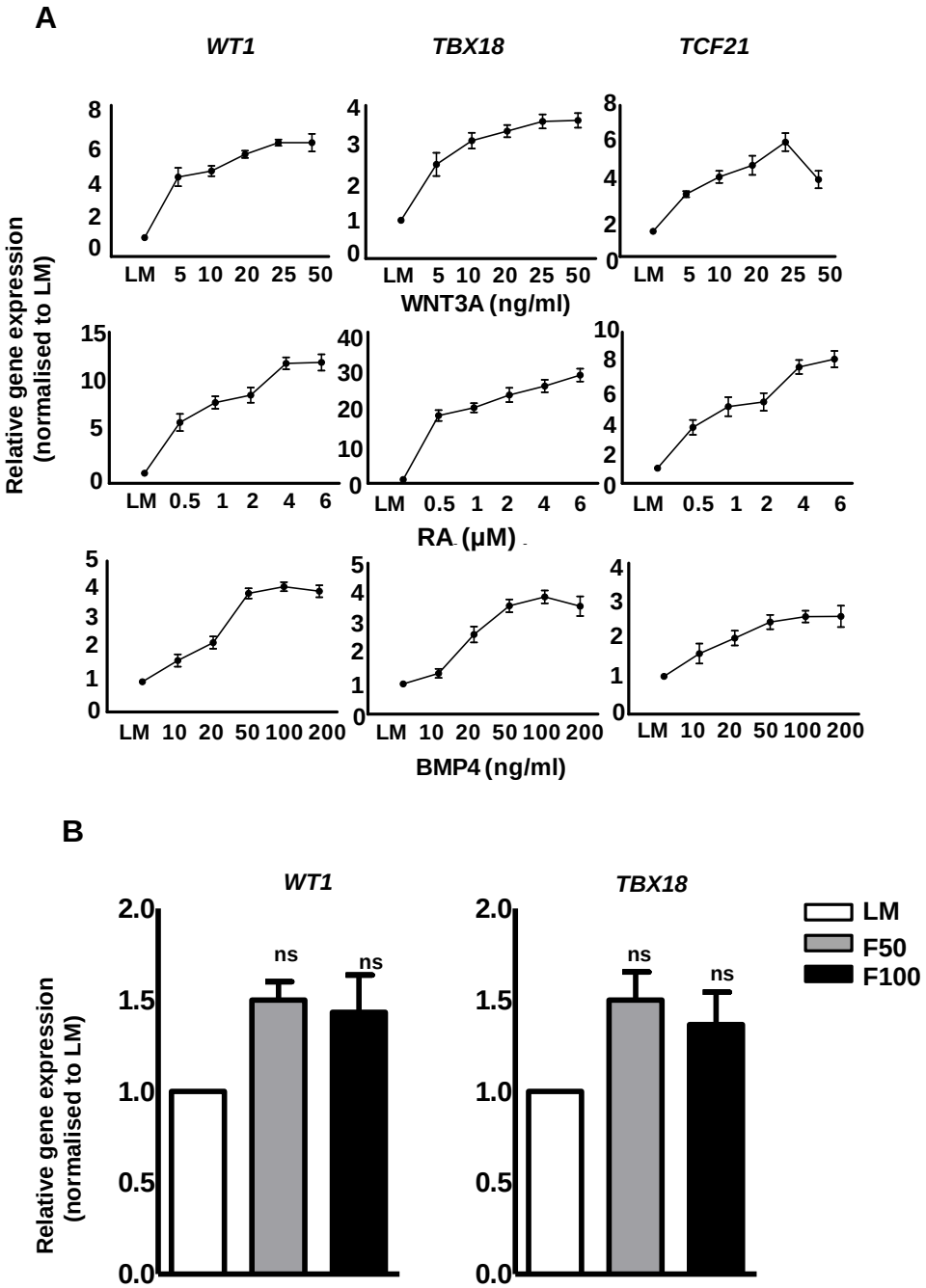



**Fig. S1 Titration of WNT3A, BMP4 and RA and their effect on epicardial gene expression**. (A) High expression of *TBX18*, *WT1* and *TCF21* was noted with increasing doses of WNT3A. Highest expression of all three epicardial genes was observed at a final concentration of 25 ng/ml. Enhanced epicardial gene expression was noted in cultures supplemented with 4 μM and 6 μM of all trans retinoic acid (RA). Enriched expression of epicardial markers was noted with increasing doses of BMP4 which plateaued at higher concentrations (50, 100 and 200 ng/ml). (B) Analysis of epicardial markers in LM cells differentiated with two different concentrations of FGF2. No significant induction in the expression of *TBX18* and *WT1* was observed.

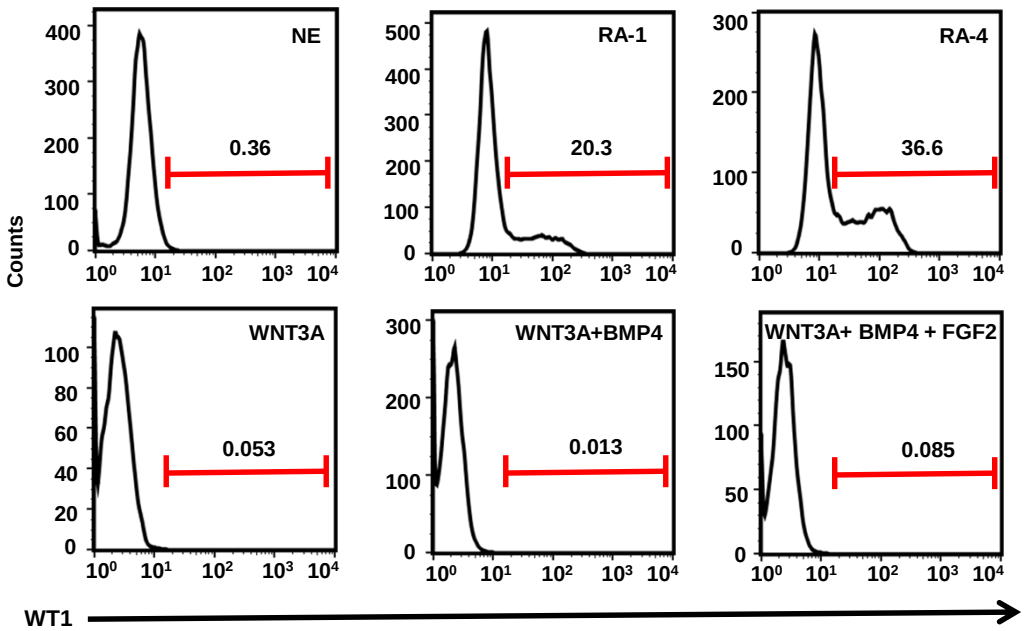



**Fig. S2. Flow cytometric analysis of WT1 expression in cells differentiated using WNT3A and RA treated cells**. Lateral plate mesoderm cells differentiated with WNT3A, WNT3A+BMP4 and WNT3A+BMP4+FGF2 failed to induce any WT1 expression in the differentiated cells after 10 days of differentiation. Addition of RA promoted expression of WT1 in the differentiated cells. A higher concentration of RA (4 μM) resulted in a larger percentage of $WT1^{+}$ cells compared to cells differentiated with a lower concentration of RA (1 μM). H9-derived neuroectoderm (NE) cells were used as a negative control.

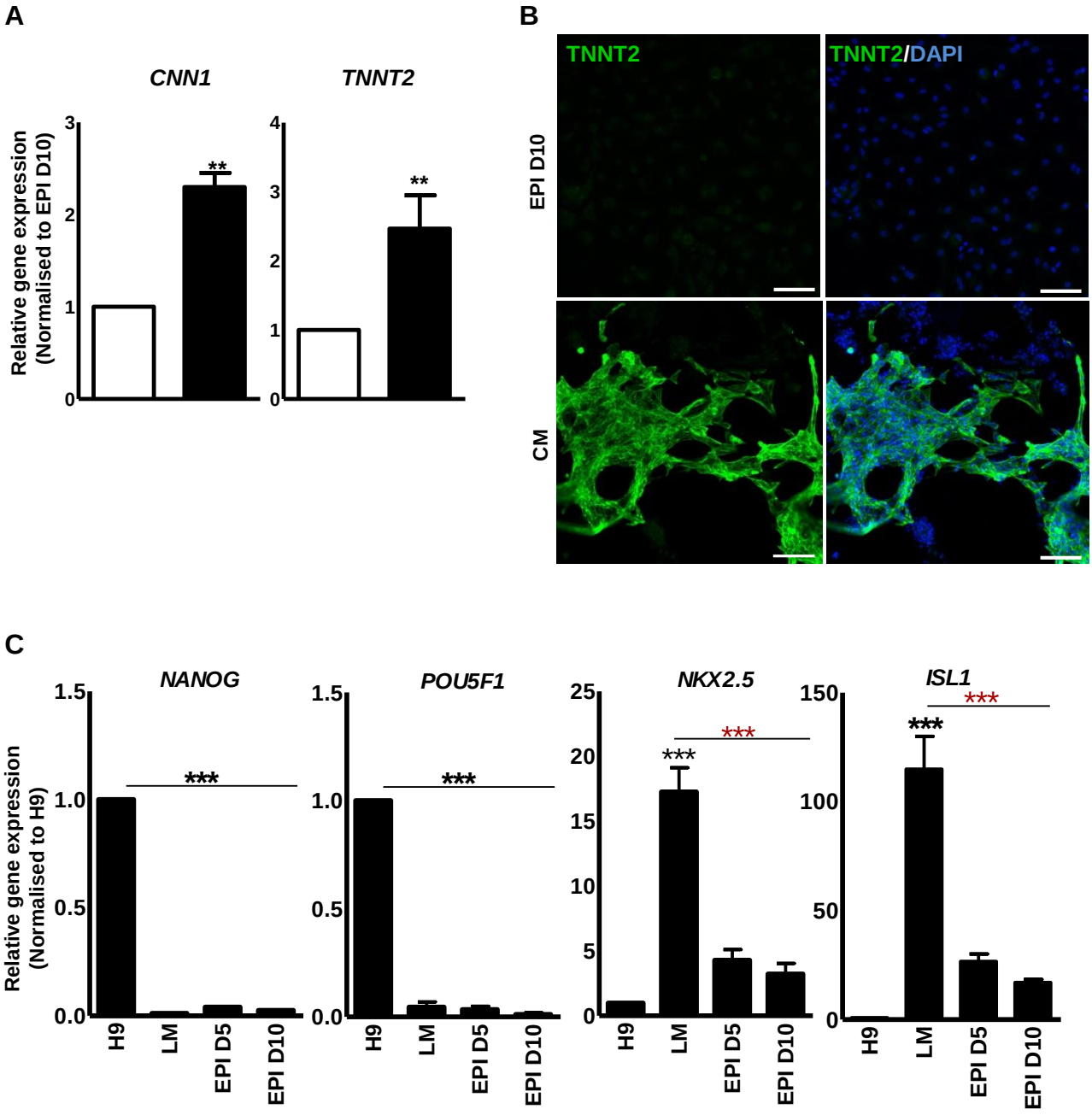



**Fig. S3. HESC-derived epicardial cells express SMC and cardiomyocyte markers at longer periods of differentiation**. (A) Analysis of SMC (*CNN1*) and cardiomyocyte (*TNNT2*) markers in the LM-derived day 10 (EPI D10) and day15 (EPI D15) epicardial cells by qRT-PCR. $^{***}P<0.001$, $^{**}P<0.01$. (B) Immunocytochemical analysis of TNNT2 in EPI D10 and H9-derived cardiomyocytes (CM). (C) Analysis of pluripotency (*NANOG*, and *POU5F1*) and LM (*NKX2.5* and *ISL1*) markers in H9, LM and epicardial cells after 5 (EPI D5) and 10 (EPI D10) days of differentiation. Significant differences compared to H9 are indicated in black, whereas differences between groups are indicated in red. $^{***}P<0.001$, $^{**}P<0.01$, $^{*}P<0.05$.

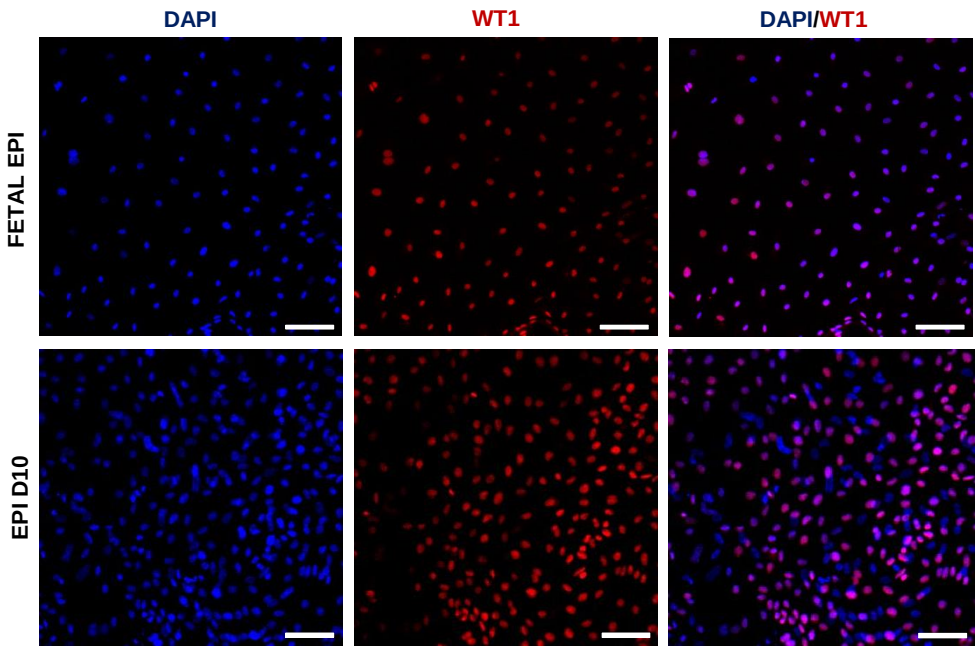



**Fig. S4. HESC-derived epicardial cells are similar to human fetal epicardium.** Human fetal epicardial outgrowths (FETAL EPI) and H9-derived epicardial cells (EPI D10) displayed similar WT1 expression. Scale bar, 100 μm.

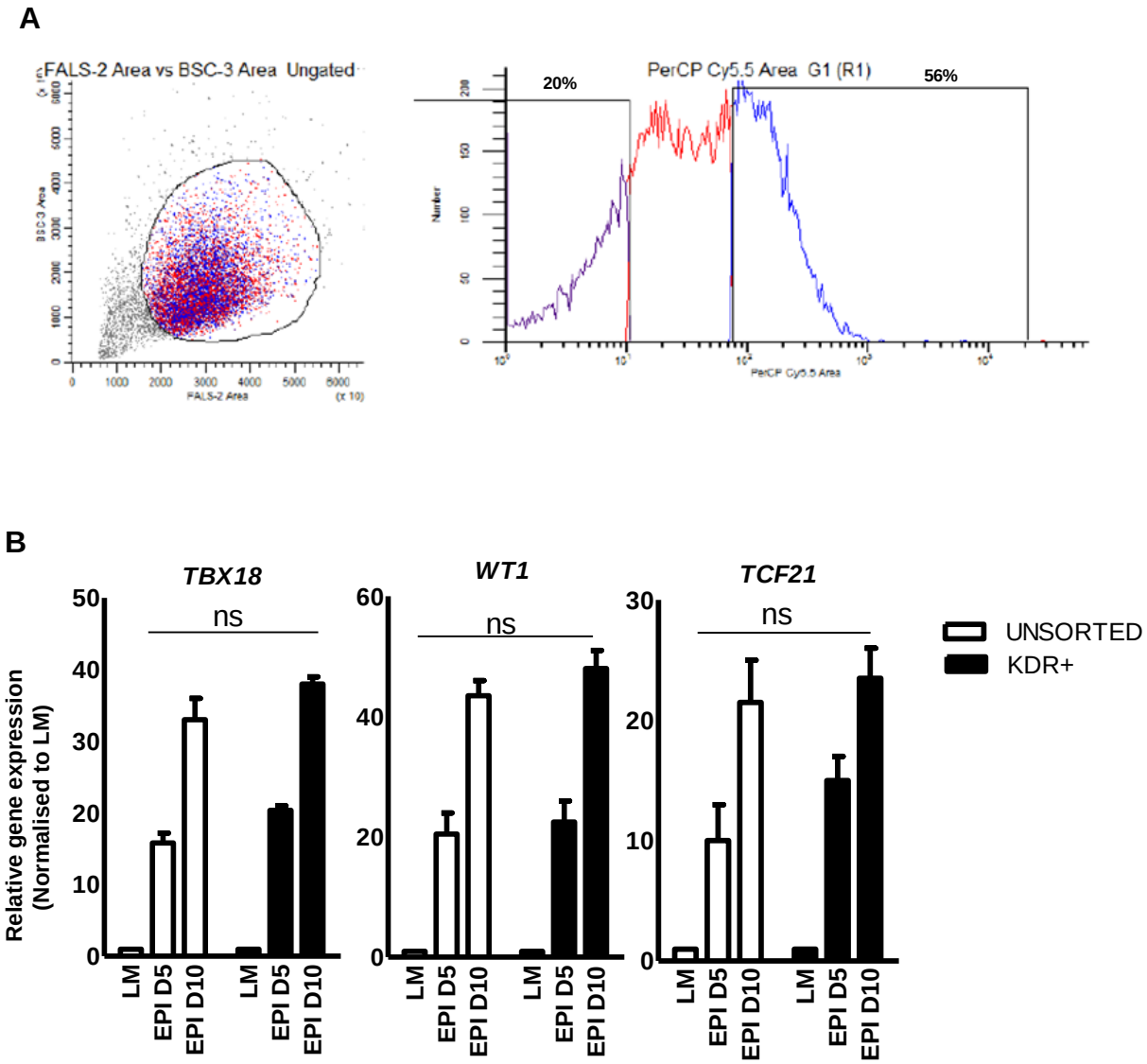



**Fig. S5. Epicardium differentiation from flow sorted lateral plate mesoderm (LM)**. (A) Sort plot demonstrating 56% KDR$^+$ (blue) and 20% KDR$^-$ (purple) LM cells. (B) Analysis of epicardial genes by qRT-PCR in epicardium-like cells derived from flow sorted and unsorted LM. Comparisons between the sorted and unsorted groups were made using Two-Way ANOVA.

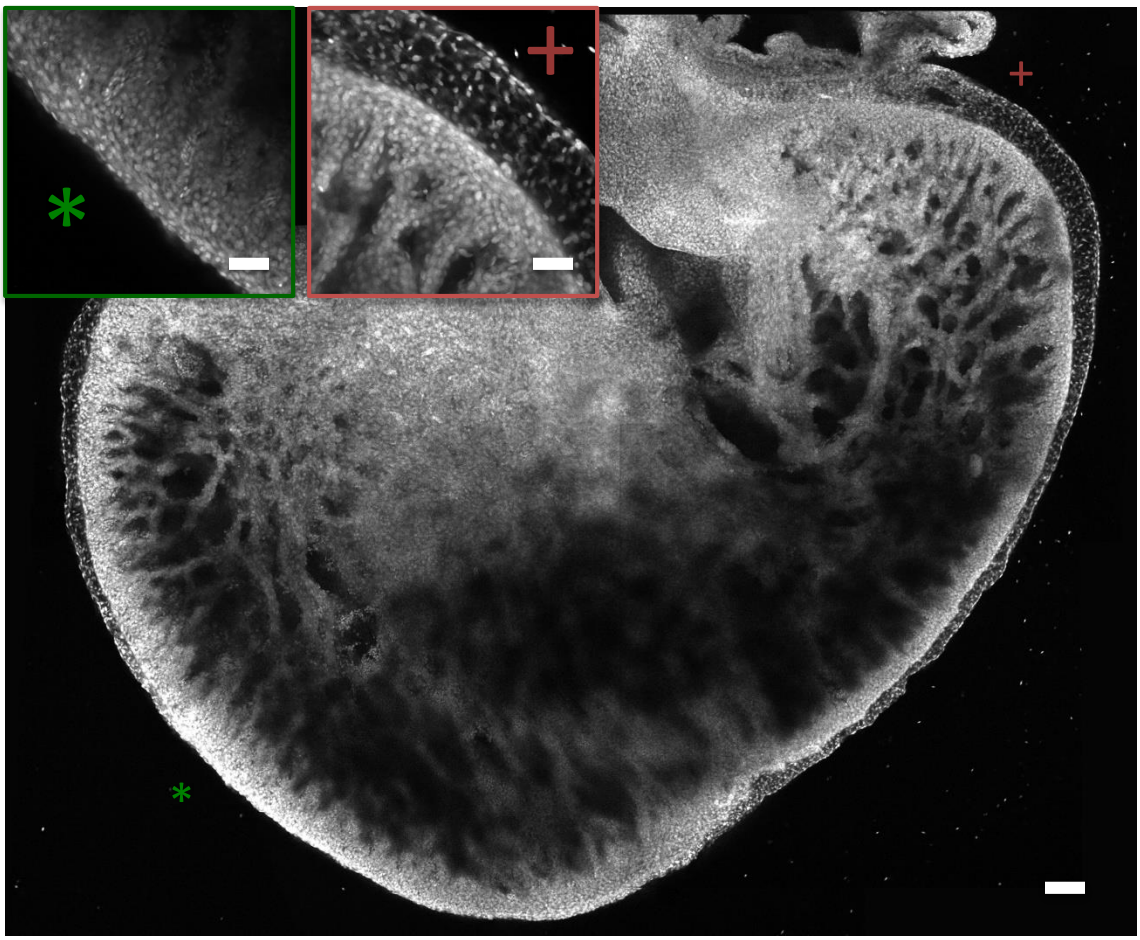


**Fig. S6. Localisation of human epicardial cells in the subepicardial/epicardial region *in vivo***. A chicken heart at HH34 reconstructed from confocal sections. $GFP^+$ or $mStrawb^+$ epicardial cells were mostly seen in the subepicardial space between the epicardium and myocardium at the base of the heart (indicated by red +). WT1 expressing epicardial cells were spotted at the apex of the heart (indicated by green*) where the epicardial/subepicardial region was relatively thinner. The scale bar represents 285 mm in the large panel and 100 mm in the two small inserted panels.

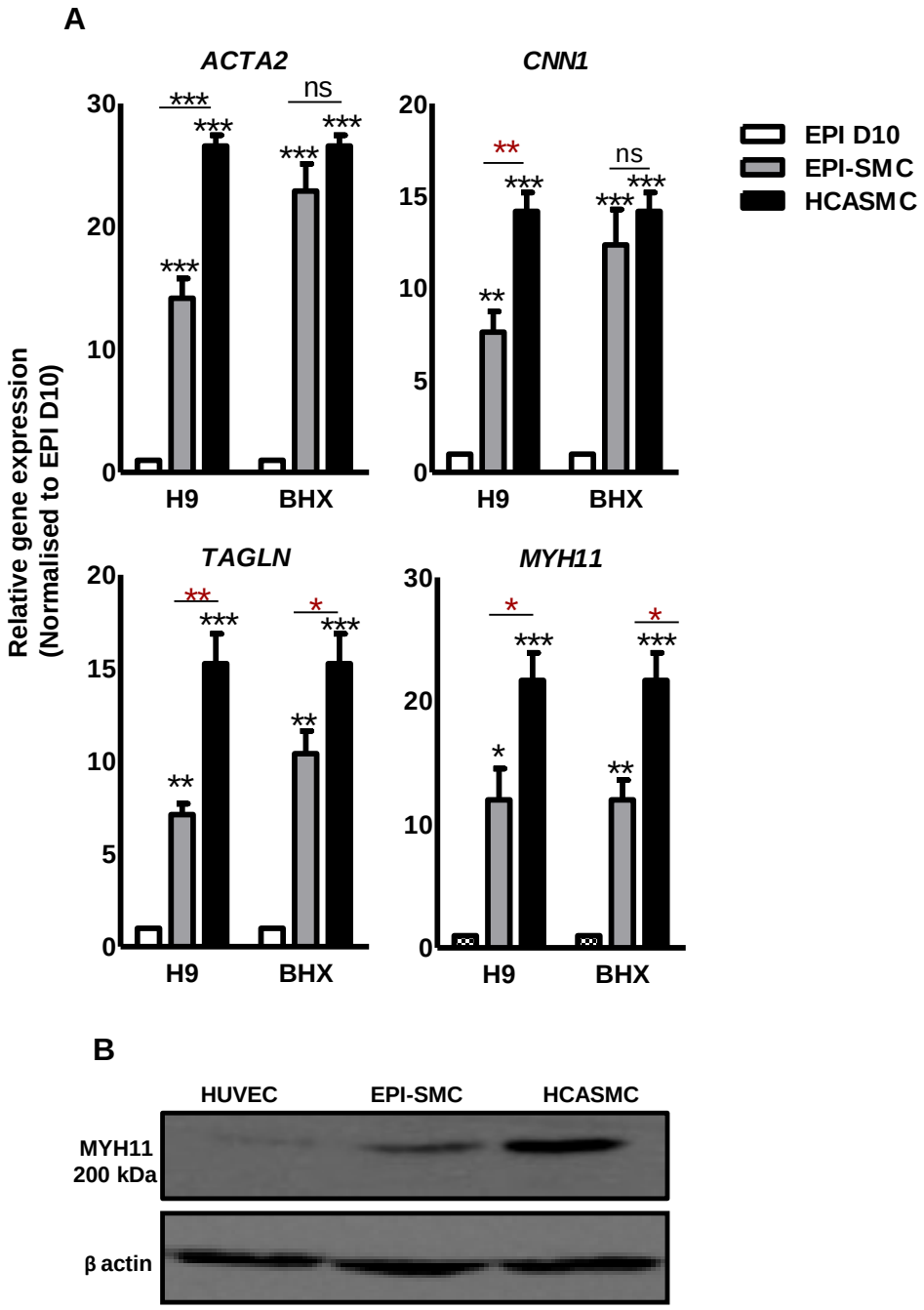
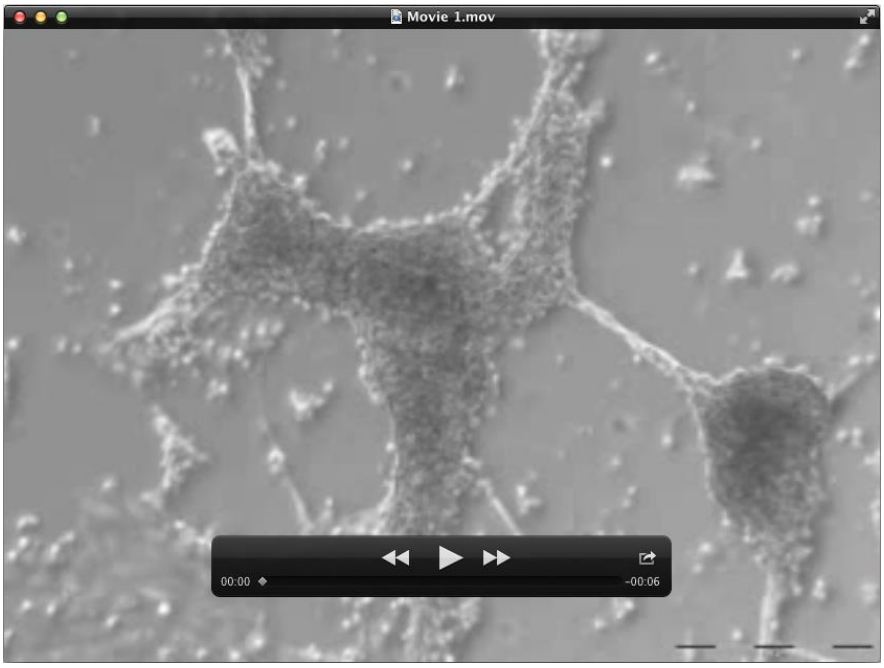



**Fig. S7. HPSC-derived epicardial cells differentiate into mature vascular SMCs.** (A) Analysis of SMC markers in epicardium-derived SMCs (EPI-SMC) generated from H9 HESCs and BHX HiPSCs and their comparison with human coronary artery SMCs (HCASMCs) by qRT-PCR. Significant differences compared to day 10 epicardium (EPI D10) are indicated in black, whereas differences between groups are indicated in red. $^{***}P<0.001$, $^{**}P<0.01$, $^{*}P<0.05$. (B) Western Blot analysis of mature SMC protein MYH11 in human umbilical vein endothelial cells (HUVECs), EPI-SMCs and HCASMCs. β-actin was used as loading control.




Movie 1 : This movie shows beating cardiomyocytes that were derived from human embryonic stem cells. These cells were used as a positive control in figure S3 to examine the expression of myocardial marker TNNT2.

**Table S1**

| Gene | Sequence |
|---|---|
| NKX2.5 | AGCCGAAAAGAAAGAGCTGTGCG<br>GACCTGCGCCTGCGAGAAGAG |
| ISL1 | GCAAATGGCAGCGGAGCCCA<br>AGCAGGTCCGCAAGGTGTGC |
| FOXF1 | GTACCCGCACCACGACAGCTC<br>ATACCGCGGGATGCCTTGCAG |
| PITX2 | GCGTGTGTGCAATTAGGCG<br>AAAGTGAGTCCGCTGCCGCC |
| PBGD | GGAGCCATGTCTGGTAACGG<br>CCACGCGAATCACTCTCATCT |
| TBX18 | ACTCCGGGCGCAACAGAATGG<br>TGGGCCCCAGATGGAAGGCA |
| WT1 | TCCGGCCCAGTTTGTAGTAGA<br>AGCAGGGCTCGCTGGGTGAG |
| TCF21 | GAAGTGGTGACCGCGAGCCG<br>AGTGTTCTCGCGGGGTGGGA |
| BNC1 | GGGAACTGTGAAGGGTCGAG<br>TCGACTGCGAACAGACGAAA |
| UPK1B | AGGACAATTGCTGTGGCGTA<br>GGAGAACCCAAAAAGTCCAGC |
| ANXA8 | ACACGAATCCATCCCAACCGAGAT<br>AACACAGTGTCCTTGGGTCAGGAA |
| ACTA2 | CACTGTCAGGAATCCTGTGA<br>CAAAGCCGGCCTTACAGA |
| CNN1 | GTCCACCCTCCTGGCTTT<br>AAACTTGTTGGTGCCCATCT |
| TAGLN | TCTTTGAAGGCAAAGACATGG<br>TTATGCTCCTGCGCTTTCTT |
| MYH11 | AGATGGTTCTGAGGAGGAAACG<br>AAAACTGTAGAAAGTTGCTTATTCACT |
| CDH1 | GGCTGGACCGAGAGAGTTTC<br>CGACGTTAGCCTCGTTCTCA |

| | |
|---|---|
| VIM | GAAGGCGAGGAGAGCAGGATT<br>CAAGGTCATCGTGATGCTGAG |
| OCLN | CGGCGAGCGGATTGGTT<br>TAGGCTGGCTGAGAGAGCATT |
| ZEB1 | CATATTGAGCTGTTGCCGCTG<br>TCTTGCCCTTCCTTTCCTGTGT |
| NANOG | TCCTGAACCTCAGCTACAAACA<br>GGTAGGTGCTGAGGCCTTCT |
| POU5F1 | GTGGGGGCAGGGGAGTTTGG<br>AGTGTGTCTATCTACTGTGTCCCAGGC |
| CD34 | CACAGGAGAAAGGCTGGGCGA<br>TGGCCGTTTCTGGAGGTGGC |
| POSTN | CCCGTGACTGTCTATAAGCCAA<br>GTGTGTCTCCCTGAAGCAGT |
| DDR2 | TTCTTTTTGGGTTGGGGAAACG<br>TGGGTCCTGGGAGGCATATCA |
| NOS3 | AAGGCTTTTGATCCCCGGGTCCT<br>TCTCCATCAGGGCAGCTGCAAAG |
| PDGFRA | ATCGGAGGAGAAGTTTCCCAGAG<br>GGTACTGCCAGCTCACTTCA |

## Table S2

| Marker | Application | Concentration | Manufacturer |
|---|---|---|---|
| ISL1 | Flow cytometry<br>Immunocytochemistry | 1:200<br>1:250 | Abcam (ab26122) |
| KDR-PerCP | Flow cytometry | 10µl added to 100µl of $10^6$ cells | R&D Systems<br>(FAB357C) |
| NKX2-5 | Immunocytochemistry | 1:200 | Santa Cruz (SC-14033) |
| WT1 | Flow cytometry<br>Immunocytochemistry<br>Western Blot | 1:100<br>1:50<br>1:1000 | Abcam (ab89901) |
| BNC1 | Immunocytochemistry | 1:50 | Dr Shiro Luchi and Professor Howard Green |
| TCF21 | Western Blot | 1:250 | Abcam (ab170395) |
| ACTA2-FITC | Flow cytometry<br>Immunocytochemistry | 1:400<br>1:400 | Sigma (F3777) |
| CNN1 | Flow cytometry<br>Immunocytochemistry | 1: 15000<br>1: 15000 | Sigma (C2687) |
| TAGLN | Immunocytochemistry | 1:1000 | Abcam (ab14106) |
| ACTA2 | Whole mount<br>Immunofluorescence | 1:200 | Dako (M0851) |
| MYH11 | Western blot | 1:5000 | Sigma (M7786) |
| DDR2 | Immunocytochemistry | 1:200 | Santa Cruz (sc7555) |
| POSTN | Flow cytometry | 1:500 | Abcam (ab14041) |

| | | | |
|---|---|---|---|
| HOECHST 33342 | Whole mount immunofluorescence | 1:250 | Sigma (14533) |
| β-ACTIN | Western blot | 1:5000 | Sigma (A1978) |