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

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

Telma Ventura[1], Ewan Joshua Egan[1], Nicola Romanò[2], Antonella Fidanza[1*]
1 Centre for Regenerative Medicine, Institute for Regeneration and Repair, University of Edinburgh, Edinburgh, UK
1 Centre for Discovery Brian Sciences, University of Edinburgh, Edinburgh, UK
*Correspondence to afidanza@ed.ac.uk

SUMMARY

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

ABSTRACT

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

INTRODUCTION

During embryonic development, the heart is the first organ to establish functionality[1], with detectable contractions since the stage of endocardial tube[2]. Circulation, and the mechanical cues mediated by the flow of blood within the vessel, controls many crucial aspects of early developmental process. Prior to fetal circulation establishment, the vasculature is organized into a primary capillary plexus; upon cardiac functioning this plexus reorganizes into venous and arterial vasculature[3]. The role of mechanical cues on the arteriovenous specification is reflected by the pan-endothelial expression of arterial and venous markers before blood flow initiation[4].

Hemodynamics not only control the development of the vasculature itself but also plays a fundamental role in the control of blood cell formation. Hematopoietic Stem and Progenitor Cells (HSPCs) emerge from specialized endothelial cells called hemogenic endothelium[5–8], present in different anatomical regions of the embryos exclusively in the early stage of development. Heart deficient models together with *in vitro* models have demonstrated that mechanical cues instruct and increase the HSPCs production from the hemogenic endothelium[9–14].

Different types of flow dynamics have been shown to differentially control cell cycle[15], known to be important in both hemogenic endothelium[16,17] and arterial cell specification[18]. Altogether, mechanical cues are critical determinants of cell identity and function during development. Novel *in vitro* fluidic devices allow us to overcome the limitations involved with studying developmental mechanobiology during human blood development *in vivo*.

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

PROTOCOL

NOTE: All cell culture techniques were performed under sterile conditions in a laminar flow hood and cells were incubated at 37 °C in humified atmosphere with 5% CO_2.

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

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

1.1 Preparation of maintenance media, growth factors and other reagents

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

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

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

be kept at 4 °C for one week.

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

1.2 hiPSCs cell line thawing

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

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

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

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

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

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

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

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

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

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

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

1.3 Maintenance and passaging of hiPSCs

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

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

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

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

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

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

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

incubation time and the tapping procedure.

1.3.7 Add 1 mL of culture media to the cells and with a pasteur pipette wash once the with the media to ensure that most of the colonies are collected.

1.3.8 Add 3 drop of the cell suspension to each well, this will provide a passage ratio of 1 well into 6.

NOTE: Immediately after thawing a new vial it is better to passage the cells at lower ratio such as 1:1 or 1:2 for one or two passages to allow them to reach a steady growing phase before passaging at a ratio of 1:6.

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

1.4 hiPSCs cell line freezing

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

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

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

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

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

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

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

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

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

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

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

2. <u>Differentiation of hiPSCs into Endothelial cells</u>

2.1 Preparation of differentiation media, cytokines, growth factors

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

[Table 1 here]

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

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

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

2.2 Endothelial cells differentiation

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

2.2.1 Day 0 – Formation of Embryoid bodies (EBs)

2.2.1.1 Prepare 18 mL of SFD media with Mix 1 cytokine according to **Table 2**, for each 6 well plate (3 mL/well).
2.2.1.2 Add 2 mL of prewarmed media in each well of a cell repellent 6-well plate.
2.2.1.3 To form EBs follow the steps described in 1.3.4 -1.3.6.
NOTE: hiPSCs must be between 80-90% confluent to start the differentiation.
2.2.1.4 Add 1 mL of prewarmed SFD media with Mix 1 cytokines to each well of detached cells' clusters.
2.2.1.5 Use a pasteur pipette to gently transfer the cells' clusters into a single well of cell repellent well for EBs formation at a ratio of 1:1.
2.2.1.6 After placing the plate in the incubator, move it forward and back, right and left, to disperse the EBs evenly in the well.

2.2.2 Day 1 – Media change to the EBs

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

2.2.2.1 Prepare 18 mL of SFD media with Mix 1 cytokine according to **Table 2**, for each 6 well plate (3 mL/well).
2.2.2.2 Swirl the plate with the EBs to move them in the center and collect them using a pasteur pipette into a 15 mL centrifuge tube.
NOTE: If the EBs look clumped together as in strings, separate them by pipetting them

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

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

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

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

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

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

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

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

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

2.2.3 Day 2 – Addition of CHIR99021

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

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

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

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

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

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

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

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

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

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

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

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

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

2.2.5.4 Carefully aspirate the supernatant from the EBs. Add 1 mL/well of SFM-34 with Mix 4 cytokines.

2.2.5.5 Distribute the EBs between the wells as described in the steps 2.2.2.7 – 2.2.2.9.

3. CD34$^+$ cells isolation and seeding into chip

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

3.1 Prepare materials and reagents

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

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

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

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

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

3.1.1 Day 8 – Dissociation of EBs and CD34$^+$ isolation

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

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

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

3.1.1.4 Incubate for 10 min in the incubator.

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

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

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

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

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

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

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

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

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

3.1.2 CD34+ Cells seeding into fluidic chips

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

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

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

3.1.2.3 Resuspend $2.5x10^5$ cells in a final volume of 150 µL supplemented SFM-34, add also 0.5 µL of iRock.

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

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

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

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

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

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

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

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

4.1 Prepare materials and reagents

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

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

4.2 Fluidic system assembly

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

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

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

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

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

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

4.2.6 Remove the tubing from the test chip.

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

4.2.7 Connect the chip containing the cells with the tubing.

4.2.8 Remove or open the clamps.

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

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

NOTE: Depending on the specific experiment that is needed, the stimulation program might need optimization. We describe here a gradual shear stress increase leading to final value of 5 dyn/cm^2 which mimics the shear stress calculated to be experienced by the wall of dorsal aorta at the onset of fetal circulation [9]. Independently of the final shear stress that will be employed, it is necessary to gradually increase over time to allow the cells to adapt to the force without causing the cells to detach from the chip.

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

4.3 Cell collection for analysis

4.3.1 Prewarm the dissociation buffer in a water bath.

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

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

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

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

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

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

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

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

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

4.3.9 Spin the tubes for 5 min at 300 g.

4.3.10 Prepare the staining solution to have 50 µL for each test tube to stain. Add the CD34 PerCP-efluor710 or CD34-PE at 1:100 and 1:200 dilution, respectively.

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

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

4.3.13 Resuspend the pellets in 100 µL of staining solution and acquire the data using a flow cytometer.

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

4.4 Cell orientation analysis

Images were analysed to quantify changes in cell orientation using FIJI[23] (Supplementary Figure 2).

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

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

4.4.3 Mesure the orientation of each ROI by choosing the “Fit ellipse” measure in the “Analyze → Set Measurements…” menu.

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

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

REPRESENTATIVE RESULTS

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

[Figure 1 here]

1. Culture of human induced Pluripotent Stem Cells

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

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

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

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

[Figure 2 here]

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

[Figure 3 here]

3. CD34$^+$ cell isolation

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

4. Seeding cells into the fluidic channel

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

FIGURE AND TABLE LEGENDS

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

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

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

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

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

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

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

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

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

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

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

DISCUSSION

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

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

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

this limitation, specific combination of cytokines might be needed during the differentiation process preceding the fluidic stimulation to obtain the desired identity or tissue-specific phenotype[26]. Furthermore, this protocol is specifically designed to study the role of shear stress mediated by laminar flow. Alternative fluidic approaches will have to be employed to study the effect of other mechanical cues, such as stretching or compression, or other type of flow such as perturbed or disturbed flow.

We previously shown that iPSCs derived endothelial cells mimic the heterogenous artero-venous cellular identities[27] similar to that observed in the fetal dorsal aorta[28–30]. This is of particular importance in the context of vessel development and cellular specification, known to be controlled by blood circulation. Studies in different models showed that lack of circulation results in altered artero-venous specification [11, 14, 31]. The mechanism that connect mechanical cues with the specification are still unknown and the pipeline described here allows for refined functional studies that cannot be tested in vivo.

The pipeline here defined describes the production, and the stimulation of endothelial cells derived from hiPSCs using commercially available fluidic channels, avoiding the need for casting the devices as for the widely used Polydimethylsiloxane (PDMS) devices[12]. Furthermore, the use of PDMS chips makes the collection of the stimulated cells particularly challenging, while with this protocol, the cells can be easily retrieved from the channel. This significantly improve the analytic power allowing for subsequent analysis such as proteomic and transcriptomic analysis, flow cytometry and functional assay which might need further culture in different culture conditions or even *in vivo* assays.

ACKNOWLEDGMENTS

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

DISCLOSURES

The authors have nothing to disclose.

REFERENCES

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

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

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