



THE UNIVERSITY *of* EDINBURGH

Edinburgh Research Explorer

## Extracardiac septum transversum/proepicardial endothelial cells pattern embryonic coronary arterio–venous connections

### Citation for published version:

Cano, E, Carmona, R, Ruiz-Villalba, A, Rojas, A, Chau, Y-Y, Wagner, KD, Wagner, N, Hastie, ND, Muñoz-Chápuli, R & Pérez-Pomares, JM 2016, 'Extracardiac septum transversum/proepicardial endothelial cells pattern embryonic coronary arterio–venous connections', *Proceedings of the National Academy of Sciences*, vol. 113, no. 3, pp. 656-661. <https://doi.org/10.1073/pnas.1509834113>

### Digital Object Identifier (DOI):

[10.1073/pnas.1509834113](https://doi.org/10.1073/pnas.1509834113)

### Link:

[Link to publication record in Edinburgh Research Explorer](#)

### Document Version:

Peer reviewed version

### Published In:

Proceedings of the National Academy of Sciences

### Publisher Rights Statement:

Author's final peer-reviewed manuscript as accepted for publication.

### General rights

Copyright for the publications made accessible via the Edinburgh Research Explorer is retained by the author(s) and / or other copyright owners and it is a condition of accessing these publications that users recognise and abide by the legal requirements associated with these rights.

### Take down policy

The University of Edinburgh has made every reasonable effort to ensure that Edinburgh Research Explorer content complies with UK legislation. If you believe that the public display of this file breaches copyright please contact [openaccess@ed.ac.uk](mailto:openaccess@ed.ac.uk) providing details, and we will remove access to the work immediately and investigate your claim.



*Classification:* BIOLOGICAL SCIENCES; Developmental Biology

## **Extracardiac septum transversum-proepicardial endothelial cells pattern embryonic coronary arterio-venous connections**

**Elena Cano, PhD<sup>a,f</sup>, Rita Carmona, PhD<sup>a,b</sup>, Adrián Ruiz-Villalba, PhD<sup>a,c</sup>, Anabel Rojas, PhD<sup>d</sup>, You-Ying Chau, PhD<sup>e</sup>, Kay D. Wagner, MD<sup>g</sup>, Nicole Wagner, MD<sup>g</sup>, Nicholas D. Hastie, PhD<sup>e</sup>, Ramón Muñoz-Chápuli, PhD<sup>a,b</sup>, and José M. Pérez-Pomares, PhD<sup>a,b\*</sup>**

<sup>a</sup>Department of Animal Biology, Faculty of Sciences, Instituto de Investigación Biomédica de Málaga (IBIMA), University of Málaga, Campus de Teatinos s/n, 29071 Málaga, Spain.

<sup>b</sup>Andalusian Center for Nanomedicine and Biotechnology (BIONAND), c/ Severo Ochoa nº25, 29590 Campanillas (Málaga), Spain.

<sup>c</sup>Department of Anatomy, Embryology and Physiology, Academic Medical Center-University of Amsterdam, Meibergdreef 15, AZ1105 Amsterdam, The Netherlands

<sup>d</sup>Centro Andaluz de Biología Molecular y Medicina Regenerativa (CABIMER), Avda. Americo Vesputio s/n. Parque Científico Isla de la Cartuja 41092 Sevilla, Spain. Centro de Investigación Biomédica en Red de Diabetes y Enfermedades Metabólicas Asociadas (CIBERDEM).

<sup>e</sup>MRC Human Genetics Unit and Institute of Genetics and Molecular Medicine at the University of Edinburgh, Western General Hospital, Crewe Road, EH4 2XU, Edinburgh, United Kingdom.

<sup>f</sup>Group of Cardiovascular Genetics, Department of Vertebrate Genomics and Cardiovascular Genetics, Experimental and Clinical Research Center, Charité-Universitätsmedizin Berlin and Max Delbrück Center (MDC) for Molecular Medicine, Berlin 13125, Germany.

<sup>g</sup>Institute for Research on Cancer and Aging, Nice (IRCAN), Faculty of Medicine, University of Nice Sophia-Antipolis, CNRS UMR7284/INSERM U1081, 06107 Nice, France.

**Short title:** Embryonic extracardiac coronary endothelium.

**Key words:** Coronary endothelium; Septum transversum; Proepicardium; *Gata4*; *Wt1*

**\*Corresponding author:** Dr. J.M. Pérez-Pomares, Department of Animal Biology, Faculty of Sciences, University of Málaga, Campus de Teatinos s/n, 29071 Málaga, Spain; Telephone: +34 952 136 653; E-mail: [jmperezp@uma.es](mailto:jmperezp@uma.es)

## **Abstract**

Recent reports suggest that mammalian embryonic coronary endothelium (CoE) develops from the sinus venosus and ventricular endocardium. The contribution of extracardiac cells to CoE is, however, regarded to be minor and non-significant for coronary formation. Using classic (*Wt1<sup>Cre</sup>*) and novel (*G2-Gata4<sup>Cre</sup>*) transgenic mouse models for the study of coronary vascular development, we show that extracardiac septum transversum-proepicardium (ST/PE)-derived endothelial cells are required for the formation of ventricular coronary arterio-venous vascular connections. Our results indicate that at least 20% of embryonic coronary arterial and capillary endothelial cells derive from the ST/PE cell compartment. Moreover, we show that conditional deletion of the ST/PE lineage-specific Wilms' tumor suppressor gene (*Wt1*) in the ST/PE (*G2-Gata4<sup>Cre</sup>*) and the endothelium (*Tie2<sup>Cre</sup>*) disrupts embryonic coronary transmural patterning, leading to embryonic death. Taken together, our results demonstrate that ST/PE-derived endothelial cells significantly contribute to and are required for proper coronary vascular morphogenesis.

## **Significance statement**

This study shows that extracardiac endothelial cells from the septum transversum/proepicardium (ST/PE) contribute, at least, to 20% of embryonic coronary endothelium (CoE), definitively proving that the endocardium (cardiac endothelium) is not the only developmental origin of CoE. Using different mouse transgenic lines, we first identified the preferential incorporation of ST/PE-derived endothelial cells into prospective coronary arteries and capillaries. We then deleted the Wilms' tumor gene (*Wt1*), an important coronary developmental regulator, from both the ST/PE and embryonic endothelial cells. The defects of both mutant mice, which die before birth, indicate that ST/PE endothelial cells are required for the establishment of coronary arterio-venous connections through the ventricular wall, and are thus necessary for the proper formation of the coronary vasculature.

## Introduction

The coronary vascular system, whose function is necessary to sustain late embryonic and postnatal cardiac function, is formed by a complex network of blood vessels, including arteries, arterioles, capillaries, venules, and veins (1). Recent reports indicate that various sources of endothelial cells contribute to the mammalian embryonic coronary system, which forms from a primary endothelial plexus (2–4). However, the specific fate and function of these different endothelial cell pools during coronary vascular morphogenesis is subject of an intense controversy (5).

Two endocardial populations have been reported to participate in the building of the embryonic coronary vascular system. The first one derives from the sinus venosus endocardium, which sprouts to give rise to the nascent, *Apelin*<sup>+</sup>, coronary vasculature (2). A careful analysis of these results suggests that the sinus venosus endocardium provides a cellular scaffold for the development of coronary veins, but its contribution to coronary artery formation is less evident. Accordingly, a second source of coronary endothelium has been identified in the ventricular endocardium (*Nfatc1*<sup>+</sup> lineage) that massively contributes to coronary arterial endothelium (3, 6).

A third disputed source of coronary endothelium (CoE) is the proepicardium (PE), a structure that comprises epicardial progenitor cells. The PE protrudes from the septum transversum (ST), a folding of lateral mesoderm that initiates the separation of thoracic and abdominal cavities in mammals (7). Although *in vivo* cell-tracing and *in vitro* culture of avian PE cells unambiguously show their robust differentiation into CoE (8, 9), data from experiments performed in mammals, which have largely relied on the use of the *Cre/LoxP* technology, claimed a minor contribution of PE-derived cells to the developing CoE (10–12). It is important to note here that the so-called ‘epicardial’ Cre constructs used in these studies are based in the expression of genes like *Gata5*, *Tbx18* or *Wilms’ tumor suppressor (Wt1)*, which are expressed by both PE and ST cells, thus confirming these two tissues form an ontogenetic and histomorphological continuum, and are therefore difficult to distinguish based on their molecular expression profile. The restricted involvement of PE cells to CoE has been recently challenged by new results indicating that the PE is constituted of different cell populations, including a significant number of endothelial progenitors

(13). Despite the suggested vascular potential of the PE, the final fate of these cells, the extent of their contribution and their specific role during coronary blood vessels morphogenesis remains unknown.

To study the extent and significance of ST/PE contribution to the developing coronary vasculature, we have used novel and classic transgenic mice to first track ST/PE cells into the developing coronary vascular system, and then identify their ontogenetic functions. Our results show that ST/PE-derived endothelial cells (*G2-Gata4<sup>CreYFP</sup>; Wt1<sup>CreYFP</sup>*) preferentially contribute to arterial and capillary CoE, and are required for the growth of the coronary vascular tree, the transmural patterning of early arterio-venous vascular connections, and the viability of the entire coronary circulation.

## Results

### **G2-*Gata4* enhancer-driven reporter expression labels septum transversum and proepicardial cells**

At embryonic days (E) 9.5, G2-*Gata4*<sup>LacZ</sup> mice display reporter activity in the septum transversum mesenchyme, including the PE, but not in heart tissues (myocardium, endocardium) (Fig.1A). At E11.5, G2-*Gata4*<sup>LacZ</sup> expression remains mainly confined to the mesenchyme surrounding the liver (14); weak X-gal staining is also observed in the myocardium of sinus venosus horns (Fig. 1B). Cre recombinase protein is only found in the sinus venosus myocardium and aortic walls (Fig.S1A), and is absent from the epicardium, myocardium and endocardium of other cardiac chambers (Fig.S1B-D).

### **G2-*Gata4* enhancer-lineage tracing reveals an extensive contribution to the epicardium, EPDCs (E9.5-E12.5) and CoE (E12.5-18.5)**

To trace G2-*Gata4* ST/PE cells throughout embryonic development, we crossed the G2-*Gata4*<sup>Cre</sup> line with *Rosa26-YFP* reporter mice, the resulting offspring displaying permanent YFP expression (from here onwards, G2<sup>CreYFP+</sup>) in the ST/PE (Fig.1C,D) and their derivatives, i.e. the epicardium and epicardial-derived cells (EPDCs) (Fig.1E-F'). GATA4 protein is detected in E9.5 G2<sup>CreYFP+</sup> ST/PE and some epicardial cells (Fig.1C). The majority of G2<sup>CreYFP+</sup> ST/PE cells express WT1 protein (Fig.1D), while some ST/PE (Fig.1E) and epicardial (Fig.1E,F,F') WT1<sup>+</sup> cells are G2<sup>CreYFP-</sup>, suggesting that *Wt1* expression may occur in epicardial cells that do not belong to the G2-*Gata4* population. WT1 protein expression is progressively reduced as G2<sup>CreYFP+</sup> cells migrate from the interventricular and atrio-ventricular subepicardium (E11.5 to E12.5) into the myocardial layers (Fig.1F'). At these stages *Wt1* gene expression remains confined to the epicardium and early EPDCs (Fig.1G). From E12.5 onwards, G2<sup>CreYFP+</sup> cells are found in the epicardium, subepicardium, and the myocardial walls and septa; numerous ST/PE-derived G2<sup>CreYFP+</sup> intramyocardial cells are also endothelial CD31<sup>+</sup> (Fig.1H-J) and isolectin B4<sup>+</sup> cells of the developing coronary blood vessels (IB4, Fig.1K,L). No G2<sup>CreYFP+</sup> cells were found in the sinus venosus endocardium (Fig.S1E); however,

some  $G2^{CreYFP+}/CD31^+$  cells are observed in the ventricular endocardium, suggesting an early contribution of ST/PE cells to this tissue (Fig.S1F). Chimeric transplantation of quail proepicardia into chick host hearts confirms ST/PE endothelial cell incorporation to the developing endocardium is a normal event (Fig.S1G,H).

$G2^{CreYFP+}$  cells can be found in the coronary endothelium and medial coronary smooth muscle (CoSM) layers, always intermingled with  $G2^{CreYFP-}$  cells in a ‘salt and pepper’ pattern (Fig.1I-L, see also Movie S1). To verify the vascular potential of the ST/PE, angioblastic/endothelial specific *Scl/Tall* and *Vegfr2* expression was confirmed in ST/PE cells by semiquantitative-PCR (Fig.S2A), and the vasculogenic potential of the tissue tested *in vivo* and *in vitro* (Fig.S2B-F). Most  $G2^{CreYFP+}$  coronary endothelial cells are found in coronary vessels of the ventricular compact layer. Since the activation of NOTCH signaling pathway via the nuclear translocation of the NOTCH1 intracellular domain (N1ICD) is known to reveal arterial endothelium fate, we studied N1ICD nuclear accumulation in  $G2^{CreYFP+}$  cells, and found that many endothelial (IB4<sup>+</sup>)  $G2^{CreYFP+}$  cells are also N1ICD<sup>+</sup> (Fig.1K,L).

### ***Wt1*-driven GFP expression is a *bona fide* marker for early epicardial cells, EPDCs (E10.5-12.5) and coronary blood vessels (E12.5-18.5)**

Since *Wt1* is also known to be a marker of ST/PE cells (15, 16), we first studied its expression pattern in *Wt1<sup>GFP</sup>* knock in mouse embryos. At E10.5, *Wt1* protein and *Wt1*-driven GFP expressions overlap in space and time, and are restricted to the primitive epicardium (Fig.2A). These results confirm that the *Wt1<sup>GFP</sup>* knock-in mouse faithfully recapitulates native *Wt1* gene activity. Between E11.5 and E12.5, *Wt1* expression is detected in epicardial cells and EPDCs, which accumulate at the subepicardium of the ventricles (Fig.2B), atrio-ventricular and interventricular grooves (not shown). In accordance with the results from *G2-Gata4* cell lineage tracing, no CD31 expression is detected in *Wt1<sup>GFP+</sup>* epicardial cells or EPDCs before E11.5 (Fig.2C). At E12.5, a primary subepicardial coronary vascular plexus has formed in the ventricles. Starting at this stage, GFP expression is identified in a significant proportion of subepicardial and intramyocardial CD31<sup>+</sup> cells of the developing atrio-ventricular ventricular and interventricular coronary vasculature (Fig.2D,E-E’’).

Some of these cells display the typical spindle-shaped morphology of migratory mesenchymal cells, with their major axis oriented orthogonally with respect to the epicardial surface (Fig.2F). Between E13.5-14.5 some  $Wt1^{GFP+}/CD31^+$  cells can be identified in the forming intramyocardial blood vessels (Fig.2G), their number decreasing at perinatal stages (E18.5) (Fig.2H).

### ***Wt1* cell lineage tracing into developing coronary blood vessels**

To further confirm ST/PE contribution to the developing coronary vasculature, we selected a  $Wt1^{Cre}$  mouse line which has been previously used to study PE and coronary development (15, 16). Crossing these mice with the *Rosa26-YFP* reporter line allows for the tracing of the *Wt1* cell lineage (from here onwards,  $Wt1^{CreYFP}$ ). At E9.0-9.5 no  $Wt1^{CreYFP+}$  cells are seen in heart, with the exception of a few isolated myocardial ( $Wt1^{CreYFP+}/\alpha\text{-SMA}^+$ ) ones that formed part of the developing cardiac chamber walls (Fig.S3A). None of these cells express the vascular marker CD31 (Fig.S3B). Between E10.5-11.5, almost all epicardial cells and the majority of subepicardial EPDCs were  $Wt1^{CreYFP+}$  (Fig.2I). A fraction of  $Wt1^{CreYFP+}$  epicardial cells were apparently detaching and migrating from the epicardial lining towards the subepicardial mesenchyme (Fig.2I', arrowheads). Active epicardial epithelial-to-mesenchymal transition was confirmed by time-lapse analysis of  $Wt1^{CreYFP+}$  whole heart explants (Movie S2). At these stages, a minor number of  $Wt1^{CreYFP+}/CD31^+$  cells could be identified in the endocardial layer (Fig.S3C). From E12.5 onwards, the abundance of subepicardial and intramyocardial  $Wt1^{CreYFP+}$  cells increases, being these cells especially frequent within the interventricular septum (Fig.2J), and the ventricular walls (Fig.2K).  $Wt1^{CreYFP+}$  cells differentiate into CoE ( $Wt1^{CreYFP+}/CD31^+$ ) (Fig.2L) and CoSMCs ( $Wt1^{CreYFP+}/\alpha\text{-SMA}^+$ ) (Fig.2M) of intramyocardial coronary vessels (prospective coronary arteries, CoA). Neonatal arterial CoE was found to be a mosaic of  $Wt1^{CreYFP+}$  and  $Wt1^{CreYFP-}$  cells, with  $Wt1^{CreYFP+}$  cells displaying a characteristic 'salt and pepper' distribution pattern. Perivascular cells closer to the CoE expressed  $\alpha\text{-SMA}$ , but only part of them was  $Wt1^{CreYFP-}$  (Fig.2N,O). To confirm the ST/PE  $Wt1^+$  cell population contributes to the forming coronary vessels,  $Wt1^{CreERT2}$  mice were crossed with the *Rosa26-YFP* line and recombination induced with tamoxifen at proepicardial stages (E9.0). All embryos (E14.5) showed a reduced but evident contribution to the developing coronary endothelium (Fig.2P-R).



## **FACS and image analysis reveals a differential contribution of *Wt1* and G2-*Gata4* cell lineages to CoE**

To further quantify the contribution of PE cells to the embryonic CoE, we analyzed G2<sup>CreYFP</sup> and *Wt1*<sup>CreYFP</sup> dissociated ventricles by FACS. CD31 was used as pan-endothelial marker. From midgestation to birth, *Wt1*<sup>CreYFP+</sup> cells account for 6.5% (E12.5)-8% (neonates) of total ventricular cells. At E12.5 the percentage of G2<sup>CreYFP+</sup> cells is 9.1%, this percentage decreasing to 4% of total ventricular cells by the end of gestation (Fig.2S). Interestingly, the cytometric analysis reveals that the percentage of CD31<sup>+</sup>/G2<sup>CreYFP+</sup> cells is higher than CD31<sup>+</sup>/*Wt1*<sup>CreYFP+</sup> cells: by E17.5, 22.7±4.2% of all CD31<sup>+</sup> cells are G2<sup>CreYFP+</sup> also (n=4), this percentage reaching 35.7±5.0% in neonates (n=3). Instead, only 11.3±1.9% of CD31<sup>+</sup> cardiac cells are *Wt1*<sup>CreYFP+</sup> by E18.5 (n=6) (Fig.2T). This differential contribution of both lineages to the CoE was confirmed by quantitative image analysis performed on confocal micrographs from E18.5 G2<sup>CreYFP</sup> and *Wt1*<sup>CreYFP</sup> embryos. Considering only the compact ventricular layer (i.e., excluding endocardium, epicardium and trabeculae), 49.3±13.9% of CD31<sup>+</sup> cells are G2<sup>CreYFP+</sup>, while only 25.1±4.1 % of CD31<sup>+</sup> cells are *Wt1*<sup>CreYFP+</sup> and therefore at least a 20% of embryonic coronary endothelial cells derive from the ST/PE. The percentages of endothelial cells obtained after image analysis are higher than those from the cytometry analysis, mainly due to the exclusion of the endocardial cells, but the 2:1 proportion between *Wt1*<sup>CreYFP+</sup> and G2<sup>CreYFP+</sup> cells remains evident.

## ***Wt1* expression in G2-*Gata4* cells is required for CoA development**

G2<sup>Cre</sup>-driven *Wt1* deletion (G2<sup>Cre</sup>;*Wt1*<sup>LoxP/LoxP</sup>) severely impairs the development of the coronary vasculature causing embryonic lethality around E15.5 (compare Fig. 3A-C with Fig.3D-F). 3D reconstruction of coronary endothelium (CD31<sup>+</sup>) shows that mutant embryos, as compared to wild types, develop tortuous and sinusoidal irregular vessels that fail to progress transmurally and to complete ventricular myocardium invasion (compare Fig. 3A with Fig.3D and Movie S3 with Movie S4). Normal CoA are missing in the mutants, which also display a thin ventricular compact myocardium (Fig.3 B,E). Effective *Wt1* deletion was confirmed by the absence of WT1 protein and

the decrease of RALDH2, a known direct *Wt1* target (17), in mutant mice (Fig. S4A-D). However, the epicardium remains intact in the mutants (Fig.3F, arrowheads).

### **Notch1 signaling is active in G2<sup>CreYFP</sup> cells**

Prompted by the characteristic N1ICD expression of endothelial G2<sup>CreYFP+</sup> cells, we decided to test whether the N1ICD<sup>+</sup> endothelial compartment is affected after G2-mediated *Wt1* deletion (G2-*Gata4*<sup>Cre</sup>; *Wt1*<sup>LoxP/LoxP</sup>). N1ICD was found to be present in many wild type coronary endothelial cells, mainly capillaries and putative coronary arteries; however, such cells do not form normal transmural coronary blood vessels in the mutants (compare Fig. 3C with Fig.3F). A detailed analysis of the ventricular wall reveals that N1ICD<sup>+</sup> endothelial cells form abnormal intramyocardial vessels that fail to connect to the deeper vascular elements of the developing coronary vasculature (Fig. 3F).

### **Early systemic *Wt1* deletion phenotype is more severe than that of G2-*Gata4*<sup>Cre</sup>; *Wt1*<sup>LoxP/LoxP</sup> mutants**

Since the E10.5 epicardial *Wt1*-expressing population encompasses all epicardial G2-*Gata4* cells, we decided to cross tamoxifen-inducible *CAGG*<sup>CreERT2</sup> and *Wt1*<sup>LoxP/GFP</sup> mouse lines to generate systemic *Wt1* mutants at early epicardial stages. *CAGG-Cre*-mediated *Wt1* deletion at E10.5 results in embryonic death by E13.5. Mutant embryos were found to display a severe phenotype characterized by the impairment of EPDC migration into the compact ventricular myocardium and intramyocardial coronary vessel morphogenesis disruption, as revealed by the *GFP* copy carried by mutant mice. However, the subepicardial coronary vasculature is still formed (compare Fig. 3G with Fig.3H-I').

### **Conditional *Wt1* deletion in endothelial cells reproduces the coronary defects of G2-*Gata4*<sup>Cre</sup>; *Wt1*<sup>LoxP/LoxP</sup> mutants**

To prove that coronary vascular defects in G2-*Gata4*<sup>Cre</sup>; *Wt1*<sup>LoxP/LoxP</sup> mutants are not secondary to disrupted epicardial signaling, we crossed *Tie2*<sup>CreERT2</sup> (18) and *Wt1*<sup>LoxP/LoxP</sup> mice to create endothelial cell-specific *Wt1* knockouts (19) (tamoxifen injected at E10; specificity of tamoxifen-

induced recombination is shown in Fig.S5). Heart morphology is similar in wild type and mutant animals (E16.5), which do not show compact myocardial thinning (Fig.4A,B). WT1 protein is found in some coronary vascular structures and isolated EPDCs of wild type animals, but their number decreases in *Tie2<sup>CreERT2</sup>;Wt1<sup>LoxP/LoxP</sup>* mutants (compare Fig.4C with 4D; WT1 protein expression in wild type and mutants has been quantified in Fig.4E). *Wt1* gene expression is also significantly downregulated in mutants (Fig.4F). CD31 immunostaining of wild type and mutant samples reveals a marked reduction in the number of coronary vessels, most especially in the transmural vessels that connect the endocardial and epicardial elements of the coronary vasculature (Fig.4G,H). Reduced coronary vascularization in mutant hearts was confirmed by the analysis of the area occupied by CD31<sup>+</sup> cells (Fig.4I) and *CD31* gene downregulation (Fig.4J).

## Discussion

To fully understand the morphogenesis of the coronary vascular system we need to unveil the molecular and cellular mechanisms that integrate different endothelial compartments (i.e. the embryonic arterial and venous coronary vasculature) into a single, continuous vascular bed (1). In this regard, the identification of the origin of coronary endothelial progenitors becomes crucial to the understanding of coronary blood vessel morphogenesis. Thus, while some reports discarded the PE and its derivatives as a significant source of CoE (12), or identified the endocardium as the major contributor to CoE (2, 3), Katz and collaborators proved that the PE comprises several cell compartments (identified by either *Scleraxis* or *Semaphorin3D* gene expression) which differentially contribute to CoE (13). However, the specific developmental function of this ST/PE-derived CoE during coronary morphogenesis remains unknown.

Despite all these important evidences, which indicate that ‘one molecule-based’ genetic strategies to trace certain embryonic tissues can be misleading, recent reviews still regard ST/PE contribution to CoE as quantitatively negligible and non-significant from a developmental standpoint (5). To reconcile different hypotheses on the origin of CoE, we aimed at resolving the controversy on ST/PE contribution to CoE and identifying the function of this specific cell population during coronary development. To tackle these goals, we have analyzed coronary formation using the *Cre/LoxP* system to track cells identified in origin by their G2-*Gata4* enhancer or *Wtl* expression. Moreover, we have used this same technology to conditionally delete the *Wtl* gene in ST/PE lineage cells ( $G2^{Cre}$ ) and endothelial cells ( $Tie2^{Cre}$ ) and then characterize the relevance of these cells during coronary blood vessel formation.

Our results indicate that the G2-*Gata4* enhancer, used here for the first time to study coronary vascular development, is as a bona fide marker of ST/PE cells, since this transgene is active at the extracardiac ST/PE location only (E9.5), and not in heart tissues proper. G2-*Gata4* cell lineage tracing reveals a significant contribution of ST/PE cells to CoE, migrating from their extracardiac location to the heart surface to form the primitive epicardial epithelium. After epicardial EMT is initiated,  $G2^{CreYFP+}$  cells progressively colonize the subepicardial matrix and the myocardial layers. Along this process G2-*Gata4*-driven reporter expression is confined to the epicardium and its

mesenchymal derivatives (1, 20–22). Specifically, early G2-*Gata4*-derived CoE is originally found at the subepicardial coronary vascular plexus, while from E13.5 onwards they mainly accumulate at the intramyocardial coronary vasculature (prospective CoA and capillaries), but not significantly to prospective coronary veins.

*Wt1*<sup>CreYFP+</sup> ST/PE cells also contribute to CoE, although they are less abundant than G2-*Gata4*<sup>CreYFP+</sup> ones, suggesting an early developmental specification of this endothelial lineage. This finding also explains why other studies, mostly relying on *Wt1* cell lineage genetic tracking, deemed ST/PE contribution to CoE as negligible. It is possible that the differences between the relative abundance of *Wt1*<sup>CreYFP+</sup> and G2-*Gata4*<sup>CreYFP+</sup> cells in the developing heart could relate to the reported *de novo* expression of Wt1 protein in cardiac vessels following myocardial infarction (23, 24). Whether this Wt1 activation is linked to the developmental origin of these cells (25) or rather represents an ectopic activation of the gene in response to hypoxia (26) has been extensively discussed (27). However, the lack of activation of the G2 enhancer within heart tissues strongly suggests an ST/PE, extracardiac, origin for G2-*Gata4*<sup>CreYFP+</sup> cells, whose endothelial differentiation potential is higher than that shown by *Wt1*<sup>CreYFP+</sup> cells. Our most restrictive estimation of G2-*Gata4*<sup>CreYFP+</sup> and *Wt1*<sup>CreYFP+</sup> cell incorporation to CoE (at least a 20%) is compatible with the reported variable endocardial contribution to coronary vasculature, roughly ranging from 70 to 40% of coronary embryonic coronary endothelial cells (3).

Our cell tracing analysis shows a preferential incorporation of ST/PE endothelial cells to intramyocardial prospective coronary arteries, and capillaries, pointing to a different developmental origin for coronary arteries and veins. This concept is further supported by the coronary arterial (but not venous) endothelial phenotype of mouse embryos with deficient epicardial NOTCH signaling (15). We thus decided to inspect whether ST/PE cells play role in coronary arterio-venous connection. Intramyocardial arterial CoE, identified by the nuclear accumulation of NOTCH intracellular domain (N1ICD) is anomalous in G2-*Gata4*<sup>Cre</sup>; *Wt1*<sup>LoxP/LoxP</sup> mutants. In detail, endothelial structures connecting the inner, intramyocardial coronary vasculature (prospective arteries) and the outer, subepicardial one (prospective coronary veins), are unable to cross the myocardial wall and form aberrant vascular structures. Coronary anomalies recorded in

*Tie2*<sup>CreERT2</sup>;*Wt1*<sup>LoxP/LoxP</sup> mutants, which show a reduction in both WT1<sup>+</sup> and CD31<sup>+</sup> cells in the ventricular myocardial walls and a disruption of the transmural organization of embryonic coronary vessels, confirm the loss of Wt1 in ST/PE cells after G2-*Gata4*-mediated deletion primarily affects coronary endothelial cells.

In summary, our work unambiguously show that the ST/PE, a complex population of extracardiac splanchnic mesoderm constituted of various cells populations (*Wt1*<sup>+</sup> and G2-*Gata4*<sup>+</sup>), significantly contributes cells to the CoE that are necessary for proper coronary vascular morphogenesis (Fig. 5). Our results also suggest that the ST/PE-derived component of CoE is mechanistically related to CoA rather than to CoV, and reveal a functional role for these cells in transmural arterio-venous patterning of the coronary vascular tree, most likely via the segregation of NOTCH<sup>+</sup> and NOTCH<sup>-</sup> endothelial domains. In summary, our findings support the concept of CoE as a developmental mosaic forming from different sources of endothelial cells that actively contribute to the patterning of coronary blood vessels, and open new perspectives to the understanding of congenital coronary anomalies and adult coronary endothelium malfunction.

## **Methods**

### **Mouse lines, and embryo extraction**

The animals used in our research program were handled in compliance with the institutional and European Union guidelines for animal care and welfare. The procedure was approved by the Committee on the Ethics of Animal Experiments of the University of Malaga (procedure code 2009-0037) and the French Coordination Committee on Cancer Research guidelines and local Home Office regulations. All embryos were staged from the time point of vaginal plug observation, which was designated as E0.5. Embryos were excised and washed in PBS before further processing. Details on the transgenic mouse lines used in this paper are included in the SI.

### **Tissue sampling for fluorescent reporter analysis**

For fluorescent reporter expression analysis and immunofluorescence the embryos were fixed in 2-4% fresh paraformaldehyde solution in PBS for 2–8 h, washed in PBS, cryoprotected in sucrose solutions, embedded in OCT (Tissue-Tek), and frozen in liquid N<sub>2</sub>-cooled isopentane. Samples were sectioned on a cryostat (10µm) and cryosections were stored at -20°C until use. Quantification of cells was performed as indicated in the SI.

### **Quantitative and semiquantitative (reverse-transcriptase) PCR**

Total RNA was isolated from embryonic hearts using the Trizol reagent (Invitrogen). First-strand cDNA synthesis was performed with 0.5 µg of total RNA using oligo(dT) and random primers and superscript III reverse transcriptase (Invitrogen). PCRs were performed as described in the SI.

### **Immunofluorescence and X-Gal staining**

Immunofluorescence staining was performed as described elsewhere (see supplemental information for a detailed protocol). All images were captured on a Leica SP5 confocal microscope. β-Galactosidase expression in *G2-Gata4<sup>LacZ</sup>* transgenic embryos or tissues was detected by X-gal

staining, which was performed as described (28). Transverse and sagittal sections from X-gal-stained embryos and tissues were prepared and counterstained with Neutral Fast Red.

### **Immunohistochemistry (peroxidase)**

Immunohistochemistry on embryo tissue sections were performed as previously described (23). The primary antibodies used and the procedure applied for CD31<sup>+</sup> and WT1<sup>+</sup> cell quantification can be found in the SI.

### **Wt1<sup>CreYFP</sup> whole mount and time-lapse analysis**

For time lapse analysis, E11.5 *Wt1<sup>CreYFP</sup>* embryos were dissected in PBS supplemented with 10% FBS, penicillin (100U/ml) and streptomycin (100µg/ml). Hearts were isolated and transferred to HEPES buffered DMEM-F12 medium supplemented with 2% FBS and penicillin-streptomycin. An Alexa660-conjugated rat anti-mouse CD31 antibody (eBiosciences, #17-0311) was injected into the hearts through the outflow tract using a microinjector (PicospritzerII). Hearts were then embedded into a 1.5 mg/ml collagen gel (BD Bioscience) in a glass-bottom culture dish (MatTek). For CD31 whole mount immunofluorescence embryonic hearts were fixed in 4% paraformaldehyde and incubated in Alexa660-conjugated rat anti-mouse CD31 (eBiosciences, #17-0311). Images were captured in a Leica SP5 laser confocal microscope every 10 min for 12 h. During the image capture, the culture chamber was maintained at 37°C in a 5% CO<sub>2</sub> humidified atmosphere.

### **Flow cytometry (Fluorescence-Activated Cell Sorting, FACS)**

Hearts from *Wt1<sup>CreYFP</sup>* and *G2-Gata4<sup>CreYFP</sup>* were dissociated for 15 min at 37°C in a pre-warmed collagenase solution (0.1% in HBSS, 3mM CaCl<sub>2</sub>) (Sigma), and homogenized by repeated pipetting. Cell suspension was washed in PBS plus 2% FBS and 10mM HEPES. The cells were then incubated for 20 min on ice with Alexa660-conjugated rat anti-mouse CD31 (eBioscience #17-0311). After washing, the cell suspension was analyzed in a MoFlo cell sorter. Damaged cells were excluded from the analysis by propidium iodide staining. The standard deviation of replicates is indicated.



### **Author contributions**

EC, RC, ARV, AR, YYC, NW and KDW performed research; NW, NDH, RMC and JMPP designed research, analyzed data; JMPP wrote the manuscript.

### **Disclosures**

None.

### **Acknowledgments**

We thank Dr. John Burch and Dr. Brian Black for providing Wt1Cre and the G2-Gata4-LacZ mice, respectively. We also thank Dr. José Luis de la Pompa and Dr. Robert Kelly for fruitful discussions and David Navas (UMA) and John Pearson (BIONAND) for their technical support. This research was supported by MINECO grants BFU2012-35799 & ISCIII-RD12/0019-0022 (to JMPP), BFU2011-25304 (to RMC), EU FP7-Marie Curie-ITN actions PITN-GA-2011-289600 (to JMPP), Junta de Andalucía CTS-7564 (to RMC and JMPP), PI14/00814 ISCIII, co-funded by Fondos FEDER (to AR), the French Government (National Research Agency, ANR) through the "Investments for the Future" LABEX SIGNALIFE program (reference ANR-11-LABX-0028-01 to KDW), the Association pour la Recherche sur le Cancer (R13026AA-ARC-WAGNER), and Fondation de France (FDF-U1081-WAGNER) (KDW).

## References

1. Pérez-Pomares JM, de la Pompa JL (2011) Signaling during epicardium and coronary vessel development. *Circ Res* 109(12):1429–42.
2. Red-Horse K, Ueno H, Weissman IL, Krasnow M (2010) Coronary arteries form by developmental reprogramming of venous cells. *Nature* 464(7288):549–53.
3. Wu B, et al. (2012) Endocardial cells form the coronary arteries by angiogenesis through myocardial-endocardial VEGF signaling. *Cell* 151(5):1083–96.
4. Tian X, et al. (2014) De novo formation of a distinct coronary vascular population in neonatal heart. *Science* 345(6192):90–94.
5. Tian X, Pu WT, Zhou B (2015) Cellular Origin and Developmental Program of Coronary Angiogenesis. *Circ Res* 116(3):515–530.
6. Zhang Z, Zhou B (2013) Accelerated Coronary Angiogenesis by Vegfr1-Knockout Endocardial Cells. *PLoS One* 8(7):1–11.
7. Männer J, Pérez-Pomares JM, Macías D, Muñoz-Chápuli R (2001) The origin, formation and developmental significance of the epicardium: a review. *Cells Tissues Organs* 169(2):89–103.
8. Guadix JA, Carmona R, Muñoz-Chápuli R, Pérez-Pomares JM (2006) In vivo and in vitro analysis of the vasculogenic potential of avian proepicardial and epicardial cells. *Dev Dyn* 235(4):1014–1026.
9. Mikawa T, Fischman D a (1992) Retroviral analysis of cardiac morphogenesis: discontinuous formation of coronary vessels. *Proc Natl Acad Sci U S A* 89(20):9504–8.
10. Merki E, et al. (2005) Epicardial retinoid X receptor alpha is required for myocardial growth and coronary artery formation. *Proc Natl Acad Sci U S A* 102(51):18455–60.
11. Cai C, et al. (2008) A myocardial lineage derives from Tbx18 epicardial cells. *Nature* 454(5):104–108.
12. Zhou B, et al. (2008) Epicardial progenitors contribute to the cardiomyocyte lineage in the developing heart. *Nature* 454(7200):109–13.
13. Katz TC, et al. (2012) Distinct compartments of the proepicardial organ give rise to coronary vascular endothelial cells. *Dev Cell* 22(3):639–50.
14. Rojas A, et al. (2005) Gata4 expression in lateral mesoderm is downstream of BMP4 and is activated directly by Forkhead and GATA transcription factors through a distal enhancer element. *Development* 132(15):3405–3417.
15. Del Monte G, et al. (2011) Differential Notch signaling in the epicardium is required for cardiac inflow development and coronary vessel morphogenesis. *Circ Res* 108(7):824–36.
16. Wessels A, et al. (2012) Epicardially derived fibroblasts preferentially contribute to the parietal leaflets of the atrioventricular valves in the murine heart. *Dev Biol* 366(2):111–124.

17. Guadix JA, et al. (2011) Wt1 controls retinoic acid signalling in embryonic epicardium through transcriptional activation of Raldh2. *Development* 138(6):1093–7.
18. Forde A, Constien R, Gröne HJ, Hämmerling G, Arnold B (2002) Temporal Cre-mediated recombination exclusively in endothelial cells using Tie2 regulatory elements. *Genesis* 33:191–197.
19. Wagner K-D, et al. (2014) The Wilms' tumour suppressor Wt1 is a major regulator of tumour angiogenesis and progression. *Nat Commun* 5:5852.
20. Gittenberger-de Groot A, Vrancken Peeters MP, Mentink MM, Gourdie RG, Poelmann RE (1998) Epicardium-derived cells contribute a novel population to the myocardial wall and the atrioventricular cushions. *Circ Res* 82(10):1043–1052.
21. Pérez-Pomares JM, Macías D, García-Garrido L, Muñoz-Chápuli R (1998) The origin of the subepicardial mesenchyme in the avian embryo: an immunohistochemical and quail-chick chimera study. *Dev Biol* 200(1):57–68.
22. Männer J (1999) Does the subepicardial mesenchyme contribute myocardioblasts to the myocardium of the chick embryo heart? A quail-chick chimera study tracing the fate of the epicardial primordium. *Anat Rec* 255(2):212–26.
23. Wagner K, et al. (2002) The Wilms' tumor suppressor Wt1 is expressed in the coronary vasculature after myocardial infarction. *FASEB J* 16(9):1117–1119.
24. Duim SN, Kurakula K, Goumans M-J, Kruithof BPT (2015) Cardiac endothelial cells express Wilms' tumor-1. *J Mol Cell Cardiol* 81:127–135.
25. Wagner N, et al. (2005) Coronary vessel development requires activation of the TrkB neurotrophin receptor by the Wilms' tumor transcription factor Wt1. *Genes Dev* 19:2631–2642.
26. Wagner K, et al. (2003) Oxygen-regulated expression of the Wilms' tumor suppressor Wt1 involves hypoxia-inducible factor (HIF-1). *FASEB J* 17(10):1364–1366.
27. Rudat C, Kispert A (2012) Wt1 and epicardial fate mapping. *Circ Res* 111:165–169.
28. Anderson JP, et al. (2004) HRC Is a Direct Transcriptional Target of MEF2 during Cardiac, Skeletal, and Arterial Smooth Muscle Development In Vivo. *Mol Cell Biol* 24(9):3757–3768.

## Figure legends

**Figure 1. ST/PE G2-*Gata4* cells along cardiac development.** G2-*Gata4*<sup>LacZ</sup> mice show reporter activity in the septum transversum (including the PE, E9.5, **A**) and inflow myocardium (E11.5, **B**, arrowheads). Immunohistochemistry of G2-*Gata4*<sup>CreYFP+</sup> samples show the expression of GATA4 and WT1 proteins in G2-*Gata4*<sup>CreYFP+</sup> E9.5 proepicardial cells (**C**, **D**, respectively). Between stages E10.5 and E14.5, G2-*Gata4*<sup>CreYFP</sup> mice show an increasing number of G2-*Gata4*<sup>CreYFP+</sup> cells from the developing epicardium to subepicardial and intramyocardial areas (**E-F'**). Note that the epicardium comprises WT1<sup>+</sup>/G2-*Gata4*<sup>+</sup> (arrowheads) and WT1<sup>+</sup>/G2-*Gata4*<sup>-</sup> cells (arrows) (**F**). A few EPDCs retain WT1 expression transiently (**F'**, white arrow), whereas other EPDCs do not (**F'**, black arrows). *Wt1* gene expression is conspicuous in the epicardium (**G**) but restricted to a few EPDCs (**G**, arrowheads). Progressive expansion of EPDCs through the myocardial walls (E14.5-18.5) parallels G2-*Gata4*<sup>CreYFP+</sup> incorporation to developing coronary blood vessels (**H**, arrows). 3D reconstructions (**I**) and tissue section (**J**) analysis of the developing coronary vasculature allows distinguishing perivascular (**I**, **J**, arrows) from G2-*Gata4*<sup>CreYFP+</sup> CoE cells (**I**, **J**, arrowheads). Identification of active Notch1 signaling by Notch1 intracellular domain (NICD) nuclear localization confirms the arterial nature of these vessels (**K**, **L**, arrows). **Abbreviations:** A: atrium; AVC: atrio-ventricular canal; ENDO: endocardium; EPI: Epicardium; IVS: Interventricular Septum; MYO: myocardium; PE: proepicardium; ST: septum transversum; V: ventricle. **Bars:** **A**, **B**=100μm; **C-H**=50μm; **I**=40μm; **J**=10μm; **K**=25μm; **L**=5μm.

**Figure 2. *Wt1*-expressing cells and their progeny contribute to coronary endothelium.** WT1 protein is ubiquitously expressed in early (E10.5-12.5) epicardial cells and EPDCs, extensively overlapping with *Wt1* promoter-driven *GFP* expression (**A**, **B**, arrowheads). Reporter expression in *Wt1*<sup>GFP</sup> embryos is frequently observed in subepicardial and intramyocardial coronary vasculature (CD31<sup>+</sup>) between E12.5-E14.5 (**D**, **G**, arrows), but not at earlier developmental stages (**C**). A number of *Wt1*<sup>GFP+</sup>/CD31<sup>+</sup> cells are still found at perinatal stages (**H**, arrows). Early *Wt1*<sup>CreYFP+</sup> cells form the epicardium (**I**, arrowheads; the area marked with a black arrowhead is magnified in **I'**) and first

EPDCs (**J**, arrows). At E11.5 many *Wt1<sup>CreYFP+</sup>* epicardial cells show morphological features of EMT (**I'**, arrowhead). Between E12.5-E15.5, the lineage reporter co-localizes with the vascular marker CD31 in the subepicardial and intramyocardial coronary plexus (**J**, arrows). Perinatal and neonatal coronary arteries show *Wt1<sup>CreYFP+</sup>* cells incorporated to both the CoE (**L**, **O**, arrowheads) and CoSM layers (**M**, **O**, arrowheads). **P-R**. Tamoxifen-induced (E9.5) *Wt1<sup>CreERT2</sup>;Rosa26-YFP* embryos show YFP<sup>+</sup> cell incorporation (E.14.5) to coronary vessels (arrowheads). **S**. The total percentage of cardiac *Wt1<sup>CreYFP+</sup>* and *G2<sup>CreYFP+</sup>* cells along development is shown. **T**. Representative cytograms of dissociated ventricles from midgestation embryos and neonates. Quadrant limits were established with fluorochrome-conjugated isotypes. Numbers indicate percentages on total events. Both *Wt1<sup>CreYFP+</sup>* and *G2<sup>CreYFP+</sup>* populations include CD31<sup>+</sup> in cells. **Abbreviations**: A: atrium; AVC: atrioventricular canal; CoA: coronary artery; CoV: coronary veins; ENDO: endocardium; EPI: epicardium; IVS: interventricular septum; V: ventricle. **Bars**: **I**=100μm; **A**, **C**, **D**, **J**, **M**, **N**, **P**=50μm; **B**, **F**, **G**, **H**, **K**, **L**, **R**=25μm; **E-E''**, **O**=10μm; **Q**=5μm.

**Figure 3. G2-Gata4 and conditional systemic Wt1 deletion disrupt coronary artery formation.**

*Wt1* deletion in *G2-Gata4* cells disrupts intramyocardial CD31<sup>+</sup> coronary blood vessels. Embryonic coronary endothelial structures are dysmorphic and fail to contact the endocardium (double-headed arrows, compare **A** with **D**, movie S3 with S4), and mutants show a dramatic reduction of compact ventricular myocardium thickness (double-headed arrows, compare **B** with **E**). N1ICD immunohistochemistry identifies dysmorphic intramyocardial vessels as coronary arteries (arrowheads, **C**, **F**), whereas subepicardial vascular structures are N1ICD<sup>-</sup> (prospective coronary veins). Tamoxifen-induced (E10.5) systemic *Wt1* deletion has similar effects to *G2-Gata4*-driven *Wt1* deletion. At E13.5, the number of developing intramyocardial blood vessels is even lower than in the former (**G**, **H**). Despite the sharp thinning of the compact ventricular myocardium, subepicardial blood vessels still form (**I-I''**, arrowheads). **Abbreviations**: CoA: coronary artery; CoV: coronary vein; ENDO: Endocardium; EPI: Epicardium; V: ventricle. **Bars**: **A**, **B**=50μm; **C**=25μm; **D**, **E**, **G**, **H**, **I**=50μm; **F**=25μm.

**Figure 4. Endothelial *Wt1* expression is required for coronary vessel formation.** Hematoxylin-eosin stained sections from control *Wt1<sup>LoxP/LoxP</sup>*+Tamoxifen (A) and mutant *Tie<sup>CreERT2</sup>;Wt1<sup>LoxP/LoxP</sup>*+Tamoxifen (B) E16.5 embryos. WT1<sup>+</sup> cell contribution (C, arrows) to coronary vessels is reduced in the mutants (D) as confirmed by quantification of the area density occupied by WT1<sup>+</sup> cells (E) and *Wt1* gene expression (F) in E16.5 mutant and control embryonic hearts. CD31 staining of E16.5 control and mutant embryonic hearts shows a decrease in compact ventricular wall coronary endothelial cells in the experimental group (arrow in H; compare with G). I. Quantification of the area density occupied by CD31<sup>+</sup> cells and *CD31* gene expression (Fig.4J) from E16.5 hearts further supports CoE depletion in mutant animals. **Abbreviations:** A: atrium; EPI: epicardium; V: ventricle. **Bars:** 50µm. Data are mean ± SEM. \*p<0.05, \*\*\*p<0.001.

**Figure 5. A model on EPDC contribution to CoE.** G2-*Gata4<sup>CreYFP+</sup>/Wt1<sup>CreYFP+</sup>* ST/PE cells (green) are transferred to the surface of the heart to form the epicardium; and then epicardial-derived cells invade heart walls (A-C). The boxed area in C is magnified in D. G2-*Gata4<sup>CreYFP+</sup>/Wt1<sup>CreYFP+</sup>* cells (green) preferentially incorporate to intramyocardial coronary arterial and capillary endothelium. Color arrows indicate the major transmural endothelial cell flows. Note that the arrow size estimates the frequency of the events. **Abbreviations:** A: atrium; A-V: arterio-venous; CoA: coronary arteries; CoV: coronary veins; EPI: epicardium; PE: proepicardium; ST: septum transversum; SV: sinus venosus; V: ventricle.