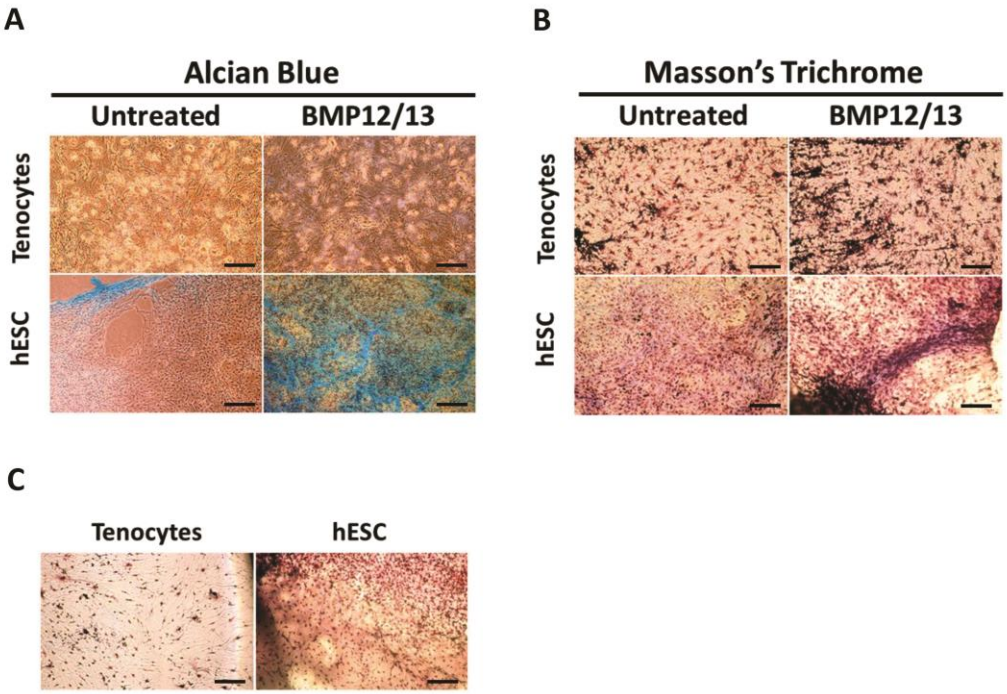

Figure 3. Matrix compositional changes induced by BMP12/13 supplementation. A) Primary rat tenocytes (Top panels) and hESC (Bottom panels) with and without BMP12/13 supplementation. Samples were fixed and stained with Alcian blue after 40 days in continuous culture without passaging. B) Samples matched to 3A) fixed and stained with Masson's Trichrome. C) Representative image from BMP12/13 supplemented hESC (right hand panel) indicated shared morphological features with primary rat tenocytes (left hand panel). Scale bar indicates 200µm.

Figure 4

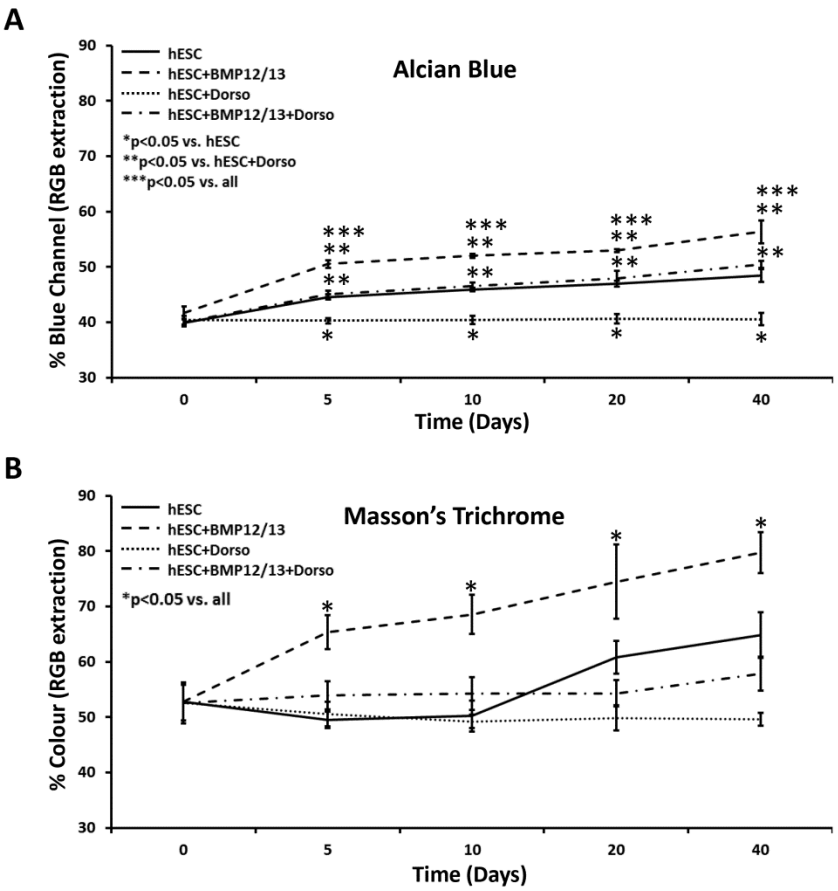

Figure 4. Dorsomorphin blocks BMP12/13 supplementation-induced matrix deposition in hESC. A) ImageJ driven analysis of Alcian blue-stained hESC differentiation over 40 days. Y-axis indicates % Blue Channel (RGB extraction) of randomly selected fields of view. X-axis indicates Time (days). Solid line indicates hESC, dotted line indicates hESC+dorsomorphin (Dorso), dashed line indicates hESC + BMP12/13, and hatched line indicates hESC + BMP12/13+Dorso. * indicates $p<0.05$ vs. hESC, ** indicates $p<0.05$ vs. hESC+Dorso, *** indicates $p<0.05$ vs. all. B) ImageJ driven analysis of Masson's Trichrome-stained hESC over 40 days differentiation. Y-axis indicates % Colour (RGB extraction) of randomly selected fields of view. X-axis indicates Time (days). Legend labelling is consistent with (4A) above. Error bars indicate standard deviations. n=5 at each time point.

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