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# Tenogenic Differentiation of Human Embryonic Stem Cells

Dale, Tina P.; Mazher, Shazia; Webb, William R.; Zhou, Jing; Maffulli, Nicola; Chen, Guo Qiang; El Haj, Alicia J.; Forsyth, Nicholas R.

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1 **Tenogenic differentiation of human embryonic stem cells**

2

3 Tina P Dale, PhD [(1)], Shazia Mazher, BSc [(1)], William R Webb, PhD [(1)], Jing Zhou, MSc [(2)],

4 Nicola Maffulli, MD, PhD [(3)], Guo-Qiang Chen, PhD [(2)], Alicia J El Haj, PhD [(1)] and Nicholas R

5 Forsyth, PhD[(1,4)]

6

7 [(1)] Guy Hilton Research Centre, Keele University, Thornburrow Drive, Stoke-on-Trent ST4

8 7QB, UK.

9 Tel. +44 (0) 1782 674 087

10 Dr Tina Dale – t.p.dale@keele.ac.uk

11 Ms Shazia Mazher - shaziamazher452@hotmail.com

12 Dr William R Webb - william_r_webb@sky.com

13 Prof Alicia J El Haj – a.j.el.haj@keele.ac.uk

14 Prof Nicholas R Forsyth – n.r.forsyth@keele.ac.uk

15

16 [(2)] School of Life Science, Tsinghua University, Beijing, 100084, China.

17 Tel: +86 10 62783844

18 Dr Jing Zhou - jingzhou2010@gmail.com

19 Professor Guo-Qiang Chen - chengq@mail.tsinghua.edu.cn

20

21 [(3)] Centre for Sport & Exercise Medicine, Queen Mary, University of London, UK.

22 + 44 20 8223 8839

23 n.maffulli@qmul.ac.uk

24 (4) Corresponding author

25

26 **Abstract**

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

46

47 **Introduction**

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

65

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

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

73

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

88 **Materials and Methods**

89 ***Culture of Primary Rat tenocytes and hESCs***

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

102

103 hESC were cultured according to the Matrigel substrate and mouse embryonic fibroblast
104 (MEF)-conditioned media (CM) protocol (30). CM was obtained by placing Knockout
105 (KO)-DMEM (Gibco), 20% Serum Replacent (SR) (Gibco), 1% NEAA, 1% L-Glutamine,
106 4ng/ml bFGF (Peprotech) and 50mM β-mercaptoethanol (Gibco) for 24 hours on 50-60%
107 confluent MEFs. After 24 hours, the culture medium was removed, and 4ng/ml bFGF added
108 prior to filtration. SHEF-1 hESCs were cultured in either ambient (21% $O_2$) or physiological,
109 low-oxygen (2% $O_2$) conditions. Differentiation was performed in the 2% $O_2$ condition only.

110 ***Tenogenic differentiation of hESCs***

111 SHEF-1 cells were seeded into 6-well plates at ($2\times10^3$ cells/cm$^2$) in CM. After 24 hours CM

112 was removed and replaced with differentiation media which consisted of KO-DMEM, 10%

113 FBS, 1% NEAA, 1% L-glutamine, 50mM β-mercaptoethanol and 10mM AA (Sigma) with or

114 without BMP12 (R & D Systems) and BMP13 (Peprotech) both at 10ng/ml. To evaluate the

115 role of SMAD signalling in tenogenic differentiation, hESC were seeded and differentiated as

116 above, with the exception that during differentiation hESC were further supplemented with

117 1μM dorsomorphin (31,32) (Sigma).

118

119 ***Reverse transcription PCR (RT-PCR)***

120 RNA was collected from undifferentiated hESC at Day 0, and subsequently at Days 5, 10 and

121 20 in the presence of differentiation media in both 2% $O_2$ and 21% $O_2$. In addition to above

122 RNA was collected from hESC in differentiation media supplemented with either BMP12/13 or

123 BMP12/13/dorsomorphin supplementation at days 5, 10, 20 and 40. RNA was collected by first

124 washing with PBS followed by the addition of cell lysis buffer (Qiagen), scraping, collection,

125 and homogenisation with a QIAshredder spin column (Qiagen). RNA extraction was performed

126 with the RNeasy Mini kit (Qiagen) following manufacturer's instructions. RT-PCR was

127 performed with Superscript III One-Step HiFi RT-PCR kit (Invitrogen) again following

128 manufacturer's instructions. The genes analysed were representative of a tenocyte-like

129 phenotype and were *COL1A2*, *COL3A1*, *DCN*, *TNC*, *TNMD* and *THBS4* (33), primers used are

130 shown in Table 1. *GAPDH* level was used as an internal control. Electrophoresis was

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

132 Gel UV illuminator.

133

134 ***Immunocytochemical analysis***

135 Cells were fixed in 95% methanol for 15 minutes before being washed with PBS. Cells were

136 permeabilised with 0.5% Triton-X for 5 minutes, washed with PBS, and incubated in a 3%

137 albumin solution (Sigma) for 1 hour at room temperature. Primary tenomodulin antibody

138 (C-terminus) (SC98875, Santa Cruz Biotechnologies, Germany, 1:500 dilution in PBS) was

139 then added to each well followed by a 30 minute incubation at 37$^{o}$C and PBS washes.

140 Secondary antibody (SC2090 Santa Cruz Biotechnologies, Germany, 1:500 dilution in PBS)

141 was then added to the appropriate wells followed by a further incubation at 37$^{o}$C for 30 minutes,

142 PBS washes, and DAPI (1:500 dilution, Sigma) counterstaining. Images were captured via

143 appropriate filter sets on Nikon Eclipse T1 microscope using a Nikon DSi 1 camera.

144

145 ***Histological analysis***

146 *Alcian Blue staining*

147 Cells were fixed at Days 0, 2, 5, 10, 20 and 40 using 95% methanol for 15 minutes followed by

148 PBS washes. Cells were then stained with Alcian blue (A3157-10G, Sigma Aldrich, UK) for 24

149 hours at room temperature on an R100 Rotateck shaker (Luckham). After 24 hours, the Alcian

150 blue solution was aspirated, and each well washed with sterile double filtered d$H_2O$. Once all

151 the excess Alcian blue stain had been removed, the plates were dried at room temperature

152 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

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

176 determined.

177 ***Statistical Analysis***

178 The significance of difference between groups (n=6 per group) was determined by one-way

179 ANOVA single factor one-tailed comparison analysis. A p value less than 0.05 was considered

180 to indicate statistical significance. Data are presented as mean ± standard deviation (SD). All

181 statistical analysis was performed using Minitab® 16 (Minitab Inc., Pennsylvania, USA).

182

183 **Results**

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

185 SHEF-1 cells cultured in a 21% $O_2$ environment in differentiation media without BMP
186 supplementation showed continued expression of *GAPDH*, *COL1A2*, and *TNC* over 20 days
187 (Figure 1, Left panel). *COL3A1* and *DCN* expression was apparent by Day 10 and thereafter
188 whereas *THBS4* displayed sequential downregulation over the 20 day timecourse. *TNMD*
189 expression was not detected. Similarly, SHEF-1 cells cultured in 2% $O_2$ environment in
190 differentiation media without BMP supplementation again showed continued expression of
191 *GAPDH*, *COL1A2* and *TNC* over 20 days (Figure 1, Right panel). *TNMD* expression was noted
192 on Day 5 only and *COL3A1* and *DCN* on Day 10. In contrast to the observed expression pattern
193 in 21% $O_2$, *THBS4* underwent sequential upregulation of expression in 2% $O_2$.
194
195 SHEF-1 treated with BMP12/13 over 40 days in 2% $O_2$ resulted in continuous expression of
196 *GAPDH*, *COL1A2*, *COL3A1*, *TNC*, and *THBS4*. *DCN* underwent an apparent upregulation over
197 the first 20 days while *TNMD* expression was maintained to Day 20 and reduced thereafter
198 (Figure 2A Left panel). Differentiation media supplemented with both BMP12/13 and
199 dorsomorphin showed several distinct differences when compared to BMP12/13 supplemented
200 differentiation media (Figure 2A ). *COL3A1*, *DCN*, and *TNC* all underwent substantial
201 downregulation of expression, whereas *COL1A2*, *THBS4*, and *TNMD* transcripts displayed
202 sustained expression throughout the timecourse.
203
204

205 ***BMP-12/13 induced tenomodulin expression in hESCs***

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

212

213 **Histological staining and colorimetric quantification**

214 **Alcian blue**

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

227 hESC cultures resulted in a complete blockage of GAG deposition, while its addition in the

228 presence of BMP12/13 resulted in a 26% increase in GAG deposition over 40 days which was

229 comparable to untreated control cells (Figure 4A).

230

231 **Masson's Trichrome**

232 The primary component of tendon matrix is collagen. Masson's trichrome is a convenient stain

233 in the identification of collagen deposition. Differentiated hESCs revealed distinct cord-like

234 patterning of collagen deposition after 40 days supplementation with BMP12/13 (Figure 3B).

235 In contrast, control unsupplemented cultures displayed a diffuse faint relatively ubiquitous

236 patterning. Primary rat tenocytes, again included for observation, displayed evidence of a

237 distinct pattern of collagen deposition when compared to differentiated hESC. Over 40 days

238 BMP12/13 supplementation resulted in a 51% increase in collagen deposition vs. 23% for

239 control cultures (Figure 4B). Conversely dorsomorphin supplementation of control cells

240 resulted in a complete block on collagen deposition which was only marginally improved to a

241 10% increase in the presence of BMP12/13.

242

243 **Discussion**

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

252

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

265 tenomodulin protein in association with cell-cell synapses. In agreement with this, the
266 combined addition of BMP12, BMP13, and AA, to a basic hESC differentiation media resulted
267 in a controlled hESC differentiation towards a tenogenic lineage.
268
269 There are relatively few reports of supplement-directed tenogenic differentiation which allow
270 comparisons to be made. *SCX* expressing, endogenous or ectopic, hESC-MSCs or
271 hESC-Connective Tissue Progenitors (CTPs) were allowed to be become confluent before
272 being rolled into a sheet and mechanically conditioned for application in an *in vivo* repair model
273 (10,11,40,41). Histology and mechanical properties of *in vitro* and *in vivo* tissue was consistent
274 with tendon, but tendon-linked marker gene expression was either lost after 2 weeks *in situ* or
275 not explored post-transplant and suboptimal regeneration was frequently observed.
276
277 BMP12 and BMP13 signalling are transduced by the BMP Type Ia receptor via
278 receptor-regulated SMADS (SMAD1, 5 and 8) association with the common mediator,
279 SMAD4, followed by complex translocation into the nucleus to activate gene transcription
280 (20,29,42–45). BMP signaling has been suggested to be inhibitory to tendon development by
281 decreasing the pool of available tendon progenitor cells and restricting tendon-linked gene
282 expression (40). In this instance, and in agreement with studies documenting an association of
283 BMP12 and/or 13 with tenogenesis (13,18,19,21–29,44), we noted that BMP12 and BMP13
284 supplementation was required for maintenance of tendon-specific gene expression including
285 *TNMD* and *THBS4*. Berasi *et al* similarly found sustained expression of *THSB4* in response to
286 BMP12/13 supplementation in ectopic tissue in a rat model and a mouse mesenchymal cell

287 line with no evidence of SMAD1, 5 and 8 activation (46). We noted that dorsomorphin, an
288 inhibitor of BMP signaling, did not inhibit transcription of *TNMD* or *THBS4*, but did inhibit
289 *COL3A1*, *DCN*, and *TNC* to some extent. This is suggestive of an alternative, BMP-signalling
290 independent, control of tendon-specific gene expression. It is also notable that although *TNMD*
291 gene expression was maintained in the presence of dorsomorphin the protein was virtually
292 undetectable via immunofluorescence, indicating the likelihood of a BMP-signalling driven
293 translational machinery or key factor in post-translational stability. A deficiency in
294 extracellular structure or matrix composition was also apparent following on from
295 dorsomorphin treatment, with significant reduction in matrix-associated GAG, collagen and
296 elastin. Taken together, these data indicate a complex scenario of BMP signaling requirements
297 in the development and maintenance of tendon gene expression and tendon tissue.
298
299 In this study, we have demonstrated that hESCs are responsive to tenogenic induction via
300 BMP12/13 and ascorbic acid supplementation at 2% $O_2$. However, the mechanisms by which
301 BMP12/13 maintain tendon-linked and tendon-specific gene expression and histology remain
302 unclear, but appear to dissociate into BMP-dependent (*COL3A1*, *DCN*, *TNC*, tenomodulin
303 immunofluorescence, tendon-like matrix) and BMP-independent (*TNMD* and *THBS4*). These
304 results will help provide greater insight into BMP12/13 driven tenogenesis of hESC and new
305 directions of exploration in the design of hESC based treatments for tendon healing.
306

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313

314 **Author Disclosure Statement**

315 No competing financial interests exist.

316

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443 Corresponding author;

444 Professor Nicholas R. Forsyth,

445 The Guy Hilton Research Laboratories,

446 Institute of Science and Technology in Medicine,

447 Faculty of Medicine and Health Sciences,

448 Keele University,

449 Thornburrow Drive,

450 Stoke on Trent, ST4 7QB.

451 UK.

452 email; n.r.forsyth@keele.ac.uk

453 tel; 0044(1)782674388

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 | | |

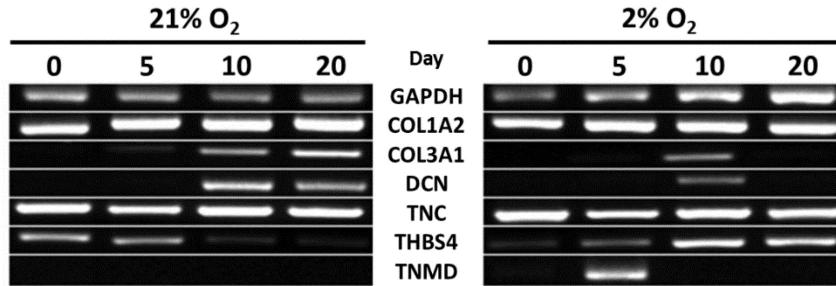
457 Gene names, primer pair sequences, annealing temperatures, and expected amplicon sizes are

458 shown.

459

460

Figure 1

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462 Figure 1. Tendon-linked gene expression in spontaneously differentiated hESC. Expression of

463 RT-PCR amplified tendon-linked genes including *COL1A2*, *COL3A1*, *DCN*, *TNC*, *THBS4*, and

464 *TNMD* is shown. *GAPDH* is included as an internal control. Primer sequences used are

465 described in Table 1. The left-hand and right-hand panels indicate hESC spontaneously

466 differentiating in 21% $O_2$ and 2% $O_2$, respectively, over days 0, 5, 10 and 20.

467

Figure 2

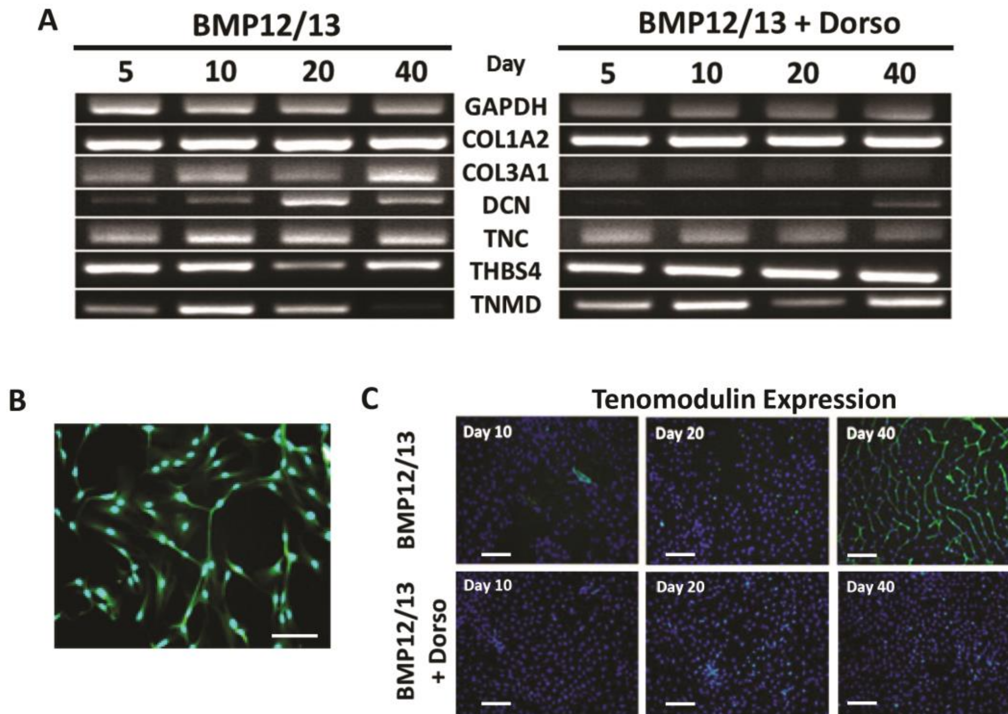


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Figure 2. BMP12/13 supplementation and 2% $O_2$ culture promotes stable tenomodulin expression. A) RT-PCR amplification of the tendon-linked genes described in Figure 1. The left-hand and right-hand panels indicate hESC differentiating in 2% O2 with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

Figure 3

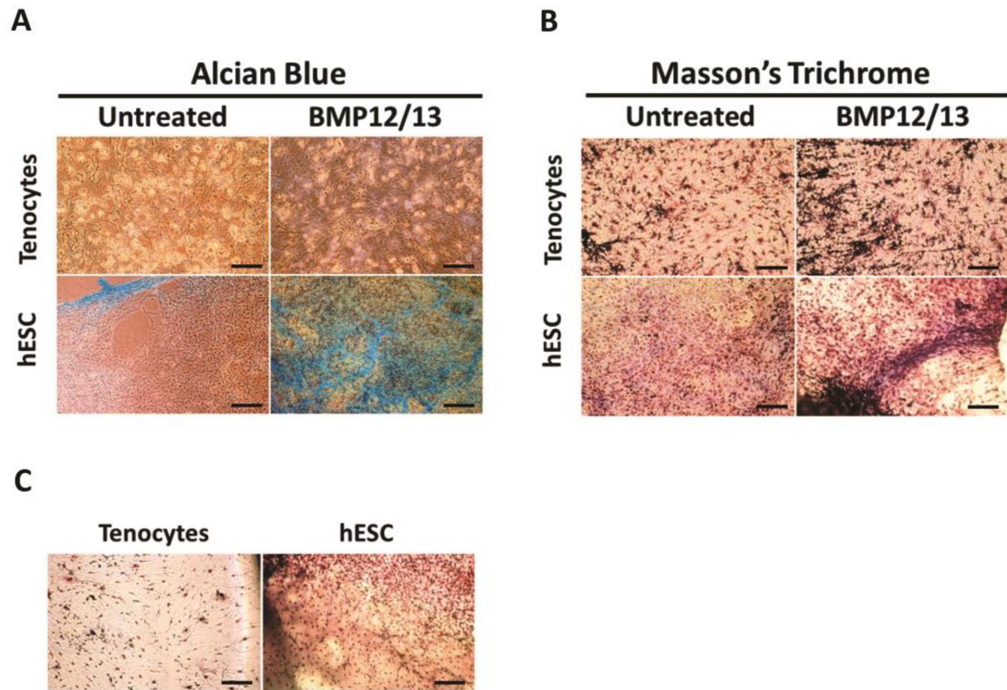


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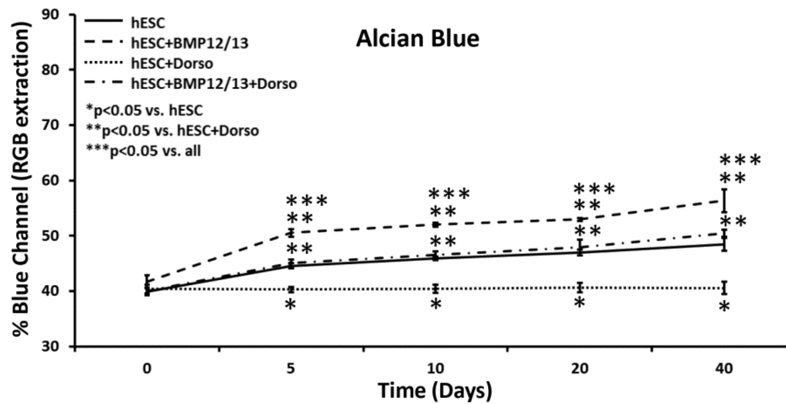
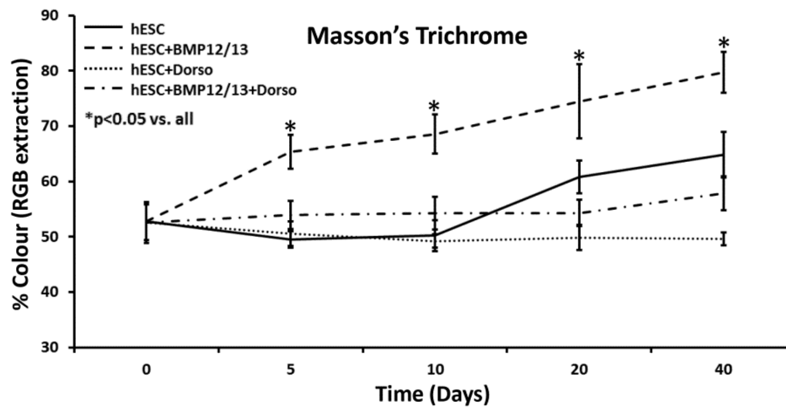
Figure 3. Matrix compositional changes induced by BMP12/13 supplementation. A) Primary rat tenocytes (Top panels) and hESC (Bottom panels) with and without BMP12/13 supplementation. Samples were fixed and stained with Alcian blue after 40 days in continuous culture without passaging. B) Samples matched 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 A




B

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487 Figure 4. Dorsomorphin blocks BMP12/13 supplementation-induced matrix deposition in
488 hESC. A) ImageJ driven analysis of Alcian blue-stained hESC differentiation over 40 days.
489 Y-axis indicates % Blue Channel (RGB extraction) of randomly selected fields of view. X-axis
490 indicates Time (days). Solid line indicates hESC, dotted line indicates hESC+dorsomorphin
491 (Dorso), dashed line indicates hESC + BMP12/13, and hatched line indicates hESC +
492 BMP12/13+Dorso. * indicates $p<0.05$ vs. hESC, ** indicates $p<0.05$ vs. hESC+Dorso, ***
493 indicates $p<0.05$ vs. all. B) ImageJ driven analysis of Masson's Trichrome-stained hESC over
494 40 days differentiation. Y-axis indicates % Colour (RGB extraction) of randomly selected
495 fields of view. X-axis indicates Time (days). Legend labelling is consistent with (4A) above.
496 Error bars indicate standard deviations. n=5 at each time point.

497