# A human cell model of valvulogenesis

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**Method Article**

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# Abstract

Genetically modified mice have advanced our understanding of valve development and related pathologies. Yet, little is known regarding human valvulogenesis in health and diseases. Genuine human in vitro models that reproduce valvular \(patho)biology are thus needed. We here developed a human pluripotent stem cell-derived model fit to decode the early steps of human valvulogenesis and to recapitulate valve disease traits in a dish. Using cellular based, single cell omics-informed and in vivo-validated approaches, we derived a population of pre-valvular endocardial cells from a pluripotent stem cell source. These human prevalvular cells \(HPVCs) expressed gene patterns conforming to the atrio-ventricular canal \(AVC) endocardium signature originally established in E9.0 mouse embryos. In fact, HPVC treated with BMP2, cultured onto mouse AVC cushions, or transplanted into the AVC of embryonic mouse hearts, underwent endothelial-to-mesenchymal transition and expressed markers of valve interstitial cells of different valvular layers demonstrating tissue functionality. HPVCs also differentiated into tendinous/chondrogenic cells in line with the valvular repertoire. Extending this valvulogenic model to patient specific iPS cells, we recapitulated features of mitral valve prolapse and uncovered that dysregulation of the SHH pathway is likely to be at the origin of the disease thus providing a putative therapeutic target. Human pluripotent stem cells recapitulate early valvulogenesis and provide a powerful model to systematically decipher the origin and lineage contribution of different valvular cell types in humans as well as to study valve diseases in a dish.


# Introduction

Cardiac valves are affected in up to one third of cardiac congenital diseases1. Valves emerge early in mouse and human embryos \(E9.5 and 4 weeks, respectively) in two restricted heart territories, the atrioventricular canal and the outflow tract from a process of endothelio-to-mesenchymal transition \(EMT) of endocardial cells. Mouse models have provided a tremendous knowledge as to biological processes and cell lineages that contribute to valve formation2-3. However, these in vivo models could not allow to easily studying signaling interaction, cell metabolism, epigenetic regulatory mechanisms, and mechano-transduction. In addition, human valve formation could at least in part be different from mouse. A human specific cell model of valvulogenesis that could be extended to cells derived from individual patients would definitively advance the understanding of developmental mechanisms driving valvulogenesis in health and disease. Herein we use pluripotent stem cells and report a protocol of cell differentiation toward endocardial cells and in turn to valvular cell types.

# Reagents

Human CD15 positive selection kit \(Stem cell technologies 18651) Anti SSEA1 antibody-alexa488 \(clone MC480) \(Biolegend 125610) Anti-vimentin dylight 594 \(Abcam ab139878) CD31microbeads kit human \(Miltenyi 130-91-95) DMEM high glucose \(Gibco 11965-084) Endothelial Cell Growth Medium MV 2 \(Promocell C-22022) KO-DMEM \(Gibco 10829-018) KnockOut Serum Replacement \(Gibco 10828028/) RPMI 1640 \( Gibco 61870010) B27 \(Gibco 17504044). Recombinant mouse Wnt3a \

(Peprotech. 315-20) Human recombinant BMP2 \(Gibco PHC7145 ) Recombinant Human FGF-basic \(146 a.a.) \(Peprotech 100-18) Recombinant Human VEGF165 \(Peprotech 100-20) Recombinant Human TGF-β1 \(Peprotech 100-21C) MEM Non-Essential Amino Acids \(NEAA) Solution \(100X) \(Gibco 11140050) Glutamax supplement 100X \(Gibco 35050061 ) ascorbic acid-2-phosphate \(Sigma-Aldrich A8960) CHIR 99021 \(Stem cell technologies) 2-Mercaptoethanol \(Sigma-Aldrich M-6250): Make up a 10-4 M solution by adding 0.1 ml of stock solution to 13.9 ml of DPBS. Keep the solution at 4°C for 1 month Fetal Calf serum \(Eurobio CVFSVF00-01) Mitomycin C: \(Santa-cruz sc-3514) SU5402 \(Stem cell technologies 73912) IWR-1 \(stem cell technologies 7256) Fibronectin from bovine plasma \(Sigma-Aldrich F1141-1MG) Gelatin \(Sigma-Aldrich G-9391): make up a 0.1% solution in distilled H20 and sterilize Collagenase IV \(Gibco 17104019 ITS-G Insulin Transferin Selenium \(Gibco 17104019) Dexamethasone \(Sigma-Aldrich D4902) Heparin sodium salt \(Sigma-Aldrich 84020) Reagents setup: HUES and iPS cells propagation medium: 500 ml KO-DMEM+ 15% SR + 5 ml NEAA + 5 ml Glutamax + 0.5 ml 2-Mercaptoethanol \(10-4M) + 5 \(HUESC) or 10 \(iPSC) ng/ml FGF2 Cell differentiation medium: 500 ml RPMI 1640 added with B27, 0.5 ml 2-Mercaptoethanol \(10-4M), 5 ml NEAA, and appropriate growth factors Cell sorting buffer: DPBS \(Gibco 141190-094) added with 0.5% BSA \(SIGMA A-7030) and 2 mM EDTA \(stock 0.5 M in Vitrogen 15575-036)

# Equipment

Tissue culture equipment: Incubator at 37°C with 95% air and 5% Laminar flow hood Bench centrifuge to spin down cells at 80g Microscopes for cell imaging 5 ml polypropylene round bottom tubes \(Falcon cat. no. 352063) 15 ml polystyrene conical tubes \(Falcon cat. no. 352095) 50 ml polypropylene conical tubes \(Falcon cat. no. 352070). 6 wells plates \(Sarstedt 83.3920.005) 150 mm culture \(Sarstedt 83.3903.300) Miltenyi QuadroMACS™ Separator \(Miltenyi 130-090-976) Easy step magnet \(Stem cell technology 18000).

# Procedure

Feeder cells are mouse embryonic fibroblast \(MEF) from E14.5 embryos. Cells are cultured in DMEM added with 10% FCS for 4 passages. Cells are split 1/4 every 4-5 days when 80-90 % confluency is reached. MEF at P4 are treated for 1H30-2H with 10 ug/ml mitomycin C, then washed twice with DPBS and collected after 5 min Trypsin. Cells are plated at 40 000 cells/cm2 for pluripotent stem cells propagation on gelatin-coated plates or 10 000 cells/cm2 for cell differentiation on fibronectin-coated plates \(coating for 30 min at 37°C). TIP: this culture protocol is important as MEF go to replicative senescence easily if grown at a wrong density. TIP: MEF can be frozen at any passage between P0 and P4 in DMEM/50%FCS/10%DMSO Culture of pluripotent stem cells: 1. Culture the pluripotent stem cells in the propagation medium on a layer of mitotically-arrested mouse embryonic fibroblasts \(40 000 cells/cm2). Critical: change every 24 hrs the medium with prewarmed \(37°C) medium and check the absence of mycoplasma in the culture on a regular basis 2. To start cell differentiation, plate the pluripotent stem cells on low density MEF \(10 000 cells/cm2). Critical: Start with cells featuring small

colonies \(200-500 cells) the next day after passaging or two days post-passaging 3. Prepare a 35 mm plate as a control plate to follow the differentiation and for ant-CD15alexa \(MC488) alive staining the day before sorting. 4. Day 0: Add to the differentiation medium either 100ng/ml Wnt3a or 5 uM CHIR 99021 \(prewarm CHIR in your hands before adding it to the medium) and then change cell medium. TIP: both procedures will activate wnt signaling pathways in the cells. 5. Day 1: No later than 24Hrs later, change differentiation medium and add 100ng/ml Wnt3a or 5 uM CHIR99021, BMP2 10 ng/ml and 1 uM SU5402. 6. Day 2: first check your cells and make sure they started to differentiate: the colonies should not be compact any longer; cells should spread out \( see figure 1a below) TIP: Make sure that all media are warmed at 37°C before adding to cells TIP: All stocks reagents growth factors, inhibitors should be stored at -20°C and should not be left at 4°C for more than 5 days TIP: Test cell differentiation by staining your 35 mm plate with 1/100 anti SSEA1 alexa488 \(clone MC480) in 400 ul PBS \( see Figure 1b). If at least roughly 30% of cells are CD15+, you can sort out the cells the next day. 7. Day 3: anti-CD15 cell sorting \(according to stem cell technologies protocol) 8. Plate sorted cells on feeders \(10 000 MEF/cm2) on fibronectin-coated plates \(fibronectin 1mg/ml Sigma diluted 1/100 in PBS to coat plates for 1hr at 37°C) in RPMI-B27 added with 30 ng/ml VEGF and 10 ng/ml FGF8 \(and 2 ng/mL FGF2). TIP: 300000 cells /B6 plate is a good number to start the culture 9. Days 4 to 9: change medium at D6 and D8 with the same medium +VEGF/FGF8/\(FGF2) 10. Day 9: Sort the cells with anti-CD31 \(Miltenyi) according to the manufacturer protocol and plate them in ECGM with VEGF 30 ng/ml on collagen-coated plate \(plates are coated at RT for 1 hrs with collagen \(stock 3 mg/ml diluted 1/60 in acetic acid) . Wash twice the collagen coated plate with PBS before plating the cells. Plate 300 000 cells/well of a 6 wells plate. Let grow the cells for 5 more days, change medium every 2 days. Cells can be split and will remain “endothelial” for 2-3 passages TIP: add 1U/ml heparin to the ECGM medium. This will help preventing spontaneous EMT. TIP: Cells can be tracked by Anti-CD31 staining \(Immunofluorescence \(Figure 2 or FACS) 11. Day 9 or later: induce EMT with 100 ng/ml BMP2 in differentiation medium. The next day 100% of cells are vimentin+ \(Figure 3) Tendinous/chondrogenic differentiations of HPVCs. 12. Chondrogenic differentiation can be performed according to established methods4. Endocardial cells are isolated from MEF using collagenase IV, transferred into 15 mL of polypropylene centrifuge tubes \(500 000 cells/tube) and gently centrifuged at 80 g. The resulting pellets have to be statically cultured for 3 weeks in DMEM high-glucose medium with glutamine, penicillin/streptomycin and chondrogenic supplements \(1X ITS-G, 1 μmol/L dexamethasone, 100 μmol/L ascorbic acid-2-phosphate), and 10 ng/mL TGF-b1. 13. Pellets can be embedded in paraffin and cut in 4 μm sections for immunofluorescence.

# Timing

9 days to get endocardial cells 11 days to get valvular cells up to 1 month to get chondrogenic cells

# Anticipated Results

This protocol gives rise to highly enriched endocardial specific endothelial cells. These cells feature the potential to give rise to any valvular endothelial cells and valvular interstitial cells. The protocol is based on two cell sorting steps which allows for a robust cell phenotype.

## References

1. Combs, M.D. & Yutzey, K.E. Heart valve development: regulatory networks in development Circ. Res. \(2011) 105:408-21 2. Van Vliet, P., Wu, S.M., Zaffran, S. & Puceat, M. Early cardiac development: a view from stem cells to embryos. Cardiovasc Res \(2012) 96\(3):352-62. 3. Pucéat, M. Embryological origin of the endocardium and derived valve progenitor cells: from developmental biology to stem cell-based valve repair. BBA Mol Cell Res 1833,917-22. \(2013). 4. Wylie-Sears, J., Aikawa, E., Levine, R.A., Yang, J.H. & Bischoff, J. Mitral valve endothelial cells with osteogenic differentiation potential. Arterioscler Thromb Vasc Biol 31, 598-607 \(2011).

## Acknowledgements

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## Figures

**Figure 1: differentiating cells.**

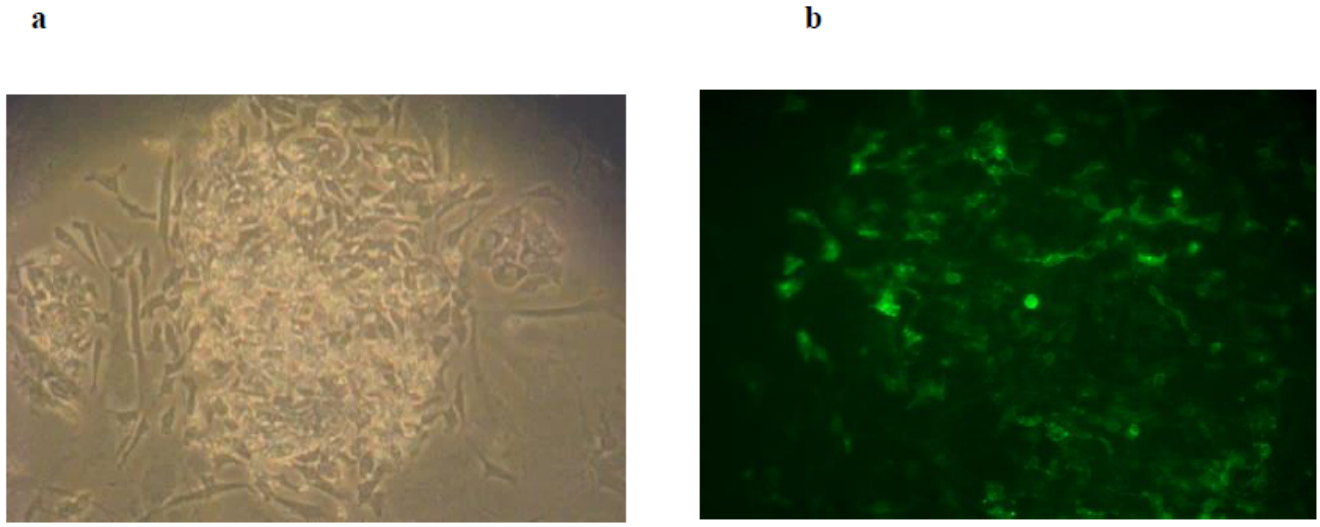



**Figure 1**

differentiating cells Fig 1: differentiating cells a: Bright field image at differentiation day 2. b: anti-SSEA1-alex488 live staining at differentiation day 2

Figure 2

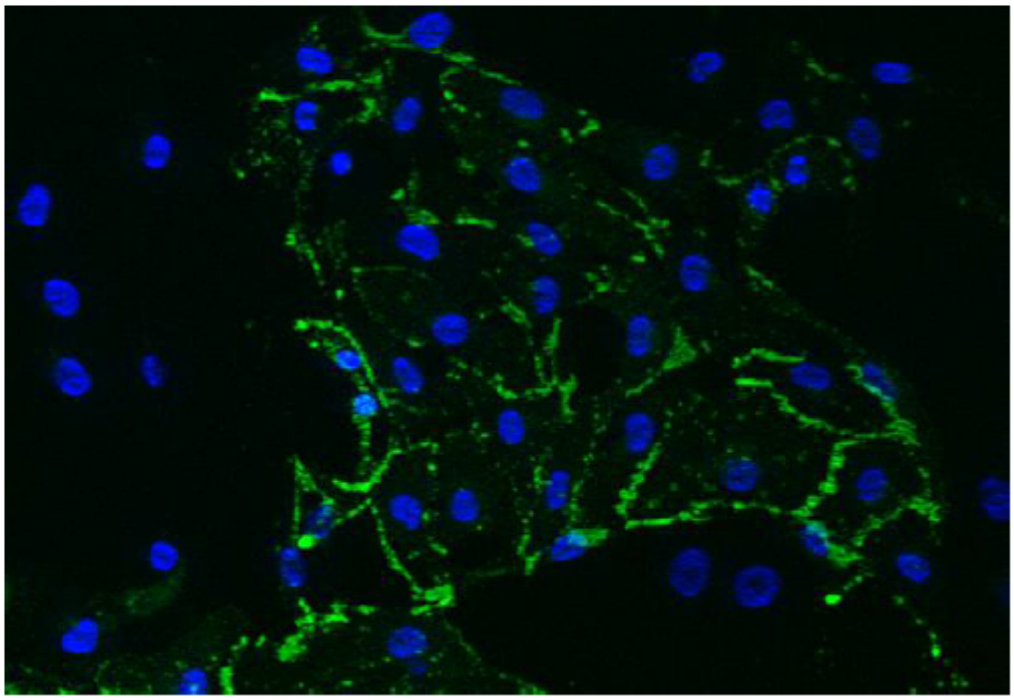
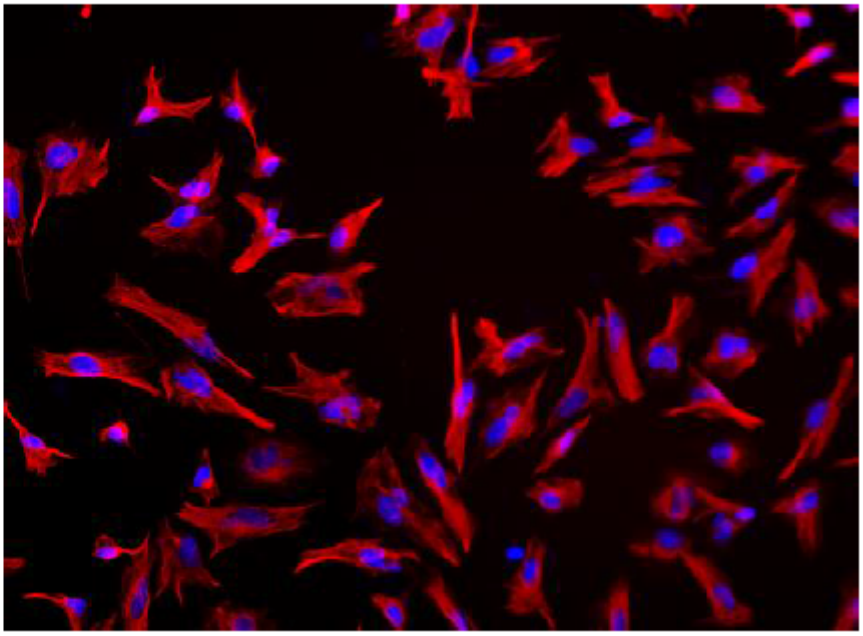


**Figure 2**

anti-CD31 staining of sorted cells (endocardial cells): cells were stained overnight with anti-CD31 antibody and then a secondary alexa488-conjugated antibody and DAPI.

Figure 3



## Figure 3

Post-EMT valvular interstitial cells Cells were stained with vimentin-dye light594 and DAPI