



THE UNIVERSITY *of* EDINBURGH

Edinburgh Research Explorer

In vitro model of fetal human vessel on-chip to study developmental mechanobiology

Citation for published version:

Ventura, T, Egan, E, Romanò, N & Fidanza, A 2023, 'In vitro model of fetal human vessel on-chip to study developmental mechanobiology', *Journal of Visualized Experiments (JoVE)*. <https://doi.org/10.3791/65492>

Digital Object Identifier (DOI):

[10.3791/65492](https://doi.org/10.3791/65492)

Link:

[Link to publication record in Edinburgh Research Explorer](#)

Document Version:

Peer reviewed version

Published In:

Journal of Visualized Experiments (JoVE)

General rights

Copyright for the publications made accessible via the Edinburgh Research Explorer is retained by the author(s) and / or other copyright owners and it is a condition of accessing these publications that users recognise and abide by the legal requirements associated with these rights.

Take down policy

The University of Edinburgh has made every reasonable effort to ensure that Edinburgh Research Explorer content complies with UK legislation. If you believe that the public display of this file breaches copyright please contact openaccess@ed.ac.uk providing details, and we will remove access to the work immediately and investigate your claim.



1 **TITLE: *In vitro* model of fetal human vessel on-chip to study developmental mechanobiology**

2
3 Telma Ventura¹, Ewan Joshua Egan¹, Nicola Romanò², Antonella Fidanza^{1*}

4 1 Centre for Regenerative Medicine, Institute for Regeneration and Repair, University of
5 Edinburgh, Edinburgh, UK

6 1 Centre for Discovery Brain Sciences, University of Edinburgh, Edinburgh, UK

7 *Correspondence to afidanza@ed.ac.uk

8
9 **SUMMARY**

10
11 We describe here a simple workflow to differentiate endothelial cells from human pluripotent
12 stem cells and to stimulate them with mechanical cues allowing the study of developmental
13 mechanobiology. The approach described in this protocol is compatible with downstream assays
14 of live cells after mechanical stimulation following cell collection from the culture chip.

15
16 **ABSTRACT**

17
18 The heart is the first organ to be functionally established during development, thus initiating
19 blood circulation very early in gestation. Besides transporting oxygen and nutrients to ensure
20 fetal growth, fetal circulation controls many crucial developmental events taking place within the
21 endothelial layer through mechanical cues. This includes inducing blood vessel structural
22 changes, establishing artero-venous specification, and controlling the development of
23 hematopoietic stem cells. The understanding of the instructive role of circulation early in human
24 development is limited due to the inaccessibility of the tissues, therefore, *in vitro* models are
25 pivotal tools for the study of vessel mechanobiology. Here, it is described the protocol for
26 differentiating endothelial cells from human iPSCs and their subsequent seeding into a fluidic
27 pump device to study their response to mechanical cues. This approach allows for long-term
28 culture of endothelial cells under mechanical stimulation, and it allows the subsequent retrieval
29 of the endothelial cells for phenotypical and functional characterization. The *in vitro* model
30 established in here will be instrumental in elucidating the intracellular molecular mechanisms
31 that transduce the signaling mediated by mechanical cues which ultimately orchestrate vessel
32 development during human fetal life.

33
34 **INTRODUCTION**

35
36 During embryonic development, the heart is the first organ to establish functionality¹,
37 with detectable contractions since the stage of endocardial tube². Circulation, and the
38 mechanical cues mediated by the flow of blood within the vessel, controls many crucial aspects
39 of early developmental process. Prior to fetal circulation establishment, the vasculature is
40 organized into a primary capillary plexus; upon cardiac functioning this plexus reorganizes into
41 venous and arterial vasculature³. The role of mechanical cues on the arteriovenous specification
42 is reflected by the pan-endothelial expression of arterial and venous markers before blood flow
43 initiation⁴.

44

45 Hemodynamics not only control the development of the vasculature itself but also plays
46 a fundamental role in the control of blood cell formation. Hematopoietic Stem and Progenitor
47 Cells (HSPCs) emerge from specialized endothelial cells called hemogenic endothelium⁵⁻⁸,
48 present in different anatomical regions of the embryos exclusively in the early stage of
49 development. Heart deficient models together with *in vitro* models have demonstrated that
50 mechanical cues instruct and increase the HSPCs production from the hemogenic endothelium⁹⁻
51 ¹⁴.

52
53 Different types of flow dynamics have been shown to differentially control cell cycle¹⁵,
54 known to be important in both hemogenic endothelium^{16,17} and arterial cell specification¹⁸.
55 Altogether, mechanical cues are critical determinants of cell identity and function during
56 development. Novel *in vitro* fluidic devices allow us to overcome the limitations involved with
57 studying developmental mechanobiology during human blood development *in vivo*.

58
59 The overall goal of the protocol in this manuscript is to describe, step-by-step the analytic
60 pipeline to study the effect of shear stress on human endothelial cells derived *in vitro* from
61 human induced pluripotent stem cells (hiPSCs). This protocol contains detailed instructions on
62 the differentiation of human pluripotent stem cells into endothelial cells and their subsequent
63 seeding into fluidic chips and stimulation protocol. This will allow other laboratories to address
64 questions about the response to shear stress and its functional consequences on different
65 endothelial cell identities, as well as testing the mechanosensitivity by analysing the orientation
66 to the direction of the flow.

67 68 **PROTOCOL**

69
70 **NOTE:** All cell culture techniques were performed under sterile conditions in a laminar flow hood
71 and cells were incubated at 37 °C in humidified atmosphere with 5% CO₂.

72
73 Instructions for all cytokine preparation for both maintenance (rhbFGF) and those for the
74 differentiation protocol (rhBMP4, rhVEGF, rhbFGF, rhIL6, rhFLT3L, rhIGF1, rhIL11, rhSCF, rhEPO,
75 rhTPO, rhIL3) are in the **Supplementary Table 1**.

76 **1. Culturing of hiPSCs – Thawing, maintenance and freezing of cells**

77 **1.1 Preparation of maintenance media, growth factors and other reagents**

78 **Culture media** - Add the whole hESCs Serum Free Medium supplement, 36 mL of 25% of Bovine
79 Serum Albumin (BSA) and 1 mL of 55 mM of β-Mercaptoethanol to the Dulbecco's Modified Eagle
80 Medium/F12 (DMEM/F-12) basal medium (**See Table of materials**).

81
82 **Human Recombinant Basic Fibroblast Growth Factor (rhbFGF)** – See **Supplementary Table 1**.

83
84 **Rho Kinase inhibitor (iRock)** - Resuspend 1 mg of iRock in 1 mL of DMSO and make aliquots of 50
85 μL and store at -20 °C. These aliquots can be kept at -20°C for one year. Once thawed they can

86 be kept at 4 °C for one week.

87

88 **Vitronectin (VTN-N) solution** - Thaw the Vitronectin solution on ice and aliquot 60 µL per vial
89 prior to storing at -80 °C. Just before coating the plates, dilute the 60 µL stock in 6 mL of DPBS,
90 final concentration of 5 µg/mL.

91

92 **1.2 hiPSCs cell line thawing**

93

94 **The human pluripotent stem cells SFCi55 were previously derived in-house and extensively**
95 **used for differentiation into various cell types and different embryonic lineages^{19–22}.**

96 1.2.1 Coat one well of a 6-well plate with 1 mL of Vitronectin (VTN-N) solution for 1 h at
97 37 °C.

98 **NOTE:** After incubation, coated plates can be stored at 4 °C for up to one week.

99 1.2.2 Aspirate the Vitronectin solution with an aspiration pipette and add 1 mL of
100 prewarmed culture media supplemented with 20 ng/mL of rhbFGF.

101 1.2.3 Quickly defrost the vial containing the hiPSCs in a water bath and transfer the cell
102 into 5 mL of prewarmed culture media.

103 1.2.4 Spin the cells down for 3 min at 300g at room temperature.

104 1.2.5 Resuspend the cell pellet in 0.5 mL of culture media supplemented with 20 ng/mL of
105 rhbFGF.

106 1.2.6 Transfer the cells to one coated well containing already 1 mL of media.

107 1.2.7 Add 5 µl of iRock into the wells containing the cells in a total of 1.5 mL of media.

108 1.2.8 Culture the cells in the incubator, change the media daily during the week and double
109 feed the cells once over the weekend.

110 **NOTE:** Cells are grown in presence of Rock Inhibitor for 24 h only.

111

112 **1.3 Maintenance and passaging of hiPSCs**

113 1.3.1 Change the media daily with fresh prewarmed culture media supplemented with 20
114 ng/mL of rhbFGF.

115 1.3.2 Passage the cells when they reach approximately 80% of confluency, generally twice
116 a week.

117 1.3.3 To passage the cells, coat a plate with Vitronectin as described before in steps 1.2.1
118 and 1.2.2.

119 1.3.4 Aspirate the media from the wells with cells and wash them with DPBS.

120 1.3.5 Aspirate the DPBS and add 1 mL of dissociation reagent (**See Table of materials**) and
121 incubate for 1 min.

122 1.3.6 Aspirate the dissociation reagent and incubate for further 3 min. Firmly tap the plate
123 10 times on each side.

124 **NOTE:** The dissociation step might need cell-type specific optimization in the

125 incubation time and the tapping procedure.
126 1.3.7 Add 1 mL of culture media to the cells and with a pasteur pipette wash once the with
127 the media to ensure that most of the colonies are collected.
128 1.3.8 Add 3 drop of the cell suspension to each well, this will provide a passage ratio of 1
129 well into 6.
130 **NOTE:** Immediately after thawing a new vial it is better to passage the cells at lower
131 ratio such as 1:1 or 1:2 for one or two passages to allow them to reach a steady growing
132 phase before passaging at a ratio of 1:6.
133 1.3.9 Culture cells in the incubator, change the media daily during the week and double
134 feed the cells once over the weekend.

135

136 **1.4 hiPSCs cell line freezing**

137 **NOTE:** Freeze cell within their first two passages after thawing to ensure to maintain a constant
138 low passage batch of frozen vial to start the culture. Freeze the cells when they reach a
139 confluency of approximately 80%.

140

141 1.4.1 Change the media to fresh prewarmed culture media supplemented with 20 ng/mL
142 of rhbFGF and 5 µl of iRock and incubate for at least 1 h.

143 1.4.2 Detach the cells as described in steps 1.3.4 – 1.3.7.

144 1.4.3 Collect the detached cells in a 15 mL centrifuge tube containing 5 mL of culture
145 media.

146 1.4.4 Centrifuge for 3 min at 300g at room temperature.

147 1.4.5 Aspirate the supernatant and add 1 mL of cryopreservation solution (**See Table of**
148 **materials**).

149 1.4.6 Using a pasteur pipette, gently pipette the cells up and down to mix them in the
150 cryopreservation solution.

151 **NOTE:** Avoid excessive pipetting which might result in dissociating the cells' cluster

152 1.4.7 Divide the cell suspension into two cryopreservation vials with 0.5 mL each.

153 1.4.8 Transfer the cryopreservation vials to a cryopreservation container pre-cooled at 4°C.

154 1.4.9 Transfer the container with the cells to a -80 °C freezer for 24 h before transferring
155 the vials in liquid nitrogen for long term storage.

156

157 **2. Differentiation of hiPSCs into Endothelial cells**

158

159 **2.1 Preparation of differentiation media, cytokines, growth factors**

160

161 **Serum Free Differentiation Medium (SFD)** – Prepare the media according to **Table 1**. This
162 medium is used from Day 0 to Day 5 of differentiation

163

164 [Table 1 here]

165

166 **Serum Free Medium for CD34+ cells (SFM-34)** – Prepare the media by adding 34 nutrient
167 supplement and 5 mL of L-Glutamine supplement to the 34 SFM Basal Medium (**See Table of**
168 **materials**). This medium will be used from Day 6 of differentiation onwards.

169

170 **CHIR99021** – Resuspend 1 mg of CHIR99021 in 716 µL of DMSO to obtain a 3 mM solution.
171 Incubate at room temperature until fully resuspended, if needed warm up quickly at 37 °C. Make
172 20 µL aliquots and store them at -20 °C for up to 6 months. Use immediately after thawing and
173 do not freeze again or store.

174

175 **Cytokines** – Resuspend the cytokines according to the instruction in **Supplementary Table 1**. All
176 cytokines' aliquots are stored at -80 °C.

177

178 **2.2 Endothelial cells differentiation**

179

180 For each day of the differentiation prepare 18 mL (3 mL media/well) of prewarmed SFD media,
181 according to the cytokines' mixes described in **Table 2**.

182 [Table 2 here]

183

184 **2.2.1 Day 0 – Formation of Embryoid bodies (EBs)**

185 2.2.1.1 Prepare 18 mL of SFD media with Mix 1 cytokine according to **Table 2**, for each 6
186 well plate (3 mL/well).

187 2.2.1.2 Add 2 mL of prewarmed media in each well of a cell repellent 6-well plate.

188 2.2.1.3 To form EBs follow the steps described in 1.3.4 -1.3.6.

189 **NOTE:** hiPSCs must be between 80-90% confluent to start the differentiation.

190 2.2.1.4 Add 1 mL of prewarmed SFD media with Mix 1 cytokines to each well of detached
191 cells' clusters.

192 2.2.1.5 Use a pasteur pipette to gently transfer the cells' clusters into a single well of cell
193 repellent well for EBs formation at a ratio of 1:1.

194 2.2.1.6 After placing the plate in the incubator, move it forward and back, right and left,
195 to disperse the EBs evenly in the well.

196

197 **2.2.2 Day 1 – Media change to the EBs**

198 **NOTE:** This step is only necessary if by day 1 of differentiation there are a lot of single cells in
199 suspension alongside with the EBs.

200

201 2.2.2.1 Prepare 18 mL of SFD media with Mix 1 cytokine according to **Table 2**, for each 6
202 well plate (3 mL/well).

203 2.2.2.2 Swirl the plate with the EBs to move them in the center and collect them using a
204 pasteur pipette into a 15 mL centrifuge tube.

205 **NOTE:** If the EBs look clumped together as in strings, separate them by pipetting them

206 up and down with a P1000 before collecting them into the 15 mL centrifuge tube.

207 2.2.2.3 Wait 5-10 min for the EBs to settle at the bottom of the tube.

208 **NOTE:** If the EBs are too small centrifuge them for 5 min at 100 g to help them settle.

209 2.2.2.4 Wash the cell repellent plates with sterile water or DPBS to remove any single cell
210 or debris.

211 2.2.2.5 Add 2 mL of SFD with Mix 1 cytokines to each well of the cell repellent plates.

212 2.2.2.6 Carefully and slowly aspirate the supernatant from the EBs without dislodging
213 them.

214 2.2.2.7 Resuspend the EBs using 1 mL of SFD media with Mix 1 cytokines for each starting
215 well – for a 6 well plate, add 6mL of media.

216 2.2.2.8 Transfer the EBs to the cell repellent plates 1 mL per well, which already contains
217 the 2 mL of SFD media.

218 2.2.2.9 After placing the plate in the incubator, move it forward and back, right and left,
219 to disperse the EBs evenly in the well.

220

221 2.2.3 Day 2 – Addition of CHIR99021

222 2.2.3.1 Swirl the EBs in the center of the plate and add CHIR99021 according to **Table 2**
223 on the side of the well to avoid direct contact with the cells.

224 **NOTE:** If the media was not changed at Day 1, replace the whole media instead of
225 adding CHIR alone. Prepare 18 mL of SFD media with Mix 2 according to **Table 2**, for
226 each 6 well plate (3 mL/well).

227 2.2.3.2 After placing the plate in the incubator, move it forward and back, right and left,
228 to disperse the EBs evenly in the well.

229 2.2.4 Day 3 – Media change to the EBs and addition of Day 3 cytokines

230 2.2.4.1 Prepare 18 mL of SFD media with Mix 3 cytokines according to **Table 2**, for each 6
231 well plate (3 mL/well).

232 2.2.4.2 Collect the EBs as described on steps 2.2.2.2 – 2.2.2.4.

233 2.2.4.3 Add prewarmed 2 mL of SFD media with Mix 3 cytokines to the cell repellent
234 plates.

235 2.2.4.4 Carefully aspirate the supernatant from the EBs. Add 1 mL/well of SFD with Mix 3
236 cytokines.

237 2.2.4.5 Distribute the EBs between the well as described in the steps 2.2.2.7 – 2.2.2.9.

238

239 2.2.5 Day 6 – Media change for SFM-34 and addition of Day 6 cytokines

240 2.2.5.1 Prepare 18 mL of SFD media with Mix 4 cytokines according to **Table 2**, for each 6
241 well plate (3 mL/well).

242 2.2.5.2 Collect the EBs as described on steps 2.2.2.2 – 2.2.2.4.

243 2.2.5.3 Add 2 mL of prewarmed SFM-34 media with Mix 4 cytokines to the low adherent
244 plates.

245 2.2.5.4 Carefully aspirate the supernatant from the EBs. Add 1 mL/well of SFM-34 with
246 Mix 4 cytokines.
247 2.2.5.5 Distribute the EBs between the wells as described in the steps 2.2.2.7 – 2.2.2.9.
248

249 **3. CD34⁺ cells isolation and seeding into chip**

250 **NOTE:** CD34⁺ cell isolation is done using a positive isolation approach with CD34 Microbead kit
251 (See **Table of materials**), that contains CD34 Microbeads conjugated to monoclonal mouse
252 antibodies anti-human CD34 antibodies and FcR Blocking reagent (Human IgG).

254 **3.1 Prepare materials and reagents**

255
256 **Washing buffer** – Add 5 mL of 5% BSA solution and 200 µL of EDTA 0.5 M to 45 mL of DPBS to
257 obtain PBS + 0.5% BSA + 2 mM EDTA. Prepare fresh for each isolation, filter sterilize and keep
258 refrigerated until use.

259
260 **Fluidic chips** - Coat the fluidic chips with Laminin solution prepared by diluting the rhLaminin 521
261 1:50 in PBS containing Ca⁺⁺/Mg⁺⁺. Coat each chip with the appropriate volume for the chip in use
262 and incubate in the incubator for 2 h ahead of seeding.

263 **NOTE:** Other matrix can be employed for the coating and should be tested for the specific cell
264 type/experiment.

265
266 **Mix 4 SFM-34 medium** - Prepare 18 mL of SFM-34 medium supplemented with Mix 4 cytokines
267 according to **Table 2** and place it in a 50 mL tube in the incubator with the lid slightly unscrewed
268 to facilitate gas exchange.

269
270 **Perfusion set and any other tubing for the fluidic set up** - Place the selected perfusion sets are
271 planning to use in the incubator to degas.

272 273 **3.1.1 Day 8 – Dissociation of EBs and CD34⁺ isolation**

274 3.1.1.1 Collect the EBs as described on steps 2.2.2.2 – 2.2.2.4.

275 3.1.1.2 Add 1 mL of cell dissociation reagent per each starting well of EBs collected (if
276 collected 6 wells add 6 mL).

277 3.1.1.3 Transfer back 1 mL of the EBs suspension in cell dissociation reagent to each well
278 of the cell repellent plate.

279 3.1.1.4 Incubate for 10 min in the incubator.

280 3.1.1.5 Gently pipette the EBs up and down against the well with a P1000, no more than
281 10 times.

282 3.1.1.6 Repeat steps 2.2.7.7 – 2.2.7.8 for a total of 3 times.

283 **NOTE:** If the EBs are difficult to dissociate repeat the above steps 4 times in total.

284 3.1.1.7 Add 5 mL of washing buffer for each well of dissociated EBs.

285 3.1.1.8 Collect the cells into a 50 mL centrifuge tube by passing them through a 40 µm

286 strainer. Take 10 μL of the cell suspension to count the cells.

287 **NOTE:** For a 6 well plate, around 10^6 cells should be collected after filtration.

288 3.1.1.9 Spin the cells down for 10 min at 300 g.

289 3.1.1.10 Resuspend the cells into 300 μL of washing buffer, gently pipetting a few times
290 to make sure that no clumps are present. Continue following the manufacturer protocol (**See**
291 **Table of materials**).

292

293 3.1.2 CD34+ Cells seeding into fluidic chips

294 **NOTE:** The fluidic chip used in the protocol has a channel height of 0.6 mm and length of 50 mm,
295 for a total growth area of 2.5 cm^2 . This type of chip is seeded with a total volume of cell
296 suspension of 150 μL . Different chips can be used, and the volume of seeding and the cell density
297 should be adapted according to the growth area. Additional optimisation might be needed
298 depending on the cell line used and its growth.

299

300 3.1.2.1 Resuspend the isolated CD34⁺ cells in 300 μL of prewarmed SFM-34 media with
301 Mix 4 cytokines.

302 3.1.2.2 Take 10 μL of the cell solution and count the cells.

303 3.1.2.3 Resuspend 2.5×10^5 cells in a final volume of 150 μL supplemented SFM-34, add
304 also 0.5 μL of iRock.

305 3.1.2.4 Slowly aspirate the laminin from fluidic chips by putting the tip of a P200 inside
306 the reservoir on the edge of the channel.

307 **NOTE:** If the solution is difficult to collect, slowly lift one side of the chip to help the
308 solution move to the opposite reservoir.

309 3.1.2.5 Add the cell solution steadily into the channel to make sure no bubbles are
310 formed.

311 **NOTE:** Perform steps 3.1.5 quickly but gently to avoid the laminin drying out and the
312 formation of bubbles in channel of the chip. If bubbles are formed, lift one side of the
313 chip and gently tap the slide to mobilize the bubbles, when they reach the reservoir,
314 they will rise to the air interface and should not be able to reenter the channel.

315 3.1.2.6 Transfer the chip in the incubator and leave for 3-5 h or until the endothelial cells
316 are completely attached to channel and they look elongated.

317 3.1.2.7 When the cells are fully attached, aspirate the media as in the step 3.1.4 and
318 replace with 200 μL of cytokine supplemented SFM-34.

319 3.1.2.8 From now on, replace the media daily until the cells have reached 90%-100%
320 confluency.

321

322 4. Application of continuous flow to endothelial cells – Aorta-on-a-chip

323

324 **4.1 Prepare materials and reagents**

325

326 **Mix 4 SFM-34 medium** – Prepare 18 mL of SFM-34 medium supplemented with Mix 4 cytokines
327 according to **Table 2** and place it in a 50 mL tube in the incubator with the lid slightly unscrewed
328 to facilitate gas exchange.

329

330 **Perfusion set and any other tubing for the fluidic set up** - Place the selected perfusion sets are
331 planning to use in the incubator to degas.

332

333 **4.2 Fluidic system assembly**

334

335 4.2.1 Assemble the perfusion set to the unit according to the manufacturer protocol,
336 making sure to add the media in sterile condition in the hood.

337 **NOTE:** Remember to use clamps in the system. If sliding clamps are used for this step
338 they need to be slid on the tubing before connecting to the chip.

339 4.2.2 Attach a new fluidic chip to perform the bubble removal program and the calibration
340 step.

341 4.2.3 Once the whole set up of the pump is completed, change the media to the cells
342 using cytokines supplemented SFM-34 making sure to fill up completely both reservoirs.

343 4.2.4 Remove the fluidic unit with the connected set from the incubator and transfer in
344 the hood, take also the chips containing the cells from the incubator.

345 4.2.5 Clamp the tubing on both sides of the test chip.

346 4.2.6 Remove the tubing from the test chip.

347 **NOTE:** Check that no bubbles are present in the Luer connector at the end of the
348 tubing. If bubbles are visible, carefully aspirate them using a P200 pipette and if
349 necessary add more media to make sure that the connector is filled with media.

350 4.2.7 Connect the chip containing the cells with the tubing.

351 4.2.8 Remove or open the clamps.

352 4.2.9 Transfer the system to the incubator and connect the air pump to the fluidic unit .

353 4.2.10 Start the preselected program using the pump dedicated software, with the
354 gradual increase of shear stress described in **Table 3**.

355 **NOTE:** Depending on the specific experiment that is needed, the stimulation program
356 might need optimization. We describe here a gradual shear stress increase leading to
357 final value of 5 dyn/cm² which mimics the shear stress calculated to be experienced by
358 the wall of dorsal aorta at the onset of fetal circulation ⁹. Independently of the final
359 shear stress that will be employed, it is necessary to gradually increase over time to
360 allow the cells to adapt to the force without causing the cells to detach from the chip.

361

362 **NOTE:** If the selected stimulation protocol is longer than 3 days, cytokines should be
363 topped up into the system by adding 1 mL of SFM-34 containing the Mix 4 cytokines
364 that would normally be added into 18 mL. To do this, the pump program is quickly
365 paused and 500 µL of supplemented media is added to each of the two syringes in the
366 fluidic set.

367

368 **4.3 Cell collection for analysis**

369

370 4.3.1 Prewarm the dissociation buffer in a water bath.

371 4.3.2 Remove the fluidic unit from the pump and move into the hood.

372 4.3.3 Clamp the tubing flanking the chip on both sides and remove the tubing from the
373 reservoirs on the chip.

374 4.3.4 Gently remove the media from the chip and replace with DPBS to wash the cells.

375 **NOTE:** This step of washing with PBS can be skipped if the cells start to detach.

376 4.3.5 Gently add 150 μ L of dissociation buffer and incubate for 3 minutes at 37°C.

377 **NOTE:** Check under the microscope if the cells are single and detached, otherwise
378 incubate for additional 2 minutes. It is essential that the cells are completely detached
379 from the channel before aspirating the media, as the chip does not allow to aid cell
380 detachment by pipetting. Other solutions can be employed to detach the cells, such as
381 Trypsin or EDTA based buffers.

382 4.3.6 Aspirate the dissociation buffer from one reservoir and wash the channel once with
383 DPBS.

384 4.3.7 Add 1 ml of washing buffer (step 3.1), take a 10 μ L to count the cells.

385 4.3.8 Divide the cell solution in flow cytometry tubes to have 10^5 cells per test tube.

386 4.3.9 Spin the tubes for 5 min at 300 g.

387 4.3.10 Prepare the staining solution to have 50 μ L for each test tube to stain. Add the
388 CD34 PerCP-eFluor710 or CD34-PE at 1:100 and 1:200 dilution, respectively.

389 4.3.11 Resuspend the cells in 50 μ L of staining solution and incubate for 30 min at 4 °C.

390 4.3.12 Wash the cells by adding 2 mL of washing buffer and spin for 5 minutes at 300g.

391 4.3.13 Resuspend the pellets in 100 μ L of staining solution and acquire the data using a
392 flow cytometer.

393 **NOTE:** The cells can also be lysed directly in the chip for RNA extraction using 150 μ L
394 of RNA lysis buffer or fixed for imaging using 4% Paraformaldehyde in DPBS for 10 min
395 at room temperature.

396

397 **4.4 Cell orientation analysis**

398

399 Images were analysed to quantify changes in cell orientation using FIJI²³ (Supplementary Figure
400 2).

401

402 4.4.1 Open the ROI (region of interest) manager from the Analyze -> Tools -> ROI manager
403 menu.

404 4.4.2. Draw the cell contours manually using the polygon selection tool and add them to the
405 ROI manager pressing "Add" or using the CTRL+T shortcut (Fig xxx A).

406 4.4.3 Measure the orientation of each ROI by choosing the "Fit ellipse" measure in the
407 "Analyze → Set Measurements..." menu.

408 4.4.4 Apply the measurement to all ROI through the "More >> → Multi measure"
409 command in the ROI manager. This will fit an ellipse to each ROI and generate a table
410 containing the length of the major and minor ellipse axis, as well as the angle.

411 4.4.5 Export the table to a CSV file to be imported in other software to plot, such as R.
412 The script used for the plots in figure 5 is available at
413 <https://gist.github.com/nicolaromano/708b3231d730ee7f70763a7cf8850ddc>.
414
415

416
417

418 **REPRESENTATIVE RESULTS**

419

420 We describe here a protocol for the differentiation and mechano-stimulation of endothelial cells
421 derived from hiPSCs that allows the study of their response to mechanical cues (Figure1). This
422 protocol results in the production of functionally mechanosensitive endothelial cells. We provide
423 here representative results and describe the expected phenotype to assess how the cells respond
424 to the cytokine stimulation during the differentiation.

425 **[Figure 1 here]**

426

427 **1. Culture of human induced Pluripotent Stem Cells**

428

429 It is important to start the protocol from hiPSCs that are growing correctly in self-renewal
430 conditions. A good indication of the quality of the culture is the speed of their growth. After
431 thawing, the cells might need a couple of weeks to reach the right phase of growth that will
432 ensure a good differentiation. When the cells can be passaged twice a week at the ratio of 1:6
433 reaching almost full confluency, this is the time that they are ready to be differentiated on the
434 same day they need to be passaged.

435

436 **2. Differentiation of human induce Pluripotent Stem Cells into Endothelial cells**

437

438 The first step of the differentiation, consisting of the formation of Embryoid Bodies (EB),
439 this process is cell line dependent, and it might need some optimization for the specific cell line
440 in use. The dissociation step described in 2.2.2 can be modified by both reducing/extending the
441 incubation with the dissociation reagent and the subsequent dissociation with the pasteur
442 pipette. Furthermore, other dissociation reagent can be used for this step as well as physical
443 dissociation of the colonies with a cutting tool or a P100 pipette tip.

444 EBs of good quality show a defined edge by day 2 of the differentiation and appear clear and
445 bright when observed using a microscope; darker area might indicate cell death within the EBs
446 (Figure2).

447 **[Figure 2 here]**

448

449 At day 2 the EBs are exposed to Wnt Pathways manipulation by addition of CHIR99021
449 which by inhibiting the GSK-3 protein mediate activation of the pathway. Different cell lines have
450 a different response to CHIR treatment, and this should be tested by quantifying the number of
451 CD34⁺ cells obtained at day 8 by using different concentrations (Figure 3).

452 **[Figure 3 here]**

453

454

455 **3. CD34⁺ cell isolation**

456

457 It is important to validate that the CD34⁺ enrichment using the magnetic beads provides at least
458 80% CD34⁺ after elution of the column. To ensure sufficient purity, an aliquot of cells obtained
459 from the magnetic isolation can be analysed by flow cytometry making sure to use a different
460 antibody clone than the one used for the magnetic enrichment. We used the clone 4H11 and we
461 obtain around 85% purity post enrichment (Figure 4).

462 **[Figure 4 here]**

463

464

465 **4. Seeding cells into the fluidic channel**

466

467 When seeding the cells in the fluidic channel it is crucial to track the adhesion and
468 proliferation of the endothelial cells. After seeding, the cells take around 5 hours to fully adhere
469 to the channel (Figure 5A). Alternative coating solution might also be tested to improve adhesion
470 at this stage. To validate that the tested cells are mechanosensitive and able to respond to the
471 mechanical stimulation the cell orientation can be tested over time. Cells before the stimulation
472 show random orientation (Figure 5A and 5C) and they reorient parallel to the direction of the
473 flow (Figure 5B-C). The protocol here described allows to collect the cells from the channel to
474 perform downstream analysis, like flow cytometry for the study of their membrane profile,
475 providing the endothelial identity of the stimulated cells (Figure 5D-E).

476 **[Figure 5 here]**

477

478

479

480 **FIGURE AND TABLE LEGENDS**

481

482 **Figure 1 – Schematic of the differentiation and mechanical stimulation protocol.** Schematic of
483 the differentiation protocol showing the timing of the different mixes of cytokines, the CD34⁺ cell
484 isolation, fluidic chip seeding and final analysis of the mechanically stimulated cells.

485

486 **Figure 2 – Embryoid Bodies structure.** A – Day 2 Embryoid Bodies showing well defined outer
487 hedge and of consistent size. B – Day 2 Embryoid Bodies of poor quality showing extensive cell
488 death leading to disaggregation of the structure.

489

490 **Figure 3 – Endothelial cells differentiation with different CHIR treatments.** Endothelial cells
491 commitment quantified by flow cytometry at day 8 of differentiation by CD34 membrane
492 expression, following CHIR treatment at day 2 at 3 μ M, 5 μ M and 7 μ M (A, B, C, respectively).
493 Flow cytometry data were obtained using 5 laser cytometer and dedicated software (see Table of
494 Materials).

495

496 **Figure 4 – Membrane expression of CD34 before and after enrichment by magnetic sorting.** Day
497 8 dissociate Embryoid Bodies (Grey) and cells after magnetic enrichment (Green) were stained for
498 CD34 expression and analysed by flow cytometry, showing successful enrichment post sorting

499 *Flow cytometry data were obtained using 5 laser cytometer and dedicated software (see Table of*
500 *Materials).*

501

502 **Figure 5 – hiPSCs are mechanoresponsive.** *Confluent layer of Isolated CD34⁺ cells 48 hour before*
503 *post seeding (A). Reoriented layer of endothelial cells 3 days under dynamic culture (B).*
504 *Orientation analysis of the endothelial cells after 5 days of dynamic culture (C). CD34 expression*
505 *profile of cells cultured under flow for 5 days (D). Percentage of CD34⁺ cells of cell population*
506 *retrieved from the fluidic channel (E). Images were taken using an inverted in-incubator*
507 *microscope; flow cytometry data were obtained using 5 laser cytometer and dedicated software*
508 *(see Table of Materials).*

509

510 **Supplementary Figure 1 – Geometry and dimension of the chip and tubing used for this**
511 **protocol.**

512 **Supplementary Table 1 - Unit size, resuspension volume and stock concentrations for cytokines**
513 **used in differentiation protocol.**

514 **Table 1 - Composition and recipe for 500 mL of Serum Free Differentiation (SFD) media.**

515 **Table 2 – Mixes of cytokines used for endothelial cells differentiation, days in which they are**
516 **added to the SFD media and final concentration.**

517 **Table 3 - Shear stress values for the dynamic culture and length of their application.**

518

519 **DISCUSSION**

520

521 The protocol that we describe here allows for the generation of mechanosensitive
522 endothelial cells from human pluripotent stem cells and the study of their response to
523 mechanical stimulation mediated by controlled shear stress. This protocol is entirely cytokine-
524 based and fully compatible with GMP reagents for potential translation into production of cells
525 for cell-therapy.

526

527 The derivation of hiPSCs provided scientist with a potent model for early stages of
528 embryonic development provides that enable the study of processes that are difficult to study *in*
529 *vivo*²⁴. The approach here described enables for the live-imaging and real-time study of cell
530 response to shear stress, which is not possible otherwise. The combination of hiPSCs and fluidic
531 set-up overcome both the limited availability and the inaccessibility of the tissue during
532 development, when the initiation of circulation remodels and control the establishment of the
533 cardiovascular and blood system^{3, 9, 10, 25}. In fact, the interruption of circulation in human
534 embryonic tissues available for research might significantly impacts the understanding of the
535 molecular signature controlled by mechanical cues.

536

537 A limitation of the protocol is that the endothelial cells derived from this protocol might
538 not be reflect the various identities of endothelial cells that are present after birth. To overcome

539 this limitation, specific combination of cytokines might be needed during the differentiation
540 process preceding the fluidic stimulation to obtain the desired identity or tissue-specific
541 phenotype²⁶. Furthermore, this protocol is specifically designed to study the role of shear stress
542 mediated by laminar flow. Alternative fluidic approaches will have to be employed to study the
543 effect of other mechanical cues, such as stretching or compression, or other type of flow such as
544 perturbed or disturbed flow.

545
546 We previously shown that iPSCs derived endothelial cells mimic the heterogenous artero-
547 venous cellular identities²⁷ similar to that observed in the fetal dorsal aorta²⁸⁻³⁰. This is of
548 particular importance in the context of vessel development and cellular specification, known to
549 be controlled by blood circulation. Studies in different models showed that lack of circulation
550 results in altered artero-venous specification^{11, 14, 31}. The mechanism that connect mechanical
551 cues with the specification are still unknown and the pipeline described here allows for refined
552 functional studies that cannot be tested *in vivo*.

553
554 The pipeline here defined describes the production, and the stimulation of endothelial
555 cells derived from hiPSCs using commercially available fluidic channels, avoiding the need for
556 casting the devices as for the widely used Polydimethylsiloxane (PDMS) devices¹². Furthermore,
557 the use of PDMS chips makes the collection of the stimulated cells particularly challenging, while
558 with this protocol, the cells can be easily retrieved from the channel. This significantly improve
559 the analytic power allowing for subsequent analysis such as proteomic and transcriptomic
560 analysis, flow cytometry and functional assay which might need further culture in different
561 culture conditions or even *in vivo* assays.

562 **ACKNOWLEDGMENTS**

563
564 This work was supported by the Research Advanced Grant 2021 from the European Hematology
565 Association, the Global Research Award 2021 from the American Society of Hematology and the
566 Internal Strategy Support Fund ISSF3 funded by the Wellcome Trust and the University of
567 Edinburgh.

568 **DISCLOSURES**

569
570 The authors have nothing to disclose.

571 **REFERENCES**

- 572 1. Copp, A.J. Death before birth: clues from gene knockouts and mutations. *Trends in*
573 *Genetics*. **11** (3), 87–93, doi: 10.1016/S0168-9525(00)89008-3 (1995).
- 574 2. Ji, R.P., Phoon, C.K.L., Aristizábal, O., McGrath, K.E., Palis, J., Turnbull, D.H. Onset of cardiac
575 function during early mouse embryogenesis coincides with entry of primitive erythroblasts
576 into the embryo proper. *Circulation Research*. **92** (2), 133–135, doi:
577 10.1161/01.RES.0000056532.18710.C0 (2003).
- 578 3. Peacock, H.M., Daems, M., Jones, E.A.V. Hemodynamic Control of Endothelial Cell Fates in
579
580
581
582

- 583 Development. *Cardiac and Vascular Biology*. **8**, 127–166, doi: 10.1007/978-3-030-63164-
584 2_6/FIGURES/4 (2021).
- 585 4. Chong, D.C., Koo, Y., Xu, K., Fu, S., Cleaver, O. Stepwise arteriovenous fate acquisition
586 during mammalian vasculogenesis. *Developmental Dynamics*. **240** (9), 2153–2165, doi:
587 10.1002/DVDY.22706 (2011).
- 588 5. Jaffredo, T., Gautier, R., Eichmann, A., Dieterlen-Lièvre, F. Intraaortic hemopoietic cells are
589 derived from endothelial cells during ontogeny. *Development (Cambridge, England)*. **125**
590 (22), 4575–83, at <http://www.ncbi.nlm.nih.gov/pubmed/9778515> (1998).
- 591 6. Zovein, A.C. *et al.* Fate Tracing Reveals the Endothelial Origin of Hematopoietic Stem Cells.
592 *Cell Stem Cell*. **3** (6), 625–636, doi: 10.1016/j.stem.2008.09.018 (2008).
- 593 7. Bertrand, J.Y., Chi, N.C., Santoso, B., Teng, S., Stainier, D.Y.R., Traver, D. Haematopoietic
594 stem cells derive directly from aortic endothelium during development. *Nature*. **464**
595 (7285), 108–111, doi: 10.1038/nature08738 (2010).
- 596 8. Boisset, J.C., Van Cappellen, W., Andrieu-Soler, C., Galjart, N., Dzierzak, E., Robin, C. In vivo
597 imaging of haematopoietic cells emerging from the mouse aortic endothelium. *Nature*.
598 **464** (7285), 116–120, doi: 10.1038/nature08764 (2010).
- 599 9. Adamo, L. *et al.* Biomechanical forces promote embryonic haematopoiesis. *Nature*. **459**
600 (7250), 1131–1135, doi: 10.1038/nature08073 (2009).
- 601 10. Diaz, M.F. *et al.* Biomechanical forces promote blood development through prostaglandin
602 E2 and the cAMP-PKA signaling axis. *Journal of Experimental Medicine*. **212** (5), 665–680,
603 doi: 10.1084/jem.20142235 (2015).
- 604 11. North, T.E. *et al.* Hematopoietic Stem Cell Development Is Dependent on Blood Flow. *Cell*.
605 **137** (4), 736–748, doi: 10.1016/j.cell.2009.04.023 (2009).
- 606 12. Lundin, V. *et al.* YAP Regulates Hematopoietic Stem Cell Formation in Response to the
607 Biomechanical Forces of Blood Flow. *Developmental Cell*. **52** (4), 446-460.e5, doi:
608 10.1016/j.devcel.2020.01.006 (2020).
- 609 13. Li, J. *et al.* Mimicry of embryonic circulation enhances the *hoxa* hemogenic niche and
610 human blood development. *Cell Reports*. **40** (11), 111339, doi:
611 10.1016/J.CELREP.2022.111339 (2022).
- 612 14. Azzoni, E. *et al.* The onset of circulation triggers a metabolic switch required for endothelial
613 to hematopoietic transition. *Cell reports*. **37** (11), doi: 10.1016/J.CELREP.2021.110103
614 (2021).
- 615 15. Li, Y.S.J., Haga, J.H., Chien, S. Molecular basis of the effects of shear stress on vascular
616 endothelial cells. *Journal of Biomechanics*. **38** (10), 1949–1971, doi:
617 10.1016/J.JBIOMECH.2004.09.030 (2005).
- 618 16. Batsivari, A. *et al.* Understanding Hematopoietic Stem Cell Development through
619 Functional Correlation of Their Proliferative Status with the Intra-aortic Cluster
620 Architecture. *Stem cell reports*. **8** (6), 1549–1562, doi: 10.1016/J.STEMCR.2017.04.003
621 (2017).
- 622 17. Canu, G. *et al.* Analysis of endothelial-to-haematopoietic transition at the single cell level
623 identifies cell cycle regulation as a driver of differentiation. *Genome biology*. **21** (1), doi:
624 10.1186/S13059-020-02058-4 (2020).
- 625 18. Luo, W. *et al.* Arterialization requires the timely suppression of cell growth. *Nature* **2020**
626 **589:7842**. **589** (7842), 437–441, doi: 10.1038/s41586-020-3018-x (2020).

- 627 19. Yang, C.-T. *et al.* Activation of KLF1 Enhances the Differentiation and Maturation of Red
628 Blood Cells from Human Pluripotent Stem Cells. *STEM CELLS*. **35** (4), 886–897, doi:
629 10.1002/stem.2562 (2017).
- 630 20. Lopez-Yrigoyen, M. *et al.* A human iPSC line capable of differentiating into functional
631 macrophages expressing ZsGreen: A tool for the study and in vivo tracking of therapeutic
632 cells. *Philosophical Transactions of the Royal Society B: Biological Sciences*. **373** (1750), doi:
633 10.1098/rstb.2017.0219 (2018).
- 634 21. Lopez-Yrigoyen, M. *et al.* Production and characterization of human macrophages from
635 pluripotent stem cells. *Journal of Visualized Experiments*. **2020** (158), doi: 10.3791/61038
636 (2020).
- 637 22. Fidanza, A. *et al.* Single cell analyses and machine learning define hematopoietic
638 progenitor and HSC-like cells derived from human PSCs. *Blood*. **136** (25), 2893–2904, doi:
639 10.1182/blood.2020006229 (2020).
- 640 23. Schindelin, J. *et al.* Fiji: an open-source platform for biological-image analysis. *Nature*
641 *Methods* 2012 9:7. **9** (7), 676–682, doi: 10.1038/nmeth.2019 (2012).
- 642 24. Takahashi, K. *et al.* Induction of pluripotent stem cells from adult human fibroblasts by
643 defined factors. *Cell*. **131** (5), 861–872, doi: 10.1016/J.CELL.2007.11.019 (2007).
- 644 25. North, T.E. *et al.* Hematopoietic Stem Cell Development Is Dependent on Blood Flow. *Cell*.
645 **137** (4), 736–748, doi: 10.1016/j.cell.2009.04.023 (2009).
- 646 26. Nguyen, J., Lin, Y.Y., Gerecht, S. The next generation of endothelial differentiation: Tissue-
647 specific ECs. *Cell Stem Cell*. **28** (7), 1188–1204, doi: 10.1016/J.STEM.2021.05.002 (2021).
- 648 27. Petazzi, P. *et al.* Arterial cells support the development of human hematopoietic
649 progenitors in vitro via secretion of IGFBP2. *bioRxiv*. 2022.10.04.510611, doi:
650 10.1101/2022.10.04.510611 (2022).
- 651 28. Crosse, E.I. *et al.* Multi-layered Spatial Transcriptomics Identify Secretory Factors
652 Promoting Human Hematopoietic Stem Cell Development. *Cell Stem Cell*. **27** (5), 822, doi:
653 10.1016/J.STEM.2020.08.004 (2020).
- 654 29. Calvanese, V. *et al.* Mapping human haematopoietic stem cells from haemogenic
655 endothelium to birth. *Nature* 2022 604:7906. **604** (7906), 534–540, doi: 10.1038/s41586-
656 022-04571-x (2022).
- 657 30. Zeng, Y. *et al.* Tracing the first hematopoietic stem cell generation in human embryo by
658 single-cell RNA sequencing. *Cell Research*. **29** (11), 881–894, doi: 10.1038/s41422-019-
659 0228-6 (2019).
- 660 31. Hwa, J.J., Beckouche, N., Huang, L., Kram, Y., Lindskog, H., Wang, R.A. Abnormal arterial-
661 venous fusions and fate specification in mouse embryos lacking blood flow. *Scientific*
662 *Reports*. **7** (1), doi: 10.1038/S41598-017-12353-Z (2017).
- 663
664