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Avian coronary endothelium is a mosaic of sinus venosus- and ventricle-derived endothelial cells in a region-specific manner

Short title: Origin of coronary endothelial cells

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Abstract

The origin of coronary endothelial cells (ECs) has been investigated in avian species, and the results showed that the coronary ECs are thought to originate from the proepicardial organ (PEO) and developing epicardium. Genetic approaches in mouse models showed that the major source of coronary ECs is the sinus venosus endothelium or ventricular endocardium. To clarify and reconcile the differences between avian and mouse species, we examined the source of coronary ECs in avian embryonic hearts. Using an enhanced green fluorescent protein-Tol2 system and fluorescent dye labeling, four types of quail-chick chimeras were made and quail-specific endothelial marker (QH1) immunohistochemistry was performed. The developing PEO consisted of at least two cellular populations, one was sinus venosus endothelium-derived inner cells and the other was surface mesothelium-derived cells. The majority of ECs in the coronary stems, ventricular free wall, and dorsal ventricular septum originated from the sinus venosus endothelium. The ventricular endocardium contributed mainly to the septal artery and a few cells to the coronary stems. Surface mesothelial cells of the PEO differentiated mainly into a smooth muscle phenotype, but a few differentiated into ECs. In avian species, the coronary endothelium had a heterogeneous origin in a region-
specific manner, and the sources of ECs were basically the same as those observed in mice.

Key words: avian heart, coronary artery development, origin, endocardium, EGFP-Tol2

Introduction

Coronary vessels function to supply oxygen and nutrients to the cardiac muscle to maintain the heart beat. During heart development in the early embryonic stages, oxygen and nutrients are supplied to the myocardium by diffusion through the inner endocardium. At the onset of the fetal stage, endothelial cells (ECs) from the peritruncal endothelial plexus invade into the aortic sinuses to form coronary stems, thereafter coronary circulation starts and continues throughout life (Ando et al. 2004). Disruption of coronary circulation causes ischemic heart disease including angina pectoris and myocardial infarction. Improved understanding of the developmental biology of coronary vessels is necessary to develop therapeutic strategies for revascularization of the ischemic heart.

The coronary artery consists of three distinct layers, the tunica interna, tunica media, and tunica externa. It is well accepted that not only vascular smooth muscle cells of the tunica media but also interstitial cells of the tunica externa originate from the
developing epicardium. The developing epicardium is a derivative of the proepicardial organ (PEO), which is a cauliflower-shaped protrusion extending from the mesothelial layer covering the ventral surface of the sinus venosus (SV) (Hiruma et al. 1989; Mikawa & Fischman 1992; Mikawa & Gourdie 1996; Dettman et al. 1998). The developing epicardium undergoes epithelial-to-mesenchymal transition to seed subepicardial mesenchymal cells, which later give rise to vascular smooth muscle and interstitial cells (Mikawa & Gourdie 1996; Dettman et al. 1998). Genetic cell-tracing experiments in mouse embryos showed that the major source of coronary ECs is the SV endothelium and/or ventricular endocardium (Red-Horse et al. 2010; Wu et al. 2012; Tian et al. 2013; Chen et al. 2014; Tian et al. 2015; Zhang et al. 2016). In the neonatal mouse heart, ventricular endocardium contributes to form the subendocardial coronary vasculatures (Tian et al. 2015). In avian hearts, retrovirus cell-tracing, quail-chick chimera, and dye-marking experiments showed that cells from the PEO differentiate into the epicardium, vascular smooth muscle, and coronary endothelium (Mikawa & Fischman 1992; Männer 1999; Pérez-Pomares et al. 2002). The discrepancy of the origin of coronary ECs between mouse and avian species as well as among mouse models is an unresolved issue.

The PEO is a transient tissue consisting of surface mesothelium and inner
mesenchymal-like populations. Matured PEO adheres to the dorsal surface of the atrioventricular groove and spreads over the heart in a dorsal-to-ventral direction to generate the epicardium (Hiruma et al. 1989; Nakajima & Imanaka-Yoshida 2013). In the mouse PEO, a Scleraxis-/Semaphorin3D-expressing population gives rise to coronary ECs, whereas Tbx18/Wt1-positive surface mesothelial cells provide the epicardium, vascular smooth muscle, and myocardial interstitial cells (Katz et al. 2012). In the avian PEO, hematopoietic- and endothelial-marker positive cells are observed just before attaching to the ventricle (Poelmann et al. 1993; Kattan et al. 2004; Guadix et al. 2006; Niderla-Bielinska et al. 2015). The origin and nature of the inner hemangioblast-like population remain uncertain.

In the present study, using enhanced green fluorescent protein (EGFP)-Tol2 and fluorescent dye labeling in combination with quail-chick chimera, we examined the origin of coronary ECs and the relative contributions of their distinct sources for coronary vessels. The results showed that cells from the SV endothelium migrated into the PEO, subepicardial space, and ventricular wall, and contributed to coronary vessels in the ventricular free wall including coronary stems. Ventricular endocardium contributed mainly to the septal coronary vessels. The surface mesothelial cells of the PEO differentiated into coronary smooth muscle cells but a few of them also
contributed to the coronary endothelium.

Materials and methods

Chick and quail embryos

Fertilized eggs (chick \textit{Gallus gallus}, Shiroyama Farm, Kanagawa, Japan; quail \textit{Coturnix japonica}, Quail Cosmos, Aichi, Japan) were incubated at 37°C and 60% humidity. After an appropriate incubation period, 4 mL (1 mL in quail) of egg albumin was removed and a fenestration (1.5×2 cm) (1×1 cm in quail) was made, followed by injection of 10% carbon ink/Tyrode’s solution into the yolk sac beneath the embryo, and staged in accordance with Hamburger and Hamilton \textit{(Hamburger & Hamilton 1951)}.

Embryos were subjected to EGFP-Tol2 or dye labeling as well as chimera generation.

Animal handling and procedures were approved by the Osaka City University Animal Care and Use Committee, as set forth in the NIH Guide for the Care and Use of Laboratory Animals (Eighth Edition).

Preparation of EGFP-Tol2 transfection mixture

To label the target cells across the cell cycles, transfection of the EGFP-Tol2 system was attempted. The transfection mixture was prepared using Lipofectamine
In accordance with the manufacturer's instructions with a minor modification (https://www.thermofisher.com/jp/ja/home/references/protocols/cell-culture/transfection-protocol/lipofectamine-2000.html#procedure). Lipofectamine 2000 was diluted with Opti-MEM I (0.8 μL/μL, Thermo Fisher Scientific Inc.) and incubated for 5 minutes at room temperature. The same amount of CAGGS-transposase (pCAGGS-T2TP) (kindly donated by Dr Kawakami, National Institute of Genetics) and Tol2-flanked CAGGS-EGFP (pT2K-CAGGS-EGFP) (kindly donated by Dr Takahashi, Kyoto University) (Sato et al. 2007) were diluted with Opti-MEM I (0.28 μg/μL). The same amounts of diluted Lipofectamine 2000 and DNA were mixed gently and incubated for 20 minutes at room temperature. The resulting transfection mixture, which contained pCAGGS-T2TP (0.14 μg/μL), pT2K-CAGGS-EGFP (0.14 μg/μL) and Lipofectamine 2000 (0.4 μL/μL) (0.075%), was used for labeling experiments.

**Fluorescent labeling in ovo**

To label the SV ECs, 2 μL of acetylated low-density lipoprotein labeled with 1,1’-dioctadecyl-3,3,3’,3-tetramethylindo-carbocyanine perchlorate (DiI-LDL; 100 μg/mL in 0.05% fast green/phosphate-buffered saline [PBS]; Biomedical Technologies Inc., MA,
USA) was slowly injected into the peripheral vitelline vein of stage 14–15 embryos using a sharpened pulled-glass needle (10–20 μm in external diameter) equipped with a pressure injector (NARISHIGE, Tokyo, Japan). To label the surface mesothelial cells of the PEO, 2 μL of 5- and 6-carboxyfluorescein diacetate succinimidyl ester (CFSE; 100 μmol/L in Tyrode’s solution, Bioquest, CA, USA) (Kruithof et al. 2006) or 0.1–1 μL of EGFP-Tol2 transfection mixture was slowly microinjected into the pericardial cavity of stage 13–14 embryos using the pressure injector. To label the chick ventricular endocardium or quail SV endothelium, 0.1–0.2 μL of EGFP-Tol2 transfection mixture was slowly microinjected into the ventricle, atrium, or SV of cold-induced arrested hearts at stage 12. Embryos were left for 1 hour at room temperature. After an additional incubation period at 37°C, embryos were subjected to quail-chick chimera or organ culture. Fluorescently labeled target cells (ECs and mesothelial cells) and the labeling efficiency of EFGP-Tol2 system were shown in Fig. S1.

### Quail-chick chimera

Four types of quail-chick chimera were prepared in accordance with a modified method described by Männer (Fig. 1) (Männer 1999). To define the cranio-caudal orientation of the PEO, a small long pentagonal-shaped eggshell membrane was
inserted into the stage 16–17 quail SV posterior to the PEO. SV with the PEO, and
eggshell membrane were carefully extirpated and transplanted orthotopically into the
host chick embryo. To avoid contamination of host SV ECs and PEO cells into the
chimeric heart, the host chick PEO was cauterized using a small vessel cauterizer before
transplantation (FST Inc., CA, USA). To trace the ECs of SV, stage 16–17 quail SV with
the PEO, in which ECs had been labeled with EGFP-Tol2 or DiI-LDL at stage 14, was
orthotopically transplanted into the PEO-cauterized host chick embryos at stage 17
(n=26, Fig. 1A). To trace the surface mesothelial cells of the PEO, stage 16–17 quail SV
with PEO, in which mesothelial cells had been labeled with EGFP-Tol2 or CFSE at
stage 12–13, was orthotopically transplanted into the PEO-cauterized host chick
embryos at stage 17 (n=22, Fig. 1B). To trace both surface mesothelial cells of the PEO
and ECs of the SV, stage 16–17 quail SV with the PEO, in which mesothelial cells had
been labeled with CFSE at stage 12–13, followed by reincubation, and SV labeling with
DiI-LDL at stage 14, was orthotopically transplanted into PEO-cauterized host chick
embryos at stage 17 (n=7, Fig. 1C). To trace the ventricular endocardium, stage 16–17
unlabeled quail SV with the PEO was orthotopically transplanted into PEO-cauterized
host chick hearts, in which the endocardium had been transfected with EGFP-Tol2 at
stage 12 (n=3, Fig. 1D). Number of chimeric hearts examined was summarized in Table
S1. There was no obvious cardiac anomaly except an adherent transplanted tissue on the dorsal surface of the atrioventricular region. We reported previously that PEO-deleted embryos die before stage 31 because of defective epicardium/coronary vessels and thin myocardium; therefore, coronary vessels observed in the chimeric heart mainly originated from the transplanted SV with the PEO (Takahashi et al. 2014).

**Organ culture**

SV with the PEO or PEO alone was prepared from stage 16–17 chick or quail embryos, in which SV endothelial cells or surface mesothelial cells were labeled with fluorescent dye as described above, were resected and cultured on an 8-well chamber slide (Thermo Fisher Scientific, MA, USA) supplemented with 200 μL of serum-free medium (75% Dulbecco’s modified Eagle’s medium [DMEM], 25% McCoy’s medium, 10^−7 mol/L dexamethasone, and penicillin-streptomycin, Sigma-Aldrich, MO, USA) (Yanagawa et al. 2011). After 48–96 hours in culture, cultures were fixed with 4% paraformaldehyde in PBS and subjected to immunohistochemistry.

**Immunohistochemistry**

Embryos or hearts were fixed in 4% paraformaldehyde in PBS for 30 minutes at
room temperature (for anti-WT1 staining) or 3 hours at 4°C (for other antibodies). After extensive washing in PBS, samples were equilibrated in graded series of sucrose in PBS (7%, 15%, 20% W/V) and embedded in optimum cutting temperature (OCT) compound (Sakura, Tokyo, Japan) and frozen in liquid nitrogen. Frozen sections were cut using a cryostat and mounted on slides. After being rinsed in PBS, sections were blocked with 1% bovine serum albumin (BSA)/PBS for 1 hour at room temperature, incubated with a primary antibody for 2 hours at room temperature, rinsed with PBS, and incubated with a secondary antibody for 2 hours at room temperature followed by nuclei staining with 4’,6-diamino-2-phenylindole dehydrochloride (DAPI) for 20 minutes. After washing in PBS, samples were mounted and observed using a confocal laser microscope (Leica, Wetzlar, Germany) or conventional fluorescence microscope equipped with cooled CCD camera (Olympus, Tokyo, Japan).

Cultures were drained of medium, rinsed with PBS, and fixed with 4% paraformaldehyde/PBS for 30 minutes at room temperature. After washing with PBS, samples were blocked with 1% BSA/PBS containing 0.1% Triton X-100 (PBST) for 1 hour and incubated with a primary antibody for 2 hours at room temperature. Samples were rinsed with PBS, incubated with a secondary antibody, and the nuclei stained with DAPI. After washing, the samples were mounted in mounting medium and observed.
Using a Fluo Render (University of Utah, UT, USA), confocal images were stacked in the z-axis direction and cross-sectional images were reconstructed.

**Antibodies**

The following primary antibodies were used: mouse monoclonal anti-quail-specific endothelial marker QH1 (supernatant, 20x, Developmental Studies Hybridoma Bank, University of Iowa, IA, USA), anti-WT1 (rabbit polyclonal, SC192, 100x, Santa Cruz, Dallas, TX, USA), anti-smooth α-muscle actin (SMA, mouse monoclonal, clone 1A4, 500x, Sigma-Aldrich, MO, USA), anti-SM22α (rabbit serum, 200x, kindly donated Dr Kobayashi, Kagawa University, Kagawa, Japan) (Shishibori et al. 1996), and anti-calponin (mouse monoclonal, clone CP93, 150x, Sigma-Aldrich). Secondary antibodies were: TRITC-conjugated goat anti-mouse IgG1 (cat #1070-03, 100x, Southern Biotech, AL, USA), TRITC-conjugated goat anti-mouse IgG2a (cat #1080-03, 100x, Southern Biotech), Alexa Fluoro 405-conjugated goat anti-mouse IgG (cat#ab175661, 500x, Abcam, Cambridge, UK), TRITC-conjugated donkey anti-rabbit IgG (cat #AP182R, 100x, Millipore, Darmstadt, Germany), FITC-conjugated donkey anti-rabbit IgG (cat #AP182F, 100x, Millipore), and FITC-conjugated donkey anti-mouse IgG (cat #AP192F, 100x, Millipore).
Counting labeled cells and statistical analysis

Using an ImageJ (NIH), number of cells of interest (maker-positive, marker + EGFP [or fluorescent dye]-positive, EGFP [or fluorescent dye]-positive cells) was manually counted in histological images which were photographed under a 20× (or 40×) objective lens. Percentage of cells of interest in a certain cellular population was calculated. In stage 35 (E9 [embryonic day 9]) coronary artery, percentages of QH1-positive, EGFP-positive, and unlabeled cells were calculated in endothelial lining (more than 100 μm in length). Statistical analysis was performed by non-parametric Mann-Whitney U test and Bonferroni correction was used for multiple comparison. The significance level was set at <5%.

Results

The PEO consists of at least two cellular populations

At first we examined whether ECs of the SV contributed to the cellular population of the developing PEO. ECs in stage 14–15 chick embryos were labeled with DiI-LDL that had been injected via the peripheral vitelline vein to avoid mesothelial labeling. The embryos were reincubated and the PEO was inspected as to whether it
contained DiI-LDL-positive cells at stage 19. As shown in Fig. 2, DiI-LDL-positive but
WT1-negative cells were observed in the PEO core-mesenchyme (white arrowheads in
Fig. 2A). In histological sections, 76 ± 12% of the core-mesenchymal cells had DiI-
LDL (Fig. S2). Mesothelial cells of the PEO, which expressed WT1, were unlabeled
with DiI-LDL (yellow arrowheads in Fig. 2A). Similar experiments using quail embryos
showed that both DiI-LDL- and QH1-positive cells were observed in the PEO (Fig.
S1A). We next examined the distribution of mesothelial-derived cells in the PEO.
Mesothelial cells of the pericardial cavity were labeled with CFSE at stage 13, at which
point mesothelial protrusion began to take place, then embryos were reincubated, and
the distribution of CFSE-positive cells in the PEO was examined at stage 16–17. CFSE-
labeled cells, which expressed high levels of WT1, were distributed on the surface of
the PEO and its subjacent region (yellow arrowheads in Fig. 2B). CFSE-negative/WT1-
negative cells were observed within the PEO (white arrowheads in Fig. 2B). These
results suggested that the PEO consisted of at least two cellular populations, one
comprising SV endothelium-derived cells and the other mesothelium-derived cells.

SV-derived ECs contribute to coronary vessels in the
ventricular free wall
To investigate whether SV-derived ECs contributed to coronary vessels, quail PEOs with SV, in which the ECs had been labeled with EGFP-Tol2 or DiI-LDL, was orthotopically transplanted into PEO-cauterized host chick embryos (Fig. 1A). After reincubation, the hearts of chimeric embryos were subjected to anti-QH1 (ECs) or anti-smooth muscle α-actin (SMA) immunohistochemistry. Coronary vessels with SMA-positive tunica media are identified as the coronary artery before stage 39 (E 13) (Vrancken Peeters et al. 1997). In stage 23 (E4) hearts, QH1-positive cells, some of which had EGFP, were observed in the subepicardial space and ventricular myocardium of the dorsal atrioventricular and ventricular regions (arrowheads in Fig. 3A). At stage 27–29 (E5–6), QH1-positive cells, some of which had EGFP, showed a strand-like structure, and these vessel-like structures were found in the subepicardial space and myocardium of the atrioventricular canal and ventricular free wall (arrowheads in Fig. 3B, C). At stage 31–34 (E7–8), coronary arteries (stems) connected with the aortic sinuses. QH1- and/or EGFP-positive cells were found in ECs of both the coronary artery stem (arrowheads in Fig. 3D) and cardiac vein (arrows in Fig. 3E). Coronary vessels develop closely associated with cardiac lymphatics, however PEO does not contribute to lymphangioblasts (Wilting et al. 2007). Observations suggested that quail SV ECs contributed to coronary ECs in the ventricular free wall including the coronary
artery stem and cardiac vein in the host chick heart.

Cultured SV ECs migrate through the PEO and maintain endothelial character

We next investigated whether ECs of SV were capable of migrating through the PEO and differentiating into ECs in culture. PEOs with SV, in which ECs had been labeled with DiI-LDL, were cultured for 24–96 hours and stained with the anti-QH1 antibody. QH1-positive cells migrated through the PEO and expanded on the culture dish (Fig. 4A–C). High magnification images showed that these migrating QH1-positive cells contained DiI-LDL (arrowheads in Fig. 4D–F), indicating that SV ECs were capable of migrating through the PEO to expand on the culture dish. After 48 hours in culture, DiI-LDL- and QH1-positive cells showed a mesenchymal appearance with cellular processes (Fig. 4G–I), and 37 ± 2% of DiI-LDL-positive cells was stained with QH1 (Fig. 4M). After 72–96 hours in culture, these cells showed a cobblestone appearance (Fig. 4J–L), and 79 ± 3% of DiI-LDL-positive cells expressed QH1 after 96 hours (Fig. 4M). These results suggested that ECs migrating from the SV through the PEO maintained their endothelial nature/lineage, but some of the migrating SV-derived cells were unlabeled with QH1 at the beginning of/during migration.
Surface mesothelial cells of the PEO contribute to the coronary smooth muscle but few of them to ECs

To examine whether PEO mesothelial cells contributed to coronary ECs, quail PEOs, in which mesothelial surface cells had been labeled with EGFP-Tol2 or CFSE, was transplanted orthotopically into PEO-cauterized host chick embryos (Fig. 1B). The chimeric embryos were reincubated and heart sections were stained with an anti-QH1 antibody or anti-SMA antibody. In stage 23 (E4) chimeric hearts, EGFP-positive cells and QH1-positive cells were observed in the epicardium/subepicardial layer of the dorsal atrioventricular groove. Some of EGFP-positive cells had a QH1 epitope (arrowheads in Fig. 5A). In stage 29 (E6) hearts, EGFP-positive cells were observed in the epicardium/subepicardial layer at the atrioventricular groove (Fig. 5B), dorsal ventricular wall, and conotruncal base. Some EGFP-positive cells with a QH1 epitope were recruited into the QH1-positive vessel-like structures (arrowheads in Fig. 5B). In stage 34 (E8) hearts, EGFP-positive cells were observed in the epicardium, subepicardial, and myocardial layers (Fig. 5C, D). Many of these cells expressing SMA were incorporated into the SMA-positive smooth muscle layer of the coronary artery (arrows in Fig. 5C). Some EGFP/QH1-positive cells were incorporated in to the
coronary artery and cardiac vein (arrowheads in Fig. 5D). In tissue sections of stage 34 hearts, the percentage of SMA-positive cells in EGFP-positive cells was much greater than that of QH1-positive cells (Fig. S3). These results suggested that most surface mesothelial cells of the PEO differentiated into smooth muscle cells but some of them also differentiated into ECs.

Cultured PEO mesothelial cells differentiate into cells with smooth muscle markers but only a small population gained endothelial character

We next examined whether mesothelial cells of the PEO were capable of differentiating into ECs in culture. CFSE-labeled chick or quail PEOs (without SV) were cultured for 24–72 hours and stained with an anti-QH1 antibody and/or antibodies against smooth muscle antigens. Almost all the cells spread over the culture dish had CFSE, and more than 80% of these cells expressed smooth muscle markers (SM22α, SMA, calponin). On the other hand, approximately 5% of CFSE-positive cells were positively stained with the QH1 antibody (Fig. 6). Some CFSE/QH1-positive cells showed vessel-like strands (Fig. 6D–H), in which a luminal structure was detected using z-stack confocal images (Fig. 6H–K). The results suggested that CFSE-positive surface
mesothelial cells of the PEO mainly differentiated into cells with smooth muscle markers, but only a few of them had endothelial characteristics.

**QH1-positive subepicardial cells originated from SV endothelium**

The above experiments suggested that SV ECs contributed to coronary endothelium rather than surface mesothelial cells of the PEO. We next examined the ratio of SV-derived cells (or PEO mesothelial-derived cells) in QH1-positive cells in a region where the PEO adhered to the dorsal atrioventricular region. Quail PEO with SV, in which ECs and surface mesothelial cells had been labeled with DiI-LDL and CFSE, respectively, was orthotopically transplanted into PEO-cauterized chick embryo at stage 17 (Fig. 1C). In stage 24 (E4) hearts, CFSE-positive cells were distributed in the epicardium and its subjacent region, whereas DiI-LDL-positive cells were observed in the subepicardial and myocardial regions (Fig. 7A). QH1 staining showed that QH1-positive cells with DiI-LDL (arrowheads in Fig. 7B) were more predominant than those with CFSE (arrows in Fig. 7C). To examine the percentage of SV-derived or PEO-mesothelial derived cells in QH1-positive cell, quail PEO with SV, in which ECs or surface mesothelial cells had been labeled with fluorescent dye (Fig. 1A, B), was
transplanted. At stage 24 (E4), the percentage of DiI-LDL- or CFSE-positive cells in the QH1-positive cells were counted manually in tissue sections. The percentage of DiI-LDL-positive cells (44.5 ± 6.1%) in the QH1-stained cells was significantly higher than that of CFSE-positive cells (5.1 ± 0.9%) (P=0.0495, Mann-Whitney U test, Fig. 7D).

Results suggested that the majority of QH1-positive cells in the region where the PEO attached to the ventricle originated from the SV endothelium.

Ventricular endocardium contributes to the septal artery

We next examined whether ventricular endocardium contributed to coronary vessels in the chick heart. Unlabeled quail PEO with SV was orthotopically transplanted into PEO-cauterized chick hearts, in which the endocardium had been transfected with EGFP-Tol2 (Fig. 1D). In stage 35 (E9) chimeric hearts, QH1-positive cells were distributed in a compact myocardial layer in the left and right ventricular free wall, whereas EGFP-positive cells were observed in the endocardial lining of the trabecular layer (Fig. 8A, B). In the ventricular septum, QH1-positive cells were densely distributed in the dorsal region adjoining the ventricular free wall (Fig. 8C), whereas QH1-positive cells were sparse and EGFP-positive cells were observed in the middle to ventral aspect of the septum (arrowheads in Fig. 8C2, 3). At this stage, we observed
well-developed coronary stems as well as the septal artery (major coronary artery originating from the coronary stem in chick hearts) (Lindsay & Smith 1965), which had an SMA-positive medial layer (Fig. 8D1, F1). The endothelium of these main coronary arteries had both QH1-positive (quail SV origin, arrows in Fig. 8E, G) and EGFP-positive ECs (chick ventricle origin, arrowheads in Fig. 8E, G). The percentages of QH1- or EGFP-positive ECs in the coronary endothelium were calculated, in which the vascular segment was more than 100 μm in histological sections (Fig. 9). The result showed that 50–100% (mean 72%) of ECs in the coronary stems had QH1 immunoreactivity (SV derived cells) and the percentages of QH1-positive ECs were significantly high in both right and left coronary stems. In the dorsal segment of the septal artery 47% of ECs had QH1, whereas the rates were 14% in medial and 20% in the ventral segments. Less than 20% of ECs contained detectable amounts of EGFP in either the coronary stem or septal artery, and unlabeled ECs were predominant in the medial and ventral septal arteries. The origin of unlabeled coronary ECs was uncertain; however, the ventricular endocardium was a candidate for the source of unlabeled ECs rather than the host SV/PEO, because the host PEO was cauterized and 90% of the coronary ECs in the ventricular free wall were transplanted quail SV in origin (Fig. S4). These observations suggested that SV ECs were the major source of coronary stem and
coronary vessels in the ventricular free wall, whereas the ventricular endocardium contributed mainly to the ventricular septum. Schematic representations of the sources of coronary ECs were shown in Fig. 10.

**Discussion**

**PEO consists of at least two distinct tissue compartments in origin**

Our dye-labeling experiments showed that the mature PEO consisted of at least two distinct cellular populations, which were CFSE-labeled surface mesothelial cells highly expressing WT1, and DiI-LDL-labeled SV-derived inner mesenchymal-like population. In the mouse PEO, there are three genetically distinct cellular compartments, in which Wt1/Tbx18-, Scleraxis-, and Semaphorin3D-expressing cells are identified (Katz et al. 2012). Wt1-positive surface PEO cells differentiate into epicardium, smooth muscle cells, and interstitial cells. This cellular population is also required to form the coronary arterio-venous connection (Cano et al. 2016). Scleraxis-positive but Wt1-negative mesothelial cells are observed mainly in the surface of the PEO and are competent to give rise to coronary ECs (Katz et al. 2012). Semaphorin3D-positive cells, which are observed in the mesenchymal-like compartment of the mouse
PEO, differentiate not only into coronary ECs but also SV ECs (Katz et al. 2012). The surface mesothelial cells in the mouse PEO are similar in nature to avian surface PEO cells, which were capable of differentiating to smooth muscle cells and ECs *in vivo* and *in vitro*. Semaphorin3D-positive core compartment in the mouse PEO appears to be similar in nature to the WT1-negative inner population of the avian PEO. The origin of the core mesenchymal population remains uncertain. Our dye-labeling experiment suggests that the mesenchymal-like population in the PEO mainly originate from the SV ECs, because significant amount of the inner cells were labeled with DiI-LDL, which was injected into the peripheral vitelline vein, and SV ECs expressed an epithelial-mesenchymal transition marker, Slug, at the onset of/during PEO protrusion (Fig. S2).

However, we could not rule out the possibility that the liver bud-derived ECs contribute to the PEO in avian species (Cossette & Misra 2011), because there is no specific marker to identify the liver bud-derived angioblasts. The PEO is a transient but important structure because it provides epicardium, interstitial cells, and coronary vessels to maintain myocardial growth and maturation (Gittenberger-de Groot et al. 2000; Pennisi et al. 2003; Takahashi et al. 2014). However, little is known about the molecular and cellular mechanisms underlying the specification/determination and early development of the PEO (Niderla-Bielinska et al. 2015). Our *in vivo* and *in vitro*
analyses clarified that the PEO consists of at least two cellular populations, one was an SV-derived inner population giving rise to coronary ECs in the ventricular free wall, and the other was a surface mesothelial population giving rise to epicardium, vascular smooth muscle cells, and interstitial cells.

**SV is a major source of coronary ECs in ventricular free wall**

Prior to 2000, the origin of coronary ECs had been investigated using quail-chick chimeras (co-transplantation of PEO and SV), retrovirus cell tracing, and fluorescent dye labeling in avian species. These experiments are spatial labeling but not cell- or tissue-specific labeling because the PEO contains at least two distinct compartments in origin. Therefore, results from these experiments concluded that the PEO or PEO-derived epicardium is the major source of coronary ECs (Reese et al. 2002). Our tissue-specific labeling with quail-chick chimera and culture experiments showed that the SV endothelium is the major source of coronary ECs in the ventricular free wall including the coronary stems. The surface mesothelial cells of the PEO contributed to smooth muscle cells but few of them to ECs. Our labeling experiment showed that the ratio of the SV-derived population to the PEO-mesothelium-derived population in the subepicardial QH1-positive cells was 9:1 (Fig. 7D). This ratio is consistent with mouse
lineage tracing experiments showing that 70–80% of coronary ECs in the ventricular free wall originates from the SV (Zhang et al. 2016). Mouse genetic approaches have shown that the main source of coronary ECs is the SV (Red-Horse et al. 2010; Wu et al. 2012; Tian et al. 2013; Chen et al. 2014; Zhang et al. 2016). The SV-derived ECs migrate through the subepicardial space, dedifferentiate and redifferentiate into venous and arterial ECs via unknown signaling possibly involving VEGF, Shh, FGF, and Ang1 (Red-Horse et al. 2010; Wu et al. 2012; Nakajima & Imanaka-Yoshida 2013; Tian et al. 2013; Tian et al. 2015). In our culture experiment, SV-derived cells had QH1 epitopes in 37% of cells after 48 hours in culture, subsequently they expressed QH1 in 80% of cells after 96 hours in culture. These results suggested that the SV-derived migrating cells initially dedifferentiated and then redifferentiated into ECs. Another possibility is that the cells migrating from the SV are endothelial progenitors, because the SV endothelium consists of heterogeneous populations (Arita et al. 2014). Our in vivo and in vitro observations suggested that SV ECs are incorporated into the developing PEO, spread over the ventricular surface together with the epicardium, and contribute to coronary vessels in the ventricular free wall including coronary stems.

Ventricular endocardium contributes mainly to the septal
In our quail-chick chimeras, in which unlabeled quail SV with the PEO was co-
transplanted to endocardial-EGFP-labeled chick heart, EGFP-positive ECs were found
in the septal artery and coronary stem, whereas only a few EGFP-positive cells were
observed in the ventricular compact wall. In these chimeric hearts, QH1-
negative/EGFP-negative ECs were found in the septal arteries. This may be due to
methodological limitations of the Tol2-mediated EGFP expression system, in which the
transfection efficiency was low in the ventricular endocardium (Fig. S1B, E). In these
chimeric hearts, 90% of the coronary ECs in the ventricular compact wall were QH1-
positive cells originating from the transplanted quail SV (Fig. S4); therefore, the QH1-
negative/EGFP-negative coronary ECs in the ventricular septum were thought to
originate from the host ventricular endocardium rather than the host SV. This result was
consistent with that in mice, in which ventricular endocardium contributes to ventricular
septum, whereas SV contributes to ventricular free wall (Red-Horse et al. 2010; Tian et
al. 2013; Chen et al. 2014; Zhang et al. 2016). EGFP-positive ECs were also found in
the coronary artery stem. This observation was consistent with that in mice, in which the
ventricular endocardium contributes to the ventricular free wall of the ventral cardiac
base (Zhang et al. 2016). Another mouse model showed that the ventricular
endocardium is a major source of coronary arteries not only in the ventricular septum but also the ventricular free wall (Wu et al. 2012). The discrepancy between these two observations in the mouse models may be attributed to the genes that drive Cre-recombinase in target cells of interest, because the genes that drive Cre-recombination in cells are not exclusively cell-type specific (Tian et al. 2015; Zhang et al. 2016). In the neonatal mouse heart, ventricular endocardium differentiates into subendocardial coronary vasculature (Tian et al. 2014). Taken together with these observations, it appears plausible that the ventricular endocardium contributes to the coronary vessels in the ventricular septum as well as a minor part of the coronary stems.

Despite the methodological limitation, relatively low labeling efficiency of EGFP-Tol2 system, our results strongly suggested that 1) SV are the major source of coronary ECs of the ventricular free wall including the coronary stems; 2) the ventricular endocardium contributes mainly to septal coronary vessels; and 3) the surface mesothelium of the PEO is a minor source of ECs but is the source of coronary smooth muscles and interstitial cells.

Acknowledgments

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References


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Pennisi DJ, Ballard VL, Mikawa T. 2003. Epicardium is required for the full rate of myocyte proliferation and levels of expression of myocyte mitogenic factors fgf2 and its receptor, fgfr-1, but not for transmural myocardial patterning in the embryonic chick heart. *Dev. Dyn.*, **228**, 161-172.


Figure legends

**Fig. 1.** Quail-chick chimera. Four types of quail-chick chimera were prepared. (A) To trace the ECs of the SV, stage 16–17 quail SV with the PEO, in which ECs had been labeled with EGFP-Tol2 or DiI-LDL at stage 14, was orthotopically transplanted into PEO-cauterized host chick embryos at stage 17. (B) To trace the surface mesothelial cells of the PEO, stage 16–17 quail SV with the PEO, in which mesothelial cells had been labeled with EGFP-Tol2 or CFSE at stage 12–13, was orthotopically transplanted into PEO-cauterized host chick embryos at stage 17. (C) To trace both surface mesothelial cells of the PEO and ECs of the SV, stage 16–17 quail SV with the PEO, in which mesothelial cells had been labeled with CFSE at stage 12–13, followed by reincubation, and SV ECs labeling with DiI-LDL at stage 14, was orthotopically transplanted into PEO-cauterized host chick embryos at stage 17. (D) To trace the ventricular endocardium, unlabeled quail SV with the PEO was orthotopically transplanted into PEO-cauterized host chick hearts, in which the endocardium had been transfected with EGFP-Tol2 at stage 12.

**Fig. 2.** The PEO consists of at least two types of cells. (A) Stage 14–15 chick ECs were
labeled with DiI-LDL, then reincubated, and the PEO at stage 19 was stained with an anti-WT1 antibody. Stage 19 PEO contained DiI-LDL-positive/WT1-negative cells (white arrowheads) and DiI-LDL-negative/WT1-positive cells (yellow arrowheads). (B) Stage 13 chick PEO mesothelial cells were labeled with CFSE, then reincubated, and stage 16 PEO was stained an anti-WT1 antibody. CFSE-positive/WT1-positive cells were observed on the surface of the PEO (yellow arrowheads), whereas CFSE-negative/WT1-negative cells were present within the PEO (white arrowheads). At atrium; SV, sinus venosus, V, ventricle; scale bar, 25 μm

**Fig. 3.** SV ECs contribute to coronary vessels. Stage 16–17 quail SV with the PEO, in which ECs had been labeled with EGFP-Tol2 at stage 14, was orthotopically transplanted into PEO-cauterized host chick embryos at stage 17. After reincubation, the hearts of chimeric embryos were stained with anti-QH1 or anti-smooth muscle α-actin (SMA) antibodies. (A) At stage 23, QH1-positive cells, some of which had EGFP, were observed in the subepicardial and myocardial layer (arrowheads). (B, C) At stage 29, QH1-positive cells, some of which had EGFP, were observed as endothelial strands or vessel-like structures in the ventricular free wall (arrowheads). (D, E) At stage 34, QH1-positive and EGFP-positive cells were observed in not only SMA-positive coronary
stem (arrowheads) but also the SMA-negative cardiac vein (arrows). Note that panels D and E are daughter sections. Ao, ascending aorta; LA, left atrium; LV, left ventricle, PA, pulmonary trunk; scale bar, 100 μm (A4, B4, C4, D, E); 50 μm (B1–3); 25 μm (A1–3, C1–3).

**Fig. 4.** Cultured SV ECs migrate through the PEO and maintain endothelial lineage. PEOs with SV, in which ECs had been labeled with DiI-LDL, were cultured. Cultures were fixed and stained with an anti-QH1 antibody and observed using a confocal microscope. (A–F) DiI-LDL-labeled ECs of SV migrated through the PEO and expanded on the culture dish. High magnification (white box in A–C) showed that some of DiI-LDL-positive cells had QH1 immunoreactivity (arrowheads). Note that the yellow broken line indicates the eggshell membrane inserted into the SV. (G–L) DiI-LDL/QH1-positive cells appeared as mesenchyme-like appearance after 48 hours in culture, whereas these cells showed a cobblestone appearance after 72–96 hours. (M) Percentage incidence of QH1-positive cells in DiI-LDL-positive cells. 25–90 cells were examined in each explant. *, *P < 0.05 (Mann-Whitney U test after Bonferroni correction); NS, not significant; n, number of explants examined. Scale bar, 80 μm (A–C); 20 μm (others).
Fig. 5. Surface mesothelial cells of the PEO mostly contributed coronary smooth muscles and few to ECs. Quail SV with the PEO, in which mesothelial cells had been labeled with EGFP-Tol2 was orthotopically transplanted into host chick embryos. (A) At stage 23, EGFP-positive cells were observed in the subepicardial space and some EGFP-positive cells had QH1 immunoreactivity (arrowheads). (B) At stage 29, a few EGFP-positive cells were incorporated into QH1-positive vessel-like structures (arrowheads). (C) In stage 34 hearts, EGFP-positive cells were distributed in the epicardium, subepicardial (double-headed arrow), and myocardial layer. EGFP-positive cells were incorporated into the tunica media of coronary arteries (arrows). (D) A few EGFP-positive cells were observed in the QH1-positive coronary endothelium (arrowheads). Note that panel D is a daughter section of panel C; solid arrowheads indicate coronary artery and open arrowheads cardiac vein. AVC, atrioventricular canal; LA, left atrium; OFT, outflow tract; PA, pulmonary artery; RA, right atrium; qSV, quail sinus venosus; Scale bar, 25 μm (A1–3, B1–3, C1–3, D1–3); 100 μm (A4); 250 μm (B4, C4, D4).
Fig. 6. Cultured PEO mesothelial cells differentiated into a smooth muscle phenotype but a few differentiated into ECs. Chick or quail PEOs, in which mesothelial cells were labeled with CFSE, were cultured and stained with antibodies to detect smooth muscle markers or QH1. (A-C) After 72 hours in culture, almost all the cells possessed CFSE, and these CFSE-positive cells expressed smooth muscle markers. Approximately 5% (4.7 ± 4.2%) of CFSE-positive cells expressed QH1. n, number of explants examined. (D-K) Some CFSE-positive cells generated vessel-like strands labeled with QH1 (arrowheads) and luminal structures were observed using confocal microscopic three-dimensional reconstruction (indicated broken line in H). Scale bar, 20 μm (A, B, E–K); 50 μm (D).

Fig. 7. QH1-positive subepicardial cells are mainly derived from the SV. Quail PEO with SV, in which ECs and surface mesothelium had been labeled with DiI-LDL and CFSE, respectively, was orthotopically transplanted into PEO-cauterized chick embryos at stage 17, reincubated and stage 24 (E4) hearts were stained with QH1 antibody. (A) CFSE-positive cells were distributed mainly in the epicardium (double-headed arrow), whereas DiI-LDL-positive cells were in the subepicardial space and myocardium. (B, C) QH1 staining showed that QH1-positive cells with DiI-LDL (arrowheads) were more
predominant than those with CFSE (arrows). (D) To examine the significance of DiI-LDL-positive cells in QH1-stained cells, we made type A and type B chimeras (Fig. 1). At stage 24 (E4), QH1 staining was performed, and the percentage of DiI-LDL- or CFSE-positive cells in the QH1-positive cells were counted manually in region where the PEO attached to the ventricle. In QH1-stained cells, percentage of DiI-LDL-positive cells (44.5 ± 6.1%) was significantly higher than that of CFSE-positive cells (5.1 ± 0.9%) (P=0.0495, Mann-Whitney U test). n, number of embryos examined; V, ventricle; scale bar, 25 μm.

**Fig. 8.** Ventricular endocardium contributes to septal artery. Unlabeled quail PEO with SV was orthotopically transplanted into PEO-cauterized chick hearts, in which the ventricular endocardium had been transfected with EGFP-Tol2. (A, B) In stage 35 (E9) chimeric hearts, QH1-positive cells were distributed in a compact myocardial layer of left and right ventricular free wall. EGFP-positive cells were observed in the endocardium of the trabecular layer. (C) In the dorsal ventricular septum, QH1-positive cells were densely distributed, whereas EGFP-positive cells were observed in the middle to ventral aspect of the septum (arrowheads). (D, E) Coronary stems had an SMA-positive medial layer. In the stems EGFP-positive ECs were sparse (arrowhead),
but QH1-positive ECs were predominant (arrows). (F, G) Septal artery had SMA-positive medial layer. Both EGFP- (arrowheads) and QH1-positive (arrows) ECs were observed in dorsal septal arteries. Ao, aorta; LA, left atrium; LV, left ventricle; PA, pulmonary artery; RA, right atrium; RV, right ventricle; Scale bar, 250 μm (A4, B4, C4, E4, F4); 100 μm (A1–3, B1–3, C1–3, D4, G4); 25 μm (D1–3, E1–3, F1–3, G1–3).

**Fig. 9.** Endothelial fractions in coronary stem and septal artery. Quail PEO with SV was orthotopically transplanted into PEO-cauterized chick hearts, in which the ventricular endocardium had been transfected with EGFP-Tol2. At stage 35, chimeric hearts were stained with QH1, and the percentage of QH1- or EGFP-positive ECs was counted manually in the coronary endothelium, in which the vascular segment was more than 100 μm in histological sections. *, \( P<0.05 \); **, \( P<0.01 \) (Mann-Whitney U test after Bonferroni correction); NS, not significant; Red bar, % of QH1-positive ECs; green, EGFP-positive ECs; gray, unlabeled ECs; n, number of endothelial segments examined.

**Fig. 10.** Schematic representations of the sources of coronary ECs. (A) Ventricular septum is shown from the right ventricle (right ventricular free wall is removed). (B) Cross-section of the ventricles. In the ventricular free wall, coronary ECs originate from
SV endothelium (approximately 80%) and PEO surface mesothelium. In the ventricular septum, coronary ECs originate from the ventricular endocardium (approximately 70-80%) and SV endothelium in the middle to ventral region, while SV endothelium and the ventricular endocardium evenly contribute to the dorsal region. Ao, ascending aorta; LV, left ventricle; PA, pulmonary artery; RV, right ventricle; TV, tricuspid valve.

Legends for supporting information

Fig. S1. Fluorescently labeled ECs and mesothelial cells of the PEO. (A) Stage 19 heart. ECs in the SV, atrioventricular canal and ventricle (V) as well as endocardium-derived cushion mesenchymal cells were labeled with DiI-LDL, which had been injected into the peripheral vitelline vein at stage 15. ECs and endocardium-derived cushion mesenchymal cells were stained with an anti-QH1 antibody. (B) Stage 18 heart. ECs in the SV, atrioventricular canal and ventricle were labeled with EGFP-Tol2, which had been transfected at stage 12. (C) Stage 14 PEO. Surface mesothelial cells were labeled with CFSE, which had been injected into the pericardial cavity at stage 13. (D) Stage 16 PEO and stage 19 heart. WT1-positive mesothelial cells and epicardial cells expressed EGFP, which had been transfected at stage 13. (E) Labeling efficiency of EGFP-tol2 system in vivo. In tissue sections, percentages of EGFP-positive cells in the
endocardium, PEO surface cells and epicardium was manually calculated. The labeling efficiencies were 15.4 ± 4.2% in stage 18 endocardium/SV endothelium (n=3), 12 ± 1.1% in stage 35 (E9) endocardium (n=3), 7.3 ± 0.8% in stage 16 PEO surface mesothelium (n=3), and 24% in stage 19 epicardium (n=1). PEO, proepicardial organ; SV, sinus venosus; V, ventricle.

Fig. S2. SV endothelial cells expressed Slug and contribute to PEO-core mesenchyme. (A) Stage 16-17 chick PEO was stained with anti-Slug antibody (clone 62.1E6, mouse IgG1, Developmental Studies Hybridoma Bank). SV ECs and adjacent PEO-core mesenchymal cells expressed an epithelial-to-mesenchymal transition marker, Slug. (B) PEO-core mesenchymal cells were labeled with DiI-LDL, which had been injected into the peripheral vitelline vein at stage 14 (arrows). (C) In tissue sections, the percentage of DiI-LDL-positive cells in PEO-core mesenchyme (76 ± 12%) was greater than that of DiI-LDL-negative cells (24 ± 12%, P = 0.004, paired t-test). n, number of PEO examined; PEO, proepicardial organ; SV, sinus venosus; V, ventricle.

Fig. S3. Surface mesothelial cells of the PEO contributed mainly to the coronary smooth muscle cells.
Type B chimera, in which surface mesothelial cells were labeled with EGFP. After reincubation, stage 34 (E8) heart sections were stained with an anti-SMA or anti-QH1 antibody. Number of SMA- (or QH1)/EGFP-positive cells was manually counted in tissue sections (at least 5 sections were examined in each heart, n=2). The percentage of SMA- (or QH1)-positive cells in EGFP-positive cells was calculated and compared. *, P<0.05 (Mann-Whitney U test).

**Fig. S4.** Coronary ECs in the ventricular free wall of the chimeric heart originated from transplanted quail SV. Unlabeled quail SV with PEO was orthotopically transplanted into a PEO-cauterized chick heart and reincubated. At stage 38 (E12), FITC-conjugated lectin (LCA, *Lens culinaris* agglutinin) was injected into the ascending aorta to visualize coronary ECs. Hearts were fixed in 4% paraformaldehyde/PBS and cryostat sections were stained with an anti-QH1 antibody. The percentage of QH1-positive ECs in LCA-stained ECs (170 - 400 LCA-positive ECs in 5 sections were manually counted in each region) was calculated. In the left or right ventricular free wall, 90% of LCA-positive ECs was stained with QH1. In the ventricular septum, 58% of LCA-positive ECs was stained with QH1 in the dorsal region, while 30% in the medial region and 28% in the ventral region. LV, left ventricle; RV, right ventricle.
Table S1. Number of chimera hearts examined. Number of chimeric hearts in each experiment was summarized. Details of each chimera were shown in Fig. 1.

Fig. 1

Quail SV with PEO (blue bar, eggshell membrane).

SV ECs labeled with EGFP-Tol2 or Dil-LDL.

SV ECs labeled with Dil-LDL.

PEO mesothelial cells labeled with EGFP-Tol2 or CFSE.

Green line indicates an endocardium labeled with EGFP-Tol2.

Fig. 2
Fig. 3
Fig. 7
Fig. 8
**Fig. 9**

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% of QH-1, EGFP or unlabeled ECs (mean ± SD)

- Left (n=4)
- Right (n=8)
- Dorsal (n=3)
- Medial (n=10)
- Ventral (n=4)

Coronary Stem

Septal artery

**Fig. 10**

- Red: Coronary ECs from SV endothelium
- Green: Coronary ECs from ventricular endocardium
- Brown: Coronary ECs from PEO surface mesothelium

A

PA

Ao

Dorsal

Ventral

TV

B

RV

LV

Dorsal

Ventral
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**Table S1**

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