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The heart endocardium is derived from vascular endothelial progenitors

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SUMMARY

The embryonic heart is composed of two cell layers: the myocardium, which contributes to cardiac muscle tissue, and the endocardium, which covers the inner lumen of the heart. Whereas significant progress has been made toward elucidating the embryonic origins of the myocardium, the origins of the endocardium remain unclear. Here, we have identified an endocardium-forming field medial to the cardiac crescent, in a continuum with the endothelial plexus. In vivo live imaging of quail embryos revealed that endothelial progenitors, like second/anterior heart field progenitors, migrate to, and enter, the heart from the arterial pole. Furthermore, embryonic endothelial cells implanted into the cardiac crescent contribute to the endocardium, but not to the myocardium. In mouse, lineage analysis focusing on endocardial cells revealed an unexpected heterogeneity in the origins of the endocardium. To gain deeper insight into this heterogeneity, we conditionally ablated *Flk1* in distinct cardiovascular progenitor populations; FLK1 is required in vivo for formation of the endocardium in the *Mesp1* and *Tie2* lineages, but not in the *Isl1* lineage. Ablation of *Flk1* coupled with lineage analysis in the *Isl1* lineage revealed that endothelium-derived *Isl1*$^{-}$ endocardial cells were significantly increased, whereas *Isl1*$^{+}$ endocardial cells were reduced, suggesting that the endocardium is capable of undergoing regulative compensatory growth. Collectively, our findings demonstrate that the second heart field contains distinct myocardial and endocardial progenitor populations. We suggest that the endocardium derives, at least in part, from vascular endothelial cells.

KEY WORDS: Endocardium, Endothelial cells, Heart organogenesis, FLK1 (VEGFR2, KDR), Chick, Quail, Mouse

INTRODUCTION

Heart development as a model for the study of organogenesis has attracted substantial attention owing to the complexity of the molecular and cellular developmental programs that can be linked to numerous congenital heart defects (Buckingham et al., 2005; Kirby, 2002; Olson and Schneider, 2003; Srivastava and Olson, 2000). During vertebrate gastrulation, cardiac progenitors located in the anterior portion of the primitive streak migrate anterior-laterally to form bilateral heart fields, which are also known as the cardiac crescent (Garcia-Martinez and Schoenwolf, 1993; Wei and Mikawa, 2000). As the embryo folds ventrally, the two heart fields coalesce into a linear heart tube composed of myocardial and endocardial cells.

The generation of multiple cell types in the heart can be achieved through the recruitment of a set of non-overlapping embryonic precursors of distinct origins, or via the progressive diversification of multipotent cardiovascular progenitors; conceivably, these developmental strategies for establishing multiple cell lineages within the heart are not mutually exclusive (Laugwitz et al., 2008). The second heart field (SHF) contains progenitor cells, initially located outside the heart within the pharyngeal mesoderm, that migrate into the heart and give rise to the endocardium, myocardium and smooth muscle lineages (Buckingham et al., 2005). Whether the SHF encompasses multipotent cardiovascular progenitors or distinct lineage-restricted progenitor populations is a fundamental question in embryology.

The origins of the endocardium are controversial (Harris and Black, 2010; Vincent and Buckingham, 2010). Previous lineage studies in chick (Cohen-Gould and Mikawa, 1996; Lough and Sugi, 2000; Wei and Mikawa, 2000) demonstrated that myocardial and endocardial progenitors derive from separate subpopulations within precardiac mesoderm that diverge prior to, or at, the primitive streak stage. In support of these findings, recent studies in zebrafish suggest that endocardial cells are derived from the hematopoietic and vascular lineages, and then migrate to populate the developing heart tube (Bussmann et al., 2007; Schoenebeck et al., 2007). Lineage studies in avian embryos demonstrated that a population of cells within the anterior pharyngeal mesoderm could contribute to outflow tract endocardium but not to myocardium (Noden, 1991). Together, these studies support a model in which part of the endocardium is derived from mesodermal cells that do not have myocardial potential.

In contrast to the idea that endocardial and myocardial lineages have already diverged post-gastrulation, several recent studies have suggested the existence of a common myocardial-endocardial progenitor at cardiac crescent stages, or even later. These studies, primarily performed in vitro, provide evidence that endocardial, myocardial and smooth muscle cells arise from multipotent cardiovascular progenitors that co-express Islet1 (ISL1), FLK1 (also known as VEGFR2 and KDR) and NKX2.5 (Kattman et al., 2006; Moretti et al., 2006; Wu et al., 2006). In response to temporal and positional cues from the microenvironment, these progenitors undergo progressive lineage diversification and differentiation, similar to that seen in the hematopoietic system (Laugwitz et al.,

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2008). Obviously, the existence of multipotent cardiovascular progenitor cells that contribute to all major heart lineages has clinical implications and provides the foundation for cardiac regenerative medicine.

At early stages of development, endocardial cells are distinguished from myocardium by the expression of endothelial markers, such as FLK1, CD31 (PECAM1) and VE-cadherin (cadherin 5) (Baldwin, 1996; Drake and Fleming, 2000). Although endocardial and endothelial cells share substantial molecular and functional characteristics, a recent study demonstrated molecular and lineage distinctions between these two cell types and suggested that the endocardium and myocardium derive from a common precursor, distinct from endothelial cells (Misfeldt et al., 2009). These previous in vitro studies, and lineage studies of SHF progenitors in mice (Cai et al., 2003; Moretti et al., 2006; Verzi et al., 2005), have given rise to the idea that the endocardium derives from multipotent progenitors within the first and second heart fields (Harris and Black, 2010; Vincent and Buckingham, 2010).

In this study, we have addressed a longstanding question regarding the origin(s) of the endocardium in both avian and mouse models. In DiI mapping studies in chick embryos, cells medial to the cardiac crescent at Hamburger and Hamilton stage (St.) 7 gave rise to endocardial, but not myocardial, cells, demonstrating a divergence of endocardial and myocardial progenitors at this stage. Time-lapse movies of endothelial (QH1-positive) cells in quail embryos demonstrated that endothelial cells medial to the cardiac crescent migrated upwards into the arterial pole of the heart, contributing to the endocardial population. These data are consistent with there being an endocardial progenitor population within the SHF that is distinct from myocardial progenitors. To investigate the ability of endothelial progenitors to contribute to either endocardium or myocardium we transplanted endothelial cells from St. 8 quail embryos into the cardiac region of chick host embryos. The results of these studies demonstrated that endothelial cells were able to contribute only to endocardium, not myocardium, demonstrating endothelial cell fate restriction at this stage.

In mouse, the *Isl1* lineage contributes to the SHF-derived myocardium (e.g. outflow tract, right ventricle and atria). We found that only a restricted subset of endocardial cells was derived from this lineage; we suggest that the rest of the endocardium in these regions derives from the *Isl1*$^{-}$ endothelial lineage. Finally, genetic ablation of *Flk1* in mouse in the *Mesp1* and *Tie2* (*Tek* – Mouse Genome Informatics) lineages diminished endothelial and endocardial cells. Ablation of *Flk1* in the *Isl1* lineage altered the proportion of *Isl1*$^{+}$ and *Isl1*$^{-}$ lineage-derived endocardial cells in *Isl1Cre;Flk1* mutant embryos. Taken together, our findings indicate that endocardial cells derive, at least in part, from vascular endothelial cells. These cells give rise to endocardium, but not myocardium, following a migratory pathway comparable to that described for SHF cells. Hence, the SHF contains distinct myocardial and endocardial progenitor populations.

MATERIALS AND METHODS

Preparation of chick/quail embryos

Fertilized chick/quail eggs were incubated at 38°C under 80% humidity; embryos were staged according to Hamburger and Hamilton (Hamburger and Hamilton, 1951).

Time-lapse microscopy

Live quail embryos at St. 7 were permeabilized, blocked, then incubated with QH1 primary antibody (DSHB) followed by Cy3-conjugated anti-mouse IgG1 secondary antibody (1:100; Jackson ImmunoResearch). Embryos were then placed in a humidified temperature-controlled chamber under a Nikon 90i fluorescence microscope. Images were acquired using ImageProPlus (Media Cybernetics) and assembled using Photoshop CS (Adobe).

Transplantation experiments

Quail or chick grafts of distinct tissues were dissected and inserted into an incision created in the chick or quail host, respectively. For the mouse-chick transplantations, YFP-expressing mouse endothelial cells isolated by FACS were transferred to an agar plate for 16 hours to form aggregates, which were then selected for implantation into chick embryos. All host embryos were incubated for an additional 24 hours.

In situ hybridization

Whole-mount in situ hybridization was performed using digoxigenin-labeled antisense riboprobes synthesized from the cDNA (see Table S1 in the supplementary material). Images were obtained using a Leica MZ16FA stereomicroscope attached to a digital camera (DC300F, Leica Microsystems).

Dye injection

Fate-mapping experiments were performed on St. 7-8 embryos. DiI (D282, Molecular Probes) at 5 mg/ml (ethanol) was subsequently diluted 1:2 with tetraglycol and was pressure-injected into cultured chick embryos using a micromanipulator.

Mouse lines

Conditional knockout embryos were generated using either *Mesp1Cre* (Saga et al., 1996), *Tie2Cre* (Koni et al., 2001) or *Isl1Cre* (Yang et al., 2006) mice, crossed with the *Flk1* conditional mice supplied by Genentech. Cre activity was detected using the *Rosa26R* reporter (Jackson ImmunoResearch). Endothelial cells for the transplantation studies were isolated by FACS from *VE-cadherinCre* (Alva et al., 2006) crossed with the *Rosa26YFP* reporter (Srinivas et al., 2001). As a control, neuronal YFP-expressing cells were isolated from *YFP 2.2* (Feng et al., 2000) mouse embryos.

Immunofluorescence

Cryosections were blocked with 5% horse serum and then incubated with the following antibodies: QCPN, QH1, anti-PECAM1, anti-ISL1, MF20, anti-tropomyosin (DSHB), anti-NKX2.5 (Santa-Cruz), anti-smooth muscle actin (SMA) (Sigma), anti-β-galactosidase (β-gal) (Cappel) and anti-FLK1 (gift of Philip Thorpe, University of Texas Southwestern Medical Center). Secondary antibodies were Cy5- and Cy2-conjugated anti-rabbit and anti-mouse; Cy3-conjugated anti-rat, anti-hamster, anti-mouse, anti-goat (1:100; Jackson ImmunoResearch).

Quantification of cell number

Following the immunostaining procedure, high-magnification images were taken and processed using Photoshop CS. Single cells were counted manually according to their DAPI staining and were identified according to the expression of the indicated markers in the green or red channels. FLK1 and ISL1 double-positive cells were quantified relative to the total number of ISL1-expressing mesodermal cells. To determine endocardial lineage composition, quantification of β-gal and PECAM1 double-positive cells was performed relative to the total number of PECAM1-expressing cells. The results are presented as the percentage averaged from four different images for each developmental stage.

Electroporation

Chick embryos were placed in New-Culture (Nathan et al., 2008), grown to St. 3, and placed above a platinum cathode. The pCAGG-GFP construct (0.03 μg/μl) (Nathan et al., 2008) was injected between the blastoderm and the vitelline membrane using a glass capillary. Next, the anode was placed above the embryo and two pulses of 6V for 25 mseconds and 500 mseconds were performed using an ECM830 electroporator (BTX). Embryos were analyzed under the Nikon 90i fluorescence upright microscope.

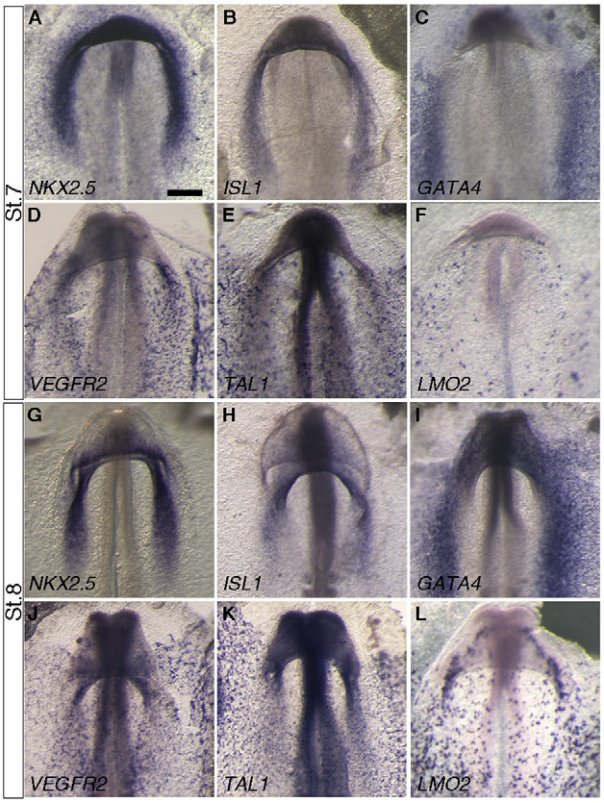

Fig. 1. Cardiac and endothelial gene expression in the chick. In situ hybridization analysis at St. 7 and 8 using cardiac and endothelial markers at cardiac crescent stages in chick embryos. (**A-C**,**G-I**) Cardiac markers are expressed in the lateral splanchnic mesoderm, which is also known as the cardiac crescent. (**D-F**,**J-L**) Vascular endothelial markers are dispersed along the entire medial-lateral axis. Scale bar: 100 μm.

RESULTS

Gene expression and fate-mapping analyses in the chick reveal a distinct endocardium-forming field adjacent to the cardiac crescent

To examine the origins of endocardial cells in chick embryos, we first mapped the expression patterns of cardiac and endothelial markers by in situ hybridization at cardiac crescent stages (St. 7-8). At these stages, *NKX2.5*, *ISL1* and *GATA4* are expressed in cardiac progenitors within the lateral splanchnic mesoderm, labeling the cardiac crescent (Fig. 1A-C,G-I) (Nathan et al., 2008; Tirosh-Finkel et al., 2006). By contrast, the endothelial markers *VEGFR2*, *TAL1* and *LMO2* are broadly expressed as a punctuate sheet, extending from the midline and spreading throughout the anterior mesoderm (Fig. 1D-F,J-L).

We next traced the fate of cells along the medial-lateral axis of the chick embryo to determine their contribution to the endocardium and myocardium. We began by labeling cardiac crescent cells with the lipophilic carbocyanine dye DiI. DiI-labeled cells could be seen in both the endocardium and the myocardium within the heart tube (Fig. 2A-C′, *n*=6/6). These findings in the chick confirm that the cardiac crescent at St. 7 (corresponding to the first heart field) contains both endocardial and myocardial progenitors. Injection of DiI into the medial mesoderm resulted in labeling of the cranial paraxial mesoderm, a subregion within the pharyngeal mesoderm (Tzahor and Evans, 2011) (Fig. 2D-F′, *n*=4/5). Subsets of these labeled cells lined the dorsal aorta, suggesting that some endothelial cells had been labeled (Fig. 2F′). Lastly, we injected DiI adjacent to the cardiac crescent, from its medial side; subsequently, labeled cells were found in the endocardium, but not in the myocardium, and were also detected in the pharyngeal mesoderm (Fig. 2G-I′, *n*=5/7). A whole-mount view of a representative embryo and serial transverse sections of its heart are shown in Fig. S1 in the supplementary material.

Endothelial cells migrate into the arterial pole of the heart and contribute to the endocardium

The fate-mapping data could suggest that cells with endothelial but not myocardial potential contribute to the endocardium. To prove that this is indeed the case, we developed a wide-field time-lapse microscopy assay (see Materials and methods) to track the migration of endothelial cells in live quail embryos (Fig. 3 and see Movie 1 in the supplementary material). This analysis enabled us to follow dynamic endothelial cell (stained with QH1 antibody, $QH1^{pos}$) movements from the cardiac crescent to linear heart tube stages. By examining the movies, and still images taken from them, we could identify the endothelial cell path (Fig. 3, green circle): endothelial cells adjacent to the inner cardiac crescent initially move towards the head, and then bifurcate medially as they enter the heart tube endocardium via the outflow tract (Fig. 3M,N). These findings further support the concept that the endocardium is formed from endothelial cells that migrate into the linear heart tube, using distinct patterns of movement. The pattern of movement of the $QH1^{pos}$ endothelial cells into the outflow tract is reminiscent of the movement of myocardial progenitors from the second/anterior heart field. Hence, we conclude that we have identified an endocardial progenitor population within the second/anterior heart field that is distinct from myocardial progenitors.

Ectopic endothelial cells contribute to the endocardium and not myocardium

We next investigated whether distinct sources of endothelial cells are capable of forming endocardium when incorporated into the cardiac environment. Accordingly, we dissected distinct quail tissues containing endothelial cells and transplanted them into the developing chick heart (Fig. 4). First, a small tissue sample from the vitelline vessel of an E4 quail embryo was transplanted to replace the cardiac outflow tract of a St. 10 chick embryo (Fig. 4A-D). At St. 12, quail-derived endothelial cells, identified by QCPN antibody staining, could be detected in the endocardium of the host chick (Fig. 4C,D; *n*=3/4). We then transplanted a somite, which contains skeletal muscle as well as endothelial progenitors (Ema et al., 2006; Kardon et al., 2002), from a St. 8 quail donor into the cardiac crescent of a St. 8 chick embryo (Fig. 4E-H). Somite-derived cells from the quail integrated with the endocardium, 24 hours later (Fig. 4G,H; *n*=2/2). In both of these transplantation assays, quail cells (stained with QCPN, red) were only found within the endocardium of the chick embryo (Fig. 4A-H). Endocardial cells derived from the transplant were positive for the quail endothelial marker QH1 (data not shown).

Next, we transplanted a small section of the cardiac crescent from a St. 8 chick embryo adjacent to the vitelline vessel of the quail embryo (Fig. 4I). Quail-derived endothelial cells ($QH1^{pos}$) migrated into the heart field explant and formed an endocardial-like structure (Fig. 4J,L; *n*=4/4). By contrast, these endothelial cells did not integrate into an explant of trunk lateral mesoderm (Fig. 4K,L).

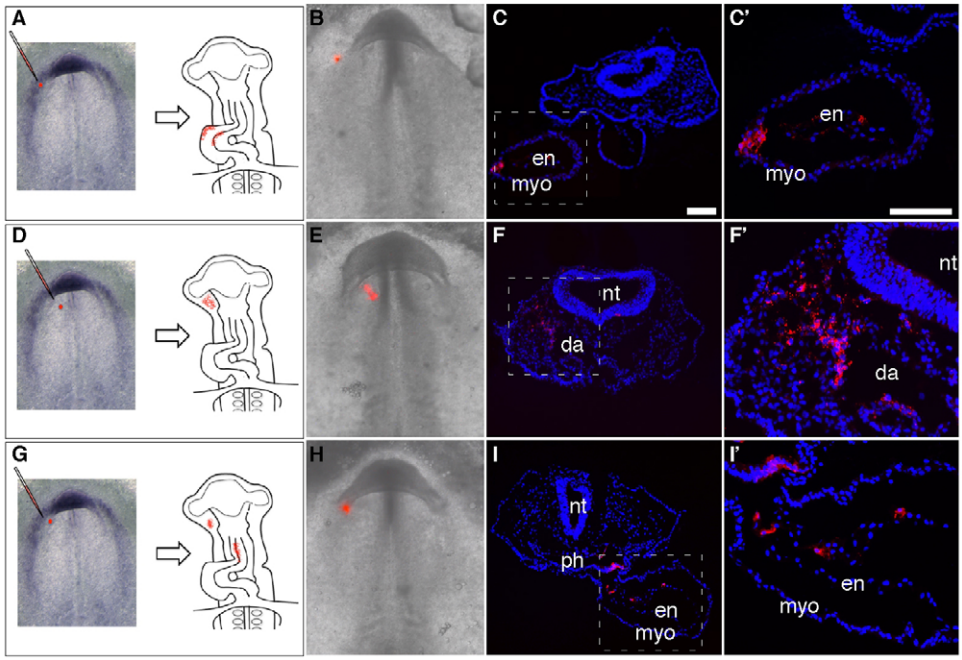

Fig. 2. Fate-mapping analyses in the chick reveal a distinct endocardium-forming field adjacent to the cardiac crescent. DiI fate-mapping experiments along the medial-lateral axis of the chick embryo at St. 7. (**A-C′**) DiI (red) injection of cells within the cardiac crescent, as illustrated in A, labeled both the myocardium and endocardium, as shown in transverse sections at St. 10. (**D-F′**) DiI injection closer to the midline of the embryo labeled head mesoderm (mostly pharyngeal mesoderm). (**G-I′**) Injection of DiI into cells adjacent to the cardiac crescent specifically labeled the endocardium, and not the myocardium. All fluorescent images are counterstained with DAPI (blue). The boxed region in C,F,I is shown at higher magnification in C′,F′,I′, respectively. en, endocardium; myo, myocardium; da, dorsal aorta; nt, neural tube; ph, pharynx. Scale bars: 100 μm.

We then considered whether mouse endothelial cells could contribute to the chick endocardium in a similar manner. To this end, we FACS purified endothelial cells obtained from E14.5 embryos derived from *VE-cadherinCre* (Alva et al., 2006) crossed with *Rosa26YFP* (Srinivas et al., 2001) mice (Fig. 4M-P). Strikingly, mouse *VE-cadherin*$^{+}$ embryonic endothelial cells implanted into the cardiac crescent of a chick embryo were detected in endocardial, but not myocardial, cells (Fig. 4O,P; *n*=4/4). In control experiments, in which FACS-sorting YFP-expressing neuronal cells were similarly transplanted into the cardiac crescent of chick, we observed no YFP expression in endocardial cells (data not shown; *n*=5/5).

Collectively, our findings in avian embryos suggest that cardiac and endothelial progenitors from distinct, non-overlapping sources give rise to myocardial and endocardial cells, respectively. Furthermore, we identified a field adjacent to the cardiac crescent that contributes to the heart endocardium. We tracked the migratory path of single endothelial cells into the outflow tract, where they

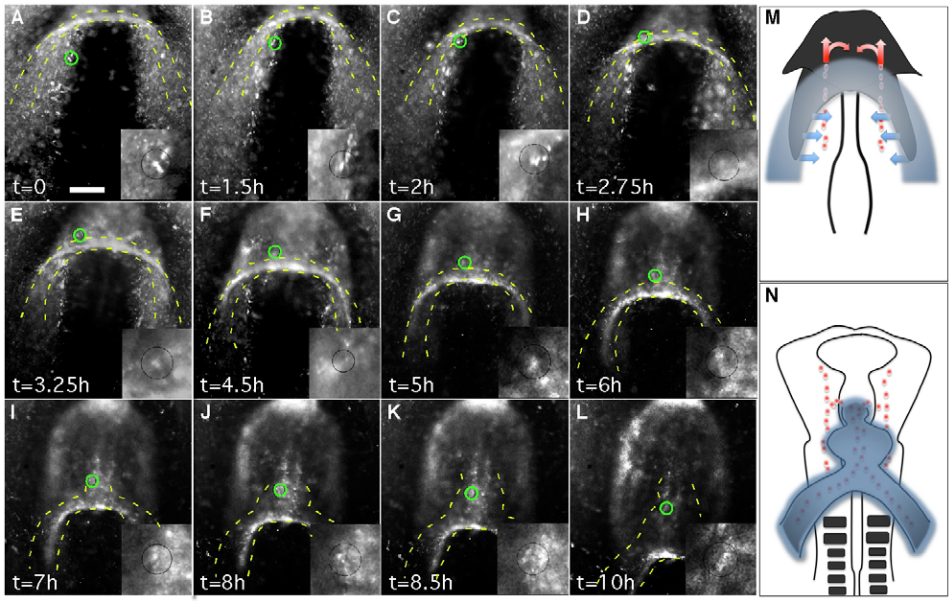

Fig. 3. Time-lapse analysis of quail endothelial cell movement into the endocardium. (**A-L**) A live quail embryo labeled with QH1 antibody was monitored under a fluorescence microscope fitted with a temperature-controlled chamber at different time points during early heart development. Single cells (green circle) are shown (magnified in each inset). The images depict QH1-positive endothelial cells outside the cardiac crescent (delineated by the dashed yellow lines) entering the heart tube. (**M**,**N**) Models describing the distinct migratory paths of endothelial (red) and myocardial (blue) progenitors. Whereas cardiac crescent cells typically converge in a zipper-like manner towards the midline, endothelial cells adjacent to the cardiac crescent initially move towards the head, where a subpopulation branches out medially. This endothelial subpopulation gives rise to the endocardium at heart looping stages. Scale bar: 100 μm.

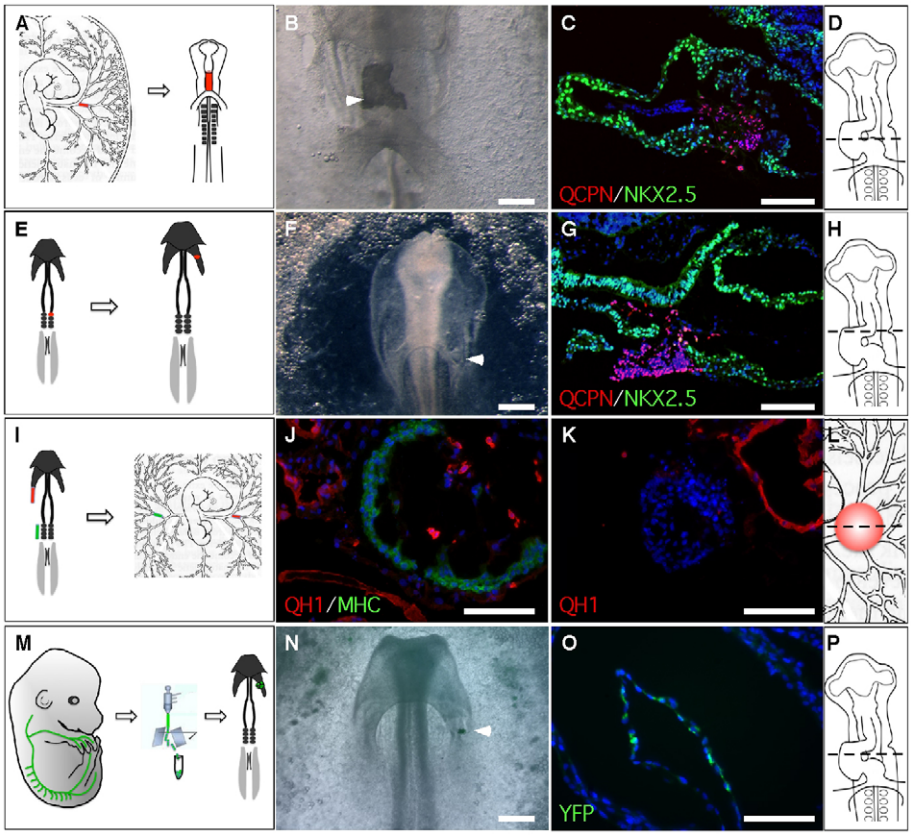

Fig. 4. Transplantation of endothelial cells in quail-chick chimeras reveals endocardial, but not myocardial, cell fate restriction. (**A-D**) A vitelline vessel explant from an E4 quail embryo was transplanted in place of the anterior pole of the heart of a St. 10 chick embryo (A,B, arrowhead). At St. 12, quail-derived cells (QCPN^{pos}) contributed to the chick endocardium (*n*=3 out of 4) (C). NKX2.5 stains the myocardium. (**E-H**) Somites from a St. 8 quail embryo were transplanted into the cardiac crescent of a St. 8 chick embryo (E,F, arrowhead). The transplanted cells specifically contributed to the endocardium (*n*=2/2) (G). (**I-L**) A small piece of the cardiac crescent from a St. 8 chick embryo was transplanted next to the vitelline vessel of a St. 12 quail embryo (I). MHC^{pos} chick cells organized around QH1^{pos} quail endothelial cells (*n*=4/4) (J) . Chick lateral plate mesoderm cells failed to form such a tubular structure with the host endothelial cells (*n*=4/4) (K) . (**M-P**) YFP^{+} endothelial cells, isolated by FACS from *VE-cadherin/R26YFP* mouse embryos, were transplanted into the cardiac crescent of a St. 8 chick embryo (M,N, arrowhead). YFP^{+} mouse cells contributed to the chick endocardium at St. 12 (*n*=4/4) (O). Schematics (D,H,L,P) indicate the plane of sectioning (dashed line) in C,G,K,O, respectively. In L, the spherical structure formed by the graft is indicated (red). All fluorescent images are counterstained with DAPI. Scale bars: 100 μm.

contribute to endocardial cells. Lastly, we provide evidence that any endothelial cells transplanted into the cardiac environment can form endocardial cells, supporting the idea that the endocardium derives from endothelial cells.

Endothelial cells in the second heart field do not express ISL1 in both avian and mouse embryos

ISL1 is a major player in the development of pharyngeal mesoderm/SHF cells during embryogenesis (Evans et al., 2010; Rochais et al., 2009; Srivastava, 2006; Tzahor and Evans, 2011; Vincent and Buckingham, 2010). This transcription factor is expressed in undifferentiated progenitors of the two heart fields, although it is only required for the normal development of the SHF (Cai et al., 2003; Prall et al., 2007; Tirosh-Finkel et al., 2006). Our data suggest that endocardial cells could be derived from vascular endothelial cells; however, we cannot exclude the possible existence of multipotent mesoderm progenitors located outside the cardiac crescent. To gain deeper insight into these issues, we performed immunostaining with QH1 and for ISL1 in St. 8 quail embryos (Fig. 5A) and for PECAM1 and ISL1 in sections of E8.0 mouse embryos (Fig. 5B). Few endothelial cells (QH1^{pos} and PECAM1^{pos}; to clarify our terminology, ^{pos} refers protein expression, whereas $^{+}$ indicates a lineage) were detected between the endoderm and the mesoderm, along the medial-lateral (quail) or dorsal-ventral (mouse) axes (Fig. 5A-C). We did not observe co-expression of QH1/PECAM1 and ISL1.

We next sought to address the existence of multipotent cardiovascular progenitors (reviewed by Laugwitz et al., 2008) expressing both FLK1 and ISL1 in E7.5-11.5 mouse embryos (Fig. 5E-G″). FLK1 expression within ISL1^{pos} mesoderm cells gradually increased, reaching a peak at E9.5; we could not detect such cells at E11.5 (Fig. 5E-G″, quantified in 5D). Three subpopulations of mesoderm cells could be detected: ISL1^{pos}, FLK1^{pos} and ISL1 FLK1 double positive at E9.5 (Fig. 5F-G″). The aortic sac, a dilation just distal to the outflow tract, serves as the primordial vasculature from which the aortic arches arise. Endothelial cells within the aortic sac lie in a continuum with the endocardium in the anterior pole of the heart, with both expressing FLK1 and PECAM1 (Fig. 5G-H). It appears that endothelial cells do not express ISL1 at cardiac crescent stages, although we do see rare

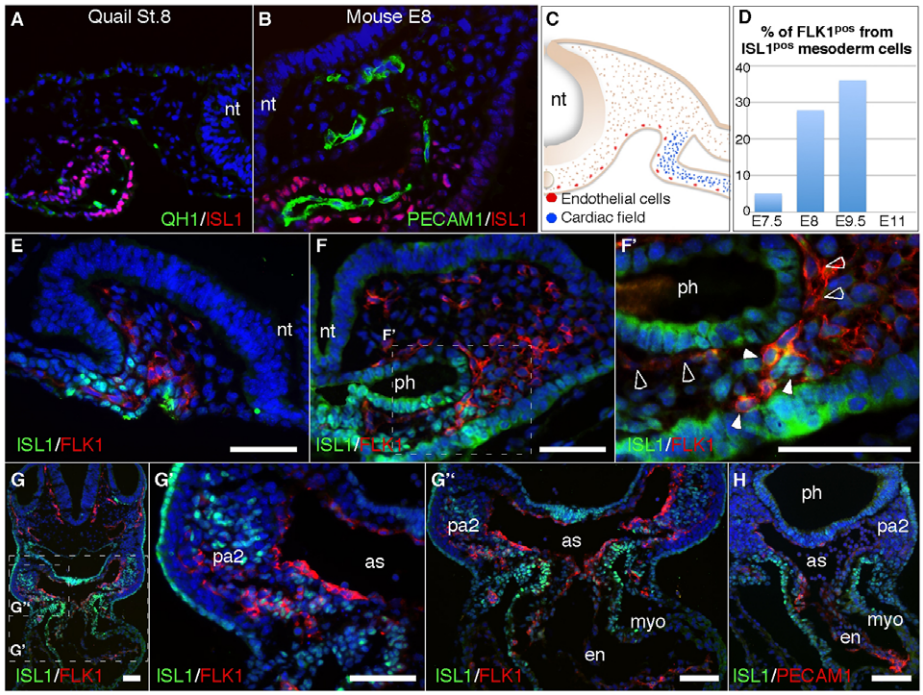

Fig. 5. Expression of cardiac and endothelial markers in the pharyngeal mesoderm. (**A**,**B**,**H**) Staining for endothelial (QH1, quail; PECAM1, mouse) and cardiac (ISL1) progenitors at the indicated stages of development. (**C**) Model depicting that endothelial and cardiac progenitors form distinct, non-overlapping populations. (**D**) Quantification of the results in E-G″ showing the percentage of FLK1^{pos} cells within the ISL1^{pos} mesoderm. (**E-G″**) Immunostaining in the mouse for ISL1 (green) and FLK1 (red) at (E) E7.5, (F,F′) E8 and (G-G″) E9.5. White arrowheads indicate cells co-expressing these markers; open arrowheads indicate FLK1^{pos} cells that do not express ISL1. All fluorescent images are counterstained with DAPI. nt, neural tube; ph, pharynx; pa2, pharyngeal arch 2; as, aortic sac; en, endocardium; myo, myocardium. Scale bars: 100 μm.

ISL1^{pos} cells in the endocardium at E9.5 (Fig. 5H). Our gene expression data, coupled with anatomical examinations, is in line with the idea that endocardial cells are derived from FLK1^{pos} ISL1^{neg} cells, rather than from the double-positive population. Importantly, we cannot rule out the possibility that some FLK1^{pos} ISL1^{pos} cells give rise to endocardial cells.

Lineage analysis of endocardial cells in the mouse reveals an unexpected heterogeneity in the origins of the endocardium

We next re-evaluated the lineage origin of the endocardium in the mouse, using *Mesp1Cre* (Saga et al., 2000) and *Isl1Cre* (Yang et al., 2006) mouse lines. MESP1 is broadly expressed in cardiac and

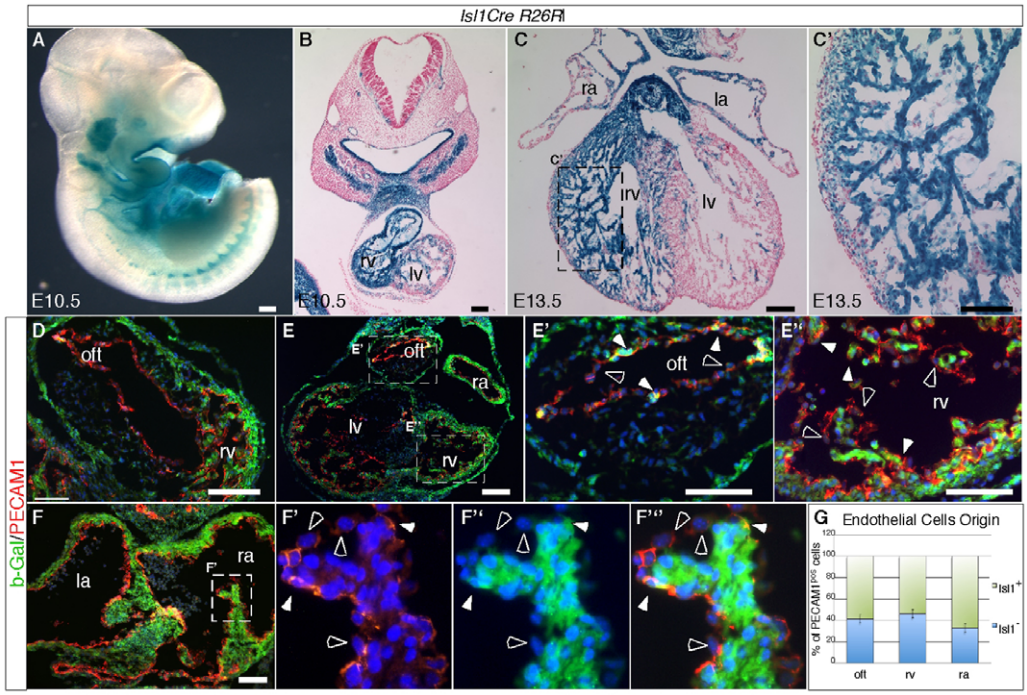

Fig. 6. Lineage analysis of endocardial cells in the mouse. (**A-C′**) *lacZ* staining of *Isl1Cre;R26R* mouse embryos at different stages of development. (**D-F‴**) Immunostaining of sections of *Isl1Cre;R26R* E12.5 embryos for PECAM1 (red) and β-gal (green). White arrowheads indicate cells that co-express these markers, indicating that they are derivatives of the *Isl1* lineage (*Isl1*$^{+}$); open arrowheads indicate PECAM1^{pos} cells that do not express β-gal (*Isl1*$^{-}$). (**G**) Quantification of *Isl1*$^{+}$ and *Isl1*$^{-}$ lineage-derived endothelial/endocardial cells. Error bars indicate s.d. All fluorescent images are counterstained with DAPI. rv, right ventricle; lv, left ventricle; ra, right atrium; la, left atrium; oft, outflow tract. Scale bars: 100 μm.

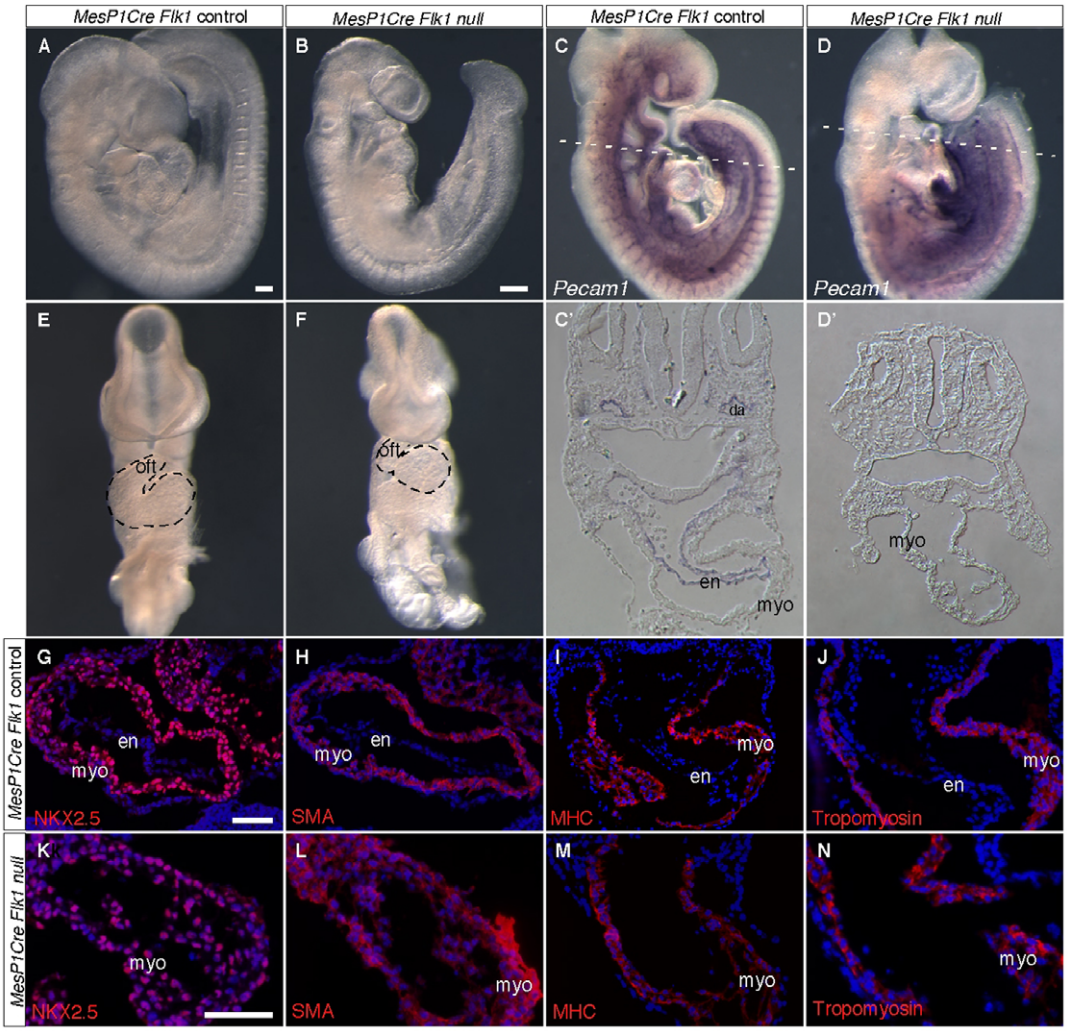

Fig. 7. Genetic ablation of *Flk1* in the *Mesp1* lineage in the mouse results in a lack of endothelial and endocardial cells, along with second heart field defects. (**A**,**B**) E9.5 mutant mouse embryos display cardio-craniofacial defects. (**C-D'**) In situ hybridization for *Pecam1* showing the absence of endothelial cells. Dashed line indicates plane of sections shown in C′,D′. (**E**,**F**) Ventral view of the same mutants as in A,B reveals abnormal heart looping, a shortened outflow tract and hypotrophic right ventricle. (**G-N**) Sections of E9.5 heart tubes from control and mutant mouse embryos immunostained for (G,K) NKX2.5, (H,L) SMA, (I,M) MHC and (J,N) tropomyosin. All fluorescent images are counterstained with DAPI. oft, outflow tract; da, dorsal aorta; en, endocardium; myo, myocardium. Scale bars: 100 μm.

craniofacial mesoderm cells at the anterior primitive streak (Saga et al., 2000). Within the heart, the *Mesp1* lineage marks all cardiac cells of mesodermal origin (see Fig. S2 in the supplementary material) (Saga et al., 1999).

The *Isl1Cre* line crossed with the *Rosa26* conditional reporter marks broad regions of the heart and the mesoderm core of the pharyngeal arches (Harel et al., 2009; Nathan et al., 2008); notably, most of the left ventricle is not marked by the *Isl1* lineage (Fig. 6A-C) (see also Cai et al., 2003; Moretti et al., 2006; Sun et al., 2007). Difficulties in distinguishing between myocardial cells and the thin endocardial lining led us to examine *Isl1Cre*-mediated *lacZ* expression in the endocardium in greater detail using anti-β-gal and PECAM1 double staining (Fig. 6D-F″; quantified in 6G). Examination of β-gal-expressing and PECAM1-expressing cells in the outflow tract, right ventricle and atria revealed that ~50-60% of endocardial cells in these regions expressed both markers. Therefore, endocardial cells are derived from two lineages: $Isl1^{+}$ and $Isl1^{-}$. These findings suggest that the endocardium is more heterogeneous than previously suggested.

FLK1 is required for formation of the endocardium in vivo in the *Mesp1* and *Tie2* lineages, but not in the *Isl1* lineage

The expression of FLK1 in early mesodermal cells marks progenitors with a broad lineage potential, although it is thought that this gene is only necessary for the formation of endothelial and hematopoietic lineages (Ema et al., 2006; Motoike et al., 2003; Shalaby et al., 1995). To further investigate the endothelial origins of the endocardium, and the necessity of FLK1 for endocardium formation in the mouse, we conditionally ablated *Flk1* in both cardiac and endothelial progenitors. To this end, we first employed an *Flk1* conditional allele crossed with the *Mesp1Cre* mouse. *Mesp1Cre;Flk1* null conditional mutants die between E9.5 and E10.5, one day later than the non-conditional *Flk1* knockout embryos, enabling us to obtain clearer insight into the cardiac phenotypes of these mutants (Fig. 7). At E9.5, the mutant embryos displayed cardio-craniofacial defects, as compared with their control littermates (Fig. 7A,B). As expected, these mutants were devoid of endothelial cells in the head and heart regions, in agreement with the

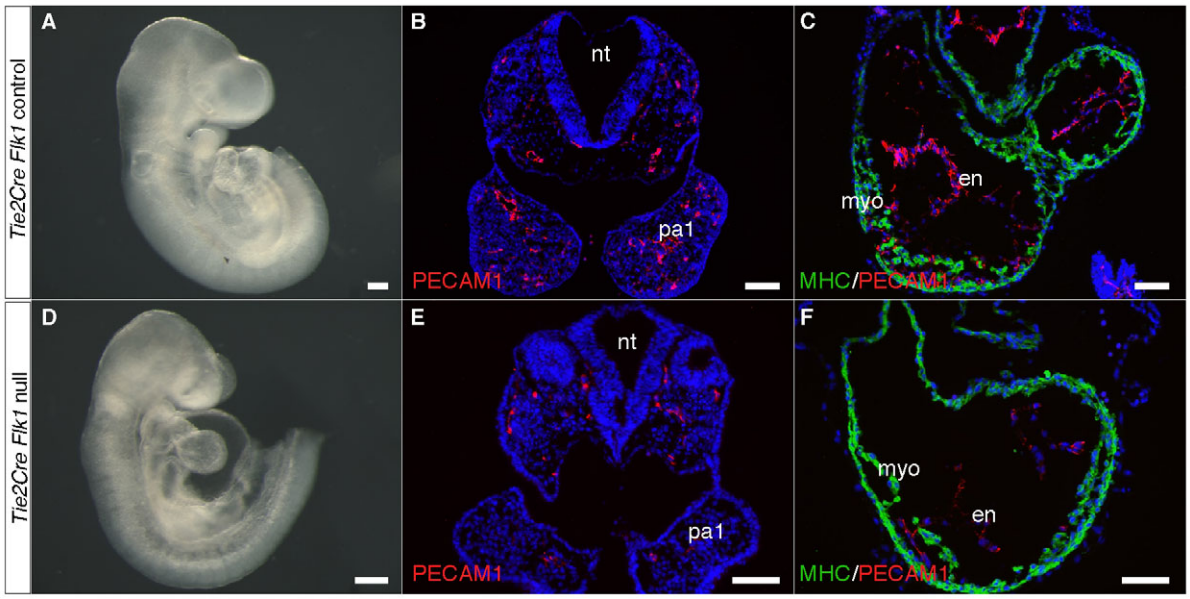

Fig. 8. *Tie2Cre;Flk1* null mouse mutants lack endothelial and endocardial cells. (**A**,**D**) Whole-mount views of control and *Tie2Cre;Flk1* null mutant embryos at E9.5. (**B,C,E,F**) Sections in the head and heart regions immunostained for PECAM1 (red) and MHC (green). Endothelial and endocardial cells are dramatically reduced in these mutants. All fluorescent images are counterstained with DAPI. pa1, pharyngeal arch 1; nt, neural tube; en, endocardium; myo, myocardium. Scale bars: 100 μm.

expression pattern of *Mesp1* lineage progenitors (Fig. 7C,D). Immunostaining of sectioned E9.5 heart tubes revealed normal myocardial gene expression; NKX2.5, SMA, myosin heavy chain (MHC) and tropomyosin were expressed throughout the entire myocardium (Fig. 7G-J). By contrast, we observed complete loss of the endocardium in the hearts of *Mesp1Cre;Flk1* null conditional mutants (Fig. 7K-N).

Because the *Mesp1* lineage gives rise to a broad range of mesodermal cells, including cardiac and endothelial progenitors, we examined the function of FLK1 in endothelial cells by deleting it using *Tie2Cre* mice (Koni et al., 2001). Although this Cre is less efficient than *Mesp1Cre*, the morphology of the *Tie2Cre;Flk1* null mutants was comparable to that of the *Mesp1Cre;Flk1* null mutants (Fig. 8A,D). In addition, the number of endothelial and endocardial cells (PECAM1^{pos}) was significantly reduced (Fig. 8B,C,E,F). Taken together, our findings in the mouse, based on ablation experiments of *Flk1* in both the *Mesp1*$^{+}$ and *Tie2*$^{+}$ lineages, confirm that FLK1 is required for the formation of endothelial and endocardial cells. The loss of endothelial cells that accompanied the loss of the endocardium in these mutants further suggests that the endocardium derives from endothelial cells in the mouse. In both *Mesp1Cre* and *Tie2Cre Flk1* null mutants, heart looping was abnormal (Figs 7 and 8): mutant embryos displayed a shortened outflow tract and hypoplastic right ventricle and pharyngeal arches, similar to classical knockouts of SHF regulators (Buckingham et al., 2005; Cai et al., 2003). These findings in both mutants suggest that endothelial cells play non-cell-autonomous roles in the incorporation of pharyngeal mesoderm/SHF cells into the heart, presumably by affecting their migration and/or survival.

To elucidate the specific requirement for FLK1 in *Isl1*$^{+}$ cardiac progenitors, we crossed the *Flk1* conditional allele with the *Isl1Cre* mouse line (Yang et al., 2006). *Isl1Cre;Flk1* null conditional mutants died at ~E14. At E9.5, the *Isl1Cre;Flk1* null conditional mutants had no apparent cardio-craniofacial defects and the endocardium was properly formed, as compared with control littermates (Fig. 9A-H). Likewise, expression of *Pecam1* at E9.5 was comparable in control and mutant embryos (Fig. 9A,E). In fact, it appears that there are more endocardial cells (PECAM1^{pos}) in sections of these mutants, as compared with controls, at E9.5-13 (Fig. 9A-H).

Owing to the observed heterogeneity in the origins of endocardial cells, we examined the proportion of *Isl1*$^{+}$ and *Isl1*$^{-}$ lineage-derived endocardial cells in controls and *Flk1* null conditional mutants (Fig. 9I-K). Although *Isl1Cre* labels endothelial cells (Fig. 6) (Moretti et al., 2006; Sun et al., 2007), many endothelial cells are *Isl1*$^{-}$ in terms of their lineage (Figs 5 and 6). Strikingly, quantification of the proportion of *Isl1*$^{+}$ (β-gal^{pos}) and *Isl1*$^{-}$ (β-gal^{neg}) lineage-derived endocardial cells (PECAM1^{pos}) revealed a decrease in the *Isl1*$^{+}$ population and an increase in the *Isl1*$^{-}$ population (Fig. 9I-K). These data suggest that ablation of *Flk1* in the pharyngeal mesoderm [*Isl1* lineage (Tzahor and Evans, 2011)] affected endocardial cell composition in the *Isl1*$^{+}$ and *Isl1*$^{-}$ lineages, and further point toward the vascular endothelial origin of the heart endocardium. In addition, we demonstrated that FLK1 plays key roles in the formation of the endocardium in *Mesp1*$^{+}$ and *Tie2*$^{+}$ (but not *Isl1*$^{+}$) cells. Notably, FLK1 is required for cardiogenesis in this lineage, as mutant embryos die at ~E14.5; the molecular pathology and underlying mechanisms of this are currently under investigation.

DISCUSSION

In this study, we addressed a controversial question in heart development concerning the origins of the endocardium (Harris and Black, 2010; Lough and Sugi, 2000; Vincent and Buckingham, 2010). We provide multiple experimental findings based on gene expression, fate mapping, time-lapse movies and transplantation assays in the avian system demonstrating that during the cardiac crescent stages, endocardial cells derive from a vascular endothelial population that is distinct from myocardial progenitors. Furthermore, lineage tracing and conditional ablation of *Flk1* in

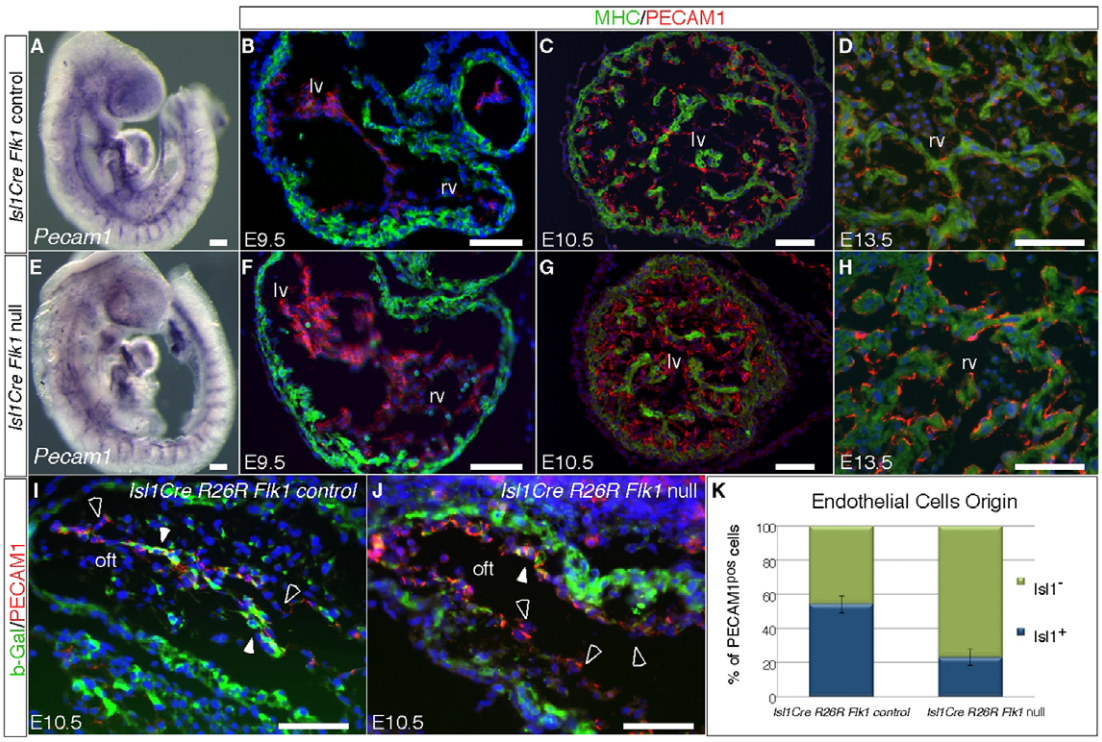

Fig. 9. The role of FLK1 in ISL1 lineage-derived progenitors in the mouse. (**A**,**E**) In situ hybridization for *Pecam1* in E9.5 control and *Isl1Cre;R26R;Flk1* null embryos. (**B-D**,**F-H**) Heart sections of control (B-D) and *Isl1Cre;R26R;Flk1* (F-H) embryos at different stages of development, immunostained for MHC (green) and PECAM1 (red). (**I**,**J**) Immunostaining of sections for PECAM1 (red) and β-gal (green). White arrowheads indicate cells that co-express these markers and hence are *Isl1* lineage derived (*Isl1*$^{+}$); open arrowheads indicate PECAM1^{pos} cells that do not express β-gal (*Isl1*$^{-}$). (**K**) Quantification of *Isl1* versus non-*Isl1* lineage-derived endothelial cells in mutant and control embryos. All fluorescent images are counterstained with DAPI. rv, right ventricle; lv, left ventricle; oft, outflow tract. Scale bars: 100 μm.

cardiovascular progenitors in the mouse demonstrated that FLK1 is required in *Mesp1*$^{+}$ and *Tie2*$^{+}$, but not *Isl1*$^{+}$, lineages, for formation of the endocardium in vivo. Ablation of *Flk1* in the *Isl1*$^{+}$ progenitors was compensated for by *Isl1*$^{-}$ endocardial cells, which are likely to be derived from endothelial cells. Taken together, we demonstrated in both avian and mouse models that the heart endocardium derives from, and is continuous with, lineage-restricted vascular endothelial cells (Fig. 10A).

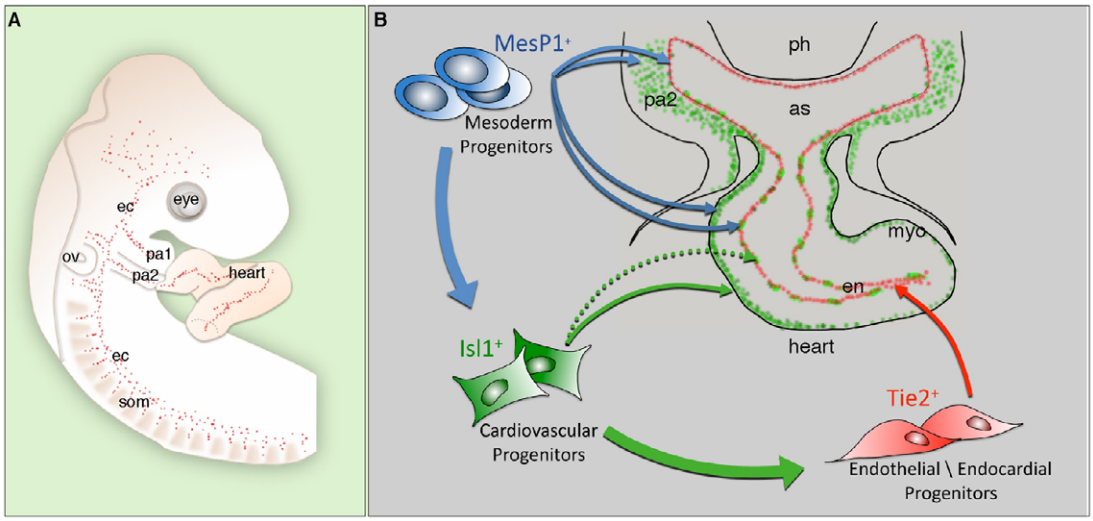

Fig. 10. The heart endocardium is derived from vascular endothelial progenitors. (**A**) E9.5 mouse embryo showing that the heart endocardium is physically and functionally linked to the early endothelial network. (**B**) Cardiovascular lineage tree highlighting the hierarchy of MESP1, ISL1 and TIE2 cardiovascular progenitor populations and their respective contributions to the embryonic heart. ec, endothelial cells; ov, otic vesicle; pa1, pharyngeal arch 1; pa2, pharyngeal arch 2; som, somites; ph, pharynx; as, aortic sac; en, endocardium; myo, myocardium.

We suggest that pharyngeal mesoderm cells within the second/anterior heart field harbor distinct myocardial and endocardial progenitor populations; the *Mesp1* lineage marks pan-mesodermal cardiovascular progenitors. These cells stand at the top of the hierarchy, upstream of the ISL1 and TIE2 progenitor populations (Fig. 10B). *Mesp1* lineage-derived cells give rise to both myocardial and endocardial progenitors (as well as to other mesodermal derivatives). Myocardial cells are also derived from the *Isl1* lineage (Fig. 10B, green), whereas endocardial cells originate from the *Tie2* endothelial lineage (Fig. 10B, red; data not shown). However, ~50% of the endocardium derives from the ISL1 lineage. Because *Isl1* lineage-derived cells mark some endothelial cells (Fig. 6), we suspect that the *Isl1*$^{+}$ endocardial cells are also derivatives of vascular endothelial cells.

In vitro studies in chick and mouse have provided evidence for a bipotential progenitor for myocardial and endocardial cells (Hutson et al., 2010; Kattman et al., 2006; Moretti et al., 2006). This observation, combined with Cre recombinase studies of SHF lineages (Cai et al., 2003; Moretti et al., 2006; Verzi et al., 2005), suggest that bipotential ISL1 FLK1 progenitors within the SHF give rise to both myocardial and endocardial lineages in vivo. In the mouse, we observed the existence of FLK1 and ISL1 co-expressing pharyngeal mesoderm/SHF cells at ~E7.5-9.5 (see also Moretti et al., 2006), although they seem to be distinct from the FLK1^{pos} and PECAM1^{pos} endothelial/endocardial populations (Fig. 5). The fact that ~50% of endocardial cells within outflow tract, right ventricle and atria derive from an ISL1 lineage does not necessarily imply that these cells are derivatives of a bipotential progenitor of the SHF. Instead, our DiI labeling and in vivo imaging data suggest that these cells might originate from committed vascular endothelial progenitors within, or adjacent to, the SHF. Because FLK1 expression is not absolutely required for the formation of the endocardium in the *Isl1* lineage, we suggest that progressive lineage diversification of cultured pluripotent embryonic stem cells into distinct heart progenitor populations might not follow the same developmental paths that occur in vivo.

Activation of Cre recombinase in *Mesp1Cre* mice precedes its activation in *Isl1Cre* mice, a finding which could suggest that FLK1 expression is required for the endocardium only within an early developmental window. Thus, FLK1 is required for the formation, but not for the maintenance, of endocardial cells. The absence of endothelial cells in *Mesp1Cre;Flk1* null and *Tie2Cre;Flk1* null, as compared with *Isl1Cre;Flk1* null, mutants (Figs 7-9) could account for the complete loss of endocardial cells.

Finally, using novel in vivo imaging technology we observed that cells with endothelial identity adjacent to the cardiac crescent migrate inwards to give rise to the endocardium. These observations demonstrate that, in vivo, myocardial and endocardial lineages have already diverged by the time that ISL1 is first expressed in cardiac progenitors. Along these same lines, we performed single-cell labeling experiments in the anterior primitive streak in the chick and identified separate populations of endocardial and myocardial progenitors (see Fig. S3 in the supplementary material), consistent with previous studies (Cohen-Gould and Mikawa, 1996; Wei and Mikawa, 2000). In summary, this work provides strong evidence that the endocardium is derived, at least in part, from vascular endothelial cells that have diverged from myocardial lineages in SHF progenitors.

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Competing interests statement
The authors declare no competing financial interests.

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