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Long-term self-renewing human epicardial cells generated from pluripotent stem cells under defined xeno-free conditions

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Abstract

The epicardium contributes both multi-lineage descendants and paracrine factors to the heart during cardiogenesis and cardiac repair, underscoring its potential for cardiac regenerative medicine. Yet little is known about the cellular and molecular mechanisms that regulate human epicardial development and regeneration. Here, we show that the temporal modulation of canonical Wnt signaling is sufficient for epicardial induction from 6 different human pluripotent stem cell (hPSC) lines, including a WT1-2A-eGFP knock-in reporter line, under chemically-defined, xeno-free conditions. We also show that treatment with transforming growth factor beta (TGF-β)-signalling inhibitors permitted long-term expansion of the hPSC-derived epicardial cells, resulting in a more than 25 population doublings of WT1+ cells in homogenous monolayers. The hPSC-derived epicardial cells were similar to primary epicardial cells both *in vitro* and *in vivo*, as determined by morphological and functional assays, including RNA-seq. Our findings have implications for the understanding of self-renewal mechanisms of the epicardium and for epicardial regeneration using cellular or small-molecule therapies.

The epicardium is the outermost mesothelium layer of the heart, and contributes both multi-lineage descendants and trophic signals to the myocardium and coronary vessels[1,2]. During

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AUTHOR CONTRIBUTIONS

X.B. conceived and designed this study, undertook experimentation, data analysis and prepared the manuscript. X.L. contributed to study design and assisted in writing the manuscript. T.A.H. and E.G.S. designed and performed the *in vivo* study. T.H, V.J.B, T.Q. and M.S. assisted in differentiation experiments and data analysis. L.D., A.P., Q.W. and M.G. isolated and provided the human primary donor samples for RNA-seq. S.P.P. designed the study and prepared the manuscript.

COMPETING FINANCIAL INTERESTS

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cardiogenesis, epicardial cells undergo epithelial-to-mesenchymal transition (EMT) and invade the myocardium to form epicardial-derived cells (EPDCs) including cardiac fibroblasts, coronary smooth muscle cells and at least a subset of endothelial cells[3,4]. In addition, mounting evidence attributes to the epicardium both a paracrine role in modulating injury responses and a source of progenitor cells in facilitating neovascularization and myocardial repair[5,6]. Particularly, epicardial cells were shown to contribute to the cardiomyocyte lineage, highlighting their cardiogenic potentials in cardiac regeneration and repair[7]. Therefore, understanding the molecular mechanisms that control the specification and self-renewal of epicardial lineages from naïve progenitor cells is of fundamental importance to elucidate the regulatory mechanisms underlying both human heart development and cardiovascular diseases.

The advent of human pluripotent stem cells (hPSCs) significantly advanced the study of early human embryonic development, disease modeling, and cell-based therapy strategies[8]. Recently, there have been substantial advances in elucidating the regulatory mechanisms that control cardiomyocyte[9–12], endothelial cell[13–15], and smooth muscle cell[16,17] specification from hPSCs. More recently, epicardial cells have also been generated from hPSCs via embryoid bodies (EBs) or monolayer differentiation with growth factor cocktails in processes that manipulate signaling pathways which regulate epicardial differentiation[18,19]. However, these hPSC models have not clearly elucidated developmental signaling mechanisms that are necessary and sufficient to specify epicardial cell formation from hPSCs. In addition, human epicardial cells spontaneously undergo epithelial-mesenchymal transition(EMT) in culture[20], and little is known about signaling pathways that regulate epicardial cell self-renewal both *in vivo* and *in vitro*, limiting the ability to expand these hPSC-derived epicardial cells for clinical applications.

To address these issues, we generated self-renewing epicardial cells from hPSCs and investigated key signaling pathways during epicardial development and regeneration. We generated a WT1-2A-eGFP knockin stem cell line and for the first time showed that temporal modulation of canonical Wnt signaling via small molecules is sufficient for epicardial induction from human pluripotent stem cells (hPSCs) under chemically-defined, xeno-free conditions, and that TGF-β signaling inhibitor treatment allows long-term self-renewal of hPSC-derived epicardial cells. In addition, these hPSC-derived epicardial cells retain many characteristics of primary epicardial cells, including formation of a polarized epithelial sheet, expression of key epicardial genes *WT1*, *TBX18* and *ALDH1A2*, and the ability to form smooth muscle cells and cardiac fibroblasts both *in vitro* and *in vivo*. RNA-seq expression data and gene set enrichment analysis (GSEA) confirm a similarity of hPSC-derived epicardial cells to primary human epicardial cells. These findings improve our understanding of differentiation and self-renewal mechanisms of the epicardium and have implications for stimulating epicardial regeneration via small molecules or cell-based therapies.

Albumin-free conditions to generate cardiac progenitors

We previously demonstrated that temporal modulation of canonical Wnt signaling in RPMI basal medium (GiWi protocol) is sufficient to generate functional cardiomyocytes from

hPSCs[21]. We found that ISL1+NKX2.5+FLK-1+ cardiac progenitors are generated as intermediates during the GiWi protocol (Fig. S1A). When re-passaged on gelatin-coated plates in LaSR basal medium[14] on day 6, these hPSC-derived cardiac progenitors expressed progenitor markers including ISL1, NKX2.5 and FLK-1, as well as a proliferation marker Ki67 (Fig. S1B). Molecular analysis of cardiac progenitor differentiation from hPSCs revealed dynamic changes in gene expression, with down-regulation of the pluripotency markers *OCT4* and *NANOG*, and induction of the primitive streak like gene *T*[14,22] in the first 24 hr after CHIR99021 (CHIR) addition (Fig. S1C). Expression of cardiac progenitor markers *ISL1*, *NKX2.5* and *FLK-1* was first detected between days 3 and 5, and was significantly up-regulated at day 6 (Fig. S1C).

Wnt/β-catenin signaling regulates epicardial specification

Pro-epicardium arises from ISL1+NKX2.5+ second heart field progenitors *in vivo*[1,23]. To identify signaling mechanisms regulating cardiac progenitor specification to epicardial cells, we treated day 6 cardiac progenitors with different small molecules and protein modulators of developmental signaling pathways for 48 hours (day 7 to 9) (Fig. 1A, Table S1). hPSC-derived cardiac progenitors formed more than 85% WT1+ putative epicardial cells following CHIR99021 treatment (Fig. 1B &C, Movie S1), demonstrating that Wnt signaling induction between days 7 and 9 is sufficient to generate epicardial cells from cardiac progenitor cells in the absence of other exogenous signaling. In the absence of CHIR, robust beating sheets of cTnT+ cardiomyocytes were observed (Fig. 1B &C, Movie S2), suggesting that the activation status of canonical Wnt signaling at day 7 toggles epicardial vs. cardiomyocyte differentiation. Interestingly, untreated and bone morphogenetic protein 4 (BMP4)-, dorsomorphin (DM)- and retinoic acid (RA)-treated cells also yielded about 10% WT1+ cells that were distinct from cTnT+ cells (Fig. 1B &C, Fig. S2A). Compared to the untreated condition, BMP4 did not significantly increase the WT1+ cell population up to 100 ng/ml, when applied from days 7–9 (Fig. S2B).To exclude the potential low quality of BMP4 protein, we tested the function of BMP4 used in our study by inducing brachyury expression according to a previous report[24]. As expected, 5 ng/ml BMP4 treatment for 2 days in mTeSR1 resulted in robust expression of brachyury (Fig. S2C). In the presence of CHIR, BMP4 treatment did not generate cardiomyocytes, but instead yielded an unknown population at the expense of WT1+ cells. Inhibition of BMP4 signaling via DM resulted in a similar purity of WT1+ cells as CHIR treatment alone (Fig. 1B, Fig. S2A), suggesting that BMP4 signaling is dispensable at this stage of epicardial development.

Construction of WT1-2A-eGFP knock-in reporter hPSC line

WT1 is required for the development of epicardium[25] and the formation of cardiovascular progenitor cells[26]. In order to better monitor the epicardial cell differentiation process and purify hPSC-derived epicardial cells *in vitro*, we engineered the ES03 cell line via CRISPR/Cas9-catalyzed homology-directed repair (HDR) and generated a homozygous WT1-2A-eGFP knock-in reporter cell line (Fig. 2A). Two 2-kilobase homologous arm sequences located just upstream and downstream of the *WT1* stop codon were inserted into the Oct4-2A-eGFP donor plasmid[27] and replaced the *Oct4* homologous arms. We then introduced the 2A-eGFP sequence into the target sites by transfecting hESCs with the

WT1-2A-eGFP donor plasmid and the Cas9/sgRNA plasmids. After puromycin (Puro) selection, PCR genotyping and sequencing showed that ~50% (21/44) of the clones were targeted in one (heterozygous) and ~25% (12/44) in both alleles (Fig. 2B) similar to a previous report[28]. The homozygous clones were then subjected to TAT-Cre recombinase treatment and the PGK-Puro cassette was excised from WT1-2A-eGFP (Fig. 2C). WT1-2A-eGFP-targeted hPSCs after Cre-mediated excision of the PGK-Puro cassette were subjected for CHIR treatment, and eGFP was detected at day 10 and elevated at day 12 (Fig. 2D). Dual immunostaining with anti-WT1 and anti-GFP antibodies detected expression of eGFP in WT1+ cells (Fig. 2E), demonstrating the success in generating WT1 reporter cell line for potential cell tracking or purification.

Chemically-defined conditions to generate epicardial cells

We next optimized the concentration of CHIR and initial seeding density of cardiac progenitors at day 6 in LaSR basal medium, and found that 3 μM CHIR with an initial density of 0.06 million cells/cm^2 yielded more than 95% WT1+ cells (Fig. S3A-D), while the no CHIR control resulted in less than 10% WT1-2A-eGFP cells. However, LaSR basal medium, which contains bovine serum albumin, adds xenogenic components to the medium which would not be desirable for the generation of epicardial cells that meet clinical requirements. In order to develop a xeno-free protocol, we systematically screened 4 commercially available basal media supplemented with 1 μg/mL human recombinant insulin and 100 μg/mL ascorbic acid (Vc) as these two factors were shown to improve the culture of cardiac cell lineages[29–31]. DMEM, DMEM/F12 and RPMI generated more than 95% WT1+ putative epicardial cells from hPSC-derived cardiac progenitors (Fig. S3E). To simplify the differentiation pipeline, we employed RPMI as the basal medium, referring to epicardial cell generation from hPSCs as the “GiWiGi” (GSK3 inhibitor - WNT inhibitor - GSK3 inhibitor) protocol.

Epicardial cell differentiation is β-catenin dependent

Selectivity is a concern when using chemical inhibitors of signaling pathways. Therefore, we tested other GSK3 inhibitors including BIO-acetoxime and CHIR98014 in the GiWiGi protocol, and found that 0.3 μM CHIR98014 and BIO-acetoxime generated WT1+ cells as effectively as 3 μM CHIR99021 (Fig. S4A). In addition, we treated day 6 cardiac progenitors with Wnt3a, up to 500 ng/ml, and found that Wnt3a significantly increased the WT1+ cell population compared to the no Wnt3a control, although Wnt3a was less effective than small molecule GSK3 inhibitors in generating WT1+ cells (Fig. S4B).To further investigate the role of β-catenin in our GiWiGi epicardial differentiation, we employed an iPSC cell line (19-9-11 ischcat-1) expressing β-catenin shRNA under the control of a tet-regulated inducible promoter described in earlier work[10]. Upon doxycycline (dox) treatment, the shRNA efficiently down-regulated β-catenin expression[10]. We also showed that the induction of NKX2.5+ISL1+ cardiac progenitors from hPSCs is β-catenin dependent[10]. In this study, we therefore focused on the examination of the stage-specific roles of β-catenin during differentiation of epicardial cells from cardiac progenitors stimulated by GSK3 inhibition. We found that β-catenin knockdown at day 6 yielded significantly fewer WT1+ cells, instead generating robust beating sheets of cTnT+ cardiomyocytes at the expense of

WT1+ cells (Fig. S4C–D, Movie S3). This finding is consistent with reports that Wnt/β-catenin inhibition is necessary for cardiomyocyte formation from cardiac progenitors both *in vitro* and *in vivo*[10,18,32,33], and further supports the notion that Wnt/β-catenin signaling regulates epicardial vs. cardiomyocyte specification from cardiac progenitors. The effects of β-catenin knockdown on decreasing WT1+ cell generation gradually diminished after day 6, with no inhibition after day 9 (Fig. S4C–D, Movie S3–4).

Molecular characterization of hPSC-derived epicardial cells

Pro-epicardial cells are marked by the expression of TBX18, WT1 and TCF21[34–36]. Molecular analysis of epicardial cell differentiation from hPSCs-derived cardiac progenitors (Fig. 3A) revealed dynamic changes in gene expression, with up-regulation of *WT1* and *TBX18*, and undetectable *TNNT2* (Fig. 3B). This gene upregulation was consistent with the increased WT1-eGFP signals (Fig. 2D), and was also confirmed by western blot analysis of WT1, TBX18 and TCF21 expression (Fig. 3C, Fig. S5A). Immunofluorescent analysis revealed expression of pro-epicardial markers WT1, TBX18, and TCF21 (Fig. 3D–E). After passage at a low density, these cells adopted a cobblestone-like organization typical of cultured primary epicardium[20,37] (Fig. 3E). In addition, the cells displayed intense β-catenin and ZO1 localization at sites of cell-to-cell contact. Taken together, these data confirm the epithelial nature of these cells. These post-passaged cells also expressed aldehyde dehydrogenase enzyme retinaldehyde dehydrogenase 2 (ALDH1A2) (Fig. S5A–C), suggesting the ability to produce retinoic acids. Therefore, we refer to the day 12, pre-passaged WT1+/ALDH1A2− cells as pro-epicardial cells (Pro-Epi) and the post-passaged WT1+/ALDH1A2+ cells as epicardial cells (Epi). The GiWiGi protocol was also effective in other hPSC lines, including human embryonic stem cell lines (hESC) H9 and ES03, and the 19-9-7 induced pluripotent stem cell (iPSC) line, generating more than 95% WT1+ cells (Fig. S6A, Table S2). Post-passaged epicardial cells retained the expression of WT1 and displayed strong β-catenin and ZO1 staining along the cell borders (Fig. S6B).

Long-term expansion of hPSC-derived WT1+ epicardial cells

Primary mouse epicardial cells have been cultured for more than 3 years[34], but epicardial cells isolated from the adult human heart rapidly undergo EMT in culture[20]. Similar to primary human epicardial cells, hPSC-derived WT1+ epicardial cells only retained their morphology for approximately 2 weeks in culture. While in the short term, hPSC-derived WT1+ epicardial cells retained the epicardial cobblestone-like morphology, basic fibroblast growth factor (bFGF) and TGF-β-treated cells adopted a fibroid spindle or fusiform-shaped appearance typical of cultured fibroblasts and smooth muscle cells, respectively (Fig. 4A–B). The expression of calponin and smooth muscle myosin heavy chain (SMMHC) in TGF-β+bFGF-induced cultures further support their smooth muscle cell identity, and vimentin (VIM) and CD90 expression support their fibroblast identity (Fig. S7). We also cultured WT1+TBX18+ epicardial cells in endothelial cell medium, but did not detect expression of endothelial markers CD31 and VE-cadherin (Fig. S7). The expression of WT1 and ZO1 significantly decreased in both bFGF and TGF-β-treated samples, indicating the transition from epithelial towards mesenchymal-like cells (Fig. 4B). In addition, *CDH1* (E-cadherin) expression decreased whereas *CDH2* (N-cadherin) and *SNAIL2* expression increased in all

treated samples compared to untreated controls, suggesting bFGF and TGF-β induced EMT in hPSC-derived epicardial cells (Fig. 4C–D).

During long term *in vitro* culture hPSC-derived WT1+ epicardial cells spontaneously underwent EMT and lost WT1 expression after several passages, even without exogenous TGF-β or bFGF treament (Fig. 5A). To identify signaling mechanisms regulating hPSC-derived epicardial cell self-renewal, we applied small molecules (Table S1) that affect pathways that regulate cell proliferation to study their effects on WT1+ cell self-renewal. TGF-β receptor 1 antibody (α-TGF-β I) and TGF-β pan-specific antibody (TGF-β Pan) partially increased the proliferation of epicardial cells and maintained their epithelial phenotype (Fig. 5A–B). A83-01, an inhibitor of TGF-β signaling, enabled expansion of hPSC-derived epicardial cells that retained polarized epithelial morphology and WT1 expression to a significantly greater extent than the untreated control (Fig. 5A–B). To reduce the likelihood that WT1+ cell self-renewal was an off-target effect of A83-01, we tested additional TGF-β inhibitors (RepSox, SB505124 and SB431542) and found they also promoted the proliferation of hPSC-derived epicardial cells to a similar extent as A83-01 (Fig. 5B–C). Upon A83-01 or SB431542 addition, hPSC-derived epicardial cells were capable of at least 25 population doublings, generating more than 10 million cells from a single hPSC-derived epicardial cell clone (Fig. 5C). After 48 days of expansion, the TGF-β inhibitor-treated cells expressed significantly higher levels of WT1 and Ki67 than the untreated control cells (Fig. 5D). Epicardial cells generated from 19-9-11 iPSCs were also expandable after A83-01 treatment (Fig. S8A), presenting a cobblestone morphology and expressing high levels of ALDH1A2, WT1, and TBX18 (Fig. S8B–D). In addition, epicardial cells expanded for 48 days retained the potential to differentiate into SMMHC +calponin+ smooth muscle cells and VIM+CD90+ fibroblasts upon TGF-β and bFGF treatment, respectively (Fig. S8D). The day 50 epicardial cells retained normal karyotypes after long-term culture in A83-01 containing medium (Fig. S9). These findings improve our understanding of self-renewal mechanisms of the epicardium and have implications for generating large quantities of hPSC-derived epicardial cells for research or cell-based therapy applications.

hPSC-derived epicardial cells were similar to primary cells

To further confirm the identity of hPSC-derived epicardial cells, RNA from 4 different hPSC-derived epicardial cell differentiations at two different expansion time points (day 12 and day 48) and primary epicardial cells of 4 different donors were subjected to RNA-seq analysis. Hierarchical clustering analysis of RNA-seq expression data of hPSCs[38–40], hPSC-derived endoderm (Endo)[38], ectoderm (Ecto)[39], mesoderm (Mes) (GSM1112833, 915324, 915325), CMs[41], epicardial cells (Epi), and primary epicardial cells (Fig. 6A) showed that hPSC-derived epicardial cells were most closely related to primary epicardial cells and were distinct from all other cell populations together as a group. Day 12 hPSC-derived epicardial cells clustered closely with day 48 hPSC-derived epicardial cells. Next we explored the relationship between different cell types relevant for development including hPSC, mesoderm, cardiomyocytes, epicardial cells using principal component analysis (PCA) on the gene expression data. hPSCs clustered relatively closer to mesoderm cells, from which epicardial cells and CMs divergently formed, in the 3D scores plot for the first 3 principal

components (Fig. 6B). Importantly, hPSC-derived epicardial cells showed highest similarity with donor epicardial cells. We also performed gene set enrichment analysis (GSEA) to identify significantly enriched pathways ($p<0.05$) in each cell type relative to hPSCs. Hierarchical clustering of the absolute value of normalized enrichment scores (NES) of these pathways confirmed the similarity between epicardial cells from hPSCs and those from donors (Fig. S10A). As both epicardial cells and CMs are derived from mesoderm, we further compared the differences and similarities in the enriched pathways among these cell types. We observed that while 42 pathways were commonly enriched in all cell types, hPSC-derived epicardial cells shared 36 pathways with donor epicardial cells, and 22 with cardiomyocytes (Fig. S10B, Table S3–5). Microarray data analysis has shown the enrichment of cell adhesion and extracellular matrix organization genes in mouse primary epicardial cells[42]. Similarly, our hPSC-derived or donor epicardial cells also showed enrichment in extracellular matrix related pathways and keratinocyte (epithelial) differentiation, while CMs were enriched in heart development and heart contraction related pathways, as expected. Donor epicardial cells were highly enriched in endoplasmic reticulum related pathways compared to hPSC-derived epicardial cells.

Epicardial cells can undergo EMT and give rise to cardiac fibroblasts and smooth muscle cells after transplantation into chicken embryos[19] or infarcted mouse hearts[20]. To examine the ability of hPSC-derived epicardial cells to invade the myocardium and undergo EMT *in vivo*, cardiac fibroblast-derived extracellular matrix (CF-ECM) patches seeded with passage 5 eGFP-labeled hPSC-derived epicardial cells (Fig. 6C) were transferred to the heart surface (Fig. S10C) of a mouse myocardial infarction (MI) model. eGFP+ cells were detected predominantly within the CF-ECM scaffold and in the epicardium beneath the scaffold before day 6, and scattered within the mid-myocardium after 12 days (Fig. S10D&E), suggesting epicardial cells invaded the myocardium. In addition, the hPSC-derived cells underwent EMT and differentiated into αSMA+calponin+ smooth muscle-like cells (Fig. 6D&E, Fig. S10F&G) and VIM+ fibroblast-like cells (Fig. S10F) *in vivo*. We did not observe any tumor formation 12 days after hPSC-derived epicardial cell transplantation into the mouse heart compared to the patch-only control (Fig. S10H). These findings demonstrate that hPSC-derived epicardial cells can invade the myocardium and form EPDCs after infarction, underscoring their potential for cell-based therapeutic heart regeneration.

DISCUSSION

While differentiation of hPSCs to epicardial cells by applying complex mixtures of growth factors from different signaling pathways has been reported[18,19], it is largely unknown which developmental signaling pathways are necessary and sufficient to specify epicardial cell fate and to control their self-renewal, limiting their large-scale production for clinical applications. This study reports for the first time the generation of a WT1-2A-eGFP knockin stem cell line, and demonstrates efficient and robust generation of epicardial cells from multiple hPSC lines solely via stage-specific manipulation of Wnt/β-catenin signaling under chemically-defined, albumin-free, xeno-free conditions. These hPSC-derived epicardial cells retain many characteristics of primary epicardial cells, including formation of an epithelial sheet, expression of key epicardial proteins WT1, TBX18 and ALDH1A2, and the ability to generate fibroblast and vascular smooth muscle lineages both *in vitro* and *in vivo*. In

addition, their identity was further confirmed by RNA-seq expression data and gene set enrichment analysis at a global level. Using inducible knockdown hPSC lines, we showed that β-catenin is essential for epicardial cell induction from hPSC-derived cardiac progenitors during the GiWiGi protocol. Given the essential roles of β-catenin during cardiac progenitor induction from hPSCs[10,43], we conclude that β-catenin is required at multiple stages of hPSC differentiation to epicardial cells via small molecule modulation of canonical Wnt signaling. Differences between our study and previous reports[18,19], including the initial starting cardiac progenitor cells and the exposure windows for signaling modulators, may account for their conclusion that BMP4 signaling, but not Wnt alone, is required for robust epicardial differentiation.

This study also demonstrates long-term self-renewal of hPSC-derived epicardial cells via TGF-β inhibitor treatment in a chemically-defined medium. For cell-based therapeutic applications, it is highly desirable to generate homogeneous committed progenitors that can expand in culture and differentiate into various tissue-specific cells of interest, avoiding the contamination of unwanted cell lineages, especially tumorigenic hPSCs[44]. We showed that TGF-β inhibitor treatment is sufficient for the self-renewal of hPSC-derived epicardial cells, in contrast to primary mouse epicardial cells which can self-renew in the absence of a TGF-β inhibitor[34]. It will also be interesting to test whether or not TGF-β inhibitor treatment can promote the self-renewal of human primary epicardial cells *in vitro*. Recent work has demonstrated that epicardial cell lineages improved the performance of the scarred myocardium by preservation of cardiac function and attenuation of ventricular remodeling after transplantation into a MI model[45]. More recently, it's reported that the epicardium of the zebrafish heart is required for muscle regeneration and itself can regenerate upon Sonic hedgehog (Shh) treatment[46]. Our results suggest that TGF-β inhibitors may impact heart regeneration following injection into the epicardium *in vivo*, similar to strategy used to test the effect of TGF-β inhibitors on scar formation after glaucoma surgery in rabbits[47].

In summary, our findings support a model (Fig. 6F) of human epicardial development in which small molecule-mediated exogenous modulation of Wnt/β-catenin signaling is sufficient for the specification of epicardial cells from hPSCs. This finding is consistent with the report that *DKK1* and *DKK2* double null mice increase epicardial specification and display a hypercellular epicardium[48]. This completely defined, xeno-free epicardial differentiation platform is compatible with current Good Manufacturing Practice (cGMP) standards and can be employed to efficiently derive self-renewing epicardial cell lineages from hPSCs, which can thereby provide insights into mechanisms of heart development, maturation, and response to cardiac injury. Moreover, we show that hPSC-derived epicardial cells can invade the myocardium in an infarcted mouse model, suggesting potential applications in cell-based heart regeneration. Our results also point to TGF-β signaling as a regulator of epicardial cell self-renewal and differentiation, indicating the potential of TGF-β signaling modulators in heart regeneration.

METHODS

Construction of donor plasmid and sgRNA

Human codon-optimized Streptococcus pyogenes wild-type Cas9 (pCas9-2A-eGFP #) was obtained from Addgene (plasmid #44719) and chimeric guide RNA expression cassette was cloned into this Cas9-2A-eGFP plasmid with two BbsI restriction sites for rapid sgRNA cloning. Two sgRNAs targeting at or near *WT1* stop codon (1: AACTCCAGCTGGCGCTTTGAGGG and 2: GGACACTGAACGGTCCCCGAGGG) were used. To generate the WT1-2A-eGFP donor plasmid, DNA fragments of about 2kb in length were PCR amplified from the genomic DNA before and after the stop codon of *WT1* and were cloned into the OCT4-2A-eGFP donor plasmid[27] (Addgene #31938), replacing the *OCT4* homologous arms.

Maintenance of hPSCs and TAT-Cre treatment of WT1 Knock-in hPSCs

Transgene and vector-free human pluripotent stem cells (hPSCs) were maintained on Matrigel (Corning) or SyntheMax (BD Biosciences)-coated plates in mTeSR1 or E8 medium (STEMCELL Technologies) according to previously published methods[49]. To remove PGK-Puro cassette from the WT1-2A-eGFP cells, targeted homozygous clones were treated with 2 μM TAT Cre Recombinase (Excellgen, EG-1001) for 6 hours in E8 medium. After two days, cells were singularized with Accutase and seeded into Matrigel-coated 96-well plate at a density of 100 to 150 cells per well. After two weeks, cells were subjected for PCR genotyping.

Electroporation

hESCs were pre-treated with 10 μM ROCK inhibitor (Y27632) for 3 to 4 hours prior to electroporation. Cells were digested by Accutase (Innovative Cell Technologies) at 37°C for 8 min and 2.5–3 million single cells were electroporated with 3 μg gRNA1, 3 μg gRNA2 and 6 μg WT1-2A-eGFP donor plasmids in 200 μL cold PBS −/− using the Gene Pulser Xcell System (Bio-Rad) at 320 V, 200 μF and 1000 Ω (Time constant should be around 15 ms) in a 0.4 cm cuvette. Two electroporations were performed and 5–6 million cells were subsequently plated onto Matrigel-coated 10-cm dish in 10 mL mTeSR1 with 10 μM Y27632. 24 hours later, and every day afterwards, the medium was changed with fresh mTeSR1. Three days after electroporation, 1 μg/ml puromycin was added into the mTeSR1 for selection for about two weeks. Single cell clones were then picked into wells of a Matrigel-coated 96-well plate and subjected for PCR genotyping after 4 to 7 days.

Cardiac progenitor induction via modulation of canonical Wnt signaling

As described previously, hPSCs were singularized with Accutase (Innovative Cell Technologies) at 37°C for 5 min once they achieved confluence and then seeded at 100,000–250,000 cells/cm^2 in mTeSR1 or E8 supplemented with 5μM ROCK inhibitor Y-27632 (Selleckchem) (day −3) for 24 hours[14,21]. Cells were then cultured in mTeSR1 or E8, changed daily. At day 0, cells were treated with 6 μM CHIR99021 (Selleckchem) for 24 hours in RPMI medium, followed by a change with RPMI medium at day 1. 2.5–5 μM IWP2 (Tocris) was added at day 3 and removed during the medium change at day 5. Cardiac

progenitor cells can also be efficiently generated in RPMI/B27- medium using our previous GiWi protocol[11].

Epicardial cell generation via activation of canonical Wnt signaling

At day 6, cardiac progenitor cells were singularized with Accutase at 37°C for 5 min and then seeded onto a gelatin- or SyntheMax-coated cell culture plate at 20,000–80,000 cells/cm^2 (or 1:3–1:12 split) in LaSR basal medium (advanced DMEM/F12 with 100 μg/mL ascorbic acid) or RPMI/Vc/Ins medium (100 μg/mL ascorbic acid and 1 μg/mL human recombinant or bovine insulin (Sigma)) with 5 μM ROCK inhibitor Y-27632 for 24 hours. The addition of 1% human recombinant albumin (Sigma) or fetal bovine serum (FBS) can improve cell attachment and survival, but they are not required. At day 7, cells were treated with 1–9 μM CHIR99021 for 2 days in LaSR basal medium or RPMI/insulin/Vc medium. CHIR99021-containing medium was aspirated on day 9 and cells were cultured in LaSR basal medium or RPMI/insulin/Vc medium without CHIR99021 for 3–5 additional days, as explained previously[14].

Long-term maintenance of hPSC-derived epicardial cells

To expand the epicardial cells, confluent cells on day 12 of differentiation or day 4 after expansion were split 1:3 to 1:9 at a density of 0.03 to 0.09 million cells/cm^2 using Versene (Life Technologies) or Accutase and routinely passaged onto gelatin-coated plates in LaSR basal medium or RPMI/Vc/Ins medium and 0.5 μM A83-01 (Stemgent) or 2 μM SB431542 (Stemgent) with medium changed daily until the cells reached confluence. Overnight treatment of 5 μM Y27632 and 1% human recombinant albumin on single cells during passage was used to improve cell attachment and survival, but they were not required once cells attached. H13 hESC-derived day 50 epicardial cells were subjected for karotype analysis at WiCell Research Institute.

Single cell passage and EMT induction

Confluent WT1+ cells were singularized with Accutase at 37°C for 5 min and then seeded onto a gelatin-coated cell culture plate at a density of 10,000 cells/cm^2 in LaSR basal medium supplemented with 5 μM Y-27632 for 24 hr. After 24 hr, medium was changed to LaSR basal medium and cells were treated with TGF-β1 or bFGF (R&D Systems) as indicated. Medium was changed every 3 days until analysis.

Cardiac fibroblast-derived extracellular matrix (CF-ECM) to transfer epicardial cells *in vivo* to the infarcted heart

Immunodeficient mice were purchased from Harlan Laboratories and all procedures were carried out in accordance with protocols approved by the Institutional Animal Care and Use Committee. As described previously[50], myocardial infarction was induced 48 hr before the transplantation. CF-ECM scaffolds were generated according to a previous report[50]. Briefly, cardiac fibroblasts isolated from porcine hearts were plated at a density of 0.1–0.25 million cells per cm^2 in high glucose DMEM supplemented with 10% FBS and cultured at 37°C, 5% CO_2 for 10–14 days. The combined cardiac fibroblasts and extracellular matrix were removed from the culture dish by incubation with 2 mM EDTA solution at 37°C after a 10-

to 14-day culture. The resulting cell sheet was treated with molecular grade water followed by 0.15% peracetic acid (PAA buffer) for 24–48 hr at 4°C with constant agitation to denude the cells. The resulting matrix was then rinsed with sterile water followed by PBS several times. CF-ECM scaffold was then seeded with 1–5 million ES03 eGFP hPSC-derived epicardial cells and incubated for 3 hr prior to transfer to the epicardial surface of the MI area. After transplantation, the chest was closed. After 2, 6, 12 days, the mouse hearts were harvested and excised for histology.

Immunostaining analysis

As explained in a previous study[13], cells were fixed with 4% paraformaldehyde for 15 min at room temperature and then stained with primary and secondary antibodies (Table S6) in PBS plus 0.4% Triton X-100 and 5% non-fat dry milk (Bio-Rad). Nuclei were stained with Gold Anti-fade Reagent with DAPI (Invitrogen). An epifluorescence microscope (Leica DM IRB) with a QImaging® Retiga 4000R camera was used for imaging analysis.

Flow cytometry analysis

As described in a previous report[14], cells were dissociated into single cells with Accutase for 10 min and then fixed with 1% paraformaldehyde for 20 min at room temperature and stained with primary and secondary antibodies (Table S6) in PBS plus 0.1% Triton X-100 and 0.5% BSA. Data were collected on a FACS Caliber flow cytometer (Beckton Dickinson) and analyzed using FlowJo. FACS gating was based on the corresponding isotype antibody control.

Genomic DNA extraction and Genomic PCR

Quick Extract™ DNA Extraction Solution (Epicentre Cat. # QE09050) was used to rapidly extract genomic DNA from hESCs according to manufacture instructions. Genomic PCR was carried out using GoTaq Green Master Mix (Promega Cat. # M7123). PCR primer sequences are provided in the Table S7.

RT-PCR and Quantitative RT-PCR

As explained previously[14], total RNA was prepared with the RNeasy mini kit (QIAGEN) and treated with DNase (QIAGEN). 1 μg RNA was reverse transcribed into cDNA via Oligo (dT) with Superscript III Reverse Transcriptase (Invitrogen). Real-time quantitative PCR was done in triplicate with iQSYBR Green SuperMix (Bio-Rad). *GAPDH* was used as an endogenous housekeeping control. PCR primer sequences are provided in the Table S7.

RNA sequencing and data analysis

Total RNA of day 12 19-9-7 iPSC-derived epicardial cells and day 48 19-9-11, ES03, H9 hPSC-derived epicardial cells were prepared with the Direct-zol™ RNA MiniPrep Plus kit (Zymo Research) according to the manufacture instructions. Human primary epicardial RNAs from 4 different donors were provided by our collaborator (AstraZeneca, Sweden). Samples were performed in IIIumina HiSeq 2500 by Biotechnology Center at University of Wisconsin-Madison. The resulting sequence reads were mapped to the human genome (hg19) using HISAT[51], and the RefSeq transcript levels (RPKMs) were quantified using the

python script rpkmforgenes.py[52]. Hierarchical clustering of whole transcripts were then plotted using GENE-E. Fastq files of hPSCs[38–40], hPSC-derivedendoderm[38], ectoderm[39], mesoderm[40] and CMs[41] were downloaded from GEO or ArrayExpress (http://www.ebi.ac.uk/arrayexpress/). Principal component analysis (PCA) (exclude 19-9-7 iPSC-derived day 12 epicardial cells) was performed using PLS Toolbox 8.1 (Eigenvector Technologies). The whole transcripts were preprocessed using auto-scaling method (subtracting the mean from the variables and dividing by the standard deviation) to study the variance. Pathway enrichment analysis was performed using Gene Set Enrichment Analysis (GSEA) software[53] (exclude 19-9-7 iPSC-derived day 12 epicardial cells). The gene expression data for each cell type was compared with hPSCs and the significantly enriched pathways ($p<0.05$) were considered for further analysis. MATLAB 2013a (Mathworks Inc.) and Microsoft Excel (2013) were used to identify the unique and common pathways in different cell types. The absolute value of normalized enrichment score (NES) of the top 50 significantly enriched pathways for each cell type (ranked by the absolute NES) were further used for hierarchical clustering using GENE-E. To further investigate the similarity and differences in the number of enriched pathways in the three cell types: donor derived epicardial cells, hPSC derived epicardial cells and cardiomyocytes, top 150 significantly enriched pathways for each cell type were selected.

Western Blot Analysis

As described in a prior report[43], cells were lysed in M-PER Mammalian Protein Extraction Reagent (Pierce) in the presence of Halt Protease and Phosphatase Inhibitor Cocktail (Pierce). Proteins were then separated by 10% Tris-Glycine SDS/PAGE (Invitrogen) under denaturing conditions and transferred to a nitrocellulose membrane. After blocking with 5% non-fat milk in TBST, the membrane was incubated with primary antibody (Table S6) overnight at 4°C. The membrane was then washed, incubated with an anti-mouse/rabbit peroxidase-conjugated secondary antibody for 1 hr at room temperature or overnight at 4°C, and developed by SuperSignal chemiluminescence (Pierce).

Statistical Analysis

Data are presented as mean ± standard error of the mean (SEM). Statistical significance was determined by Student's t-test (two-tail) between two groups, and three or more groups were analyzed by one-way analysis of variance (ANOVA). $P< 0.05$ was considered statistically significant.

Data Availability

The final processed data and raw fastq files were submitted to Gene Expression Omnibus (GEO) with accession number GSE84085. Other data that support the findings of this study are available in figshare with the identifier doi:10.6084/m9.figshare.3971748[54].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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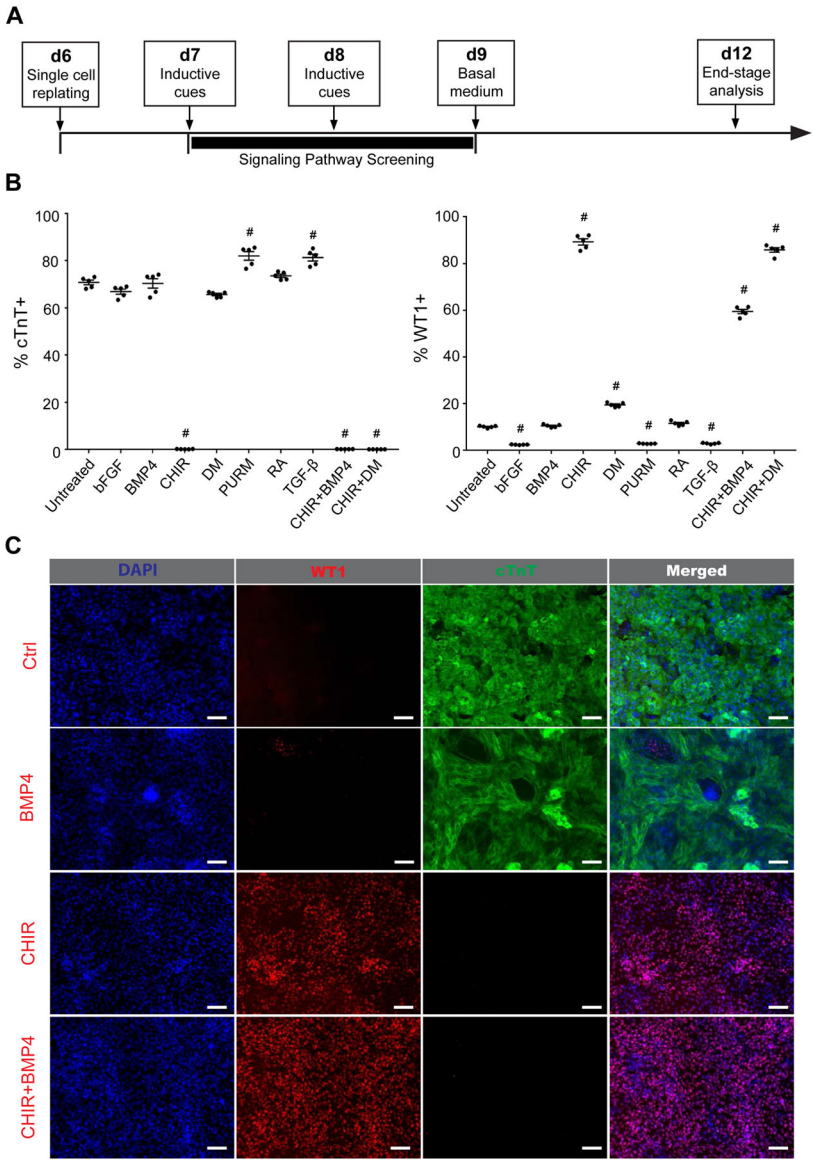

Figure 1.

Wnt/β-catenin signaling directs the specification of WT1+ epicardial lineages from hPSC-derived cardiac progenitors. (A) Schematic of the protocol to differentiate NKX2.5+ISL1+ hPSC-derived cardiac progenitors toward the epicardial lineage. H13 hESC-derived cultures differentiated as shown in (A) in the presence of the indicated molecular signaling regulators were subjected to flow cytometry analysis (B) and immunostaining analysis (C) for WT1 and cTnT at day 12. Scale bars, 100 μm. Data are represented as mean ± SEM of five independent replicates. # $p<0.05$, indicated treatment versus untreated condition. CHIR: CHIR99021; DM: Dorsomorphin; PURM: Purmorphamine; RA: Retinoic acid.

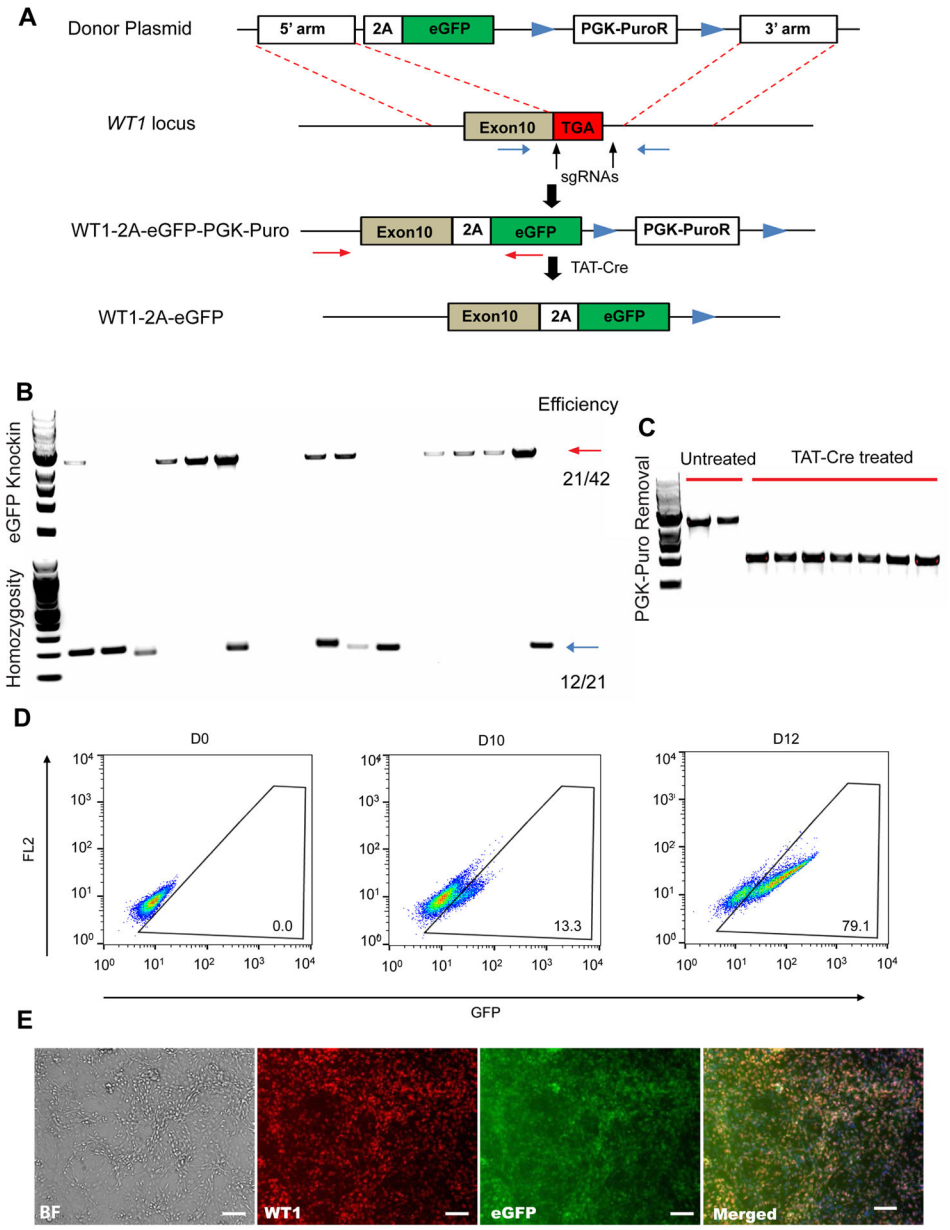

Figure 2.

Construction of WT1-2A-eGFP knockin ES03 hESC line using Cas9 nuclease. (A) Schematic diagram of the knockin strategy at the stop codon of the *WT1* locus. Vertical arrows indicate sgRNA1 and sgRNA2 targeting sites. Red and blue horizontal arrows are PCR primers for assaying *WT1* locus targeting and homozygosity, respectively. (B) Representative PCR genotyping of hESC clones after puromycin selection is shown, and the expected PCR product for correctly targeted *WT1* locus is ~3 kbp (red arrows) with an efficiency of 21/44. A homozygosity assay was performed on the knockin clones, and those without ~200 bp PCR products were homozygous (blue arrows). (C) PCR genotyping of hESC clones after TAT-Cre mediated excision of the PGK-Puro cassette. Clones with the PCR products of ~1 kbp are PGK-Puro free, and those with ~3 kbp contain PGK-Puro. (D)

Live cell flow analysis of GFP+ cells at day 0, day 10 and day 12 during CHIR treatment of WT1-2A-eGFP knockin ES03. (E) Phase contrast images and corresponding eGFP fluorescent images of WT1-2A-eGFP hPSC-derived epicardial cells after excision of the PGK-Puro cassette. Scale bars, 100 μm.

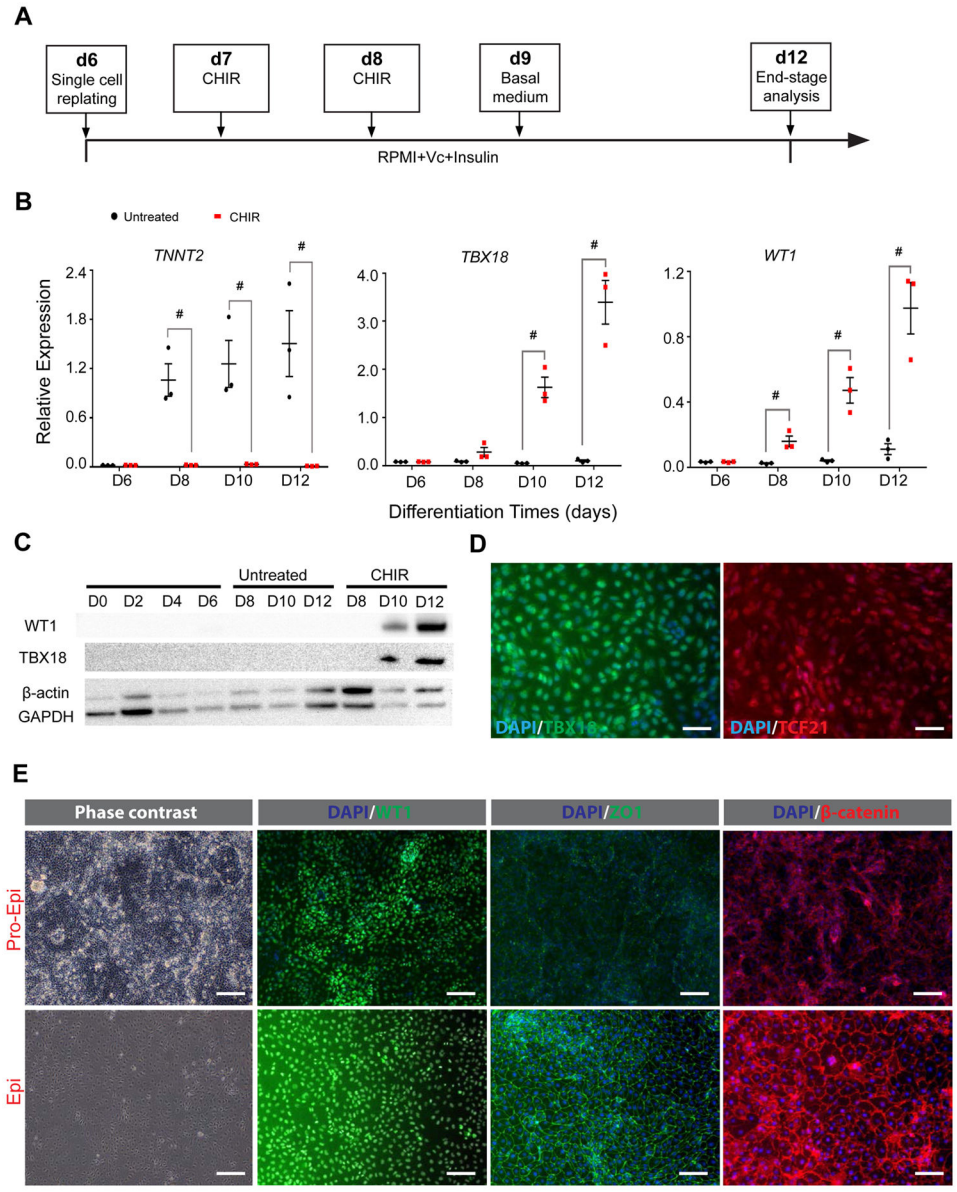

Figure 3.

Molecular analysis of hPSC-derived epicardial cells under chemically-defined, albumin-free conditions. (A) Schematic of the optimized protocol for differentiation of hPSCs to epicardial cells in RPMI basal medium. (B–D) H13 hESC-derived cardiac progenitors were differentiated as illustrated in (A). Gene expression was assessed by quantitative RT-PCR (B). Data are represented as mean ± SEM of three independent replicates. # $p<0.05$, CHIR-treated condition versus untreated condition at indicated time; t test. (C) At different time points, WT1 and TBX18 expression was assessed by western blot. (D) At day 12, immunostaining for TBX18 and TCF21 was performed. Scale bars, 50 μm. (E) Representative phase contrast microscopy and fluorescence immunostaining for WT1, ZO1 and β-catenin of day 12 pro-epicardium (Pro-Epi) and day 18 epicardium (Epi). Scale bars, 100 μm.

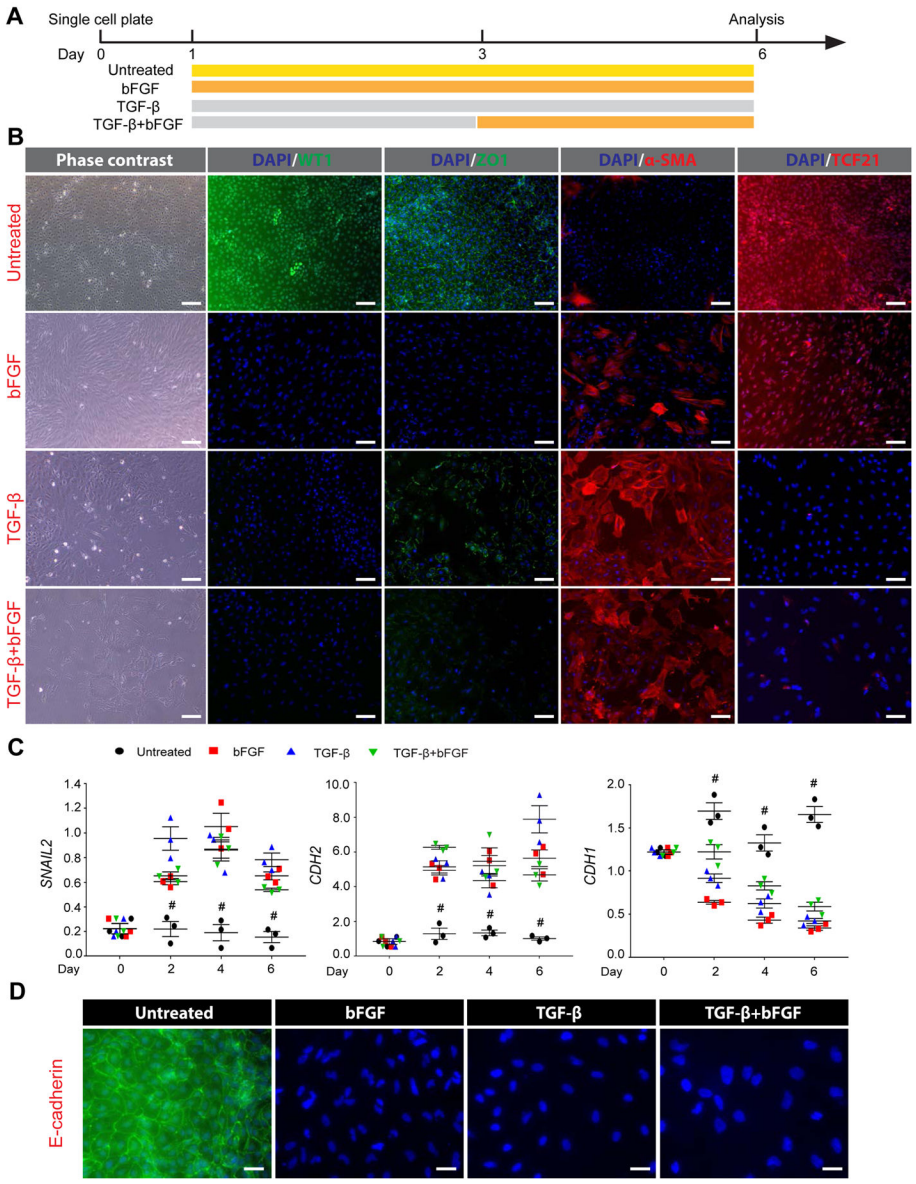

Figure 4.

hPSC-derived epicardial cells undergo EMT in response to bFGF and TGF-β1 treatment, yielding epicardium-derived cells that display characteristics of fibroblasts and vascular smooth muscle cells. (A) Schematic of the protocols used for the EMT induction of H13 hESC-derived epicardial cells with 10 ng/mL bFGF and 5 ng/mL TGF-β1. (B) At day 18, phase contrast images displaying cell morphology and fluorescence images showing the presence of WT1, ZO1, α-SMA and TCF21. Scale bars, 100 μm. (C) qPCR analysis of EMT related genes *SNAIL2*, *CDH2* and *CDH1* and (D) immunostaining analysis of E-cadherin expression after the indicated bFGF and TGF-β1 treatments. Data are represented as mean ± SEM of three independent replicates. # $p<0.05$, untreated condition versus bFGF-, TGF-β1- and bFGF+TGF-β1- treated conditions at indicated time. Scale bars, 50 μm.

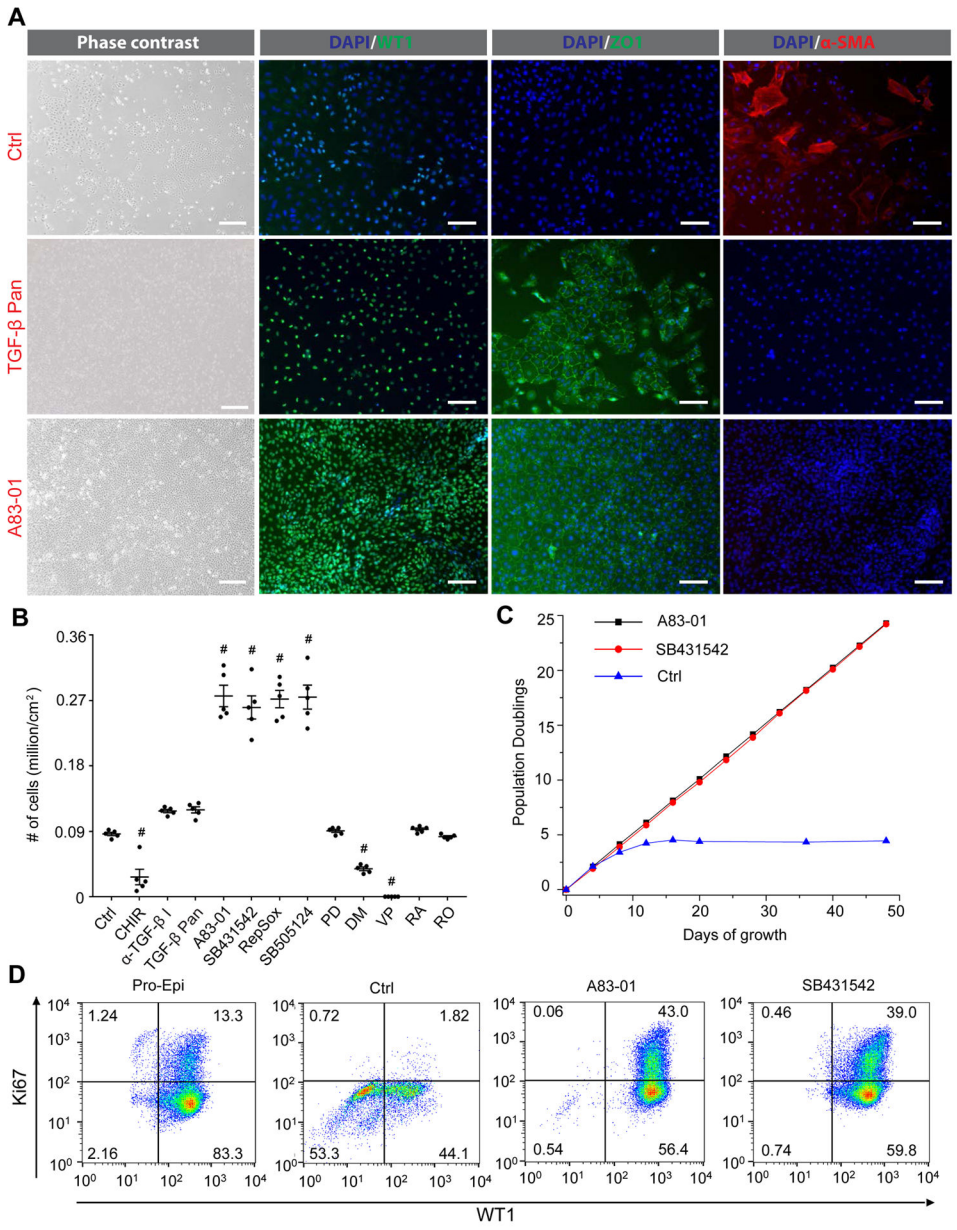

Figure 5.

Long-term expansion of hPSC-derived epicardial cells. (A–B) H13 hESC-derived day 18 epicardial cells were seeded at a density of 0.05 million cells/cm^2 and treated with the indicated small molecules for 3 days (concentrations provided in Table S1). At day 4, representative phase contrast microscopy and fluorescence immunostaining for WT1, ZO1 and α-SMA (A) and the total cell numbers were assessed (B). Data are represented as mean ± SEM of five independent replicates. # $p<0.05$, indicated treatment versus untreated condition; one-way ANOVA with Tukey's honest significant difference (HSD) as post-hoc analysis. α-TGF-β I: TGF-β receptor 1 antibody; TGF-β Pan: TGF-β pan specific antibody. Scale bars, 100 μm. (C–D) H13 hESC-derived epicardial cells were passaged and counted every four days in the absence or presence of the indicated TGF-β inhibitors: 0.5 μM

A83-01 or 2 μM SB431542. The population doublings were calculated and shown in (C), and day 48 cultures were subjected to flow analysis of WT1 and Ki67 expression (D).

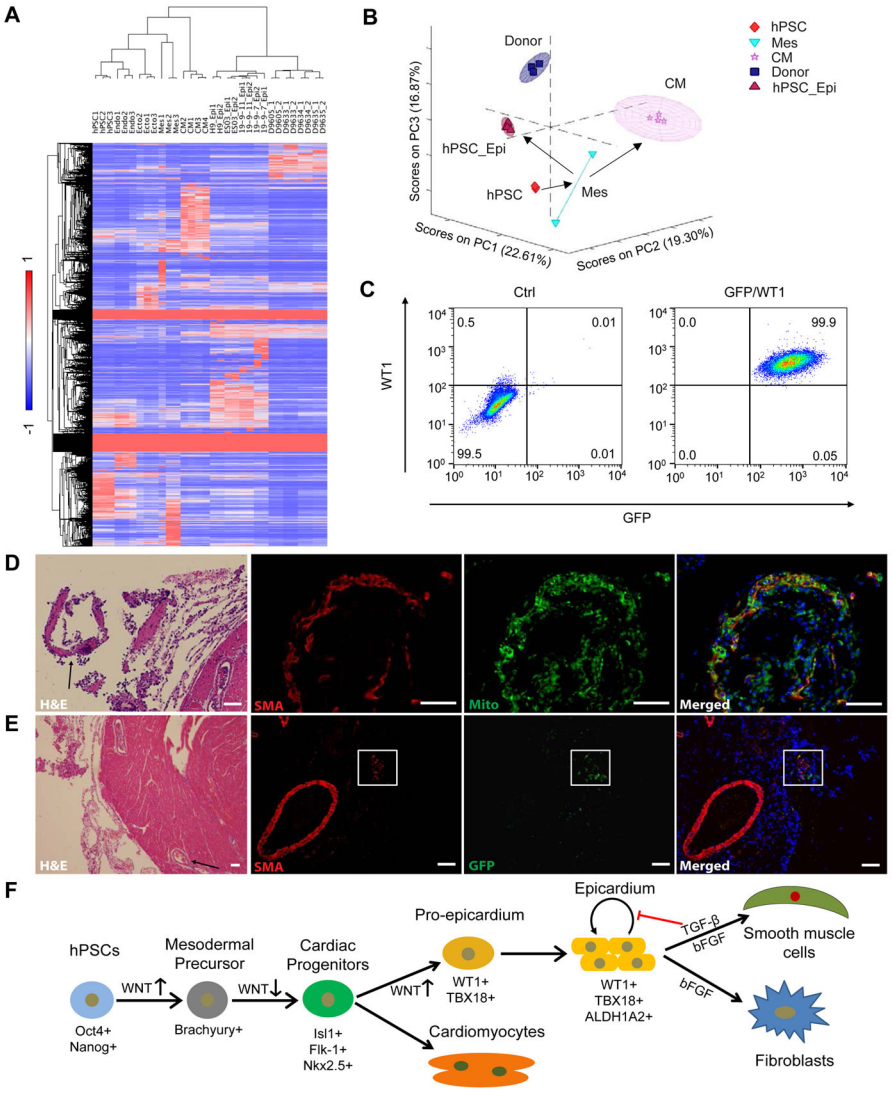

Figure 6.

hPSC-derived epicardial cells were similar to primary epicardial cells. (A) Hierarchical clustering analysis of RNA-seq expression data of hPSCs, hPSC-derived endoderm (Endo), ectoderm (Ecto), mesoderm (Mes), CMs, day 12 epicardial cells (19-9-7-Epi), day 48 epicardial cells (H9-Epi, ES03-Epi, 19-9-11-Epi), and primary epicardial cells (donor 9605, 9633, 9634, and 9635). (B) 3D scores plot of first 3 principal components (PCs) from the PCA. The ellipses show the 95% confidence limit and each data point correspond to different biological samples. Black arrows show the development transition from hPSCs to mesoderm, from which CMs and epicardial cells arise. (C) Before transplantation to mouse heart, ES03-eGFP cells were differentiated as illustrated in Figure 3A, cultured for 5 passages in A83-01 containing medium, and subjected to flow cytometry analysis for WT1 and GFP expression. (D–E) After 12 days, hearts were harvested, and representative hematoxilin and eosin stain (H&E) staining and dual immunostaining plots of smooth muscle actin (SMA) and human-specific mitochondria (Mito) (D) and SMA and GFP (E) of cross-sections of the hearts are shown. Arrows denote the corresponding sites in the H&E

images. Scale bar, 50 μm. (F) Model highlighting the specification of hPSCs to epicardial lineages by stage-specific modulation of canonical WNT signaling and the long-term maintenance of hPSC-derived epicardial cells using TGF-β signaling inhibitors.