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Somitogenesis method based on human pluripotent stem cells for in vitro generation of somite derivatives
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November 5th, 2018

Dear Editor,

Please find enclosed our manuscript entitled, "Somitogenesis method based on human pluripotent stem cells for the *in vitro* generation of somite derivatives", which I would like to submit for publication in *Journal of Visualized Experiments. This is an invited manuscript.*

Somite is a transient stem cell population that gives rise to multiple cell types such as dermatome (dermis), myotome (skeletal muscle), sclerotome (bone and cartilage), and syndetome (tendons and ligaments). The induction of myotome and sclerotome from pluripotent stem cells (PSCs) has been one of most actively investigated topics in stem cell research, because of its potential application for disease modeling and regenerative medicine, as indicated by several publications only in the recent several years (Chal et al., 2015; Loh et al., 2016; Xi et al. 2017). The same is true for syndetome and dermatome, but no report has successfully induced these two derivatives of somite from PSCs. In the submitted study, we report how to induce the differentiation of human iPSCs into somite and then all four somite derivatives (myotome, sclerotome, syndetome and dermatome).

The major findings of this study are summarized below.

1) Establishment of the induction method for dermatome and syndetome from human iPSCs by recapitulating the endogenous signaling environments, using the development of mouse and chick embryos as a roadmap. This is the first report to induce these cell types from PSCs.

2) The induction can be done in a chemically defined condition.

I and the other authors believe that these advancements greatly help researchers in various fields, including developmental biology, stem cell biology and medical biology, and that the content is appropriate for a journal with the broad spectrum of readers of *Journal of Visualized Experiments.*

I would appreciate your consideration of our manuscript for publication in *Journal of Visualized Experiments.*

Yours sincerely,

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Kyoto, Japan

1 **TITLE:**
2 **In Vitro Generation of Somite Derivatives from Human Induced Pluripotent Stem Cells**
3
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19 **KEYWORDS:**
20 induced pluripotent stem cell, differentiation, paraxial mesoderm, somite, myotome, sclerotome,
21 dermatome, syndetome
22
23 **SUMMARY:**
24 We present here a protocol for the differentiation of human induced pluripotent stem cells into
25 each somite derivative (myotome, sclerotome, dermatome, and syndetome) in chemically
26 defined conditions, which has applications in future disease modeling and cell-based therapies in
27 orthopedic surgery.
28
29 **ABSTRACT:**
30 In response to signals such as WNTs, bone morphogenetic proteins (BMPs), and sonic hedgehog
31 (SHH) secreted from surrounding tissues, somites (SMs) give rise to multiple cell types, including
32 the myotome (MYO), sclerotome (SCL), dermatome (D), and syndetome (SYN), which in turn
33 develop into skeletal muscle, axial skeleton, dorsal dermis, and axial tendon/Ligament,
34 respectively. Therefore, the generation of SMs and their derivatives from human induced
35 pluripotent stem cells (iPSCs) is critical to obtain pluripotent stem cells (PSCs) for application in
36 regenerative medicine and for disease research in the field of orthopedic surgery. Although the
37 induction protocols for MYO and SCL from PSCs have been previously reported by several
38 researchers, no study has yet demonstrated the induction of SYN and D from iPSCs. Therefore,
39 efficient induction of fully competent SMs remains a major challenge. Here, we recapitulate
40 human SM patterning with human iPSCs in vitro by mimicking the signaling environment during
41 chicken/mouse SM development, and report on methods of systematic induction of SM
42 derivatives (MYO, SCL, D, and SYN) from human iPSCs under chemically defined conditions
43 through the presomitic mesoderm (PSM) and SM states. Knowledge regarding mouse/chicken
44 SM development was successfully applied to the induction of SMs with human iPSCs. This method

45   could be a novel tool for studying human somitogenesis and patterning without the use of
46   embryos and for cell-based therapy and disease modeling.

47

48   **INTRODUCTION:**
49   Developing a directed differentiation method for a desired cell type from PSCs is a necessary step
50   for translating the study of PSC-derived cells into clinical applications. Forced expression of key
51   genes is a promising strategy for organ-cell differentiation from PSCs and has improved our
52   understanding of the genetic regulation of cell fate determination, organ morphogenesis, and
53   organization during embryogenesis[1]. In addition, recapitulating the endogenous signaling
54   environments, using the development of mouse and chick embryos as a roadmap, is considered
55   essential for the directed differentiation of PSCs. However, given the application of PSC-derived
56   cells in clinical studies such as cell-based therapies, the latter strategy is more suitable because
57   it does not require gene manipulation.

58

59   Several studies have reported the induction of mesoderm from human and mouse PSCs in
60   chemically defined conditions. Typically, these methods have relied on
61   activin/nodal/transforming growth factor β (TGFβ) signaling and bone morphogenetic protein
62   (BMP) signaling, believed to perform meso-endoderm and mesoderm differentiation, resulting
63   in a low induction efficiency of the paraxial mesoderm (approximately 20%)[2]. In other words, the
64   PSC-derived mesoderm induced by these signaling pathways was mainly lateral plate mesoderm,
65   and not paraxial mesoderm. Recently, a few studies have demonstrated the efficient production
66   of PSC-derived paraxial mesoderm based on different strategies[3-8]. In these studies, PSCs were
67   cultured with relatively high concentrations of glycogen synthase kinase 3 (GSK3) inhibitors (WNT
68   signaling activators), consequently the induction efficiency of paraxial mesoderm reached 70%–
69   95%[6,7].

70

71   In somitogenesis, the paraxial mesoderm first forms the presomitic mesoderm (PSM) posteriorly,
72   and then forms somites (SMs) in the anterior part through mesenchyme-to-epithelial
73   transition[9,10]. Notch ligand Delta-like 1 (DLL1) is known to have a pivotal role during
74   somitogenesis, as oscillatory control of DLL1 expression, both in mRNA and protein level,
75   regulates SM segmentation. SMs eventually subdivide into two parts, giving rise to the
76   dermomyotome (DM) dorsally and sclerotome (SCL) ventrally[11]. Subsequently, the DM
77   differentiates into the dermatome (D), a precursor of the dermis, and myotome (MYO), a
78   precursor of skeletal muscle; additionally, a ventral portion of SCL forms the syndetome (SYN), a
79   precursor of tendons and ligaments[12] (**Figure 1**). Some researchers have reported the induction
80   of PSC-derived SM derivatives such as MYO[4,13] and SCL[14]; however, there are several limitations
81   in these studies. Notably, since our knowledge of the signaling environments of D and SYN is
82   fragmentary, induction protocols for D and SYN have not yet been systematically established. To
83   demonstrate the full-competence of SMs induced from PSCs, it is essential to show the multi-
84   differentiation capacity of induced SMs into all four derivatives (D, MYO, SCL, and SYN), while
85   previous studies have only focused on specific SM derivatives. Here, we report on how to
86   generate all four SM derivatives, including D and SYN, through PSM and SM fates from human
87   iPSCs[15]. We believe that establishing an in vitro stepwise method that models the SM

88 development process could contribute to the study of how human SM develops during
89 embryogenesis, without using embryos.
90
91 **PROTOCOL:**
92 All experimental protocols involving human iPSCs were approved by the Ethics Committee of the
93 Department of Medicine and Graduate School of Medicine, Kyoto University.
94
95 **1. Human iPSCs preparation before induction**
96
97 NOTE: Culture human iPSCs (201B7-PAX3-GFP) on SNL feeder cells[16] with primate ES cell medium
98 supplemented with 4 ng/mL recombinant human basic fibroblast growth factor (FGF2) and 0.5%
99 penicillin and streptomycin (hereinafter referred to as hESC medium, see **Table 1**). When the
100 confluence ratio reaches 70%–80%, passage the cells as previously described[17].
101
102 1.1. Passaging of human iPSCs on SNL feeder cells[17]
103
104 1.1.1. For passaging, add PBS into the cell culturing dish and rinse cells. Then, remove the PBS
105 (hereafter, this process will be referred to as wash with PBS).
106
107 1.1.2. Add 1 mL of CTK solution (see **Table 1**) at room temperature (RT) and wait until the SNL
108 feeder cells start detaching from the bottom of the dish.
109
110 1.1.3. Remove the CTK solution and then wash twice with PBS.
111
112 1.1.4. Add 1 mL of hESC medium (see **Table 1**) into the dish, then scrape the cells using a scraper
113 and collect into a 15 mL conical tube.
114
115 1.1.5. Gently pipette the contents five times using a 1000 µL tip, and then transfer to a new dish
116 filled with hESC medium. Use a split ratio of 1:4 to 1:10, depending on the confluence ratio before
117 passaging. Also, vary the volume of hESC medium depending on the scale of the dish (*e.g.*, 3 mL
118 for a 6-cm dish, 8 mL for a 10-cm dish).
119
120 1.1.6. Incubate the human iPSCs at 37 °C and 5% CO_2.
121
122 1.1.7. Change the medium everyday (except for the day after passaging) and culture the cells until
123 the next passaging procedure.
124
125 1.2. Feeder-free culturing of human iPSCs before PSM induction
126
127 NOTE: To minimize the effect of growth factors secreted from SNL feeder cells, culture the human
128 iPSCs under feeder-free conditions with a feeder-free cell culture medium (see **Table 1**) on
129 extracellular matrix (ECM) solutions (see **Table 1**) coated 3 days prior to PSM induction.
130
131 1.2.1. Day -4 (4 days prior to starting PSM induction)

132

133 1.2.1.1. To prepare the ECM solution-coated dish, add 4 mL of ECM solution onto a 10 cm dish at
134 4 °C overnight.

135

136 NOTE: Place ECM solution on ice while preparing.

137

138 1.2.2. Day -3

139

140 1.2.2.1. First, remove the ECM solution from the dish and add 8 mL of feeder-free cell culture
141 medium.

142

143 1.2.2.2. To start feeder-free culturing, wash once with PBS to rinse the cultured cells.

144

145 1.2.2.3. Add 1 mL of CTK solution at RT until the SNL feeder cells start detaching from the bottom
146 of the dish.

147

148 NOTE: Use microscopy to confirm all feeder cells are detached from the bottom.

149

150 1.2.2.4. Remove the CTK solution and wash with PBS twice, so that all SNL feeder cells are fully
151 removed.

152

153 1.2.2.5. Add 1 mL of feeder-free cell culture medium into the dish, then scrape the cells using a
154 scraper and collect them into a 15-mL conical tube.

155

156 1.2.2.6. Gently pipette the contents three times using a 1000 μL tip, then transfer to a new ECM
157 solution-coated 10 cm dish (prepared during step 1.2.1). Use a split ratio of roughly 1:2 to 1:4,
158 depending on the confluence ratio before feeder-free culturing.

159

160 1.2.2.7. Incubate the human iPSCs at 37 °C and 5% CO_2 for 3 days, changing the medium on day
161 -1.

162

163 **2. PSM differentiation and isolation by fluorescence-activated cell sorting (FACS)**

164

165 2.1. PSM differentiation (Day 0–Day 4)

166

167 2.1.1. Aspirate the feeder-free cell culture medium and add 8 mL of PSM induction medium (CDM
168 basal medium supplemented with 10 μM SB431542, 10 μM CHIR99021, 2 μM DMH1, and 20
169 ng/mL FGF2, see **Table 1**).

170

171 NOTE: Cell confluence at the initiation of PSM differentiation is critical for induction efficiency.
172 Use microscopy to confirm the confluent ratio is approximately 30%.

173

174 2.1.2. Incubate cells at 37 °C, with 5% CO_2, for 4 days, changing the medium on Day 3.

175

176

177

178 2.2. Isolation of DLL1 positive PSM cells by fluorescence-activated cell sorting (FACS)

179

180 NOTE: Below is a procedure for cell preparation before FACS sorting of DLL1 positive cells.
181 Perform FACS sorting using a flow cytometer, according to the manufacturer's protocol.

182

183 2.2.1. Aspirate the medium, then wash with PBS. Subsequently, add 1 mL of cell dissociation
184 reagent and leave for 3 min at RT.

185

186 2.2.2. Add 4 mL of CDM basal medium, scrape the cells using a scraper, and collect them into a
187 15 mL conical tube.

188

189 2.2.3. Count the number of cells using an automated cell counter, then centrifuge at 280 x g for
190 3 min.

191

192 2.2.4. Carefully remove the supernatant by aspiration and resuspend the cells in FACS buffer (see
193 **Table 1**) at a concentration of 1.0 x 10^7 cells/mL. For a negative control sample (isotype control,
194 or in convention without antibody), transfer 50 µL into a 15-mL conical tube and then suspend
195 with 450 µL of FACS buffer.

196

197 2.2.5. Add DLL1 antibody (see **Table of Materials**) in a ratio of 1/200. Protect the tube from light
198 and keep on ice for 30 min.

199

200 2.2.6. Centrifuge at 280 x g for 3 min.

201

202 2.2.7. Carefully aspirate the supernatant and resuspend in FACS buffer (1.0 x 10^7 cells/mL)
203 supplemented with 1 mg/mL DAPI.

204

205 2.2.8. Transfer into a collection tube, incorporated with a 35-µm nylon mesh in the cap for
206 filtering, then place the tube on ice until sorting is complete. Perform the same procedure with
207 the negative control sample (step 2.2.4).

208

209 2.2.9. Perform sorting using a flow cytometer according to the manufacturer's protocol.

210

211 2.2.10. Collect the sorted DLL1-positive cells into a 15 mL conical tube, containing 4 mL of CDM
212 basal medium supplemented with 10 µM of Y27632. For total RNA extraction, centrifuge at 280
213 x g for 3 min then resuspend in RNA lysis buffer and store at -30 °C. See the RNA extraction,
214 reverse transcription, and RT-qPCR procedures (section 5.1) for more detailed information.

215

216 2.2.11. Perform SM differentiation using the sorted cells according to the below protocol (section
217 3).

218

219 **3. SM differentiation from PSM**

3.1. SM differentiation from sorted DLL1-positive PSM cells (Day 4–Day 8)

NOTE: Prepare the ECM solution-coated 12-well plates the day before FACS sorting. To prepare an ECM solution-coated 12-well plate, add 1 mL of ECM solution into each well at 4 °C and leave overnight. Keep the ECM solution on ice while preparing.

3.1.1. Following step 2.2.10, centrifuge at 280 x g for 3 min.

3.1.2. Carefully aspirate the supernatant and resuspend in 1 mL of SM induction medium (CDM basal medium supplemented with 10 µM SB431542 and 5 µM CHIR99021, see **Table 1**).

3.1.3. Count the number of cells using an automated cell counter.

3.1.4. Seed 1.0 x 10^5 cells onto each well of the ECM solution-coated 12-well plates containing 1 mL of SM induction medium supplemented with 10 µM of Y27632.

3.1.5. Incubate at 37 °C with 5% CO_2 for 4 days until Day 8. Change the medium not containing Y27632 on Day 5 (the day after FACS sorting) and Day 7.

3.1.6. Perform SM derivatives differentiation using induced SM cells according to the protocols below. For total RNA extraction from induced SM cells, collect the cells into a 15-mL conical tube and centrifuge at 280 x g for 3 min, then resuspend in RNA lysis buffer and store at -30 °C.

4. SM derivatives (DM, MYO, D, SCL, SYN) differentiation from SM

NOTE: To demonstrate the full-competence of SM cells, first perform DM (dermomyotome) and SCL (sclerotome) induction accordingly using iPSC-derived SM cells. Subsequently, perform MYO (myotome) and D (dermatome) induction using the DM cells, and conduct SYN (syndetome) induction using the SCL cells. Below are the protocols for the induction of each derivative (DM, MYO, D, SCL, and SYN) from induced SM cells in vitro.

4.1. DM differentiation from SM cells (Day 8–Day 11)

4.1.1. Aspirate the medium, then add 1 mL of DM induction medium (CDM basal medium supplemented with 5 µM CHIR99021 and 10 ng/mL BMP4, see **Table 1**).

4.1.2. Incubate the cells at 37 °C, with 5% CO_2, for 3 days until Day 11. Change the medium on Day 10 (Day 2 of DM induction).

4.1.3. Perform MYO and D differentiation using induced DM cells according to the protocols below.

4.2. MYO differentiation from DM cells (Day 11–Day 14)

264

265 4.2.1. Aspirate the medium, then add 1 mL of MYO induction medium (CDM basal medium
266 supplemented with 5 µM CHIR99021, see **Table 1**).

267

268 4.2.2. Incubate the cells at 37 °C, with 5% CO_2, for 30 days until Day 41. Change the medium every
269 3 days.

270

271 4.3. D differentiation from DM cells (Day 11–Day 20)

272

273 4.3.1. Aspirate the medium, then add 1 mL of D induction medium (CDM basal medium
274 supplemented with 5 µM CHIR99021 and 10 ng/mL BMP4, see **Table 1**).

275

276 4.3.2. Incubate the cells at 37 °C, with 5% CO_2, for 9 days until Day 20. Change the medium every
277 3 days.

278

279 4.4. SCL differentiation from SM cells (Day 8–Day 11)

280

281 4.4.1. Aspirate the medium, then add 1 mL of SCL induction medium (CDM basal medium
282 supplemented with 100 nM SAG and 0.6 µM LDN193189, see **Table 1**)[14].

283

284 4.4.2. Incubate the cells at 37 °C, with 5% CO_2, for 3 days. Change the medium on Day 10 (Day 2
285 of SCL induction).

286

287 4.4.3. Perform SYN differentiation using induced SCL cells according to the protocol below.

288

289 4.5. SYN differentiation from SCL cells (Day 11–Day 32)

290

291 NOTE: Prepare the ECM solution-coated 24-well plates the day before initiating SYN induction.
292 To prepare an ECM solution-coated 24-well plate, add 0.5 mL of ECM solution into each well at
293 4 °C and leave overnight. Keep the ECM solution on ice while preparing.

294

295 4.5.1. Aspirate the medium then wash with PBS, then add 0.2 mL of cell dissociation reagent to
296 each well and leave for 3 min at RT.

297

298 4.5.2. Add 0.8 mL of CDM basal medium to each well then scrape and collect all cells into a 15
299 mL conical tube.

300

301 4.5.3. Centrifuge at 280 x *g* for 3 min.

302

303 4.5.4. Carefully aspirate the supernatant and resuspend in 1 mL of SYN induction medium-1 (CDM
304 basal medium supplemented with 20 ng/mL FGF8, see **Table 1**), then count the number of cells
305 using an automated cell counter.

306

307 <mark>4.5.5. Seed 5.0 x 10^4 cells into each well of the ECM solution-coated 24-well plates containing 1</mark>
308 <mark>mL of SYN induction medium-1.</mark>

310 <mark>4.5.6. Incubate at 37 °C, with 5% CO_2, for 3 days.</mark>

312 <mark>4.5.7. On Day 14 (Day 3 of SYN induction), replace the medium with SYN induction medium-2</mark>
313 <mark>(CDM basal medium supplemented with 10 ng/mL BMP7 and 10 ng/mL TGFβ3, see **Table 1**).</mark>

315 <mark>4.5.8. Incubate at 37 °C, with 5% CO_2, for 18 days until Day 32. Change the medium every 3 days.</mark>

5. Characterization of iPSC-derived products

NOTE: Upon differentiation, characterize human iPSCs derivatives using quantitative real-time PCR (RT-qPCR), immunocytochemistry (ICC), enzyme-linked immunosorbent assays (ELISA), and mechanical stretch stimulation assays, accordingly.

5.1. Cell harvest, total RNA extraction, reverse transcription, and quantitative real-time PCR (RT-qPCR) analysis

5.1.1. Collect the cell samples (procedures 2.2.10, 3.1.7, 5.4.3) into a 1.5 mL tube, then centrifuge at 280 x g for 3 min.

5.1.2. Remove the supernatant, then resuspend in 350 μL of RNA lysis buffer, provided by an appropriate total RNA extraction kit.

5.1.3. Extract total RNA using the kit according to the manufacturer's protocol.

5.1.4. Reverse transcribe the isolated 1 μg of total RNA to cDNA, according to the manufacturer's protocol.

5.1.5. Perform RT-qPCR using suitable enzymes, reagents, and primers according to the manufacturer's protocol. The primer sequences used in this study are listed in **Table 2**.

5.2. Immunocytochemistry (ICC)

5.2.1. Prior to performing immunocytochemistry with antibodies, fix the cells with 2% paraformaldehyde at 4 °C for 10 min, and wash twice with PBS.

5.2.2. For permeabilization, incubate with 0.2% methanol or 0.2% polysorbate 20/PBS (hereinafter referred to as PBS-T) at 4 °C for 15 min.

5.2.3. Remove the permeabilization reagents and treat the cells with an appropriate blocking buffer or 1% bovine serum albumin/PBS at 4 °C for 60 min.

351 5.2.4. Add the first antibody diluted with 10% blocking buffer in PBS-T and place on a shaking
352 machine at 4 °C overnight.
353
354 5.2.5. Wash three times with PBS-T (add PBS-T and place on the shaking machine at RT for 10
355 min).
356
357 5.2.6. Add the second antibody, diluted with 10% blocking buffer in PBS-T and place on the
358 shaking machine at RT for 60 min. The first and second antibodies for ICC used in this study are
359 listed in **Table 3**.
360
361 NOTE: From this step onwards, protect the plate/dish from light by wrapping it in foil.
362
363 5.2.7. Wash twice with PBS-T.
364
365 5.2.8. For counter staining, add 1/5000 DAPI diluted with PBS and place on the shaking machine
366 at RT for 5 min.
367
368 5.2.9. Remove the DAPI solution and add PBS into each well.
369
370 5.2.10. Observe the cell staining using a fluorescence microscope. Alternatively, store the
371 plate/dish at 4 °C for up to 1 month.
372
373 5.3. Enzyme-linked Immunosorbent assay (ELISA) for functional analysis of iPSC-derived D
374
375 NOTE: Human dermal fibroblast cells (HDF) are commercially available. Culture HDF in DMEM
376 supplemented with 10% fetal bovine serum (see **Table 1**).
377
378 5.3.1. Seed 1.0 x 10^5 cells of iPSC-derived D and HDF onto 24-well plates containing 1 mL of each
379 culture medium (D: D induction medium, HDF: DMEM supplemented with 10% fetal bovine
380 serum).
381
382 5.3.2. After 3 days of cell culturing, collect 100 µL of each medium, place into a 1.5 mL tube, and
383 store at 4 °C.
384
385 5.3.3. Perform the series of procedures, such as the addition of detection antibodies and
386 secondary antibodies, according to the manufacturer's instruction, and quantify the number of
387 targets by generating a standard curve against the concentration of the control analyte samples.
388
389 5.4. Mechanical stretch stimulation assay for functional analysis of iPSC-derived SYN
390
391 NOTE: Adult human tenocytes are commercially available (see **Table of Materials**). Culture
392 human iPSC-derived SYN and adult human tenocytes on a cell stretching device for the
393 mechanical stretch stimulation assay as described below[18,19]. Use SYN induction medium-2 and

394 tenocytes growth medium (see **Table of Materials**) as a culturing medium for each iPSC-derived
395 SYN and adult human tenocytes, respectively.
396
397 5.4.1. At 24 h before stretching, plate 1.0 x 10^5 cells of iPSC-derived SYN and human tenocytes
398 onto ECM solution-coated multi well-type silicon rubber chambers, each with a culture surface
399 of 1.5 cm x 1.5 cm (see **Table of Materials**).
400
401 5.4.2. Set the chambers on the device for cell stretching, and force monoaxial cyclic strain (0.5
402 Hz, 5%) for 12 h.
403
404 5.4.3. For total RNA extraction, add 350 µL of RNA lysis buffer, then scrape and collect the cells
405 into a 1.5 mL tube for total RNA extraction and subsequent RT-qPCR analysis (see procedure 5.1).
406
407 **REPRESENTATIVE RESULTS**
408 All figures in this report were obtained with 201B7-PAX3-GFP iPSCs, in which EGFP replaces one
409 allele of the PAX3 coding sequence in exon 1. Establishment of 201B7-PAX3-GFP iPSCs will be
410 described elsewhere (H. Sakurai, personal communication). The statistical significance was
411 evaluated using statistical software. P-values lower than 0.05 were considered significant.
412
413 **Characterization of human iPSC-derived PSM and SM cells**
414 To assess the differentiation of human iPSCs toward SM through the PSM state (**Figure 2A**), FACS
415 analysis, ICC analysis, and RT-qPCR analysis were performed. As shown in **Figure 2B**, over 85% of
416 the cells were positive for DLL1, a marker of PSM, but negative for PAX3, a marker of SM, after 4
417 days of PSM induction with human iPSCs. Subsequently, this population became PAX3-positive
418 SM cells after 4 days of SM induction. The PSM-SM transition was also confirmed by ICC (**Figure
419 2C**) and RT-qPCR (**Figure 2D**). TBX6, MSGN1, and WNT3A, PSM markers were expressed at the
420 PSM state (day 4), but not expressed at the SM state (day 8). PARAXIS, MEOX1, and PAX3, SM
421 markers, were expressed at SM, but not expressed at PSM. Furthermore, staining of CDH11, a
422 marker of epithelialized SM, only accumulated at the cell-cell junction, following the addition of
423 SB431542 with CHIR99021 (**Figure 2E**).
424
425 **Characterization of SM derivatives induced from human iPSC-derived SM cells**
426 To evaluate the differentiation potency of human iPSC-derived SM, differentiation toward DM,
427 MYO, D, SCL, and SYN (**Figure 3A**) was assessed by ICC analysis and PAX3 (GFP)-fluorescence. As
428 shown in **Figure 3B**, DM differentiation was confirmed by ALX4 and EN1 staining, and PAX3 (GFP)-
429 fluorescence; MYO differentiation was confirmed by MYOD, MYOG, and Myosin heavy chain
430 (MHC) staining; D differentiation was confirmed by EN1 and PDGFRa staining; SCL differentiation
431 was confirmed by PAX1, PAX9, and NKX3.2 staining; and SYN differentiation was confirmed by
432 SCX, MKX, COL1A1, and COL1A2 staining.
433
434 **Characterization of induced D and SYN**
435 **1. Enzyme-linked immunosorbent assay (ELISA) for functional analysis of iPSC-derived D**
436 In the human body, one of the primary functions of dermal fibroblasts is to secrete extracellular
437 matrix (ECM) proteins, such as collagen and hyaluronic acid that hydrate the skin and help sustain

438 the skin structure. To demonstrate that a comparable amount of collagen-type 1 and hyaluronic
439 acid proteins were secreted in the culture medium of iPSC-derived D and HDF, ELISA was
440 performed, as shown in **Figure 4A**.
441
442 **2. Mechanical stretch stimulation assay for functional analysis of iPSC-derived SYN**
443 As several studies have already reported, mechanical stimulation affects tendon development
444 before and after birth, and promotes the differentiation of tenocytes from precursor cells[18,19].
445 Therefore, it is well known that reactivity to mechanical stress is one of the characteristics of
446 tenocytes. To demonstrate the comparable reactivity of human iPSC-derived SYN and human
447 adult tenocytes, a mechanical stretch stimulation assay was performed as shown in **Figure 4B**.
448
449 **FIGURE AND TABLE LEGENDS:**
450 **Figure 1: Schematic view of hierarchical differentiation of paraxial mesoderm.** Presomitic
451 mesoderm is a cell population that transiently emerges during early embryogenesis and
452 undergoes segmentation to form somites. Somites are a transient stem cell population that gives
453 rise to multiple cell types, such as sclerotome, dermomyotome, syndetome, dermatome, and
454 myotome cells, which eventually differentiate into tendon/Ligament, bone/cartilage, skeletal
455 muscle, and dermis cells.
456
457 **Figure 2: FACS, RT-qPCR, and ICC analysis of human iPSC-derived PSM and SM. (A)** Schematic
458 view of a protocol for SM differentiation through PSM. **(B)** Representative dot plot of DLL1
459 staining and PAX3 (GFP)-fluorescence on day 4 of PSM induction and day 4 (day 8 from iPSC) of
460 SM induction. **(C)** Representative immunocytochemical images and PAX3 (GFP)-fluorescence on
461 day 4 of PSM induction and day 4 (day 8 from iPSC) of SM induction. Cells were stained with anti-
462 TBX6, PARAXIS, and MEOX1 antibodies (red) and co-stained with DAPI (blue) or detected with
463 PAX3 (GFP)-fluorescence (green). **(D)** RT-qPCR analysis of markers for PSM and SM at iPSC, PSM
464 and SM. The means ± standard error (S.E.) from three sets of experiments are shown. **(E)**
465 Representative immunocytochemical images on day 4 (day 8 from iPSC) of SM, cultured in S10I10
466 (combination of SB431542 and IWR1, an inhibitor of WNT signaling), S10 (SB431542), and S10C5
467 (combination of SB431542 and CHIR99021) conditions. Cells were stained with anti-CDH11
468 antibody (red) and co-stained with DAPI (blue). iPS, induced pluripotent stem cell; PSM,
469 presomitic mesoderm; SM, somite; S10, SB431542 10 µM; C5, CHIR99021 5 µM; I10, IWR1 10
470 µM; Scale bars: 50 µm. This figure has been modified from Nakajima et al. (2018)[15].
471
472 **Figure 3: ICC analysis of DM, MYO, D, SCL, and SYN differentiated from human iPSC-derived SM.**
473 **(A)** Schematic view of protocols for SM derivatives differentiation. **(B)** Representative
474 immunocytochemical images and PAX3 (GFP)-fluorescence on day 3 (day 11 from iPSC) of DM
475 induction, day 30 (day 41 from iPSC) of MYO induction, day 9 (day 20 from iPSC) of D induction,
476 day 3 (day 11 from iPSC) of SCL induction, and day 21 (day 32 from iPSC) of SYN induction. DM,
477 cells were stained with anti-ALX4 and EN1 antibodies (red) and co-stained with DAPI (blue) or
478 detected with PAX3 (GFP)-fluorescence (green); MYO, cells were stained with anti-MYOD, MYOG
479 (red), and MHC (cyan) antibodies, also co-stained with DAPI (blue); D, cells were stained with
480 anti-EN1 (red) and PDGFRa (cyan) antibodies and co-stained with DAPI (blue); SCL, cells were
481 stained with anti-PAX1, PAX9, and NKX3.2 (red) antibodies, and co-stained with DAPI (blue); SYN,

482 cells were stained with anti-SCX, MKX, COL1A1, and COL1A2 (red) antibodies, and co-stained with
483 DAPI (blue). DM, dermomyotome; MYO, myotome; D, dermatome; SCL, sclerotome; SYN,
484 syndetome; Scale bars: 50 μm. This figure has been modified from Nakajima et al. (2018)[15].
485
486 **Figure 4: Functional assay of induced D and SYN. (A)** The amount of collagen-type 1 and
487 hyaluronic acid proteins in the culture medium were analyzed by ELISA. **(B)** The effect of
488 mechanical stretch stimulation on induced SYN and human adult tenocytes was assessed by RT-
489 qPCR. The means ± standard error (S.E.) from three sets of experiments are shown. *p < 0.05;
490 **p < 0.01; *** *p* < 0.001 by Dunnett's multiple comparisons t-test compared to Stretch (-); n.s,
491 not significant, HDF, human adult dermal fibroblast. This figure has been modified from Nakajima
492 et al. (2018)[15].
493
494 **Table 1: Media and solution recipes.**
495
496 **Table 2: Primer sequences for RT-qPCR analysis.**
497
498 **Table 3: First and second antibodies for ICC.**
499
500 **DISCUSSION**
501 A well-known method for the induction of PSC-derived SM through PSM is the combination of
502 CHIR99021 + A83-01 (TGFβ inhibitor) during PSM induction from PSC, but not during the PSM
503 maturation process[6]. In the present study, WNT/beta-catenin signaling was inhibited using C59
504 to induce SM from PSM. However, we introduced the use of CHIR99021 to activate the WNT
505 pathway during SM differentiation. This decision was made based on the finding that several
506 WNTs are expressed in the surrounding tissues of SM and given the fact that WNT reporters are
507 active in SM[20]. As a result, we observed epithelialization, a characteristic of SM in vivo, only under
508 the condition with CHIR99021, based on the accumulation of CDH11 in cell-cell junctions (**Figure
509 2E**). This observation indicates the critical involvement of WNT signaling during PSM
510 differentiation and SM epithelialization, therefore our protocol may better recapitulate the
511 endogenous signaling environment. However, it also implies a further possibility of fine-tuning
512 the WNT/beta-catenin signaling pathway during differentiation, because robustness and
513 efficiency of differentiation might vary significantly depending on the cell types, cell lines, and
514 various chemical compounds of WNT-inducers used by each researcher.
515
516 This method also allows us to generate all four SM derivatives, MYO, D, SCL, and SYN, from human
517 iPSCs. Our stepwise protocols using CDM can be used to identify the signaling requirements
518 during human somitogenesis/somite patterning, and provide important insights into human SM
519 development. For example, our methods could be useful for studying segmentation clock
520 mechanisms, a molecular oscillation system that regulates the formation of SM. It has been
521 thoroughly investigated in mice, chicks, and zebrafish, but not in humans due to the lack of
522 appropriate experimental tools.
523
524 Moreover, our method can be applicable to future clinical cell-based therapies. For example,
525 human iPSC-derived D or SYN can be transplanted into severely injured skin or ruptured tendons

for regeneration and treatment. However, several limitations need to be resolved before this method can be practically applied. Although in the present study, we used SNL feeder cells for iPSC maintenance and ECM solution, which is extracted from the Engelbreth-Holm-Swarm mouse sarcoma, as a surface coat on the dish during induction, these non-human animal-derived reagents should be removed to improve clinical quality. In addition, cell quantity and quality, which includes the purity and maturation of the desired cells, must also be improved. Furthermore, not only the cell number but also the cell strength is an important characteristic for tendon/ligament regeneration. Additionally, the development of surface markers for purification and a novel method for 3D reconstitution are indispensable in order to advance our protocols to clinical cell-based therapies.

DISCLOSURES:
The authors have nothing to disclose.

REFERENCES:

1   Tanaka, A. et al. Efficient and reproducible myogenic differentiation from human iPS cells: prospects for modeling Miyoshi Myopathy in vitro. *PLoS One.* **8** (4), e61540, (2013).

2   Sakurai, H. et al. In vitro modeling of paraxial mesodermal progenitors derived from induced pluripotent stem cells. *PLoS One.* **7** (10), e47078, (2012).

3   Chal, J. et al. Generation of human muscle fibers and satellite-like cells from human pluripotent stem cells in vitro. *Nature Protocl.* **11** (10), 1833-1850, (2016).

4   Chal, J. et al. Differentiation of pluripotent stem cells to muscle fiber to model Duchenne muscular dystrophy. *Nature Biotechnology.* **33** (9), 962-969, (2015).

5   Umeda, K. et al. Human chondrogenic paraxial mesoderm, directed specification and prospective isolation from pluripotent stem cells. *Scientific Reports.* **2** 455, (2012).

6   Loh, K. M. et al. Mapping the Pairwise Choices Leading from Pluripotency to Human Bone, Heart, and Other Mesoderm Cell Types. *Cell.* **166** (2), 451-467, (2016).

569　7　　Xi, H. et al. In Vivo Human Somitogenesis Guides Somite Development from hPSCs. *Cell*
570　　　*Reports.* **18** (6), 1573-1585, (2017).
571　8　　Chal, J., Pourquie, O. Making muscle: skeletal myogenesis in vivo and in vitro.
572　　　*Development.* **144** (12), 2104-2122, (2017).
573　9　　Tam, P. P., Beddington, R. S. The formation of mesodermal tissues in the mouse embryo
574　　　during gastrulation and early organogenesis. *Development.* **99** (1), 109-126, (1987).
575　10　Aulehla, A., Pourquie, O. Signaling gradients during paraxial mesoderm development.
576　　　*Cold Spring Harbor Perspectives in Biology.* **2** (2), a000869, (2010).
577　11　Christ, B., Scaal, M. Formation and differentiation of avian somite derivatives. *Advances*
578　　　*in Experimental Medicine and Biology.* **638** 1-41, (2008).
579　12　Brent, A. E., Schweitzer, R., Tabin, C. J. A somitic compartment of tendon progenitors.
580　　　*Cell.* **113** (2), 235-248, (2003).
581　13　Sakurai, H. et al. Bidirectional induction toward paraxial mesodermal derivatives from
582　　　mouse ES cells in chemically defined medium. *Stem Cell Research.* **3** (2-3), 157-169,
583　　　(2009).
584　14　Zhao, J. et al. Small molecule-directed specification of sclerotome-like
585　　　chondroprogenitors and induction of a somitic chondrogenesis program from embryonic
586　　　stem cells. *Development.* **141** (20), 3848-3858, (2014).
587　15　Nakajima, T. et al. Modeling human somite development and fibrodysplasia ossificans
588　　　progressiva with induced pluripotent stem cells. *Development.* **145** (16), (2018).
589　16　McMahon, A. P., Bradley, A. The Wnt-1 (int-1) proto-oncogene is required for
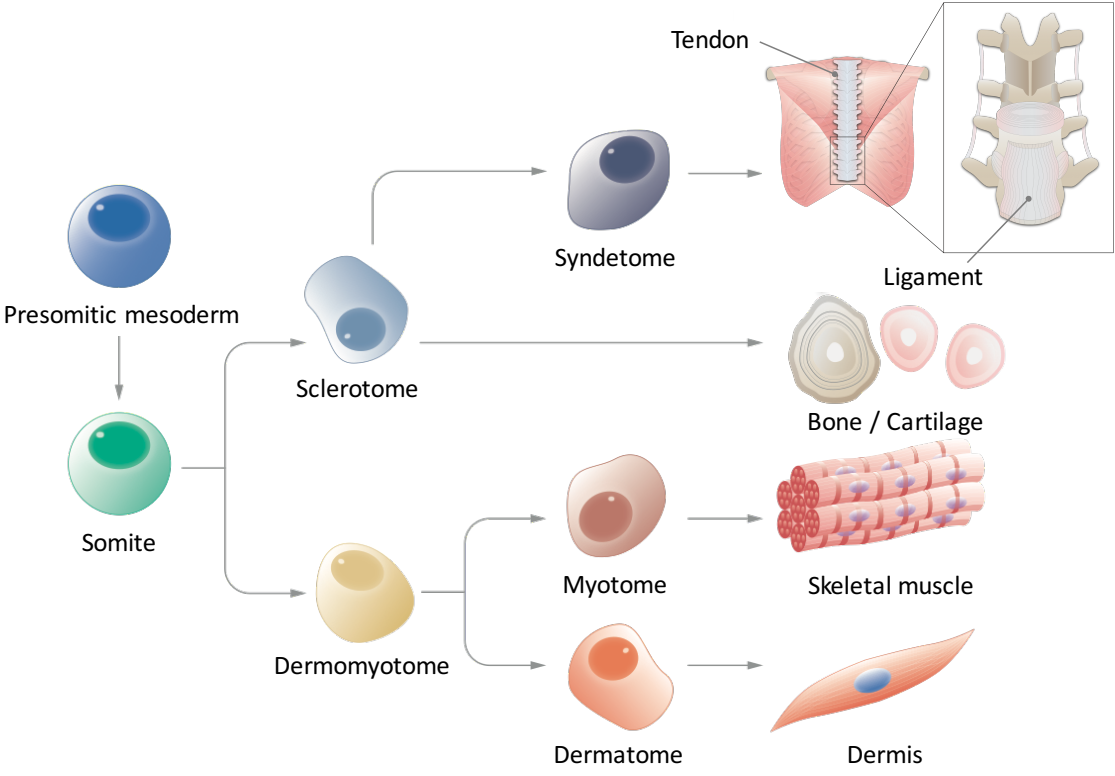590　　　development of a large region of the mouse brain. *Cell.* **62** (6), 1073-1085, (1990).
591　17　Takahashi, K. et al. Induction of pluripotent stem cells from adult human fibroblasts by
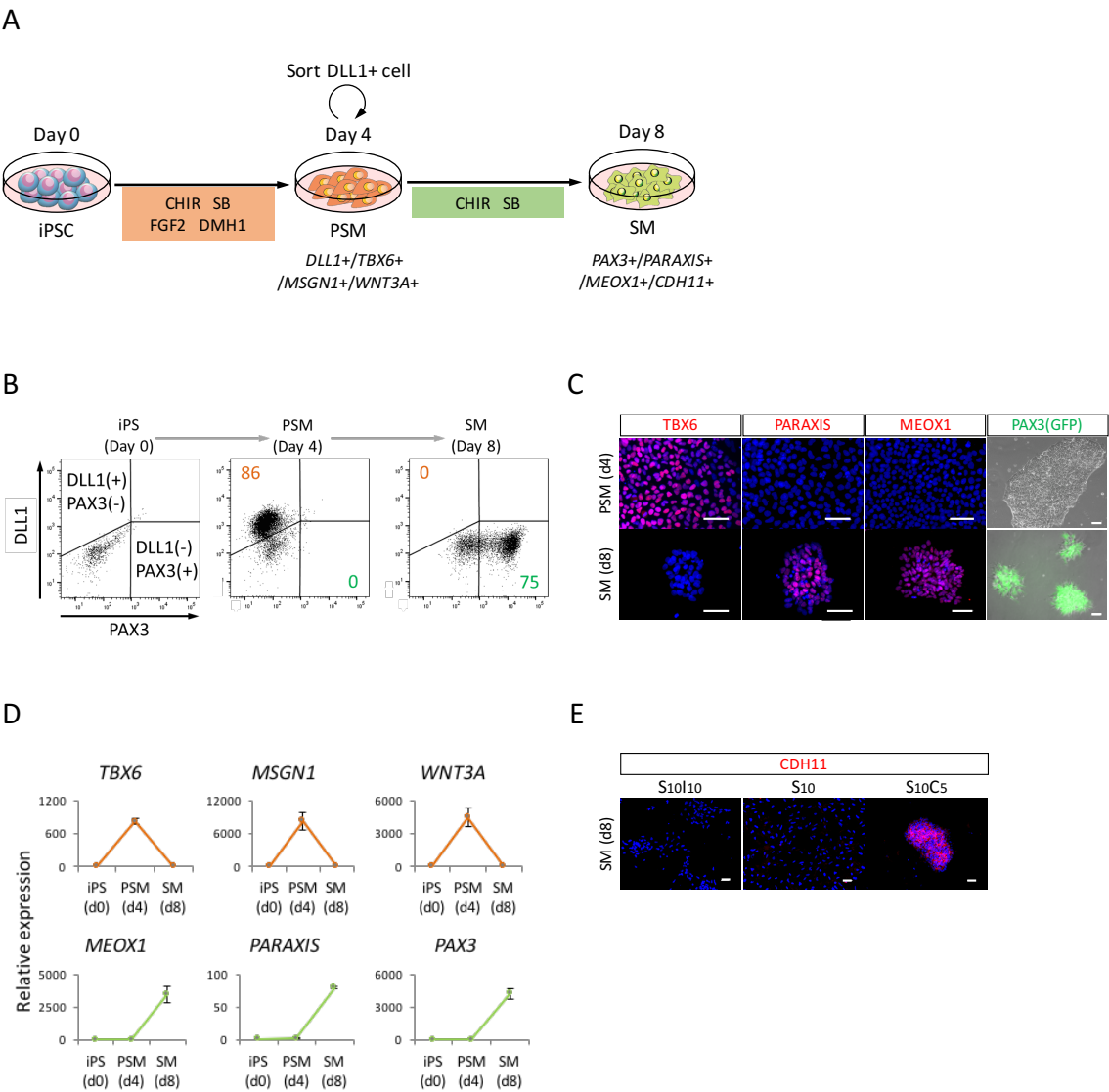592　　　defined factors. *Cell.* **131** (5), 861-872, (2007).
593　18　Suzuki, H. et al. Gene targeting of the transcription factor Mohawk in rats causes
594　　　heterotopic ossification of Achilles tendon via failed tenogenesis. *Proceedings of the*
595　　　*National Academy of Sciences of the United States of America.* **113** (28), 7840-7845,
596　　　(2016).
597　19　Marturano, J. E., Arena, J. D., Schiller, Z. A., Georgakoudi, I., Kuo, C. K. Characterization
598　　　of mechanical and biochemical properties of developing embryonic tendon. *Proceedings*
599　　　*of the National Academy of Sciences of the United States of America.* **110** (16), 6370-
600　　　6375, (2013).
601　20　Maretto, S. et al. Mapping Wnt/beta-catenin signaling during mouse development and
602　　　in colorectal tumors. *Proceedings of the National Academy of Sciences of the United*
603　　　*States of America.* **100** (6), 3299-3304, (2003).
604

Figure1 Click here to access/download;Figure;2nd-Rev_Figure 1.pdf ⤓

Presomitic mesoderm

Somite

Sclerotome

Syndetome

Tendon

Ligament

Bone / Cartilage

Dermomyotome

Myotome

Skeletal muscle

Dermatome

Dermis

Figure3   Click here to access/download;Figure;2nd-Rev_Figure 3.pdf ⬇

A

B

Figure4

Figure 4

Table 1   Click here to access/download;Table;Table_1.xlsx ⬇
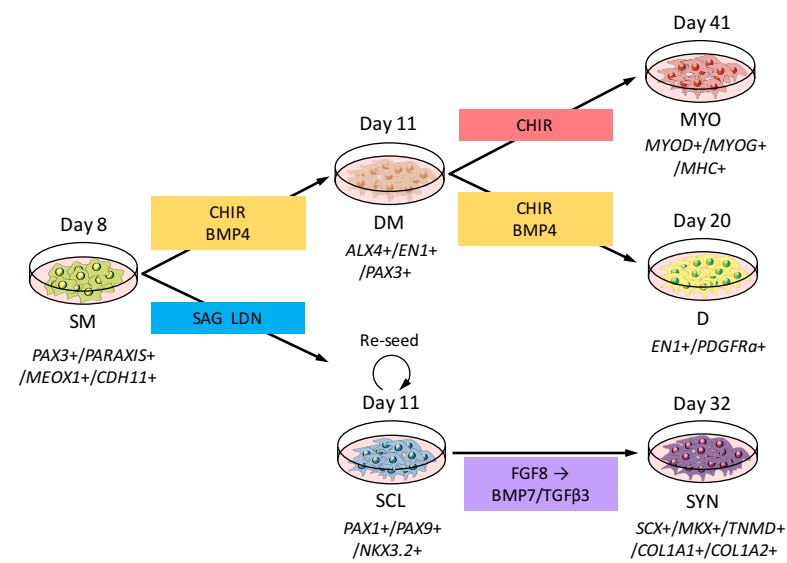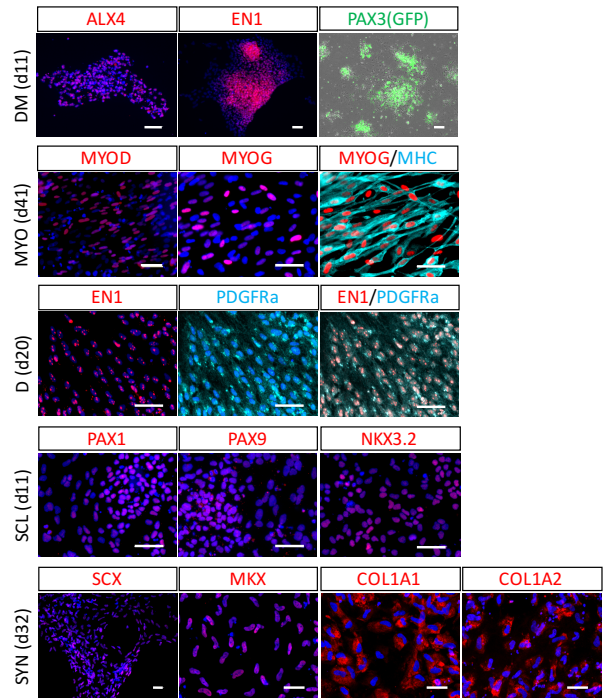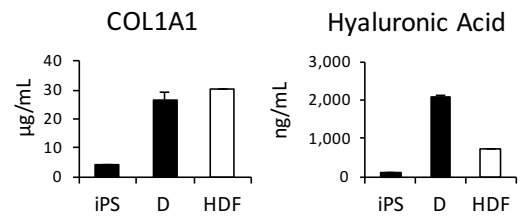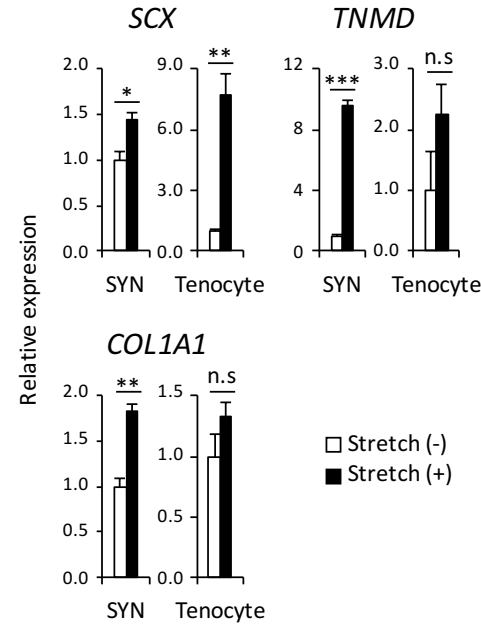
Medium/solution	Reagant
CDM basal medium	Iscove's modified Dulbecco's medium/Ham's F12
	Penicillin/Streptomycin
	Chemically defined lipid concentrate
	Apo-transferrin
	Monothioglycerol
	Bovine serum albumin
	Insulin
CTK solution	Water
	Trypsin
	Collagenase IV
	Calcium chloride
	Knockout SR
D induction medium	CDM basal medium
	CHIR99021
	BMP4
DM induction medium	CDM basal medium
	CHIR99021
	BMP4
ECM solution	Artificial extracellular matrix
	DMEM/F12
FACS buffer	PBS
	Bovine serum albumin
Feeder-free cell culture medium	mTeSR1
	Penicillin/Streptomycin
HDF culture medium	DMEM
	Fetal bovine serum
hESC medium	Primate ES cell medium
	Penicillin/Streptomycin
	FGF2
MYO induction medium	CDM basal medium
	CHIR99021
PSM induction medium	CDM basal medium
	SB431542
	CHIR99021
	DMH1
	FGF2
SCL induction medium	CDM basal medium
	SAG
	LDN193189
SM induction medium	CDM basal medium
	SB431542
	CHIR99021
SYN induction medium 1	CDM basal medium

SYN induction medium-1	FGF8
SYN induction medium-2	CDM basal medium
	BMP7
	TGFβ3

Concentration
1:1
0.5 %
1 %
15 mg/mL
450 mM
5 mg/mL
7 mg/mL
-
0.25 %
0.1 mg/mL
1 mM
20 %
-
5 µM
10 ng/mL
-
5 µM
10 ng/mL
0.3 mg/mL
-
-
0.1 %
-
0.5 %
-
10 %
-
0.5 %
4 ng/mL
-
5 µM
-
10 µM
10 µM
2 µM
20 ng/mL
-
100 nM
0.6 µM
-
10 µM
5 µM
-

20 ng/mL
-
10 ng/mL
10 ng/mL

Table 2

NAME	Forward	Reverse
ACTB	CACCATTGGCAATGAGCGGTTC	AGGTCTTTGCGGATGTCCACGT
COL1A1	GGACACAGAGGTTTCAGTGGT	GCACCATCATTTCCACGAGC
MEOX1	GAGATTGCGGTAAACCTGGA	GAACTTGGAGAGGCTGTGGA
MSGN1	GGAGAAGCTCAGGATGAGGA	GTCTGTGAGTTCCCCGATGT
PARAXIS	TCCTGGAGAGCTGTGAGGAT	CACACCCTGTCACCAACAGT
PAX3	AGGAAGGAGGCAGAGGAAAG	CAGCTGTTCTGCTGTGAAGG
SCX	CCCAAACAGATCTGCACCTTC	GCGAATCGCTGTCTTTCTGTC
TBX6	AGCCTGTGTCTTTCCATCGT	AGGCTGTCACGGAGATGAAT
TNMD	CCCTTCATGCTGAAGCCACTT	CTCACTTTCAGCAGAATTGGGG
WNT3A	CAAGATTGGCATCCAGGAGT	ATGAGCGTGTCACTGCAAAG

Table 3

		Concentration
1st Antibody	ALX4_Goat	1/50
	CDH11_Mouse	1/1000
	COL1A1_Rabbit	1/100
	COL2A1_Mouse	1-2 µg/mL
	EN1_Rabbit	1/50
	MEOX1_Rabbit	1/50
	MHC_Rabbit	1/200
	MKX_Rabbit	1/50
	MYOD_Rabbit	1/500
	MYOG_Mouse	1/400
	NKX3.2_Rabbit	1/50
	PARAXIS_Rabbit	1/50
	PAX1_Rabbit	1/50
	PAX9_Rabbit	1/50
	PDGFRa_Goat	1/100
	SCX_Rabbit	1/50
	TBX6_Goat	1/50
2nd Antibody	Donkey anti Goat IgG(H+L) secondary antibody555	1/500
	Donkey anti Goat IgG(H+L) secondary antibody647	1/500
	Goat anti Mouse IgG(H+L) secondary antibody555	1/500
	Goat anti Rabbit IgG(H+L) secondary antibody555	1/500
	Goat anti Rabbit IgG(H+L) secondary antibody647	1/500

Name of Material

ALX4_Goat antibody

Apo-transferrin

BMP4

BMP7

Bovine serum albumin

Calcium chloride

CDH11_Mouse antibody

Cell streching device

Chemically defined lipid concentrate

CHIR99021

COL1A1_Rabbit antibody

COL2A1_Mouse antibody

Collagenase IV

DLL1 APC-conjugated_Mouse antibody

DMEM

DMEM/F12

DMH1

EN1_Rabbit antibody

Fetal bovine serum

FGF2

FGF8

Human dermal fibroblast

Human tenocyte

Insulin

Iscove's modified Dulbecco's medium/Ham's F12

Knockout SR

LDN193189

Matrigel

MEOX1_Rabbit antibody

MHC_Rabbit antibody

MKX_Rabbit antibody

Monothioglycerol

mTeSR1

Multi well-type silicon rubber chamber

MYOD_Rabbit antibody

MYOG_Mouse antibody

NKX3.2_Rabbit antibody

Novex Donkey anti Goat IgG(H+L) secondary antibody555

Novex Donkey anti Goat IgG(H+L) secondary antibody647

Novex Goat anti Mouse IgG(H+L) secondary antibody555

Novex Goat anti Rabbit IgG(H+L) secondary antibody555

Novex Goat anti Rabbit IgG(H+L) secondary antibody647

PARAXIS_Rabbit antibody

PAX1_Rabbit antibody

PAX9_Rabbit antibody

PBS

PDGFRa_Goat
Penicillin/Streptomycin
Primate ES cell medium
SAG
SB431542
SCX_Rabbit antibody
TBX6_Goat antibody
Tendon cell growth medium
TGFβ3
Trypsin

Company	Cat. No	Comments
Santacruz	sc-22066	
Sigma	T1147	
R&D	314-BP-010	
R&D	354-BP-010	
Sigma	A8806	
Nacalai tesque	067730-15	
Cell signaling	13577	
Strex	STB-140	
Gibco	11905-031	
Axon	1386	
Abcam	ab34710	
Thermo scientific	MS-235	
Thermofisher	17104019	
R&D	FAB1818A	For FACS
Sigma	D6046	
Gibco	11320-082	
Tocris	4126	
Abcam	ab70993	
Nichirei	171012	
Wako	060-04543	
Peprotech	100-25	
Cell applications	160-05a	
Angio proteomie	cAP-0041	
Wako	090-06474	
Gibco	21056023	
Gibco	10828028	
Axon	1509	
BD bioscience	354230	Artificial extracellular matrix
Abcam	ab75895	
Santacruz	sc-20641	
Atlas antibodies	A83377	
Sigma	M6145	
Stemcell tech	85850	
Strex	STB-CH-4W	
Abcam	ab133627	
Santacruz	sc-12732	
Sigma	HPA027564	
Invitrogen	A21432	
Invitrogen	A21447	
Invitrogen	A21422	
Invitrogen	A21428	
Invitrogen	A21245	
Santacruz	sc-98796	
Abcam	ab95227	
Gene tex	GTX104454	
-	-	

R&D	AF307	
Invitrogen	15140-122	
Reprocell	RCHEMD001	
Calbiochem	566661	
Selleckchem	SEL-S1067-10	
Abcam	ab58655	
R&D	AF4744	
Angio-proteomie	cAP-40	Tenocytes growth medium
R&D	243-B3-200	
Gibco	15090046	

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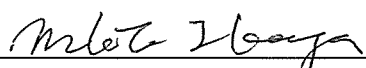
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Manuscript_JoVE59359R1

Somitogenesis method based on human pluripotent stem cells for the *in vitro* generation of somite derivatives

Rebuttal letter for 2nd_editorial comments

First of all, please let us sincerely appreciate for giving us a chance to report our recent findings in *Journal of Visualized Experiments*. We have answered all 2nd editorial comments accordingly, as described below.

Editorial comments:

< #1 >

1.2.9: Do you mean 'Day -1' here?

< Answer to #1 >

Yes, you are right. Thank you for pointing it out. We corrected it as on line 156.

< #2 >

2.2.3: How exactly are cells counted?

< Answer to #2 >

We used an automated cell counter for counting. We added this description on lines 187, 231, and 315.

< #3 >

Thank you for the diagrams in Figures 2 and 3; however, they are a little hard to reconcile with the protocol; e.g., how exactly do SB431542, CHIR99021, DMH1, and FGF2 from 2.1.1 correspond to WNT, FGF2, TGFβ-inh, and BMP-inh from Figure 2A? A table may help.

< Answer to #3 >

Thank you very much for your comments. We think these descriptions should be unified thus we used the exact names of reagents *e.g.* SB431542 or CHIR99021 in Figure 2A and 3A as well.

< #4 >

   Please remove 'Figure 1'/'Figure 2'/etc. from the Figures themselves.

< Answer to #4 >

   Thanks, we removed it from all Figures.

< #5 >

   Figures 2 and 3: Please include a space between 'Day' and the actual day; e.g., 'Day 0' instead of 'Day0'.

< Answer to #5 >

   Thanks, we corrected it.

< #6 >

   Table of Materials: There should only be 4 columns here (Name of Material, Company, Catalog Number, Comments) and it should be a unified table of all materials used. It may be best to make separate Tables for media recipes and possibly antibodies (but there still should be a Table of Materials with the above information).

< Answer to #6 >

   We agree your suggestion and we prefer to make a Table of Materials with 4 columns including all information because we think it is easier for readers to find materials arranged in alphabetical order.