Stem Cell Reports
Report

ISSCR

OPEN ACCESS

Human Pluripotent Stem Cell Differentiation into Functional Epicardial Progenitor Cells

Juan Antonio Guadix,[1,2,3] Valeria V. Orlova,[1] Elisa Giacomelli,[1] Milena Bellin,[1] Marcelo C. Ribeiro,[1,4] Christine L. Mummery,[1] José M. Pérez-Pomares,[2,3,*] and Robert Passier[1,4,*]

[1]Department of Anatomy and Embryology, Leiden University Medical Center, Einthovenweg 20, 2333 ZC Leiden, the Netherlands
[2]Department of Animal Biology, Faculty of Sciences, University of Málaga, Instituto Malagueño de Biomedicina (IBIMA), Campus de Teatinos s/n, 29071 Málaga, Spain
[3]BIONAND, Centro Andaluz de Nanomedicina y Biotecnología (Junta de Andalucía, Universidad de Málaga), Severo Ochoa 35, 29590 Campanillas (Málaga), Spain
[4]Department of Applied Stem Cell Technologies, MIRA Institute for Biomedical Technology and Technical Medicine, University of Twente, Building Zuidhorst, 7500 AE Enschede, the Netherlands
*Correspondence: jmperezp@uma.es (J.M.P.-P.), robert.passier@utwente.nl (R.P.)
https://doi.org/10.1016/j.stemcr.2017.10.023

SUMMARY

Human pluripotent stem cells (hPSCs) are widely used to study cardiovascular cell differentiation and function. Here, we induced differentiation of hPSCs (both embryonic and induced) to proepicardial/epicardial progenitor cells that cover the heart during development. Addition of retinoic acid (RA) and bone morphogenetic protein 4 (BMP4) promoted expression of the mesodermal marker PDGFRα, upregulated characteristic (pro)epicardial progenitor cell genes, and downregulated transcription of myocardial genes. We confirmed the (pro)epicardial-like properties of these cells using *in vitro* co-culture assays and *in ovo* grafting of hPSC-epicardial cells into chick embryos. Our data show that RA + BMP4-treated hPSCs differentiate into (pro)epicardial-like cells displaying functional properties (adhesion and spreading over the myocardium) of their *in vivo* counterpart. The results extend evidence that hPSCs are an excellent model to study (pro) epicardial differentiation into cardiovascular cells in human development and evaluate their potential for cardiac regeneration.

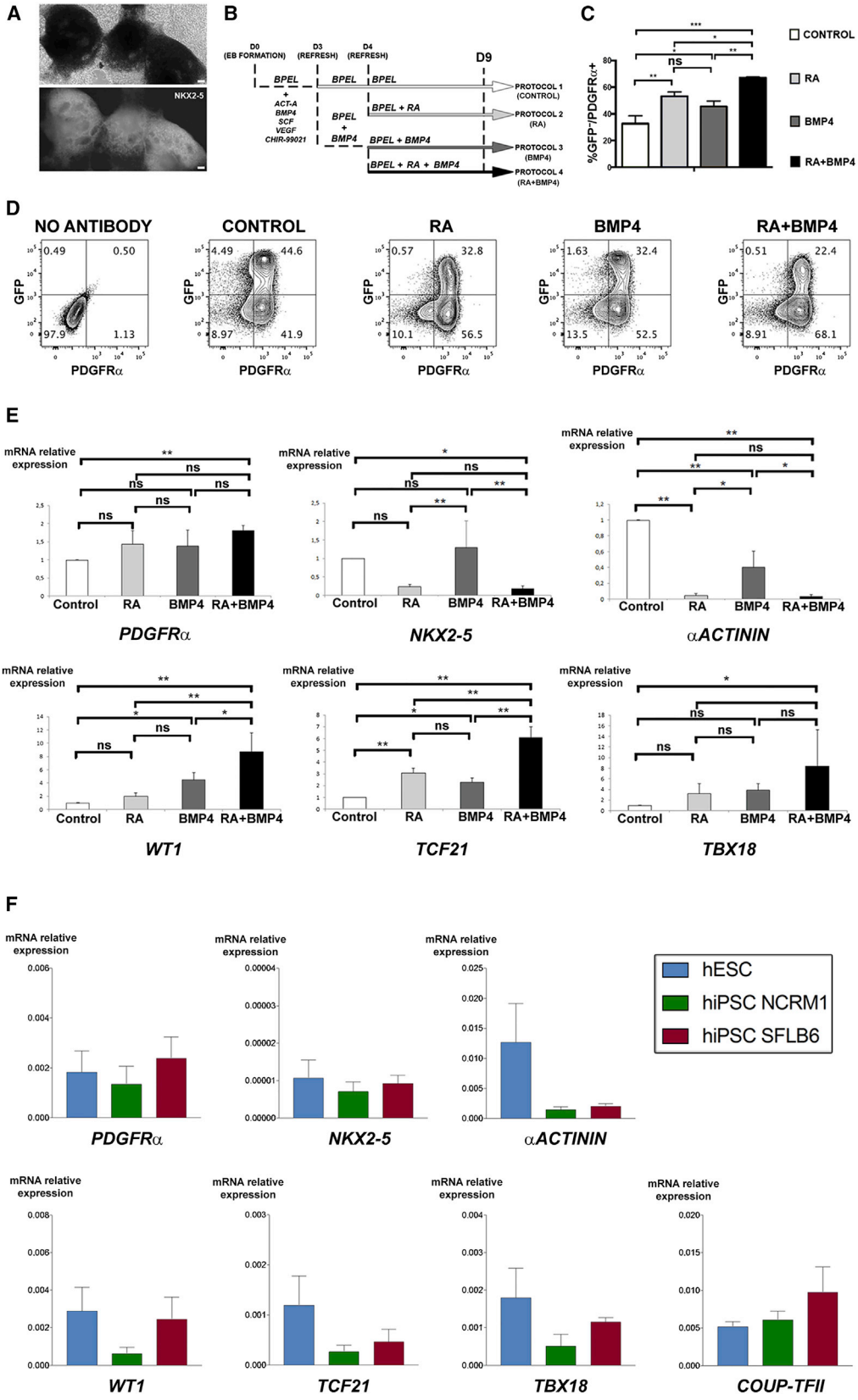
INTRODUCTION

Mature muscle cells in the mammalian heart proliferate very slowly limiting its regenerative capacity after injury. Accordingly, cardiomyocytes dying after infarction are not replaced by new ones but instead characteristic fibrotic scar tissue forms, which interferes with potential cardiomyocyte regeneration, impairs heart function, and may later result in heart failure (Jessup and Brozena, 2003). Therefore, to regenerate the injured heart, exogenous or endogenous cardiomyocytes are needed to replace those lost.

Multiple cell-based strategies to regenerate damaged post-mitotic tissues have been described (reviewed by Passier et al., 2008). Human pluripotent stem cells (hPSCs) are also considered a potential source of cardiomyocytes for transplantation strategies and are already an excellent *in vitro* model to study the complex transcriptional networks and molecular interactions that regulate cardiomyogenesis (Beqqali et al., 2006; Birket and Mummery, 2015). Recently, epicardial-derived cells have also emerged as a source of various cardiovascular cell types, including endothelial cells, smooth muscle cells, and fibroblasts (Brade et al., 2013). However, *in vivo* cardiomyocyte formation from epicardial cells remains controversial (Christoffels et al., 2009).

During embryogenesis, proepicardial (epicardial progenitor) cells form the epicardium (the monolayer of epithelium that covers the heart surface), part of the coronary vasculature, and a heterogeneous population of non-muscular cardiac interstitial cells (CICs) (Pérez-Pomares and de la Pompa, 2011; Ruiz-Villalba et al., 2015). Among epicardial-derived CICs, a platelet-derived growth factor receptor α-positive (Pdgfrα$^+$) cell subpopulation has been identified in mice, which displays cardiac stem cell properties and is able to expand clonally and differentiate into endothelial and smooth muscle cells, fibroblasts, and cardiomyocytes (Chong et al., 2011). A recent study indicated that CICs include a population of cardiac fibroblast progenitors, which massively expand after ischemic damage (Ruiz-Villalba et al., 2015). Therefore, modulation of epicardial cell differentiation into different cardiac cell types might be highly relevant in developing cell-based strategies for heart repair.

Several studies have identified some of the relevant cues that regulate cardiomyocyte differentiation and diversification. Among these, retinoic acid (RA) (Devalla et al., 2015; Niederreither et al., 2001) and bone morphogenetic protein 4 (BMP4) (Van Wijk et al., 2009) have been shown to be important in specification of cardiac inflow cardiomyocyte differentiation. Other signals, most especially WNTs, have also been involved in the regulation of cardiomyocyte differentiation (Klaus et al., 2012), but their role during early cardiogenesis remains elusive, probably due to cardiomyocyte progenitor sensitivity to WNT dose and the complexity of WNT signaling redundancy (Grigoryan



(legend on next page)

et al., 2008). Nevertheless, two recent reports have successfully linked *in vivo* information on development to an *in vitro* hPSC model and demonstrated epicardial-like cell differentiation from human embryonic stem cells (hESCs) by modulating WNT and BMP signaling (Iyer et al., 2015; Witty et al., 2014). Here, we have extended and complemented these studies by identifying developmentally relevant transitional stages between lateral plate mesoderm and the embryonic epicardium *in vitro*. In particular, we have focused on the proepicardial stage, at which cardiac inflow cardiomyocytes and fibroblasts segregate from a common progenitor in response to integrated BMP4 and fibroblast growth factor 2 signals, resulting in the loss of cardiomyocyte differentiation potential in primitive epicardial cells (Van Wijk et al., 2009).

To study mesodermal specification into the epicardial lineage while monitoring cardiomyogenic potential, we used a transgenic *NKX2-5*$^{EGFP/w}$ hESC line in which *GFP* transcription is under control of the endogenous myocardiogenic transcription factor *NKX2-5* (Elliott et al., 2011), with RA, BMP4, and RA + BMP4 at previously tested concentrations (Devalla et al., 2015; reviewed in Birket and Mummery, 2015). We found that epicardial cell-like differentiation in the presence of RA + BMP4 was at the expense of cardiomyocyte formation, as demonstrated by the failure to express *GFP*, which was also confirmed in human induced pluripotent stem cells (hiPSCs). Since WNT signaling disruption after *β-catenin* ablation in the proepicardium/epicardium does not affect proepicardial or epicardial formation, but rather affects epicardial differentiation into coronary blood vessel cells (Zamora et al., 2007), and WNTs appeared dispensable for epicardial differentiation of hESCs in an earlier study (Iyer et al., 2015), we did not include WNT in our protocols.

Our findings indicated that BMP4 and RA synergistically induce hPSC differentiation into proepicardial/epicardial cells by blocking cardiomyocyte differentiation and promoting proepicardium-specific gene expression. The hPSC-derived epicardial progenitor cells showed similar adhesion and migration properties as embryonic proepicardium, most strikingly when grafted into the prospective pericardial cavity of chick embryos. This demonstrated their functional integrity as a model for further understanding of the epicardium in the human heart.

RESULTS AND DISCUSSION

RA + BMP4 Synergistically Promote *NKX2-5*$^{EGFP/w}$ hESCs Differentiation into Proepicardial-like Cells in the Absence of Exogenous WNTs

Previously, various cytokines and small molecules have been used sequentially to initiate cardiac differentiation of *NKX2-5*$^{EGFP/w}$ hESCs cultured as aggregates/embryoid bodies (EBs) (Elliott et al., 2011). Under these conditions, *NKX2-5*$^{EGFP/w}$ hESCs robustly differentiate into cardiomyocytes after 9–10 days in culture (Figure 1A). To test whether *NKX2-5*$^{EGFP/w}$ hESCs differentiate into proepicardial-like cells, we modified this protocol by adding BMP4 and RA at different time points (Figure 1B).

Morphologically, BMP4- and/or RA-treated EBs (protocols 2–4) were similar to control EBs (protocol 1) (data not shown). However, adding BMP4 and/or RA on days 3 and 4 (protocols 2–4) increased the appearance of cells expressing the mesodermal/epicardial marker PDGFRα (Guadix et al., 2011; Hwang et al., 2013) in GFP$^-$*NKX2-5*$^{EGFP/w}$ hESCs, as assessed by fluorescence-activated cell sorting (FACS) analysis at day 9 (Figure 1C); this effect was greatest after protocol 4 (RA + BMP4) (Figures 1C–1E). Quantification of GFP$^+$ cells in each of the four treatments (day 9) confirmed a significant decrease (but not abrogation) of *NKX2-5*$^{EGFP/w}$ hESC differentiation into GFP$^+$ cardiomyocytes after protocol 4 (RA + BMP4; Figures 1C and 1D). Gene expression analysis of cells emerging from protocols 2 and 3 (Figure 1B) indicated that RA alone induced a small increase in epicardial/proepicardial-associated genes such as *WT1*, *TCF21*, and *TBX18*, while robustly suppressing cardiomyocyte-associated genes such as *NKX2-5* and *α-ACTININ* (Figure 1E). Thus, RA is not only able to activate epicardial/proepicardial genes, but is also sufficient to suppress *NKX2-5* and cardiac *α-ACTININ* expression. In

Figure 1. RA + BMP4 Synergistically Promote Proepicardial Cell Differentiation from *NKX2-5*$^{EGFP/w}$ hESCs

(A) *NKX2-5*$^{EGFP/w}$ hESCs differentiate into GFP$^+$-bearing embryoid bodies (EBs) after protocol 1 (B).

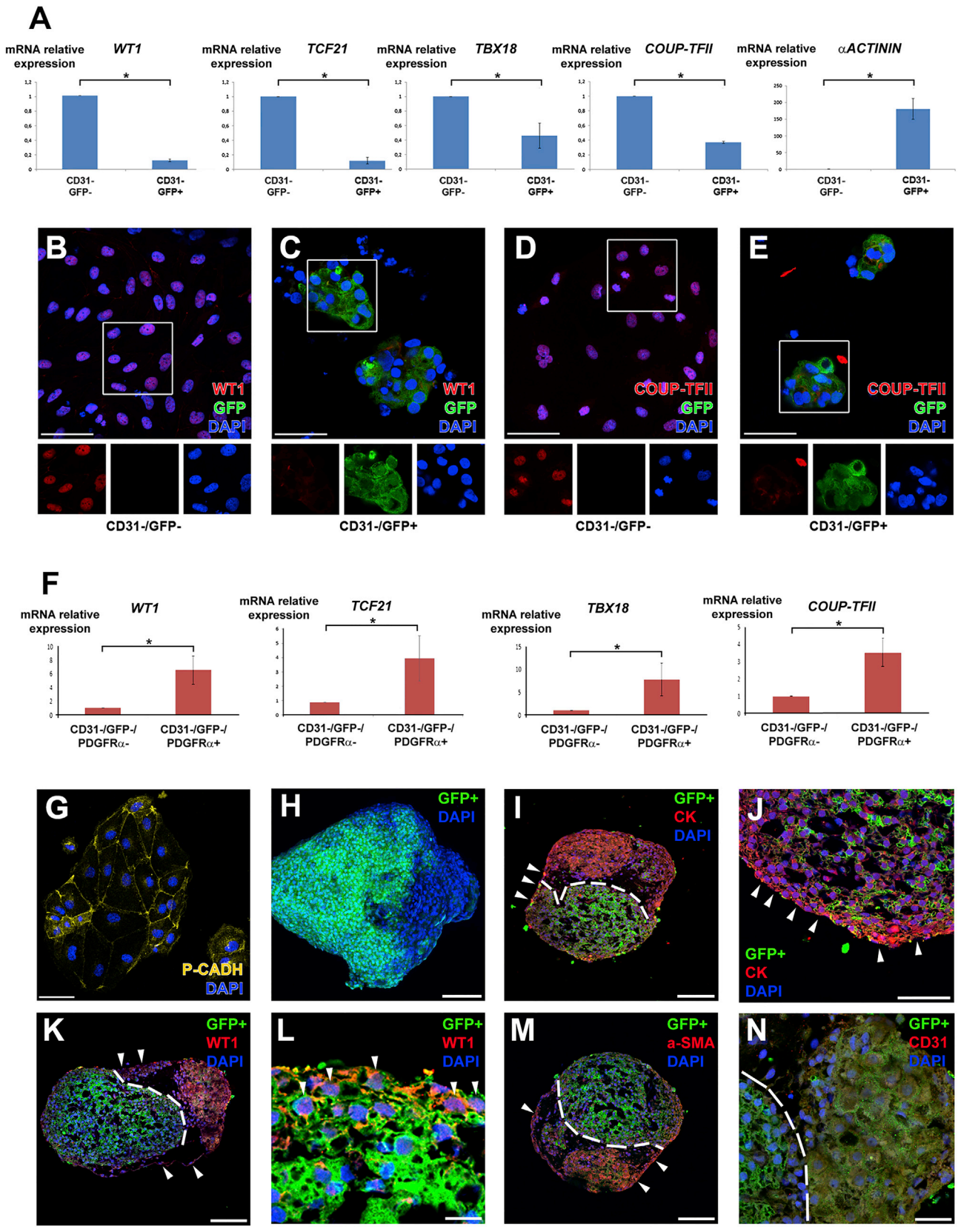
(B) Culture supplements are indicated as related to specific protocols.

(C) Average percentages (n = 7 independent experiments) of GFP$^-$ and PDGFRα$^+$ FACS analyzed cells (culture day 9). Data represent mean ± SD (ns, non-significant; *p < 0.05; **p < 0.01; ***p < 0.001).

(D) Representative FACS plots depicting GFP$^+$ and PDGFRα$^+$ cell percentages after protocols 1–4 (culture day 9).

(E) qRT-PCR validation of cardiac versus proepicardial-like gene expression in hESCs after protocols 1–4 (culture day 9, n = 4 independent experiments). Data are presented as mean ± SD (ns, non-significant; *p < 0.05; **p < 0.01).

(F) qRT-PCR validation of cardiac versus proepicardial-like gene expression in hPSCs after monolayer differentiation (culture day 9, n = 3 independent experiments). Data represent mean ± SEM (all differences between groups are non-significant, p < 0.05). Scale bars, 100 μm in (A).



(legend on next page)

accordance with these results, RA signaling in zebrafish anterior lateral plate mesoderm has also been shown to restrict the size of the cardiac progenitor pool (Keegan et al., 2005). These findings suggested that RA-dependent cardiac differentiation from hESC *in vitro* recapitulated development *in vivo* (Niederreither et al., 2001). Interestingly, BMP4, as compared with RA, increased epicardial/proepicardial gene expression (*WT1*, *TCF21*, and *TBX18*), but did not reduce the expression of cardiomyocyte genes *NKX2-5* and *α-ACTININ* (Figure 1E). The combination of BMP4 and RA further increased the expression of epicardial/proepicardial genes, such as *WT1*, *TCF21*, and *TBX18*, while efficiently reducing the expression of *NKX2-5* and *α-ACTININ* (Figure 1E) in 9 days only (for comparison, the previously reported WNT3 + BMP4 combination promoted epicardial differentiation in 15 days, Witty et al., 2014). These results indicated that BMP4 and RA synergistically activate an "epicardial lineage-like" gene program at the expense of cardiomyocyte differentiation, but without fully abrogating cardiomyocyte (Figure 1D) or endothelial cell differentiation in culture (data not shown). In our protocol, EBs were supplemented with BMP4 from day 3, and with RA + BMP4 from days 4 to 9, i.e., during the temporal window marked by the transient expression of the cardiac mesoderm specification marker *MESP1* (day 4) and the initiation of cardiovascular lineage commitment as marked by the expression of *NKX2-5*, *GATA4*, and *MEF2C* genes (day 9) (Den Hartogh et al., 2015).

RA + BMP4 Synergistically Promote Differentiation of hPSCs into Proepicardial-like Cells in Monolayer Culture

In addition to 3D hPSC differentiation in EBs, we also examined the effect of RA + BMP4 on the induction of proepicardial-like cells from hPSCs in 2D monolayer culture by adapting a protocol we recently described for simultaneous differentiation of cardiomyocytes and endothelial cells from common cardiac mesoderm (Giacomelli et al., 2017) (Figure S1A and S1A′). In agreement with our previous results in EBs, the presence of RA + BMP4 from day 3 was again critical for the induction of GFP$^-$/PDGFRα$^+$ epicardial/proepicardial cells and for the upregulation of *WT1* expression in 2D differentiations (Figures S1B and S1C). Gene expression profiling revealed comparable expression levels of epicardial/proepicardial genes (*WT1*, *TCF21*, *TBX18*, and *COUP-TFII*) at day 9 of differentiation in all the different hPSC lines (the *NKX2-5*$^{EGFP/w}$ hESCs and the wild-type hiPSC lines NCRM1 and SFLB6, see Zhang et al., 2014) used in this study (Figure 1F), indicating the robustness of the protocol. Notably, inhibition of WNT signaling by XAV supplementation at day 3 had little or no effect on the differentiation of epicardial/proepicardial cells in 2D monolayer differentiation conditions (Figures S1A′–S1C). We also tested the effect of inhibition of WNT signaling using cultured embryonic day 9.5 (E9.5) mouse proepicardia *in vitro*. Importantly, WNT antagonist IWP2 also did not affect expression of the proepicardial genes *WT1* and *COUP-TFII* in these experiments (Figure S2).

CD31, GFP, and PDGFRα Expression Profiles Define Three Cell Phenotypes after *NKX2-5*$^{EGFP/w}$ hESC Treatment with RA + BMP4

Since cardiomyocyte differentiation from *NKX2-5*$^{EGFP/w}$ hESCs can be monitored through GFP expression, we refined the characterization of cell phenotypes after culture protocol 4 in EBs (Figure 1B) using GFP as a landmark for cardiac versus non-cardiac cell differentiation. Cells were sorted by FACS based on their GFP and CD31 expression, and only the CD31$^-$ fractions (non-endothelial cells) were selected for further characterization. As expected, cardiogenic (*NKX2-5/TBX5*) gene expression in CD31$^-$/GFP$^-$ cells was almost undetectable when compared with CD31$^-$/GFP$^+$ cells (Figure S3A). However, the expression of *WT1*, *TCF21*, *TBX18*, and *COUP-TFII* genes was significantly increased in the CD31$^-$/GFP$^-$ cell fraction compared with the CD31$^-$/GFP$^+$ population (Figure 2A), as was the expression of other epicardial/proepicardial genes, such as *RALDH2*, *E-CADHERIN*, and *α4 INTEGRIN*

Figure 2. Proepicardial Gene Expression in RA + BMP4-Treated *NKX2-5*$^{EGFP/w}$ hESC-Derived Cells

(A) qRT-PCR of selected gene transcripts (culture day 9) confirms increase in cardiomyocyte and decrease in proepicardial markers in CD31$^-$/GFP$^+$ with respect to CD31$^-$/GFP$^-$ cells (n = 4 independent experiments). Data are presented as mean ± SD (*p < 0.05 by unpaired t test).

(B–E) *WT1* and *COUP-TFII* mRNA expression in RA + BMP4-treated *NKX2-5*$^{EGFP/w}$ hESCs (CD31$^-$/GFP$^-$) is paralleled by WT1 (B and C) (red) and COUP-TFII (D and E) (red) protein expression *in vitro*.

(F) qRT-PCR analysis of proepicardial markers (*WT1*, *TCF21*, *TBX18*, and *COUP-TFII*) in CD31$^-$/GFP$^-$/PDGFRα$^+$ versus CD31$^-$/GFP$^-$/PDGFRα$^-$ cells (n = 5 independent experiments). Data are presented as mean ± SD (*p < 0.05 by unpaired t test).

(G–N) CD31$^-$/GFP$^-$/PDGFRα$^+$ are epithelial, PAN-CADHERIN$^+$ cells. CD31$^-$/GFP$^-$/PDGFRα$^+$ and CD31$^-$/GFP$^+$/PDGFRα$^\pm$ cell co-culture (n = 12) results in the formation of cells aggregates showing a segregation between GFP$^+$ and GFP$^-$ cells (white dashed lines separate these two cell types); CD31$^-$/GFP$^-$/PDGFRα$^+$ cells grow over GFP$^+$ cells (H), forming a CK$^+$ epithelium (I and J) (arrowheads) and expressing epicardial markers such as WT1 (K and L) (arrowheads) and α-SMA (M) (arrowheads). All the cells remain CD31$^-$ (N). Scale bars, 50 μm in (B)–(E), (G), and (J); 100 μm in (H), (I), (K), and (M); 10 μm in (L); and 25 μm in (N).

(Figure S3A). Expression of *COUP-TFII*, an orphan nuclear receptor critical for mesodermal differentiation into multiple cell types (Xie et al., 2011), was of special interest since it is strongly expressed by proepicardial cells but only at relatively low levels by epicardial cells *in vivo* (Lin et al., 2012). This suggests that *COUP-TFII* expression identifies a more primitive set of cells in the septum transversum-proepicardium-epicardium continuum (Cano et al., 2016), and we thus refer to these cells as proepicardial/epicardial-like cells.

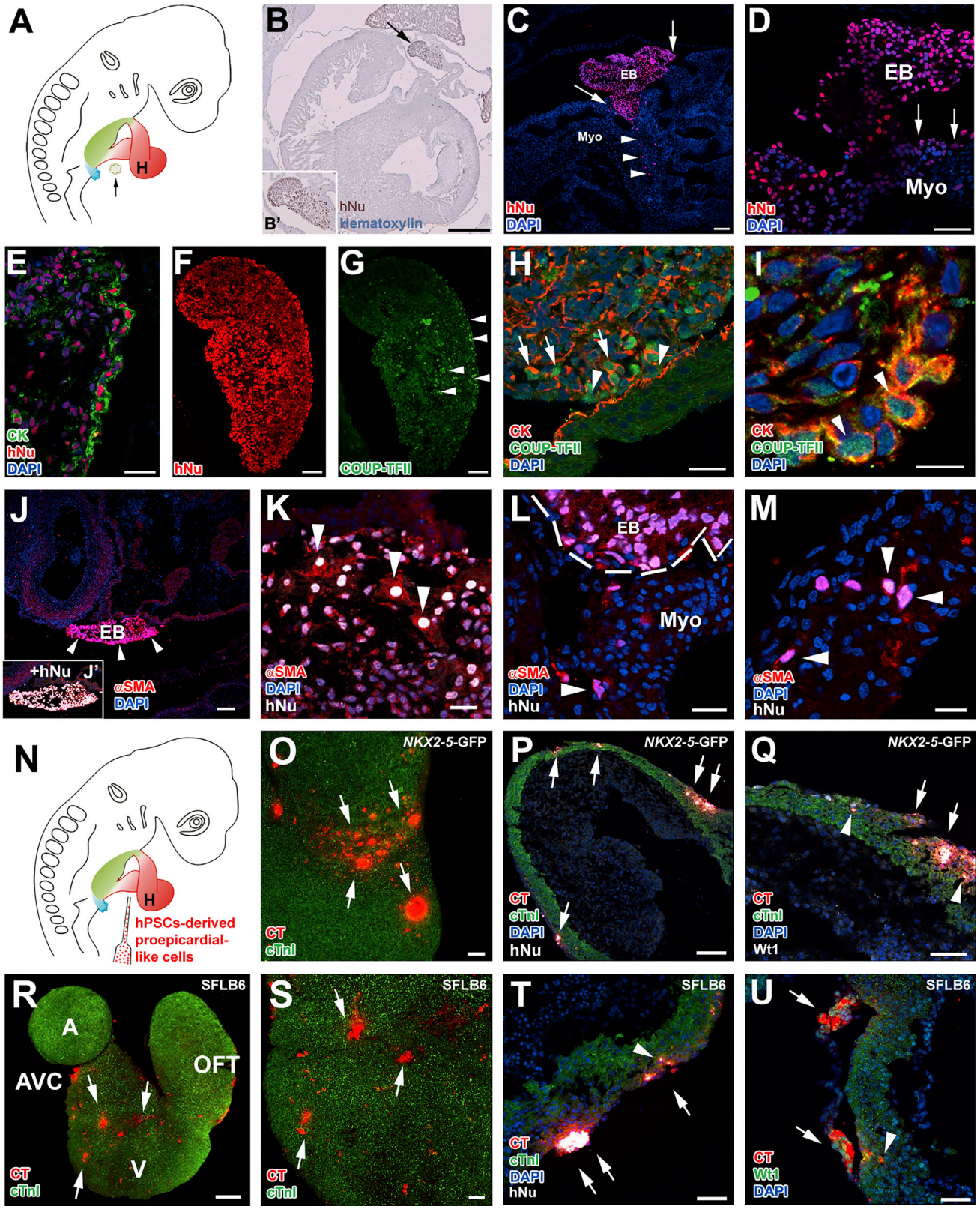
Immunocytochemistry confirmed WT1 and COUP-TFII nuclear protein accumulation in $CD31^-/GFP^-$ cells, but not in $CD31^-/GFP^+$ (Figures 2B–2E), suggesting that the identity of hESC-derived $CD31^-/GFP^-$ cells was more similar to epicardial progenitors (proepicardial cells) than to epicardial cells. To precisely define the nature of $CD31^-/GFP^-$ cells, we sorted the cells on the basis of the PDGFRα mesodermal/epicardial marker (Guadix et al., 2011; Hwang et al., 2013). Our results confirmed that the expression of the proepicardial-related genes *WT1*, *TCF21*, *TBX18*, and *COUP-TFII* (Figure 2F) was higher in $CD31^-/GFP^-/PDGFR\alpha^+$ than in $CD31^-/GFP^-/PDGFR\alpha^-$ cells. 2D monolayer differentiation resulted in robust induction of proepicardial-like cells from hPSCs without prior need for enrichment of the $PDGFR\alpha^+$ population (Figure S3B). These cells also expressed typical proepicardial-associated genes *WT1*, *TCF21*, *TBX18*, and *COUP-TFII* (Figure S3C).

Immunocytochemical analysis of PAN-CADHERIN expression confirmed that these cells were epithelial in phenotype (Figure 2G), as expected for epicardial cells, which are a subtype of visceral mesothelium. Furthermore, proepicardial-like cells expanded from the monolayer differentiation protocol exhibited typical cobblestone-like morphology with nuclear localization of WT1 and COUP-TFII (Figures S4A and S4B). We have shown that RA + BMP4-treated *NKX2-5*$^{EGFP/w}$ hESCs mostly differentiate into $PDGFR\alpha^+/GFP^-$ non-cardiomyocyte cells, which express proepicardial genes such as *WT1*, *TCF21*, *TBX18*, and *COUP-TFII*. However, some RA + BMP4-treated *NKX2-5*$^{EGFP/w}$ hESCs still differentiated into $PDGFR\alpha^+/GFP^+$ cardiomyocytes. Further research is required to ascertain whether those cardiomyocytes could secondarily derive from $PDGFR\alpha^+/GFP^-/TBX18^+/WT1^+/COUP\text{-}TFII^+$ proepicardial-like cells.

$CD31^-/GFP^-/PDGFR\alpha^+$ Cells Display Embryonic Proepicardial-like Behavior *In Vitro* and *In Ovo*

To test the functional properties of (pro)epicardial-like $CD31^-/GFP^-/PDGFR\alpha^+$ cells *in vitro*, *NKX2-5*$^{EGFP/w}$ hESCs were cultured following protocol 4 (see Figure 1B). $CD31^-/GFP^-/PDGFR\alpha^+$ (proepicardial), and $CD31^-/GFP^+/PDGFR\alpha^\pm$ (myocardial) cell populations were sorted at day 9 of differentiation (D9, see Figure 1B), and then mixed (1:1) to form aggregates *in vitro*. At day 13 of differentiation, two different cell phases were evident in the aggregates, only one of them expressing GFP (Figure 2H). Immunohistochemical analysis of the aggregates (Figures 2I and 2J) identified cytokeratin (CK) (an epithelial intermediate filament) in the $CD31^-/GFP^-/PDGFR\alpha^+$ cell population, which also expressed WT1 (Figures 2K and 2L), and smooth muscle-specific α-actin (α-SMA) (Figure 2M), but remained $CD31^-$ (Figure 2N). The characteristic distribution of some $CD31^-/GFP^-/PDGFR\alpha^+/CK^+$ cells over the surface of the structure suggested that this cell population had grown and spread over the $CD31^-/GFP^+/PDGFR\alpha^\pm$ cells (Figure 2J), recapitulating normal proepicardial cell growth over the embryonic myocardium to form the primitive epicardium. Previous studies indicated that epicardial α4β1 integrin (or VLA-4) plays a pivotal role in migration and adhesion to the heart (Yang et al., 1995). Therefore, we utilized a small-molecule α4β1 antagonist (BIO5192) to confirm the importance of this interaction for the formation of *in vitro* aggregates. BIO5192-treated aggregates displayed a marked disruption of proepicardial-like $CD31^-/GFP^-/PDGFR\alpha^+$ cell aggregation with the cells dispersing in the culture, while myocardial $CD31^-/GFP^+/PDGFR\alpha^\pm$ cells remained as a coherent cell mass in control and BIO5192-treated aggregates (Figure S4C).

Functional properties of $CD31^-/GFP^-/PDGFR\alpha^+$ cells were also tested *in ovo*. RA + BMP4-treated *NKX2-5*$^{EGFP/w}$ hESC EBs (6 days of culture, protocol 4) were grafted into chick embryo hosts (HH17) (Figure 3A). After 7 days, these EBs were identified by their expression of a human pan-nuclear (hNu) marker (Figures 3B–3D). Some of the donor EBs were attached to, and partially spread over, the myocardial surface (Figures 3C and 3D). Groups of grafted cells migrated into the myocardial layers (Figure 3D). The outer surface of the EBs was covered by CK-expressing epithelial cells (Figure 3E). All cells in the EBs expressed the hNu antigen (Figure 3F), while only some expressed the proepicardial markers COUP-TFII (Figure 3G) and WT1 (Figure S4D). No GFP^+ cells were detected in the host myocardium at any time (Figure S4D). $COUP\text{-}TFII^+$ cells were located in the inner layers of the EBs (Figure 3H) or in their epithelial surface (Figure 3I); in both cases the cells expressed the epithelial marker CK (Figures 3H and 3I). Groups of hNu^+ cells on the surface and inside the EBs expressed the α-SMA marker (Figures 3J–3M); some of these $\alpha\text{-}SMA^+$ cells were able to invade the myocardium (Figures 3L and 3M). In addition, we examined the *in ovo* functional properties of proepicardial-like cells derived from the optimized 2D monolayer differentiation protocol (RA + BMP4) (Figure S1A). For this, expanded hiPSC-derived proepicardial-like cells were counterstained with the CMTPX CellTracker Red and subsequently injected as cell suspensions into the prospective



(legend on next page)

pericardial cavity of Hamburger-Hamilton stage 16–17 chick embryos (Figure 3N). Both the hESC-derived (Figures 3O–3Q) and hiPSC-derived proepicardial-like cells (Figures 3R–3U) attached and spread over the myocardial surface. Some of these cells expressed the WT1 protein (Figures 3Q and 3U) and were found to invade the myocardial layers (Figures 3Q, 3T, and 3U). These results differ from those published previously (Iyer et al., 2015), which reported the presence of intravascularly injected hESC-derived cells in the subepicardial space and the walls of coronary arteries, but not in the epicardial epithelium itself. Our finding that hPSC-derived CD31$^-$/GFP$^-$/PDGFRα^+ cells can form an epicardial-like epithelial envelope over the chick host myocardium confirms that these cells possess critical morphogenetic properties of epicardial progenitor cells, although the extent of hPSC-derived proepicardial-like cell migration over the chick myocardium is likely to be limited by intrinsic differences between the host and the donor.

In this work we have shown that RA + BMP4-treated hPSCs can differentiate into mesodermal cells displaying a gene expression profile and functional properties of proepicardial cells, a subpopulation of the lateral plate mesoderm that accumulates at the venous (posterior) pole of the embryonic heart and is essential during heart embryogenesis (Pérez-Pomares and de la Pompa, 2011). Proepicardial cells have been hypothesized to be multipotent (Wessels and Pérez-Pomares, 2004), as they can differentiate into epicardial epithelial cells, endothelium, smooth muscle cells, fibroblasts, and cardiomyocytes (Brade et al., 2013) under different *in vivo* and/or *in vitro* conditions. Interestingly, the combined, persistent expression of *PDGFRα* and *COUP-TFII* in a proportion of hPSC-derived CD31$^-$/GFP$^-$ cells suggests they still retain multipotency associated with subpopulations of the primitive lateral mesoderm including the proepicardium (Chong et al., 2011; Lin et al., 2012), as summarized in Figure 4.

We conclude that our cell culture protocol is a rapid and robust method for generating proepicardial (epicardial progenitor)-like cells in both aggregates and monolayer cultures with intrinsic cardiomyogenic potential that can be further manipulated. This cell platform could be relevant for the study of cell and molecular pathophysiological mechanisms underlying different cardiovascular diseases, and could also facilitate stem cell-based therapies to regenerate and repair injured cardiac tissues.

EXPERIMENTAL PROCEDURES

hESC Culture and Differentiation to Cardiomyocytes and Epicardial-like Cells

Undifferentiated *NKX2-5*$^{EGFP/w}$ hESCs were cultured as described in the Supplemental Information. BMP4 and RA concentrations used have been previously tested in other studies on hESC differentiation into mesodermal cells and cardiogenic progenitors (Birket et al., 2015; Devalla et al., 2015).

Monolayer Differentiation of hPSCs to Epicardial-like Cells

Cardiac mesoderm was induced in monolayer culture as described previously (Giacomelli et al., 2017). The following hPSC lines were used: *NKX2-5*$^{EGFP/w}$ hESCs and two control hiPSC lines SFLB6 (Zhang et al., 2014) and NCRM1 (NIH Center for Regenerative Medicine [NIH CRM]), obtained from RUDCR Infinite Biologics at Rutgers University. For hPSC-derived cardiomyocyte differentiation BPEL medium (BSA polyvinyl alcohol essential lipids [Ng et al., 2008]) supplemented with a mixture of cytokines was used (see Supplemental Information).

Flow Cytometry (FACS) and Cell Sorting

On day 9 of differentiation, EBs or monolayers were dissociated using TrypLE Select 1× (Gibco), washed once with FACS buffer containing 10% fetal bovine serum, and washed again in FACS buffer (Ca^{2+}- and Mg^{2+}-free PBS plus 0.5% BSA, 2 mM EDTA). Cells were analyzed with the MACSQuant VYB cytometer

Figure 3. Functional Properties of CD31$^-$/GFP$^-$/PDGFRα^+ Cells

NKX2-5$^{EGFP/w}$ hESC-derived EBs (arrow) treated under protocol 4 were grafted into chick embryos as shown in (A) (n = 10 independent experiments). Some EBs attach to the myocardium of HH17 chick host hearts (B) (arrow), the graft is magnified in (B′); donor cells spread over the myocardium (C and D) (arrows) or migrate into the myocardium (C) (arrowheads). Cells in the EB surface express the epithelial marker CK (E) (green). COUP-TFII expression in EB cells is conspicuous (F and G) (arrowheads in G); some COUP-TFII$^+$ cells express cytokeratin both inside (H) (arrows) and on the surface of EBs (H and I) (arrowheads). Many cells at the EB surface express α-SMA, arrowheads in (J) and (K); the area marked by the asterisk in (J) is magnified in (K); some α-SMA$^+$ cells invade the myocardium; arrowheads in (L) and (M) and the dashed line in (L) mark the border between the EB and the host myocardium. hPSC-derived proepicardial/epicardial-like cells were stained with CellTracker Red and injected into the pericardial cavity of chick embryo hearts as shown (N) (NKX2-5$^{EGFP/w}$, n = 10 independent experiments; SFLB6, n = 10 independent experiments). Some proepicardial/epicardial-like cells (red staining) attached to the myocardium (cTnT, green tissue) of HH17 chick host (arrows in O, R, and S); some donor cells spread over the myocardium (P, Q, T, and U) (arrows) and some other cells migrated into the myocardium (Q, T, and U) (arrowheads). CellTracker Red$^+$ cells expressed a human nuclear-specific marker (hNuclei) (P and T) (white). WT1 expression is also conspicuous in human donor cells (Q) (white) and (U) (green). EB, embryoid body; H, heart; Myo, myocardium. Scale bars, 500 μm in (B); 25 μm in (C), (D), (E), (H), (K), and (L); 50 μm in (F), (G), (O), (Q), (S), (T), and (U); 10 μm in (I); 100 μm in (J) and (P); 15 μm in (M); and 200 μm in (R).

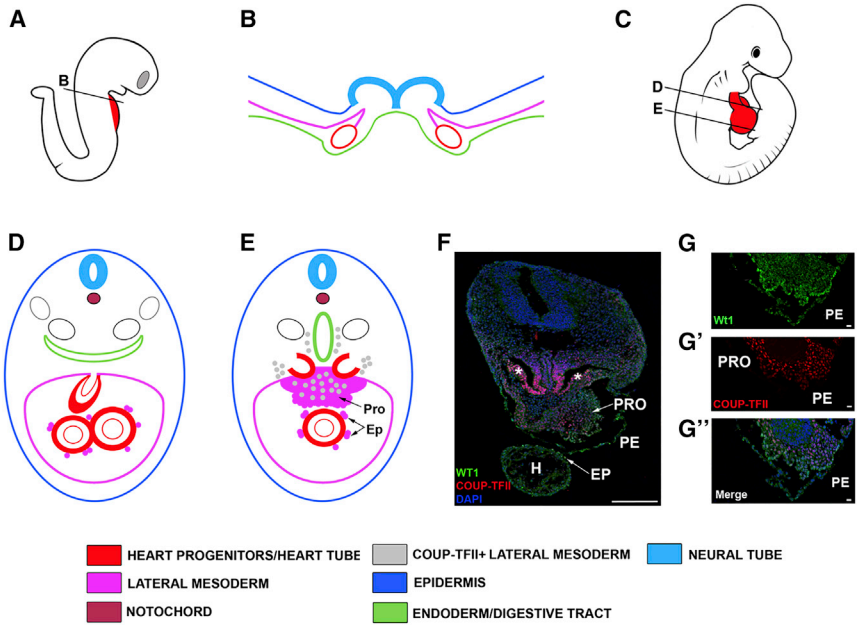


Figure 4. The Proepicardium and the Lateral Plate Mesoderm

Blastodermal derivatives in E8.5 (A–C) and E9.5–E10.0 (C–G) mouse embryos. WT1 (F) (green) is expressed in the majority of splanchnic lateral mesodermal cells, including the proepicardium (PRO), the primitive epicardium (EP), and the pericardium (PE). On the contrary, COUP-TFII expression (F) (red) is strong in cardiac inflow myocardial progenitors (asterisks), proepicardial cells (PRO), but not epicardial (EP) or pericardial (PE) cells. Scale bars, 200 μm in (F) and 10 μm in (G)–(G″).

(Miltenyi) and sorted on FACSAria III flow cell sorter (BD Biosciences) using antibodies as listed in the Supplemental Information.

Gene Expression Analysis

Extraction of RNA from EB samples (protocols 1–4, day 9 of culture) was performed using the RNeasy-Micro Kit (QIAGEN), whereas the NucleoSpin RNA XS Kit (MACHEREY-NAGEL) was used in sorted cell populations and monolayer cultures. RNA was submitted to reverse transcription to obtain cDNA (1 μg) using the Iscript cDNA Kit (Bio-Rad). For details on the full procedure and primers used see the Supplemental Information and Table S1.

Immunocytochemistry

Cell pools were sorted according to their cell surface expression profile (CD31; GFP; PDGFRα) and grown in regular BPEL medium. After 6 days of culture, cells were fixed in 4% paraformaldehyde and processed for immunohistochemistry as described in the Supplemental Information.

EB *In Ovo* Transplantations

NKX2-5$^{EGFP/w}$ hESC-derived EBs were generated using protocol 4 (RA + BMP4; Figure 1A), cultured for 7 days, and grafted into the prospective pericardial cavity of HH16-17 chick embryos. Twelve transplantations were performed; only ten grafts (those attached to the myocardium) were analyzed.

hPSC *In Ovo* Transplantation

NKX2-5$^{EGFP/w}$ hESCs and SFLB6 hiPSCs were differentiated and cultured using a monolayer differentiation protocol (Figure S1A). Differentiated cells were sorted and PDGFRα^{+} cells were expanded, stained with CellTracker Red, and then injected into the prospective pericardial cavity of HH16-17 chick embryos. Donor hearts were directly examined under a fluorescence dissecting scope and further characterized by immunohistochemistry. The full protocol is available in the Supplemental Information.

Culture of Cell Aggregates

NKX2-5$^{EGFP/w}$ hESCs from protocol 4 (RA + BMP4; Figure 1A) were used to form cell spheroids. Cells were selected and cultured as described in the Supplemental Information.

Statistical Analysis

Statistical significance was assessed by Student's t test (comparison of two groups) or one-way ANOVA with Tukey's multiple comparison test (comparison of four groups). Significance by Student's t test was attributed to comparisons with values of $p < 0.05$. All error bars represent SD.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, and two tables and can be found with this article online at https://doi.org/10.1016/j.stemcr.2017.10.023.

AUTHOR CONTRIBUTIONS

J.M.P.-P. and R.P. contributed equally to this work. J.A.G., conception and design, provision of study material, collection of data, data analysis and interpretation, manuscript writing, and final approval of manuscript. V.V.O., conception and design, collection of data, data analysis and interpretation, and manuscript writing. E.G., collection of data and data analysis and interpretation. M.B., collection of data and data analysis and interpretation. M.C.R., provision of study material. C.L.M., financial support, manuscript writing, and final approval of manuscript. J.M.P.-P.

and R.P., conception and design, financial support, data analysis and interpretation, manuscript writing, and final approval of manuscript.

ACKNOWLEDGMENTS

We thank D. Ward-van Oostwaard, J.J. Monshouwer-Kloots, and F.E. van den Hil (Department of Anatomy and Embryology, LUMC) for assistance with hESC and hiPSC culture and gene expression analysis and J. Wiegant and A. Boonzaier-van der Laan (Department of Molecular Cell Biology, LUMC) and John Pearson (BIONAND) for help with laser confocal microscopy analysis. This research was supported by ZonMw-MKMD-40-42600-98-036 (to R.P.), MINECO grants BFU2015-65783-R and ISCIII-RD16/0011/0030-TERCEL (to J.M.P.-P.), EU FP7-Marie Curie-Co-Fund actions (CARDIODIFFCULT, U-MOBILITY), the UMA Postdoctoral Grant (I PLAN PROPIO) (to J.A.G.), European Community's Seventh Framework Programme (FP7/2007–2013) grant agreement 602423 PLURIMES (to V.V.O. and C.L.M.), LUMC Gisela Thier Fellowship (to V.V.O.), European Research Council (ERCAdG 323182 STEMCARDIOVASC) (to C.L.M.), European Union's Horizon 2020 research and innovation programme (TECHNOBEAT) under grant agreement no. 668724 (to C.L.M. and V.V.O.), and The Netherlands Organization for Scientific Research within the program on Mechanosensing and Mechanotransduction by Cells (NWO-FOM 09MMC02) (to M.C.R.). C.L.M. and R.P. are co-founders of PLURIOMICS BV.

Received: January 9, 2017
Revised: October 24, 2017
Accepted: October 27, 2017
Published: November 22, 2017

REFERENCES

Beqqali, A., Kloots, J., Ward-van Oostwaard, D., Mummery, C., and Passier, R. (2006). Genome-wide transcriptional profiling of human embryonic stem cells differentiating to cardiomyocytes. Stem Cells *24*, 1956–1967.

Birket, M.J., and Mummery, C.L. (2015). Pluripotent stem cell derived cardiovascular progenitors – a developmental perspective. Dev. Biol. *400*, 169–179.

Birket, M.J., Ribeiro, M.C., Verkerk, A.O., Ward, D., Leitoguinho, A.R., den Hartogh, S.C., Orlova, V.V., Devalla, H.D., Schwach, V., Bellin, M., et al. (2015). Expansion and patterning of cardiovascular progenitors derived from human pluripotent stem cells. Nat. Biotechnol. *33*, 1–12.

Brade, T., Pane, L.S., Moretti, A., Chien, K.R., and Laugwitz, K.-L. (2013). Embryonic heart progenitors and cardiogenesis. Cold Spring Harb. Perspect. Med. *3*, a013847.

Cano, E., Carmona, R., Ruiz-Villalba, A., Rojas, A., Chau, Y.-Y., Wagner, K., Wagner, N., Hastie, N., Muñoz-Chápuli, R., and Pérez-Pomares, J. (2016). Extracardiac septum transversum/proepicardial endothelial cells pattern embryonic coronary arteriovenous connections. Proc. Natl. Acad. Sci. USA *113*, 656–661.

Chong, J.J.H., Chandrakanthan, V., Xaymardan, M., Asli, N.S., Li, J., Ahmed, I., Heffernan, C., Menon, M.K., Scarlett, C.J., Rashidianfar, A., et al. (2011). Adult cardiac-resident MSC-like stem cells with a proepicardial origin. Cell Stem Cell *9*, 527–540.

Christoffels, V.M., Grieskamp, T., Norden, J., Mommersteeg, M.T.M., Rudat, C., and Kispert, A. (2009). Tbx18 and the fate of epicardial progenitors. Nature *458*, E8–E9, discussion E9–10.

Den Hartogh, S., Schreurs, C., Monshouwer-Kloots, J., Davis, R., Ellliott, D., Mummery, C., and Passier, R. (2015). Dual reporter MESP1mCherry/w-NKX2-5eGFP/w hESCs enable studying early human cardiac differentiation. Stem Cells *33*, 56–67.

Devalla, H.D., Schwach, V., Ford, J.W., Milnes, J.T., El-Haou, S., Jackson, C., Gkatzis, K., Elliott, D.A., Chuva de Sousa Lopes, S.M., Mummery, C.L., et al. (2015). Atrial-like cardiomyocytes from human pluripotent stem cells are a robust preclinical model for assessing atrial-selective pharmacology. EMBO Mol. Med. *7*, 394–410.

Elliott, D.A., Braam, S.R., Koutsis, K., Ng, E.S., Jenny, R., Lagerqvist, E.L., Biben, C., Hatzistavrou, T., Hirst, C.E., Yu, Q.C., et al. (2011). NKX2-5(eGFP/w) hESCs for isolation of human cardiac progenitors and cardiomyocytes. Nat. Methods *8*, 1037–1040.

Giacomelli, E., Bellin, M., Sala, L., van Meer, B.J., Tertoolen, L.G., Orlova, V.V., and Mummery, C.L. (2017). Three-dimensional cardiac microtissues composed of cardiomyocytes and endothelial cells co-differentiated from human pluripotent stem cells. Development *144*, 1008–1017.

Grigoryan, T., Wend, P., Klaus, A., and Birchmeier, W. (2008). Deciphering the function of canonical Wnt signals in development and disease: conditional loss- and gain-of-function mutations of beta-catenin in mice. Genes Dev. *22*, 2308–2341.

Guadix, J.A., Ruiz-Villalba, A., Lettice, L., Velecela, V., Muñoz-Chápuli, R., Hastie, N.D., Pérez-Pomares, J.M., and Martínez-Estrada, O.M. (2011). Wt1 controls retinoic acid signalling in embryonic epicardium through transcriptional activation of Raldh2. Development *138*, 1093–1097.

Hwang, Y., Suk, S., Lin, S., Tierney, M., Du, B., Seo, T., Mitchell, A., Sacco, A., and Varghese, S. (2013). Directed in vitro myogenesis of human embryonic stem cells and their in vivo engraftment. PLoS One *8*, e72023.

Iyer, D., Gambardella, L., Bernard, W.G., Serrano, F., Mascetti, V.L., Pedersen, R.A., Talasila, A., and Sinha, S. (2015). Robust derivation of epicardium and its differentiated smooth muscle cell progeny from human pluripotent stem cells. Development *142*, 1528–1541.

Jessup, M., and Brozena, S. (2003). Heart failure. N. Engl. J. Med. *348*, 2007–2018.

Keegan, B.R., Feldman, J.L., Begemann, G., Ingham, P.W., and Yelon, D. (2005). Retinoic acid signaling restricts the cardiac progenitor pool. Science *307*, 247–249.

Klaus, A., Müller, M., Schulz, H., Saga, Y., Martin, J.F., and Birchmeier, W. (2012). Wnt/β-catenin and Bmp signals control distinct sets of transcription factors in cardiac progenitor cells. Proc. Natl. Acad. Sci. USA *109*, 10921–10926.

Lin, F.-J., You, L.-R., Yu, C.-T., Hsu, W.-H., Tsai, M.-J., and Tsai, S.Y. (2012). Endocardial cushion morphogenesis and coronary vessel development require chicken ovalbumin upstream promoter-transcription factor II. Arterioscler. Thromb. Vasc. Biol. *32*, e135–e146.

Ng, E.S., Davis, R., Stanley, E.G., and Elefanty, A.G. (2008). A protocol describing the use of a recombinant protein-based, animal product-free medium (APEL) for human embryonic stem cell differentiation as spin embryoid bodies. Nat. Protoc. *3*, 768–776.

Niederreither, K., Vermot, J., Messaddeq, N., Schuhbaur, B., Chambon, P., and Dollé, P. (2001). Embryonic retinoic acid synthesis is essential for heart morphogenesis in the mouse. Development *1031*, 1019–1031.

Passier, R., van Laake, L.W., and Mummery, C.L. (2008). Stem-cell-based therapy and lessons from the heart. Nature *453*, 322–329.

Pérez-Pomares, J.M., and de la Pompa, J.L. (2011). Signaling during epicardium and coronary vessel development. Circ. Res. *109*, 1429–1442.

Ruiz-Villalba, A., Simón, A.M., Pogontke, C., Castillo, M.I., Abizanda, G., Pelacho, B., Sánchez-Domínguez, R., Segovia, J.C., Prósper, F., and Pérez-Pomares, J.M. (2015). Interacting resident epicardium-derived fibroblasts and recruited bone marrow cells form myocardial infarction scar. J. Am. Coll. Cardiol. *65*, 2057–2066.

Van Wijk, B., Van Den Berg, G., Abu-Issa, R., Barnett, P., Van Der Velden, S., Schmidt, M., Ruijter, J.M., Kirby, M.L., Moorman, A.F.M., and Van Den Hoff, M.J.B. (2009). Epicardium and myocardium separate from a common precursor pool by crosstalk between bone morphogenetic protein- and fibroblast growth factor-signaling pathways. Circ. Res. *105*, 431–441.

Wessels, A., and Pérez-Pomares, J.M. (2004). The epicardium and epicardially derived cells (EPDCs) as cardiac stem cells. Anat. Rec. A Discov. Mol. Cell. Evol. Biol. *276*, 43–57.

Witty, A.D., Mihic, A., Tam, R.Y., Fisher, S.A., Mikryukov, A., Shoichet, M.S., Li, R.-K., Kattman, S.J., and Keller, G. (2014). Generation of the epicardial lineage from human pluripotent stem cells. Nat. Biotechnol. *32*, 1026–1035.

Xie, X., Qin, J., Lin, S.-H., Tsai, S.Y., and Tsai, M.-J. (2011). Nuclear receptor chicken ovalbumin upstream promoter-transcription factor II (COUP-TFII) modulates mesenchymal cell commitment and differentiation. Proc. Natl. Acad. Sci. USA *108*, 14843–14848.

Yang, J.T., Rayburn, H., and Hynes, R.O. (1995). Cell adhesion events mediated by alpha 4 integrins are essential in placental and cardiac development. Development *121*, 549–560.

Zamora, M., Männer, J., and Ruiz-Lozano, P. (2007). Epicardium-derived progenitor cells require beta-catenin for coronary artery formation. Proc. Natl. Acad. Sci. USA *104*, 18109–18114.

Zhang, M., Aniello, C.D., Verkerk, A.O., Wrobel, E., Frank, S., Oostwaard, D.W., Piccini, I., Freund, C., Rao, J., Seebohm, G., et al. (2014). Recessive cardiac phenotypes in induced pluripotent stem cell models of Jervell and Lange-Nielsen syndrome: disease mechanisms and pharmacological rescue. Proc. Natl. Acad. Sci. USA *111*, 5383–5392.