Vascular smooth muscle cells differentiation from hPSCs

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Abstract

This protocol describes how to differentiate hPSCs into a pure population of vascular smooth muscle cells in only 6 days

Introduction

The use of human pluripotent stem cells \(hPSCs) for disease modeling and drug screening requires rapid, robust and efficient differentiation strategies. This protocol describes the differentiation of hPSCs into vascular smooth muscle cells \(VSMCs). It is segmented in 2 stages, which recapitulates the developmental processes occurring during embryogenesis. First hPSCs are cultured for 4 days as a monolayer in a chemically defined medium supplemented with a GSK3β inhibitor and BMP4 inducing commitment of the cell population to the mesodermal state. Cells are then cultured for 2 days in a different medium containing Platelet-derived growth factor subunit B \(PDGF-BB) and Activin A. After only 6 days a pure population of VSMCs can be replated and cultured in conditions enriching for either the synthetic or the contractile state.

Reagents

mTeSR Stemcell Tech 5850 Matrigel BD/Fisher 356230 accutase StemCell Tech 7920 Y-27632 dihydrochloride Santa Cruz sc-281642A DMEM/F-12 Life technologies 11320-033 Neurobasal medium Life technologies 21103049 N-2 Supplement \(100X) Life technologies 17502048 Supplement B27 Minus Vitamin A Life technologies 12587010 2-Mercaptoethanol, 50 mM Life technologies 21985023 BMP4 Peprotech 120-05 CHIR-99021 Cayman 13122 PDGF-BB Peprotech 100-14B Activin A Peprotech 120-14E CD144 MicroBeads Miltenyi Biotech 130-097-857 MACS running buffer Miltenyi Biotech 130-091-221 MACS washing buffer Miltenyi Biotech 130-092-987 PE mouse anti-human CD144 BD Pharmigen 560410 QuadroMACS starting kit Miltenyi Biotech 130-091-051

Procedure

3.2 Reagents preparation N2B27 Medium : ~1L 500ml DMEM/F12 medium + 500ml Neurobasal medium, + 20ml B27 \(1.94%) + 10ml N2 \(0.97%) + 1ml β-Mercaptoethanol \(0.097%) sterile filtration 0.22 um, store at 4°C up to 1 month CHIR-99021 \(4.3µM): Resuspend 10mg of CHIR-99021 in 5ml DMSO. Store at -80°C or -20°C for a year. CP21R7 \(10mM): Resuspend 2mg of CP21R in 630µl DMSO. Store at -80°C or -20°C for a year. BMP4 \(25µg/ml): Resuspend 10ug of BMP4 in 4ml of 5-10mM citric acid pH 3. Store at -80°C or -20°C for a year. VEGF165 \(100µg/ml): Dissolve 100µg of VEGF165 in 1ml sterile water. Store at -80°C or -20°C for a year. Forskolin \(10mM): Dissolve 10mg of forskolin in 2.436ml DMSO. Store at -20°C for a month. Activin A \(100µg/ml): Dissolve 100µg of Activin A in 1ml sterile water. Store at -80°C or -20°C for a year. PDGF-BB \(100µg/ml): Dissolve 100µg of PDGF-BB in 1ml sterile water. Store at -80°C or -20°C for a year. Note: Because they are sterile, opening and reconstitution of all recombinant proteins and other compounds should be performed under a hood using aseptic techniques. All recombinant

proteins and compounds resuspended in DMSO should be pre-warmed for a few minutes in a water bath \(37°C) before adding to the differentiation media. Recombinant proteins are highly unstable. Repeated freezing-thawing cycles is not recommended as it will result in a rapid decreasing of their biological activity. Once thawed, aliquots can be kept at 4°C for a week. 4. Differentiation protocol Note: This protocol is routinely used to differentiate different hESCs and IPSCs lines. However, some lines may require minor changes such as seeding density and GSK3β inhibitor concentration. The example below describes the procedure to follow to differentiate cells in one T175 flask \(Cat#3292, Corning). If performing differentiation in a smaller plate adjust media volume accordingly \(for instance 3ml of N2B27 in a 12 well). 4.1 Maintenance of hPSCs 1. Human pluripotent stem cells are routinely cultured on matrigel in mTeSR1 medium. Cultures are passaged every 3-5 days using Accutase. 2. An 80% confluent 10cm plate is required to start differentiation in one T175. 4.2 Day 0: Plating hPSCs 1. Coat one T175 with growth factor reduced matrigel by thawing it on ice and diluting it 1:30. 2. Incubate the plates at RT for 1h. Aspirate the matrigel, wash once with PBS and add 20ml pre-warmed mTeSR1+ ROCK-Inhibitor \(final conc. 10μM). 3. Aspirate growth medium from an 80% confluent 10 cm plate. 4. Wash with 10ml pre-warmed PBS \(Ca2+ and Mg2+ free). 5. Add 3 ml pre-warmed Accutase and incubate 3-7 min at 37°C. 6. Check cells' detachment under a microscope. 7. Add 3ml pre-warmed mTeSR1. 8. Transfer cells to a 15ml Falcon tube. 9. Count cells 10. Centrifuge the suspension at 1000 rpm \(210g) for 5min. 11. Aspirate supernatant and resuspend the cell pellet in 10 ml pre-warmed mTeSR1+ ROCK-Inhibitor \(final conc. 10μM). 12. Seed the cells at 37.000-47.000 hPSCs per cm2. 13. Incubate the cells at 37°C, 5% CO2 overnight. Note: Starting cell density influences the yield of the differentiation and should be optimized for each cell line. 4.3 Day 1-3: Lateral mesoderm induction 1. Replace media with pre-warmed N2B27 Medium supplemented with 6-8 μM CHIR-99021 or 1 μM CP21 + 25ng/ml hBMP4 \(125 ml/T175 or 3ml/12well). For 3 days without medium change\! 4.4 Day 4 and 5: Vascular smooth muscle cells induction 1. Replace Media with N2B27 medium supplemented with 10ng/ml PDGF-BB and 2ng/ml ActivinA \(2ml/12well or 100ml/T175). Change medium every day\! Day 6: Vascular smooth muscle cell replating Synthetic condition: Seed 30000cells/cm2 o Medium: N2B27+PDGF-BB \(10ng/ml) o Coating: Gelatine coated \(0,1% Gelatine for 15min at RT, remove directly before plating cells) Contractile condition: Seed 30000cells/cm2 o Medium: N2B27+ActivinA \(2ng/ml)+Heparin \(1:1000, final conc. 2ug/ml) o Coating: Collagen coated \(Biocoat, BD) Note: Other culture conditions \(e.g. FCS containing medium, higher concentrations of PDGF-BB \(100-300ng/ml), or fibronectin coating) are reported in the literature, but haven't been tested extensively for this protocol. As we aimed to generate VSMCs as close to their physiological counterpart as possible, proliferation inducing reagents such as FCS or PDGF-BB were omitted or kept at the lowest concentration possible. Therefore, under both conditions, VSMCs will not proliferate much, especially for the contractile condition. Completely pure populations of one subtype are difficult to achieve and with prolonged culture, the synthetic phenotype will become dominant. In our hands this protocol always yields a pure population of VSMCs, however one could remove any potential CD144+ cells by MACS \(see section 5 of the hPSC-ECs differentiation protocol).

Timing

6 days for differentiation and at least 4 more days for expansion.

Troubleshooting

1. Cells are less/more confluent than the protocol picture on day 1 Cell confluency on day 1 is one of the keys to differentiation efficiency. Cells should be seeded to look like day 1 picture. 2. Differentiation medium on day 4 is light yellow and there are a lot of dead cells floating. Yellow medium on day 4 is a sign of plenty of differentiated cells. Excessive cell death can be observed when cells were too confluent on day 1. 3. Differentiation efficiency is low Differentiation efficiency lower than 20% could be due to wrong or inactive recombinant protein or wrong differentiation media. Make sure that you prepared your reagents and media correctly and kept them in proper conditions. Differentiation efficiency between 20-50% could be due to an inappropriate seeding density or medium volume. Make a serial dilution series for seeding density and GSK3β inhibitor concentration to find optimal cell line specific conditions. 4. Vascular smooth musclce cell cells did not attached after replating Be sure your gelatine solution was prepared correctly and that the plates were coated for at least 15 minutes. For contractile VSMCs, make sure that the Biocoat plates \(Collagen coated) have not expired. VSMCs can be detached with Trypsin 0.01-0.05% or Accutase. When detaching the cells for replating do not incubate the cells for too long in Accutase/Trypsin as it would result in massive cell death.

Supplementary Files

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