Developmental origin of age-related coronary artery disease

Ke Wei1,2,3†, Ramon Díaz-Trelles1,2,3†, Qiaozhen Liu4, Marta Diez-Cuñado1,2,3,5, Maria-Cecilia Scimia1,2, Wenqing Cai1,2,3, Junko Sawada1,2, Masanobu Komatsu1,2, Joseph J. Boyle6, Bin Zhou4, Pilar Ruiz-Lozano5‡*, and Mark Mercola1,2,3‡*

1Sanford-Burnham Medical Research Institute, 10901 N. Torrey Pines Road, La Jolla, CA 92037, USA; 2Sanford-Burnham Medical Research Institute, 6400 Sanger Road, Orlando, FL 32827, USA; 3Department of Bioengineering, Jacobs School of Engineering, University of California, San Diego, La Jolla, CA 92037, USA; 4Institute for Nutritional Sciences, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai, China; 5Stanford University School of Medicine, 300 Pasteur Dr, Stanford, CA 94305, USA; and 6Imperial Centre for Translational and Experimental Medicine, Imperial College London, Hammersmith Hospital, London, UK

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Aim
Age and injury cause structural and functional changes in coronary artery smooth muscle cells (caSMCs) that influence the pathogenesis of coronary artery disease. Although paracrine signalling is widely believed to drive phenotypic changes in caSMCs, here we show that developmental origin within the fetal epicardium can have a profound effect as well.

Methods and results
Fluorescent dye and transgene pulse-labelling techniques in mice revealed that the majority of caSMCs are derived from Wt1+, Gata5-Cre+ cells that migrate before E12.5, whereas a minority of cells are derived from a later-emigrating, Wt1−, Gata5-Cre− population. We functionally evaluated the influence of early emigrating cells on coronary artery development and disease by Gata5-Cre excision of Rbpj, which prevents their contribution to coronary artery smooth muscle cells. Ablation of the Gata5-Cre+ population resulted in coronary arteries consisting solely of Gata5-Cre− caSMCs. These coronary arteries appeared normal into early adulthood; however, by 5–8 months of age, they became progressively fibrotic, lost the adventitial outer elastin layer, were dysfunctional and leaky, and animals showed early mortality.

Conclusion
Taken together, these data reveal heterogeneity in the fetal epicardium that is linked to coronary artery integrity, and that distortion of the coronaries epicardial origin predisposes to adult onset disease.

Keywords
Epicardium • Smooth muscle • Coronary artery disease • Pathology

1. Introduction
Ageing is a major, and poorly understood, risk factor for coronary artery disease. Age-associated changes in the vascular wall include alterations in coronary artery smooth muscle cell (caSMC) dilatory effects, proliferation, and apoptosis that have been linked to alterations in hormonal and growth factor responsiveness.1–3 Whether variations in the developmental origin of the caSMCs underlie congenital susceptibility to disease and contribute to pathogenesis has been suggested,4 but never directly tested.

The coronary vasculature derives from a transient embryonic structure, the pro-epicardium, that forms on the sinoatrial surface of the developing heart.5 Through epithelial–mesenchymal transformation (EMT) and cellular migration, pro-epicardial cells disperse to envelop the heart where it forms the epicardium.6 Some epicardial cells, through a still unidentified mechanism,6,7 undergo another round of EMT to invade the myocardium and give rise to diverse cardiac cell types, including coronary smooth muscle cells and interstitial fibroblasts.7–9

Genetic and non-genetic cell labelling have suggested the existence of cellular heterogeneity within the epicardium.7,10,11 Here, we sought to determine whether the pro-epicardial and epicardial heterogeneity is associated with functional differences. For that purpose, we characterized the temporal pattern of epicardial migration that gives rise to coronary artery smooth muscle, and showed that selective elimination...
of an early emigrating population’s ability to contribute to caSMCs leads to adult onset disease.

2. Methods

2.1 Mouse genetics

Epicardium-specific and myocardium-specific Rbpj knockout mice (Gata5-Cre+;Rbpjfl/fl and Mlc2v-Cre+;Rbpjfl/fl) were generated by crossing Gata5-Cre+ mice6 and Mlc2v-Cre+ mice13 with Rbpjfl/fl mice,15 and subsequently crossing Gata5-Cre+;Rbpjfl/+ mice and Mlc2v-Cre+;Rbpjfl/+ mice with Rbpjfl/fl mice. Conditional reporters with LacZ (ROSA26x2GFP)14, RFP or GFP (Rosa26RFP or Rosa26GFP), and ZEG15 were used in lineage tracing of Gata5-Cre, Tbx18-Cre,16 and Wt1CreERT217 lineages as indicated in the text. About 20 mg/kg of 4-OH tamoxifen was IP injected at E10.5, 12.5, or E15.5 to label early and late epicardium. Tbx18-Cre;Rbpjfl/+mTmG and Tbx18-Cre-;mTmG samples were provided by Dr Kisper.

All animal study was approved by the Institutional Animal Care and Use Committee (IACUC) of Sanford-Burnham Medical Research Institute. All animal procedures performed conform the NIH guidelines.

To minimize changes in gene expression, pregnant mice were sacrificed by the use of cervical dislocation with anaesthesia for isolation of embryos. Tribromoethanol anaesthesia is used for cervical dislocation—340 mg/kg IP until non-responsive to toe pinch.

2.2 Histology, immunofluorescent staining, and immunohistochemistry

Histological analysis was performed according to standard protocols for paraffin embedding and sectioning (10 µm). Haematoxylin and eosin, Masson’s trichrome, Van Gieson, and Von Kossa staining were performed according to standard protocols. Antibodies used were: 1: 200 α-actinin (Sigma, A7871), 1: 400 α-smooth muscle marker (SMα; Sigma, A2547), 1: 200 eGFP (Life Technologies A-6455), 1: 100 Wt1 (Santa Cruz sc-192), 1: 50 Tbx18 (Santa Cruz sc-17869), 1: 200 PECAM (BD Pharmingen, 550274), 1: 200 β-galactosidase (MP Biomedicals, 559762), 1: 200 vimentin (Abcam, ab11256), 1: 200 fibrinogen (DAKO A0080), 1: 100 Rbpj XP (Cell Signaling), 1: 1000 FITC-conjugated isoelectric B4 (ILB4, Vector Laboratories), 1: 200 Ki67 (DAKO, M7249), 1: 200 Mac-3 (BD Pharmingen 550292), and 1: 500 F4/80 (Life Technologies MF84000). Anti-R-Ras antibody was a gift from Dr J.C. Reed,18 and anti-NG2 antibody was a gift from Dr William Stallcup. Appropriate fluorescent secondary antibodies from Life Technologies were used at 1: 200 for immunofluorescent staining, and HRP anti-mouse antibody was used at 1: 1000 for immunohistochemistry.

TUNEL assay was performed according to the manufacturer’s instruction (Roche).

2.3 Ex vivo embryo culture

Embryonic hearts were dissected at E12.5 and E18.5 followed by carboxyfluorescein diacetate succinimidyl ester (CFDA-SE) labelling performed according to the manufacturer’s protocol (Life Technologies V12883). CFDA-SE readily diffuses into cells where cytoplasmic esterases remove the acetate groups yielding the fluorescent ester, CFSE, which covalently couples via its succinimidyl group to intracellular molecules and is retained for long periods. Hearts were then cultured in DMEM with 20% rat serum for 48 h prior to histological analysis.

2.4 Statistical analysis

The number of samples (n) used in each experiment is recorded in the figure legends. The values presented are expressed as means ± SEM. Statistical significances (P < 0.05) were tested using Student’s t-test (GraphPad Prism; Figure 4F and G), and one-way ANOVA followed by multiple comparison with Bonferroni’s test as post hoc test (GraphPad Prism; Figure 2H). Survival curve analysis were performed using Kaplan–Meier survival analysis (GraphPad Prism). Data from histological sections from different regions of E9.5 and E12.5 embryonic left ventricles were pooled for quantification, while apex, middle, and base sections were examined separately for later stages. However, since no significant differences were found between the different zones of these hearts, the data were merged for representation here.

3. Results

3.1 Dynamic nature of the early fetal epicardium

We used several independent methods to probe cellular diversity in the epicardium and its precursor tissue, the pro-epicardium. Labelling of the pro-epicardium (E9.5) with Gata5-Cre,6 followed by immunostaining for Wt1 protein on histological sections, revealed distinct populations of single-positive Wt1+, single-positive Gata5-Cre+ cells, as well as double-positive Wt1+/Gata5-Cre+ cells (Figure 1A).

Heterogeneity within the epicardium was examined by examining E12.5 and E18.5 embryonic hearts for co-staining of the Gata5-Cre+ lineage label with the epicardial markers Tbx18 (Figure 1B and C) and Wt1 (Figure 1D and E). At E12.5, all epicardial cells express Tbx18 and Wt1 (Figure 1B and D and refs 17,19,20). Most of the Tbx18+ and Wt1+ cells at E12.5 are also Gata5-Cre+ (>90%, Figure 1B and D). Gata5-Cre− lineage cells also express Wt1 and Tbx18, but comprise fewer than 10% of the epicardium at E12.5 (Figure 1B and D).

As development proceeds from mid to late gestation, Gata5-Cre+ lineage cells become less prevalent in the epicardium, such that fewer than 10% of the epicardial cells are Gata5-Cre+ by adulthood (Figure 1C, E, and F–K). Although largely absent from the epicardial surface, Gata5-Cre− lineage cells were apparent within the E18.5 and adult myocardium (Figure 1I and J). The nearly complete loss of Gata5-Cre+ lineage cells from the epicardial epithelium markedly contrasts Tbx18-Cre− lineage cells, which persist in the epicardium through E18.5 (Figure 1L) despite the loss of Tbx18 protein (Figure 1E). To trace the fate of early epicardial cells (as opposed to expression of the Cre), we pulse-labelled epicardial cells at E10.5 using the Wt1CreERT2;mTmG mice17 and followed their progeny. E10.5 Wt1CreERT2−labelled cells give rise to the adult epicardium (Figure 1M; see below for contribution to other lineages), again in clear contrast to the Gata5-Cre− lineage cells (Figure 1J and K). Thus, distinct lineages populate early fetal epicardium (Gata5-Cre− vs. late gestation and adult epicardium (Gata5-Cre−)).

We investigated whether invasive cellular behaviour might cause the developmental shift from Gata5-Cre− to Gata5-Cre+ cells on the epicardial surface. Ventricular explants of E12.5 and E18.5 hearts were incubated with the small-molecule fluorescent probe CFDA-SE at either E12.5 or E18.5. CFDA-SE is taken up by cells and cleaved by cytoplasmic esterases to yield the fluorescent CFDA molecule that remains visible within cells for multiple divisions (see Methods). CFDA-SE exposure labels only cells on the outermost epicardial epithelial layer of the ventricular explants, leaving myocardial cells unlabelled (Figure 1N and O, time 0). After 48 h of culture, E12.5-labelled cells efficiently entered the myocardium (Figure 1N, 48 h). Thus, the E12.5 cells, which are mostly Gata5-Cre−, are highly competent to enter the myocardium. In contrast, most E18.5-labelled cells remained in the epicardium 48 h after labelling (Figure 1O, 48 h). Quantitatively, 59.5% of E12.5 epicardial cells entered the myocardium, whereas only 10% of E18.5-labelled cells did so, corresponding to a diminished propensity to remain epicardial (Figure 1P and Q). Thus, the early
Figure 1  Gata5-Cre transgene marks an early emigrating population of epicardial cells. (A) Heterogeneity within the pro-epicardium at E9.5. Wilm’s tumour-1 (Wt1) immunostaining (red) and eGFP fluorescence (green) in Gata5-Cre^{+}\text{Z/EG} pro-epicardium. The boxed area is shown in (A’) with higher magnification. Some Wt1^{+} cells in the pro-epicardium are Gata5-Cre2 (arrowhead (red) cell). ST, septum transversum; PE, pro-epicardium; H, heart. The panel shown is a representative image of three independent immunostaining performed on different embryos. (B–E) Overlap of Gata5-Cre lineage cells (green) with Wt1 (red, B and C) and Tbx18 (red, D and E) at E12.5 (B and D) and E18.5 (C and E). Most E12.5 epicardial cells are Gata5-Cre^{+} and stain with Wt1 (B) or Tbx18 (D), although Gata5-Cre^{+} cells that express Wt1 or Tbx18 are visible (arrowheads in B and D). At E18.5, Wt1 and Tbx18 are no longer expressed. Note that the Gata5-Cre^{+} lineage cells are largely absent from the epicardial surface at E18.5 (although some are visible sub-epicardially). All images shown are representative of three independent immunostaining performed on different embryos. (F–K) Progressive depletion of Gata5-Cre lineage cells (green) from the embryonic epicardium. Gata5-Cre^{+}\text{Z/EG} hearts immunostained for α-actinin (cardiac muscle marker, red) at E9.5 (F), E12.5 (G), E15.5 (H and H’), E18.5 (I), and adult (J) ages. White arrowheads and yellow arrowheads indicate the presence and absence of Gata5-Cre lineage cells in the epicardium, respectively. Regional heterogeneity was detectable at E15.5 (H and H’). Proportion of Gata5-Cre lineage cells detected in the epicardium in embryonic and adult hearts (three animals analysed for each stage, K). The numbers of eGFP^{+} and eGFP^{2} cells in epicardium are 733/52 (E12.5), 826/163 (E15.5), 235/552 (E18.5), and 54/563 (adult), respectively. (L and M) Lineage tracing of Tbx18-Cre and Wt1CreERT2 lineage cells. The epicardium remains entirely positive for Tbx18-Cre lineage cells at E18.5 (L, green) and Wt1CreERT2 lineage cells in the adult (M, green), although it is devoid of Gata5-Cre lineage cells by these times (I and J). Mice were Tbx18Cre^{+}\text{mTmG} and Wt1^{CreERT2}^{+}\text{mTmG} pulse-labelled by 4-OH tamoxifen injection at E10.5. Tissues were fluorescently immunostained for α-actinin (red). (N–Q) CFDA-SE ex vivo tracing of embryonic epicardium in E12.5 (N) and E18.5 (O) hearts before and after 48 h ex vivo culture. Note the absence of CFDA-SE labelling of the epicardium at time 0. Forty-eight hours after labelling at E12.5 (N), CFDA^{+} cells have entered the myocardium in the E12.5 samples and in some areas the epicardium is completely devoid of CFDA label (right panel, arrowhead). Arrowhead in middle panel indicates a CFDA^{+} cell in the myocardium. Markedly fewer cells enter the myocardium following labelling at E18.5 (O), (arrowheads indicate CFDA^{+} cells surrounding a coronary artery). Quantification of the location of CFDA^{+} cells after 48 h ex vivo culture (P, n = 3 in each group). The numbers of CFDA^{+} cells in the epicardium and myocardium after 48 h culture are 303/422 (E12.5) and 686/75 (E18.5). Quantification of composition of epicardial cells after 48 h ex vivo culture (Q, n = 3 mice in each group). The numbers of CFDA^{+}/CFDA^{–} cells in epicardium after 48 h culture are 303/345 (E12.5) and 686/0 (E18.5).
Epicardium undergoes considerably more EMT and myocardial invasion than does the late (E18.5) epicardium.

3.2 Shifting contribution of epicardium to coronary artery smooth muscle

Analysis of various Cre transgenes, including Gata5-Cre, has illustrated that epicardial cells contribute to caSMCs. Quantitative analysis revealed that Gata5-Cre+ cells account for the overwhelming majority of caSMCs from late gestation through old age (Figure 2A–D). To determine whether this bias reflects the developmental shift in invasiveness (Figure 1), we pulse-labelled epicardial cells in vivo at E10.5, E12.5, or E15.5 and traced their subsequent contribution to caSMCs. Pulse-labelling with 4-OH tamoxifen induced the heritable expression of an eGFP or RFP fluorescent reporter. Wt1CreERT2 lineage cells pulse-labelled at E10.5 yielded 84% of caSMCs in the adult, declining progressively over the labelling period such that E12.5 and E15.5 epicardium contributed 42 and 12%, respectively (Figure 2E–H). Thus, early epicardium is the predominant source of caSMCs, whereas later epicardial cells normally contribute fewer caSMCs.

3.3 Genetic ablation of the Gata5-Cre+ lineage contribution to caSMCs does not impair formation of coronary arteries

We took advantage of the early epicardial expression of the Gata5-Cre transgene to reveal the functional consequences of deleting the early emigrating epicardium. Pan-epicardial deletion of the Notch transcriptional effector Rbpj with Tbx18-Cre has been shown to prevent contribution of epicardial cells to caSMCs and cause grossly enlarged coronary arteries before birth (Figure 3A and B and refs 16,21). In contrast, Gata5-Cre-mediated deletion of Rbpj resulted in mice (Gata5-Cre+;Rbpjf/f) that had morphologically normal coronary smooth muscle (Figure 3C, D, and G–J) and were indistinguishable from their wild-type littermates up to the age of 3 months. Most interestingly, the apparently normal coronary arteries in the young Gata5-Cre+;Rbpjf/f mutant mice consisted entirely of Gata5-Cre+ cells, as shown by cellular tracing with two independent indicators: Gata5-Cre+;Rbpjf/f; Z/EG (Figure 3C and D) and Gata5-Cre+;Rbpjf/f; ROSA26;LacZ (Figure 3G–J). Cre-mediated recombination was never observed in caSMCs of either lineage label, even in the adult, indicating complete...
ablation of the Gata5-Cre<sup>+</sup> lineage and substitution by Gata5-Cre<sup>-</sup> cells. The lack of Rbpj on the Gata5-Cre<sup>-</sup> lineage could alter cell fate towards a non-SMC cell type. The possibility that the caSMCs might be derived from early epicardium that had escaped deletion of the Rbpj protein in Gata5-Cre<sup>−;Rbpjf/f</sup> mice could be ruled out because all Gata5-Cre lineage cells lacked Rbpj immunostaining (Figure 3E and F) and all caSMCs in the adult coronary arteries expressed Rbpj (Figure 3K and L) together with Noch3 receptor and Jagged1 ligand (data not shown). Thus, Gata5-Cre<sup>-</sup> cells compensated for the loss of Gata5-Cre<sup>+</sup> contribution to caSMCs, resulting in no apparent embryonic lethality or discernable defect in neonatal coronary arteries.

### 3.4 Loss of contribution from early epicardium to coronary smooth muscle cells causes late onset coronary vasculature defects

Notwithstanding their apparently normal coronary vasculature after birth and into early adulthood, the Gata5-Cre<sup>−;Rbpjf/f</sup> mutants displayed significantly shortened life span, as survival decreased to 50% after 8 months of age (Figure 4A, n = 39 for Gata5-Cre<sup>−;Rbpjf/f</sup> and n = 25 for Gata5-Cre<sup>−;Rbpjf/f</sup>). Morphometric anomalies were also evident by 8 months in Gata5-Cre<sup>−;Rbpjf/f</sup> mice, consisting of increased heart weight/body weight ratios, cardiac hypertrophy (see Supplementary material online, Figure S1), mild right ventricular thickening, and lymphoid aggregates associated with bronchovascular bundles, which likely contributed to the reduced life span. At a lower rate (2/14), we also observed extremely fibrotic, constricted, and ruptured arteries in animals older than 8 months.

Histological sections of the hearts stained with Masson’s trichrome revealed no notable differences in the hearts between genotypes at 3 months of age, including comparable degrees of fibrotic deposition surrounding the coronary walls among genotypes (Figure 4B). However, coronary fibrosis increased dramatically in Gata5-Cre<sup>−;Rbpjf/f</sup> hearts by 8 months of age (Figure 4B).

Similarly, Van Gieson staining for elastin appeared normal at 3 months of age, but was defective by 8 months: in contrast to the two-layered elastin band displayed by control coronaries, the outer elastin...
layer deposited by smooth muscle cells was completely absent in Gata5-Cre<sup>−</sup>;Rbpj<sup>−/−</sup> hearts (Figure 4C). Gata5-Cre<sup>−</sup>;Rbpj<sup>−/−</sup> hearts also had a markedly increased number of vimentin<sup>+</sup> interstitial cells surrounding the coronary arteries at 8 months consistent with an increased number of mesenchymal cells (Figure 4D and F). Pericyte and calcification markers remained unchanged among genotypes (see Supplementary material online, Figure S2). No evidence of increased inflammation, apoptosis, or proliferation was detected in mutant hearts (see Supplementary material online, Figure S2). Fibrinogen, which is normally restricted to the lumen of blood vessels (Figure 4E), was apparent outside the coronary vasculature in Gata5-Cre<sup>−</sup>;Rbpj<sup>−/−</sup> hearts (Figure 4E and G), suggesting functionally defective vessels prone to leakage. To rule out the possibility that the partial myocardial domain of Gata5-Cre expression might cause these coronary defects, we examined hearts in which Rbpj was deleted throughout the ventricular myocardium (Mlc2v-Cre<sup>−</sup>;Rbpj<sup>−/−</sup>). Mlc2v-Cre<sup>−</sup>;Rbpj<sup>−/−</sup> animals survived to old age (25 months) with the same frequency as wild-type mice, and showed no evidence of coronary artery dysmorphogenesis or dysfunction (see Supplementary material online, Figure 4).
4. Discussion

Seminal lineage studies have suggested that the epicardium is discontinuous and heterogeneous, but the relevance of these findings for coronary artery disease remained unclear since phenotypic differences in adult caSMCs had never been associated with their development origin. The timing when we note the highest proportion of epicardial cell migration into the myocardium is consistent with the recent findings of Wu et al. that EMT and migration into the myocardium is highest at E13.5 and earlier, when the epicardial cells undergo the highest rate of proliferation and the mitotic spindle of dividing cells is oriented perpendicular to the plane of epicardial cell layer, whereas reduced EMT occurs after E13.5 correlating with a lower incidence of cell division and mitotic spindles oriented parallel to the cell layer.

Our study shows that the fetal epicardium is highly dynamic. An early population (marked by Gata5-Cre and Wt1CreERT2 pulse-labelled at E10.5) invades the myocardium and gives rise to the majority of caSMCs, whereas later fetal epicardium (marked by Wt1CreERT2 pulse-labelled after E15.5) normally contributes to a minority of caSMCs (Figures 1 and 2). Genetic ablation of this early and major epicardial source of caSMCs in Gata5-Cre;Rbpj−/− mutant mice did not have a discernible effect on coronary artery smooth muscle formation or function into early adulthood because Gata5-Cre+ cells compensated to form caSMCs (Figure 3). By 8 months of age, however, the coronary arteries of the Gata5-Cre;Rbpj−/− mutant mice had lost the outer elastic lamina and were surrounded by excessive fibrous and fibrinogen deposition (Figure 4). Thus, these data suggest that early epicardial-derived caSMCs are functionally distinct from later epicardial-derived caSMCs, and that distortion in the developmental source of caSMCs can underlie age-dependent onset of coronary artery dysfunction.

Understanding how developmental origin might affect caSMC function—and that compensatory caSMCs might lead to dysfunction—will likely have important implications for understanding coronary artery disease. Congenitally distorted contribution could underlie disease susceptibility. In addition, injury and disease are known to evoke changes in smooth muscle cell structure and function, including alterations in cell proliferation, contractility, cell–cell and cell–ECM contacts, and extracellular matrix composition.  Termied phenotypic modulation or switching, these changes alter the structure and function of coronary arteries and influence progression of diseases such as atherosclerosis, hypertension, and certain cancers. Although the effects of hormones and growth factors on pre-existing cells are commonly thought to trigger phenotypic switching, changes in the cellular composition of smooth muscles have also been considered. Since our results indicate that distortion of the normal epicardial contribution causes coronary artery smooth muscle to become dysmorphic and dysfunctional with age, it is tempting to speculate that injury or environmental effects might cause a similar replacement of caSMCs, perhaps by selective replication of caSMC subsets, contributing to the pathogenesis of coronary artery disease. This observation has important implications for CAD such as arteriosclerosis, which involves detrimental changes in caSMC function that are poorly understood. Based on these findings, we conclude that the developmental heterogeneity of the epicardium dictates phenotypically distinct subsets of caSMCs that persist in the adult and can influence coronary artery disease.

Supplementary material

Supplementary material is available at Cardiovascular Research online.

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