ARTICLES

Coronary arteries form by developmental reprogramming of venous cells

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Coronary artery disease is the leading cause of death worldwide. Determining the coronary artery developmental program could aid understanding of the disease and lead to new treatments, but many aspects of the process, including their developmental origin, remain obscure. Here we show, using histological and clonal analysis in mice and cardiac organ culture, that coronary vessels arise from angiogenic sprouts of the sinus venosus—the vein that returns blood to the embryonic heart. Sprouting venous endothelial cells dedifferentiate as they migrate over and invade the myocardium. Invading cells differentiate into arteries and capillaries; cells on the surface redifferentiate into veins. These results show that some differentiated venous cells retain developmental plasticity, and indicate that position-specific cardiac signals trigger their dedifferentiation and conversion into coronary arteries, capillaries and veins. Understanding this new reprogramming process and identifying the endogenous signals should suggest more natural ways of engineering coronary bypass grafts and revascularizing the heart.

There has been tremendous progress during the past two decades in elucidating the general principles of blood vessel development and the factors that control it[1–4], especially during tumour angiogenesis[5,6]. Comparatively little is known, however, about the cellular origins and developmental programs of many of our most important vessels and vascular beds, such as the coronary arteries that supply the heart muscle (myocardium) and its pacemaker[7–9]. Seven million people die each year of coronary artery disease and its sequelae, myocardial infarction and cardiac arrest, making it the leading cause of death worldwide[10]. Tens of millions of others manage the disease medically or undergo major interventions such as angioplasty or coronary bypass surgery[10]. Determining how coronary vessels arise during development and are maintained in adult life, and how they are remodelled under pathological conditions such as arteriosclerosis, should further our understanding of the disease. It could also lead to new treatments that stimulate vessel growth, and new ways of engineering bypass grafts with the flow properties and durability of healthy young vessels.

The development of coronary arteries has been studied in a variety of animals for more than a century. Early anatomical studies in humans and other mammals suggested that they bud from the aorta[11–14]. More recent experiments with chick–quail chimaeras argued against this and suggested instead that they arise from the proepicardium, a transitory structure in the embryo that contacts and spreads over the developing heart to form its epithelial covering (epicardium) and several internal tissues[15–17]. The chick experiments and subsequent studies led to the current textbook view that coronary vessels form from proepicardial cells that undergo an epithelial-to-mesenchymal transition, and then differentiate into isolated endothelial progenitors that assemble *de novo* ('vasculogenesis') into endothelial tubes[7,18]. However, not all data from chick is easily reconciled with this model[9,19], and recent lineage-tracing experiments in mouse show that, although the proepicardium gives rise to myocardial stroma and vascular smooth muscle, it gives rise to few, if any coronary endothelial cells[20–23]. Thus, the origin of coronary artery endothelial cells, which are among the most medically important cells in the body, remains an enigma.

Here we use histological and clonal analysis in mouse, and cardiac organ culture, to investigate the origins and early development of coronary arteries. The results show that coronary arteries do not derive from the proepicardium but from endothelial sprouts of the sinus venosus—the venous inflow tract of the embryonic heart—with a small contribution from endocardium lining the cardiac chambers. The patterns of migration and marker expression of the venous sprouts suggest a model in which local signals in the developing heart induce angiogenic outgrowth, dedifferentiation, and stepwise conversion into coronary arteries, capillaries and veins.

Histological analysis of coronary development

We carried out a thorough anatomical and histological analysis of coronary vessel development during mouse embryogenesis using endothelial markers (Fig. 1 and Supplementary Fig. 1). Because canonical endothelial markers also label endocardial cells and can thus complicate the analysis (Supplementary Fig. 2), we used an apelin-nlacZ knock-in mouse strain[24] that selectively expresses nuclear β-galactosidase in coronary endothelial cells but not in endocardium (Supplementary Fig. 3). X-gal staining showed an expanding vascular plexus on the heart beginning at embryonic day (E) 11.5 (Fig. 1a–j), indistinguishable from that detected by CD31 (also known as PECAM1) immunostaining (Supplementary Fig. 2)[25,26]. The plexus originated on the dorsal cardiac surface (Fig. 1a), and expanded around the atrioventricular canal (Fig. 1b) to reach the outflow tract (Fig. 1d, f) and invade the ventral interventricular groove (Fig. 1f). It also expanded caudally (Fig. 1c, e), populating the entire dorsal surface by E14.5 (Fig. 1g); during this period (E12.5–14.5), dorsal plexus sprouts invaded the underlying myocardium (Supplementary Fig. 1). Larger diameter coronary arteries appeared within the myocardium at E14.5 and subsequently matured (Fig. 1k, l, r). By E15.5, the entire ventral surface was also covered with plexus (Fig. 1j), and coronary veins began to appear on the dorsal surface (Fig. 1i). There were also isolated clusters of endothelial cells surrounding blood cells, including erythrocytes ($TER119^+$, also known as $LY76^+$), platelets ($CD41^+$, also known as $ITGA2B^+$), and

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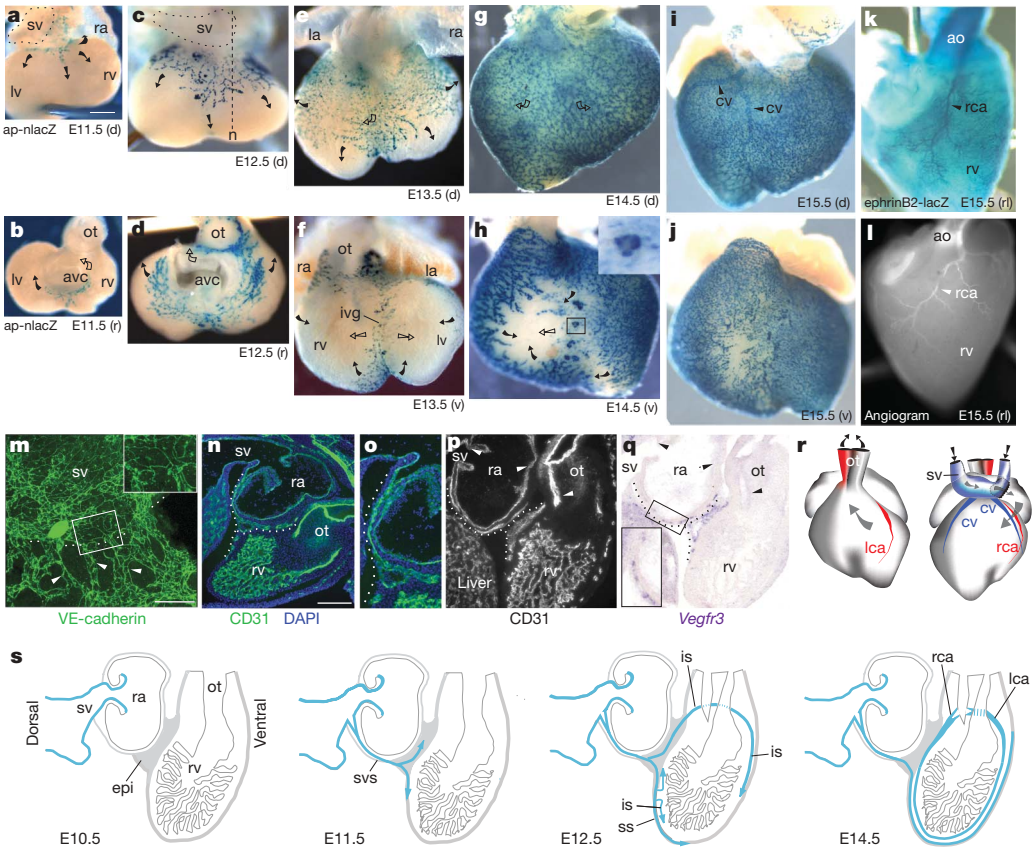

Figure 1 | Coronary vessels sprout from the sinus venosus. **a**–**j**, X-gal-stained apelin-nlacZ (ap-nlacZ) hearts from the embryonic days indicated, shown in dorsal (d), rostral (r) and ventral (v) views. Coronary vessels (blue nuclei) originate near the sinus venosus (outlined in **a** and **c**) and spread dorsally (**a**, **c**, **e**, **g**) and around the atrioventricular canal (avc) (**b**, **d**) and outflow tract (ot) (**d**) to the ventral interventricular groove (ivg) (**f**). Arrows indicate direction of new growth on the surface (filled) or in deeper layers (open). Inset in **h** is close-up of boxed blood island-like structure. la, left atrium; lv, left ventricle; ra, right atrium; rv, right ventricle. Scale bar, 200 μm (for **a**–**l**). **k**, X-gal-stained E15.5 ephrinB2-lacZ heart (right lateral (rl) view) showing right coronary artery (rca, arrowhead). ao, aorta. **l**, Angiogram of E15.5 heart showing right coronary artery (arrowhead). **m**, Frontal section through E11.5 heart immunostained for VE-cadherin. Coronary sprouts (green, arrowheads) are continuous with the sinus venosus (sv; above dashed line). Inset is close-up of boxed region showing junction between sinus venosus and coronary sprouts. Scale bar, 100 μm. **n**, Sagittal section (approximate section plane shown in **c**) through E12.5 heart stained for CD31 (green) and with the nuclear dye DAPI (blue). Coronary sprout (dotted line) extends from the sinus venosus and courses around right atrium to reach right ventricle. **o**, Close-up of coronary sprout in **n**. **p**, **q**, Adjacent sections from E11.5 heart immunostained for CD31 (**p**) and probed for *Vegfr3* messenger RNA (purple) (**q**). Coronary vessels (dotted line) express *Vegfr3*, an angiogenic sprout marker, whereas vessels formed by vasculogenesis (sinus venosus, atria, outflow tract) do not (arrowheads). Inset in **q** is close-up of boxed region. Scale bar, 200 μm (for **n**, **p**, **q**). **r**, Schematic of right and left (lca) coronary arteries and coronary veins (cv) at E15.5. Arrows show blood flow direction. **s**, Schematic sagittal sections showing coronary sprout (blue) progression during development. epi, epicardium; is, invading sprouts; ss, superficial sprouts; svs, sinus venosus sprouts.

leukocytes ($CD45^+$, also known as $PTPRC^+$), which appeared transiently at the interventricular groove (Fig. 1h, inset and Supplementary Fig. 2); these were noted previously and called 'blood islands'[14,27], but their origins and developmental fate are unknown (see later).

Close inspection of the site where the plexus originated (Fig. 1a, m–o) suggested that the source of endothelial cells is the sinus venosus. Vessels extending from the sinus venosus expressed apelin and three other angiogenic sprout markers[28–31] (*Vegfr3* (also known as *Flt4*), *Dll4* and *Pdgfb*) (Fig. 1p, q and data not shown), supporting an angiogenic origin. These sprouts grew through the inflow tract myocardium and onto the cardiac surface, traversing a stereotyped path as they extended to invest the heart (Fig. 1n, s). Apart from these sprouts and adjacent liver plexus, we did not detect any endothelial cells in or associated with the proepicardium (Supplementary Fig. 4), nor did we detect any proepicardial cells that became sinus venosus or other endothelial cells by Tbx18-Cre lineage tracing (Supplementary Fig. 5). We also detected only extremely rare $VEGFR2^+CD31^-$ cells—putative endothelial progenitors ('angioblasts') that are abundant around vessels forming by vasculogenesis in other body regions[32]—in the proepicardium or heart wall (Supplementary Fig. 6). Thus, coronary vessel progenitors seem to arise from sinus venosus angiogenic sprouts, not from the proepicardium or other vasculogenic sources.

Analysis of coronary origins *in vitro*

We tested the sinus venosus sprouting model in two ways. First, the origins and requirements for coronary sprouting were analysed in a cardiac organ culture system[26] modified to allow tissue recombination experiments (see Methods). Developing hearts were isolated from apelin-nLacZ mice at E10.5, after the proepicardium has spread over the heart surface to form the epicardium but before any coronary sprouts are present. Hearts were either left intact or dissected to separate the ventricles with their epicardial covering (V/Epi) from sinus venosus and atria (SV/A), and then cultured for 72 h and stained for coronary endothelial markers (apelin-nlacZ or CD31) to determine whether coronary vessels had formed (Fig. 2). All of the dissected chambers ($n = 39$) appeared healthy and continued to beat throughout the culture period, as did control hearts that were left intact ($n = 24$) (Supplementary Movies 1–4). However, whereas coronary sprouts formed in 22 out of 24 (92%) intact control hearts, none developed when the V/Epi ($n = 30$) or SV/A ($n = 9$) was cultured alone (Fig. 2a–c, f). In tissue recombination experiments in which a dissected SV/A from apelin-nlacZ transgenic mice was recombined with a wild-type (non-transgenic) V/Epi, coronary vessels formed on the V/Epi and expressed the apelin-nlacZ transgene (Fig. 2d; 13 of 20, 65%). By contrast, no coronary vessels expressing the transgene

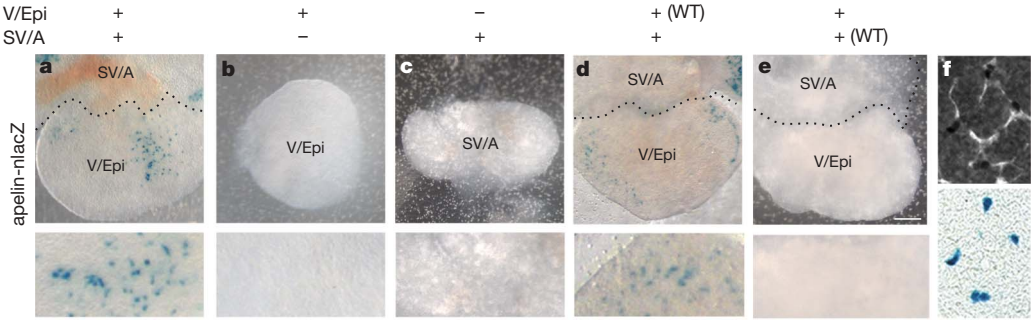

Figure 2 | Analysis of coronary vessel sprouting *in vitro*. **a**–**e**, Intact heart (**a**) or dissected sinus venosus/atria (SV/A) or ventricle/epicardium (V/Epi) as indicated from E10.5 apelin-nlacZ embryos (or wild type (WT) where noted) cultured for 3 days at 37 °C and stained with X-gal (blue nuclei) to show coronary development. Coronary sprouts formed only on intact hearts (**a**) or when dissected SV/A were recombined with V/Epi (**d**). Vessels arise from SV/A because no X-gal$^+$ sprouts were detected when SV/A was from wild-type (non-transgenic) embryo (**e**). Close-up views are shown below each panel. **f**, Close-up of a cultured intact heart as in **a** stained for CD31 (top) and with X-gal (bottom), confirming lacZ-positive (blue) nuclei are in coronary vessels. Scale bar, 200 μm.

developed when a wild-type SV/A was cultured with an apelin-nlacZ V/Epi (Fig. 2e; $n = 5$). Hence, apelin-nlacZ$^+$ coronary vessels arise from the dissected SV/A tissue, not the epicardial cells or other proepicardial derivatives in the dissected V/Epi, but they require signals provided by the V/Epi for sprouting and outgrowth.

Clonal analysis of coronary development

We next tested the sinus venosus sprouting model by clonal analysis, which allowed us to map the origin and determine the proliferation rate, outgrowth pathway, and fate of coronary endothelial progenitors *in vivo*. We used a VE-cadherin-CreER transgene that expresses a tamoxifen-inducible Cre recombinase[33], in combination with Cre-dependent marker genes (see Methods), to permanently label individual endothelial cells and their descendants. Recombination was induced by tamoxifen administration between E7.5 and E9.5 and analysed 4–7 days later. Limiting doses of tamoxifen were used, and hearts with a single cluster of marked cells (putative clone) or two or three well-isolated clusters were analysed (Fig. 3a, b). A multicolour Cre-dependent marker similar to Brainbow[34] was used to confirm clonality of clusters (Fig. 3c–h). The endothelial cell proliferation rate estimated from the clonal analysis (Supplementary Table 1) was 6–19 h for nearly all (23 of 25) of the endothelial clones.

The clonal analysis (Supplementary Table 1 and Supplementary Fig. 7) established two key aspects of the sinus venosus sprouting model. First, because coronary endothelial clones were consistently obtained after early (E7.5) induction of VE-cadherin-CreER, coronary artery progenitors must arise from differentiated (VE-cadherin$^+$) endothelial cells present at this age. This is the result expected from the sinus venosus sprouting model, and it argues against a proepicardial origin because no VE-cadherin$^+$ cells other than sinus venosus sprouts and liver plexus are associated with the proepicardial organ (Supplementary Fig. 4). Second, most coronary artery cells are clonally related to sinus venosus cells. In total, 25 out of 34 coronary endothelial clones, and all 20 that contained more than 85 coronary endothelial cells, had labelled sister cells within the sinus venosus (Fig. 3a, c–e, Supplementary Table 1 and Supplementary Fig. 7). These clones spanned the coronary plexus from the sinus venosus at the dorsal side of the heart, where plexus formation begins, to the growing front of the plexus (Supplementary Fig. 7, type I). The longer the period between clone induction and analysis, the farther the clones extended from the sinus venosus, consistent with a sinus venosus origin. At the latest times examined, clones included endothelial cells of coronary arteries, veins and capillaries, demonstrating that a single labelled cell can be reprogrammed to form not just coronary artery but all three types of coronary endothelial cells (Fig. 3e and Supplementary Table 1).

The clonal analysis revealed a minor secondary source of coronary endothelial cells. The nine coronary endothelial clones that did not contain any sinus venosus sister cells each contained sister cells in a patch of ventricular (8 out of 9) or atrial (1 out of 9) endocardium

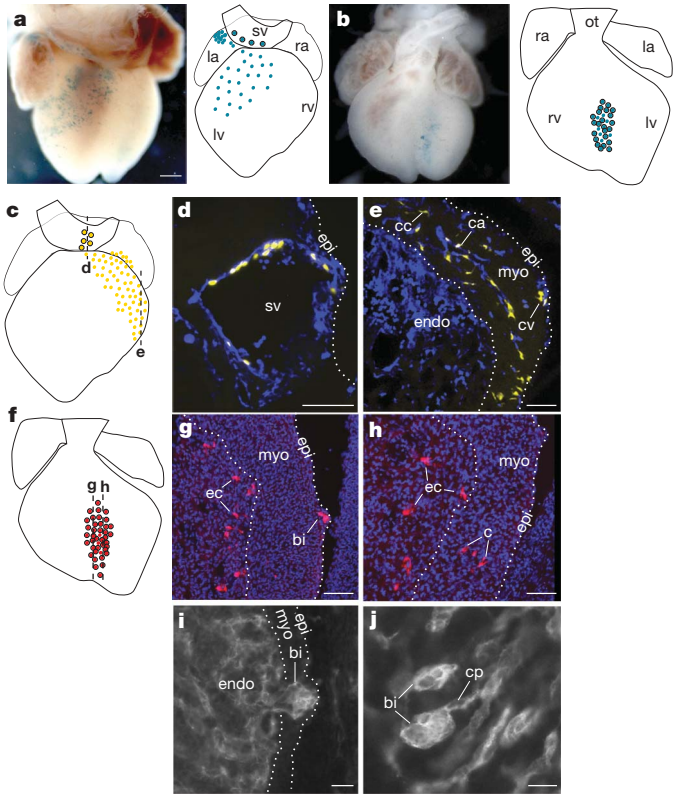

Figure 3 | Clonal analysis of coronary artery development. **a**–**h**, Micrographs and schematics of VE-cadherin-CreER-induced coronary clones at E13.5 (**b**, **f**) and E14.5 (**a**, **c**) marked with Rosa-lacZ (**a**, **b**) or multicolour (**c**–**h**) in which marked cells permanently express one of three different fluorescent reporters. Coloured dots in schematics represent 10 cells in clone. Outlined dots are sister cells in sinus venosus or endocardium. **a**, Coronary clone (no. 24 in Supplementary Table 1) with sister cells in sinus venosus. Scale bar, 200 μm (for **a**, **b**). **b**, Coronary clone (no. 34) with sister cells in ventricular endocardium. **c**–**e**, Schematic (**c**) and two sections (**d**, **e**, dashed lines in **c**) of a coronary clone (no. 25) with sister cells in sinus venosus (**d**) and in coronary artery (ca), coronary capillary (cc), and coronary vein (cv) (**e**). CD31 immunostaining (blue) shows all endothelial and endocardial cells. **f**–**h**, Schematic (**f**) and sections (**g**, **h**) of coronary clone (no. 28) with sister cells in coronary vessels (c), endocardial cells (ec), and adjacent blood island (bi). Nuclei are DAPI-stained (blue). Scale bars, 100 μm (**d**, **e**, **g**, **h**). **i**, **j**, Sections of E11.5 (**i**) and E12.5 (**j**) hearts immunostained for CD31 showing blood island-like structures budding from endocardium (endo) into myocardium (myo) (**i**) and joining the coronary vascular plexus (cp) (**j**). Scale bars, 25 μm.

underlying the clone (Fig. 3b, f–h and Supplementary Table 1). The presence of blood island-like structures in four of the ventricular clones, and their location near the interventricular groove, indicate that they correspond to the endothelial blood islands noted earlier in the histological analysis (Fig. 1h and Supplementary Fig. 2). Thus, this population of endocardium-derived coronary endothelial cells may form by endocardial budding through the myocardium, pinching-off and forming endothelial spheres with entrapped blood cells (blood islands) that later join the sinus-venosus-derived plexus. In support of this, we identified putative intermediates in which endocardial buds were seen penetrating the myocardium (Fig. 3i), and blood islands were caught joining the coronary plexus (Fig. 3j).

Marker expression during venous reprogramming

The experiments described establish that coronary artery progenitors arise primarily from VE-cadherin$^+$ cells that sprout from the sinus venosus and migrate over the heart and into the myocardium as they become coronary arteries. Because the path is stereotyped, we could analyse molecular transitions during the process. Marker analysis showed that sprouting sinus venosus cells, like other sinus venosus cells, are initially differentiated (EphB4-lacZ and *COUP-TF2*$^+$ (also known as *Nr2f2*$^+$)) venous cells. But as they extend out from the sinus venosus, expression of the markers rapidly declines and venous identity is lost (Fig. 4a, e and Supplementary Fig. 8). A day later (E12.5), vessels that have invaded the myocardium begin to express ephrinB2-lacZ (Fig. 4c)[35] and other arterial markers (*Dll4*, *Hey1*, *Notch4* and *Depp* (also known as *8430408G22Rik*)) (Supplementary Fig. 9), and subsequently assemble into mature coronary arteries (Fig. 4d and Supplementary Fig. 9). Vessels that remain superficial turn on EphB4-lacZ (Fig. 4b)[35] and other venous markers (*Vegfr3*, *Np2*, *Aplnr* and *COUP-TF2*) as they form mature coronary veins (Supplementary Fig. 9).

Discussion

Our results resolve the century-old enigma of the origin of coronary arteries and demonstrate two surprising sources of progenitors and a new program of arteriogenesis. The major source is differentiated venous endothelial cells of the sinus venosus, which sprout onto the developing heart. There they dedifferentiate, proliferate and spread to form the coronary plexus, and subsequently redifferentiate and remodel into coronary arteries, capillaries and veins (Supplementary Fig. 10). A minor secondary source is the endocardium, from which cells separate to form blood islands and then join the coronary plexus near the interventricular septum. Our data do not exclude rare contributions from other sources, or distinct origins in other species such as chick.

Coronary sprouts follow a specific outgrowth path, and dedifferentiation and specific redifferentiation events occur at stereotyped times and positions. This suggests that local signals along the outgrowth path function not only as sprout inducers and guidance cues, but also serve to sequentially deprogram sinus venosus venous endothelial cells and reprogram them to coronary artery, venous and capillary fates (Supplementary Fig. 10). Our results demonstrate that differentiated venous cells can give rise to arterial vessels[36], and we speculate that other organ-specific vascular beds, such as those of the retina and kidney, form in a similar manner: by developmental reprogramming of differentiated venous cells. Indeed, at least some venous cells in other parts of the body seem to retain plasticity because they can give rise to lymphatic vessels during development[4]. Furthermore, experimental manipulations such as flow reversal[37] and transplantation[38,39] have been shown to alter the identity of embryonic venous cells, and adult saphenous veins used in coronary bypass grafts downregulate venous markers[40]. However, venous grafts do not acquire a full arterial phenotype[40], which probably contributes to their lower success rate compared to arterial grafts[41,42].

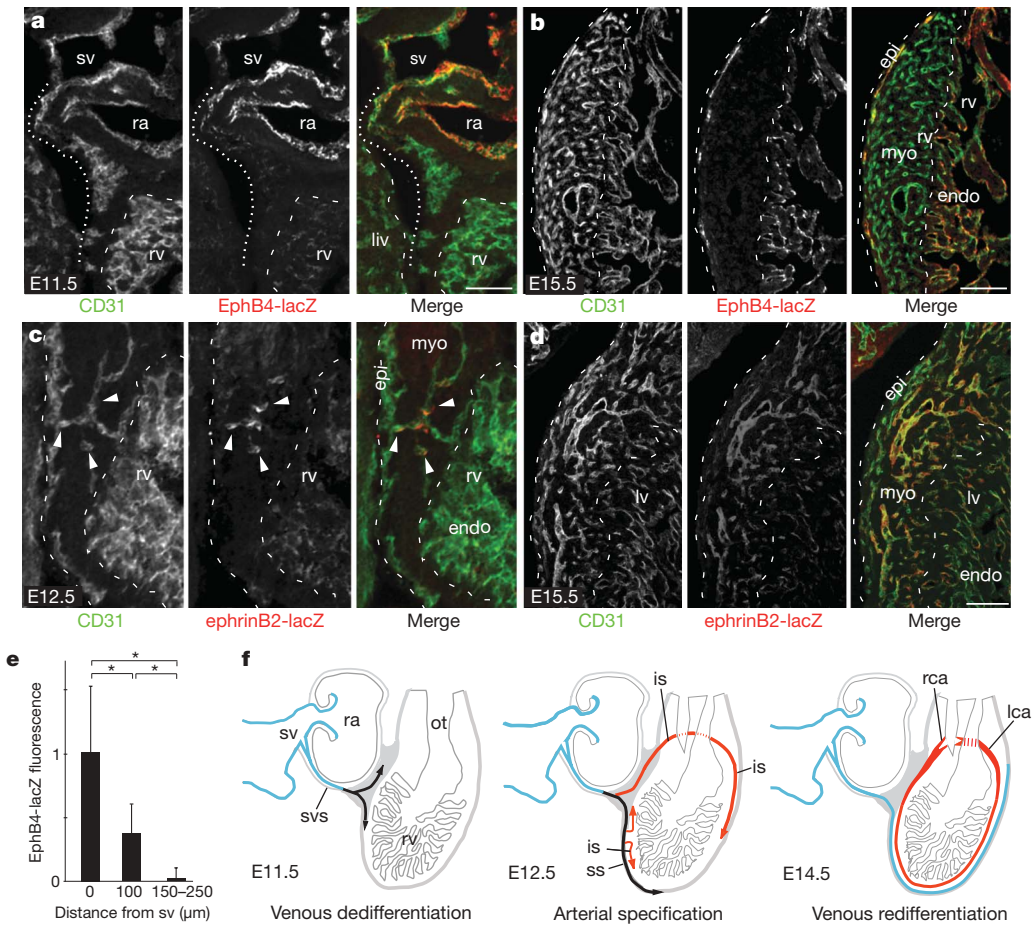

Figure 4 | Downregulation of venous markers and induction of arterial markers during coronary artery development. **a**, Sagittal section through heart of E11.5 EphB4-lacZ embryo immunostained for CD31 (green) and β-gal (EphB4-lacZ, red). Coronary sprouts (dotted line) downregulate EphB4-lacZ as they migrate away from sinus venosus. liv, liver. Scale bar, 100 μm. **b**, E15.5 EphB4-lacZ heart. Vessels on surface (epicardium) have re-acquired EphB4-lacZ expression. Scale bar, 200 μm. **c**, Sagittal section through E12.5 ephrinB2-lacZ heart. Coronary sprouts that have invaded myocardium (green, arrowheads) upregulate artery/capillary marker ephrinB2-lacZ (red), whereas surface vessels (green, dashed line at left) do not. **d**, E15.5 ephrinB2-lacZ heart. ephrinB2-lacZ is expressed by all arteries and capillaries within myocardium but not by surface vessels. Scale bar, 100 μm. **e**, Quantification of EphB4-lacZ expression in coronary sprouts budding from sinus venosus at E11.5. Values are from double-stained hearts as in **a**, normalized to CD31 staining at the same position. $n = 8$ for each position. Error bars, s.d. $^*P < 0.002$ by Student's t-test. **f**, Schematic showing changes in venous (blue) and arterial (red) marker expression during coronary development; black indicates dedifferentiated venous cells.

Identification of the endogenous signals that control coronary artery development, especially the dedifferentiation and reprogramming factors, would begin to explain the molecular basis of this new developmental process and suggest more natural ways of inducing new coronary vessels and engineering bypass grafts.

METHODS SUMMARY

Wild-type (CD1) and transgenic marker, Cre recombinase, and Cre reporter mouse strains are described in Methods. Staged embryos and hearts from timed pregnancies (the morning of vaginal plug was designated E0.5) were dissected and fixed in 4% paraformaldehyde and stored in PBS. Fixed tissues were left intact or sectioned, and then processed for whole-mount histochemistry (X-gal), immunohistochemistry, indirect or direct immunofluorescence, and RNA *in situ* hybridization with digoxigenin-labelled antisense probes as described in Methods. Specimens were imaged on a stereomicroscope (whole-mount tissue) or fluorescence compound or confocal microscopes (tissue sections); images were digitally captured and processed.

For organ culture, dissected hearts from E10.5 or E11 wild-type or apelin-nlacZ embryos were placed on polycarbonate filters at the air–liquid interface of DMEM media with supplements. Some hearts were cultured intact; others were dissected into SV/A and ventricle and the fragments cultured alone or recombined with an SV/A contacting a ventricle. Cultures were maintained at 37 °C and 5% CO_2 for 72 h, then fixed and subjected to whole-mount X-gal staining to detect apelin-nlacZ$^+$ vessels.

For clonal analysis, VE-cadherin-CreER mice were crossed to Rosa-lacZ or multicolour Cre recombination reporters. At E7.5, E8.5 or E9.5, dams received intraperitoneal injections of low tamoxifen doses to activate CreER and induce rare recombination events, generating single or well-separated clones of endothelial cells that constitutively express β-galactosidase (Rosa-lacZ) or one of three different fluorescent proteins (multicolour). At E13.5 or E14.5, embryos were dissected, fixed and stained to visualize the marked endothelial clones: embryos carrying Rosa-lacZ were stained with X-gal and the endothelial-specific antibody anti-CD31, and multicolour embryos were sectioned and stained with 4′,6-diamidino-2-phenylindole (DAPI) or anti-CD31 antibody, and analysed with fluorophore-specific filters.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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METHODS

Animals. CD1 mice (Charles River Laboratories) were used for wild-type analysis. EphB4-lacZ (Jackson Laboratories strain B6.129S7-*Ephb4*^{tm1And}/J), ephrinB2-lacZ (B6.129S7-*Efnb2*^{tm2And}/J), VE-cadherin-Cre (B6.Cg-Tg(Cdh5-cre)7Mlia/J), the Cre reporter strains Rosa-lacZ (B6.129S4-*Gt(ROSA)26Sor*^{tm1Sor}/J) and Rosa-YFP (B6.129X1-*Gt(ROSA)26Sor*$^{tm1(EYFP)Cos}$/J), apelin-nlacZ[24], VE-cadherin-CreER^{T2} (ref. 33), and Tbx18-Cre[22] mice have been described. The multicolour Cre reporter mice contain a transgene that constitutively expresses green fluorescent protein (GFP), and in the presence of Cre recombinase is randomly recombined once to express instead one of three other fluorescent proteins: mCherry, mOrange or Cerulean, similar to the Brainbow system[34]. Details of this reporter will be described elsewhere (H.U. and I.L.W., manuscript in preparation).

Immunohistochemistry. Staged embryos and hearts generated from timed pregnancies, where the morning of the vaginal plug was designated E0.5, were dissected and fixed in 4% paraformaldehyde for 1–2 h and stored in PBS at 4 °C. Whole-mount hearts were either incubated with X-gal (Sigma) to visualize β-galactosidase activity or processed for immunohistochemistry as described[43]. Tissue sections were prepared by cryoprotecting fixed tissue in 20% sucrose at room temperature for 1 h or overnight at 4 °C, snap-freezing in optical cutting temperature compound (OCT, Tissue Tek), and sectioning with a Leica CM3050 S cryostat. Sections (20 or 80 μm) were washed in PBS and incubated with primary antibodies diluted in blocking solution (5% goat serum, 0.5% Triton X-100 in PBS) at room temperature for 1–3 h or overnight at 4 °C. Sections were then washed in PBS and incubated with fluorescent-conjugated secondary antibodies for 1 h at room temperature. Antibodies used were: anti-CD31 (1:100 dilution, BD Pharmingen), anti-VEGFR2 (1:100 dilution, BD Pharmingen), fluorescein isothiocyanate (FITC)-coupled anti-CD34 (1:100 dilution, BD Pharmingen), anti-Wt1 (undiluted, DAKO), anti-β-galactosidase (1:500, Immunology Consultants Laboratory), anti-α4 integrin (1:100, BD Pharmingen), anti-VE-cadherin (1:100, BD Pharmingen), anti-GFP (1:500, Abcam), and Alexa-555- and -488-conjugated secondary antibodies (1:250, Molecular Probes).

Coronary artery angiogram. E15.5 coronary arteries were perfused by injecting FITC-conjugated tomato lectin (Vector) into the left ventricle of dissected embryos and visualized immediately afterward by fluorescence stereomicroscopy.

***In situ* hybridization.** Tissue sections (20 μm) were fixed in 4% paraformaldehyde/PBS for 10 min at room temperature, washed three times in PBS, and acetylated in 1.3% triethanolamine (Sigma), 0.25% acetic anhydride (Sigma) for 10 min at room temperature. After another three washes in PBS, sections were blocked with hybridization solution (50% formamide (Sigma), 5× saline sodium citrate solution (SSC), 5× Denhardts (Invitrogen), 0.5 mg ml^{-1} sperm DNA (Invitrogen) and 0.25 mg ml^{-1} yeast tRNA (Invitrogen)) for 1 h at room temperature in a chamber hydrated with 50% formamide, 5× SSC. Digoxigenin-labelled antisense probes (Roche) or their sense controls were diluted to 400 ng ml^{-1} in hybridization buffer, denatured at 80 °C for 5 min, cooled and added to slides, which were incubated overnight at 58 °C in a Model 241000 hybridization oven (Boekel Scientific). After hybridization, slides were washed with 2× SSC for 30 min at room temperature, 0.2× SSC three times for 40 min at 65 °C, and Tris-buffered saline containing 0.1% Tween-20 (TBST) for 5 min at room temperature. Sections were then blocked with 5% sheep serum (Invitrogen)/TBST and incubated overnight with alkaline phosphatase-conjugated anti-DIG antibodies (Roche) diluted in blocking solution. Signal was detected by NBT-BCIP (Roche) immunohistochemistry according to the manufacturer's instructions. Adjacent sections were stained with anti-CD31 antibodies.

Organ cultures. Heart cultures were carried out as described[26] with the following modifications. Embryonic hearts were from either wild-type or apelin-nlacZ embryos. The atria and attached sinus venosus were left intact or dissected from the ventricle with fine-point forceps. Intact hearts or separated SV/A or ventricles were cultured dorsal side up at the air–liquid interface on 8-mm Millicell Cell Culture Insert Filters (Millipore). For tissue recombination experiments, SV/A tissue was placed adjacent to the ventricle at the position where the original SV/A was removed. Cultures were maintained at 37 °C and 5% CO_2 in DMEM media supplemented with 2 μg ml^{-1} heparin, 10% fetal calf serum, 100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin, and 2 mM L-glutamine. After 72 h, explants were fixed with 4% paraformaldehyde and subjected to whole-mount X-gal staining. Some stained explants were then sectioned and immunostained with anti-CD31 antibodies to confirm that X-gal^{+} nuclei were coronary endothelial cells. Control littermates were fixed immediately after dissection but before culturing, and then immunostained to assess coronary vessel (anti-CD31) and epicardial (anti-α4 integrin) coverage at the beginning of the culture period.

Clonal analysis. VE-cadherin-CreER mice were crossed to either the Rosa-lacZ or the multicolour Cre reporters. At E7.5, E8.5 or E9.5, dams received an intraperitoneal injection (25G needle) of tamoxifen (Sigma), generally 0.25–0.5 mg, dissolved in 100 μl corn oil. These low doses of tamoxifen were selected to induce rare recombination events and generate single or well-separated clones. Embryos were dissected at E13.5 or E14.5. For lacZ-labelled clones, embryos were fixed and stained with X-gal as described earlier, and those containing sparsely labelled cells were further analysed. Hearts with just a single cluster or two or three well-isolated clusters of cells were sectioned, stained with an endothelial-specific antibody (anti-CD31) before cells were counted and identities assigned by marker (CD31) expression and their location and morphology. Clones marked with multicolour reporters were fixed, sectioned and stained with either DAPI or CD31 as described earlier and analysed using fluorescent filters specific to each fluorophore. Endothelial cell division rate for each clone was estimated from final cell counts, assuming that recombination occurred between 6 and 48 h[44,45] after tamoxifen injection.

Imaging. Samples were imaged on a Leica MZ16FA stereomicroscope (whole-mount tissue) or a Zeiss Axiophot upright fluorescence microscope using Axiovision software (version 4.2) or on a Leica Sp2 confocal microscope using LCS software. Adobe Photoshop was used to adjust image levels and process image overlays.

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