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Wt1 controls retinoic acid signalling in embryonic epicardium through transcriptional activation of Raldh2

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SUMMARY

Epicardial-derived signals are key regulators of cardiac embryonic development. An important part of these signals is known to relate to a retinoic acid (RA) receptor-dependent mechanism. RA is a potent morphogen synthesised by Raldh enzymes, Raldh2 being the predominant one in mesodermal tissues. Despite the importance of epicardial retinoid signalling in the heart, the molecular mechanisms controlling cardiac *Raldh2* transcription remain unknown. In the current study, we show that *Wt1*-null epicardial cells display decreased expression of *Raldh2* both in vivo and in vitro. Using a RA-responsive reporter, we have confirmed that *Wt1*-null epicardial cells actually show reduced synthesis of RA. We also demonstrate that *Raldh2* is a direct transcriptional target of Wt1 in epicardial cells. A secondary objective of this study was to identify the status of RA-related receptors previously reported to be critical to epicardial biology (PDGFRα,β; RXRα). PDGFRα and PDGFRβ mRNA and protein levels are downregulated in the absence of Wt1, but only *Pdgfra* expression is rescued by the addition of RA to Wt1-null epicardial cells. RXRα mRNA levels are not affected in *Wt1*-null epicardial cells. Taken together, our results indicate that Wt1 critically regulates epicardial RA signalling via direct activation of the *Raldh2* gene, and identify a role for Wt1 in the regulation of morphogen receptors involved in the proliferation, migration, and differentiation of epicardial and epicardially-derived cells (EPDC).

Keywords: Mouse embryo, Wilms' tumour suppressor gene, Retinoic acid, Raldh2, Epicardium

INTRODUCTION

Retinoic acid (RA) regulates a variety of crucial events during early embryonic development, acting in a specific organ-dependent manner to control the differentiation of many cell types (Duester, 2008; Bayha et al., 2009). Retinaldehyde dehydrogenases (RALDHs) are essential enzymes for RA synthesis in the developing embryo, with Raldh2 being the most broadly expressed dehydrogenase in mesodermal tissues (Niederreither et al., 1997; Niederreither et al., 2002; Moss et al., 1998).

Heart development is dramatically affected by RA deficiency, including anomalies in the compaction of ventricular myocardium (Wessels and Pérez-Pomares, 2004) and abnormal septation (Sinning, 1998). Availability of RA during cardiac development is mediated by Raldh2 expression. This is first defined by a rostrocaudal wave at the anterior lateral mesoderm, followed by a secondary wave that progresses from the cardiac inflow towards the ventricular and conotruncal myocardium, and a final one confined to the epicardium, the outermost tissue layer of the heart (Xavier-Neto et al., 2000; Niederreither et al., 2001; Hochgreb et al., 2003). Not surprisingly, *Raldh2*$^{-/-}$ embryos display early and lethal defects in cardiac development (Niederreither et al., 2001).

The epicardium and epicardially derived cells (EPDC) are pivotal to coronary vascular development (Pérez-Pomares et al., 1998; Pérez-Pomares et al., 2002; Dettman et al., 1998; Landerholm et al., 1999; Tomanek et al., 2001; Tomanek et al., 2008). However, there is not much known about the nature of the RA-dependent signals responsible for epicardial and EPDC proliferation, differentiation and involvement in myocardial growth (Kang and Sucov, 2005; Lavine et al., 2005; Merki et al., 2005). Epicardial and EPDC express the protein encoded by the Wilms' tumour suppressor gene, *Wt1* (Moore et al., 1999; Pérez-Pomares et al., 2002). *Wt1*-null mice die at midgestation with defects in the formation of several organs (Kreidberg et al., 1993), including a characteristic thinning of the myocardium reminiscent to that exhibited by RA receptor (*Rxra*) mutant mice (Kastner et al., 1994; Sucov et al., 1994). As myocardial cells do not express Wt1, this cardiac muscle phenotype is accepted to be dependent on Wt1-regulated epicardial paracrine signals (Moore et al., 1999; Chen et al., 2002). Further research on *Wt1*-null embryos has shown that coelomic cells lining the liver also display a decreased Raldh2 expression concomitant with liver hypoplasia (IJpenberg et al., 2007).

As Wt1 and Raldh2 expression patterns overlap in space and time, we have analysed RA expression in *Wt1*-null mice, and identified Wt1 as a direct regulator of *Raldh2* transcription during epicardial development. Secondary to these findings, we suggest a role for Wt1 in the regulation of RA-related morphogen receptors essential to epicardial and EPDC biology.

MATERIALS AND METHODS

The animals used in our research program were handled in compliance with the international guidelines for animal care and welfare. *Wt1*-null, *yWT470LacZ* (Wt1-LacZ) and *Wt1*-GFP mouse lines were generated as previously described (Kreidberg et al., 1993; Moore et al., 1999; Hosen et al., 2007).

Histology and immunohistochemistry

Single Raldh2, CD31 and Wt1 immunofluorescence and immunoperoxidase on 10 μM sections and cultured cells, as well as Wt1-*lacZ* embryo processing were performed as described elsewhere (IJpenberg et al., 2007). Double immunohistochemistry involving Wt1 was carried out

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as previously described (Pérez-Pomares et al., 2002) using anti-PDGFRα, anti-PDGFRβ and anti-RXRα antibodies (eBioscience 14-1401-81; Sc-432; Sc-553, respectively). A non-specific rabbit IgG was used instead of the primary antibodies for negative controls.

Immortalised epicardial cell lines (MEEC)

Hearts from control ($Wt1^{GFP/+}$) and *Wt1*-null mice ($Wt1^{GFP/-}$) carrying the 'immorto' transgene (Jat et al., 1991) were isolated at E11.5 and cultured on 24-well gelatinised dishes containing DMEM. After 24 hours, hearts were removed and epicardial cells attached to the surface were cultured and propagated as described previously Martínez-Estrada et al. (Martínez-Estrada et al., 2010). Treatments with retinoic acid (*all-trans*, Sigma) were performed at 1 μM concentration (24 hours). Control for the treatments was incubation with the RA vehicle (ethanol 100°, 1:1000).

Western blotting

Western blots were performed as described previously (Martínez-Estrada et al., 2010).

Luciferase and RA reporter assays

The Raldh2 upstream region (830 bp) was amplified by PCR from mouse genomic DNA and cloned into the pGL4.10 vector (Promega). Primer sequences were: forward, 5′-AAAGCTAGCTCTCCCACGCTATCTGG-AGT-3′; reverse, 5′-AAAAGATCTGATCTCGCTGGAAGTCATGG-3′. PCR product was digested with *Nhe*I and *Bgl*II and cloned into the corresponding sites upstream of the firefly luciferase construct in the pGL4.10 vector.

This reporter construct (100 ng) was transfected in immortalised epicardial cells, in the presence of the indicated amounts of expression construct encoding –KTS Wt1 isoform (Martinez-Estrada et al., 2010).

Site-directed Raldh2 promoter mutagenesis assays

Site-directed mutagenesis was performed with the QuikChange II XL kit (Stratagene), following the manufacturer's instructions. The oligonucleotides and their reverse-complementary sequences used were: Mut1, 5′-CTGGGCCGGCCCCCAAAGCCTGTGGGCC-3′; Mut2, 5′-AAGAAGCGAAACCCGCTTGACACCTGCC-3′; Mut3, 5′-ACAGCG-AGCGAAACCCGGCCGCGCAGCG-3′. All mutations were verified by sequencing.

ChIP assay

Chromatin immunoprecipitation (ChIP) assays were performed on immortalised epicardial cells (Martínez-Estrada et al., 2010) using an EZchIP (Upstate Biotechnology) according to the manufacturer's protocols. The primer sequences used were: positive forward, GCTGGA-AGGAGACCATCAAGAC; positive reverse, GAGCCTTAGCGC-TGGTGGGCCG; negative forward, AGCTACCTCCCCACCATTCT; and negative reverse: GCCTAAAGTGACCGAGCAAG.

Electrophoretic mobility shift assay

Electrophoretic mobility shift assay was performed using biotin 3′-end labelled 21 bp probes for the three Wt1 putative-binding sites identified in Fig. 2F′ on MEEC nuclear extracts. Probes were incubated with 6 μg of nuclear protein extracts for 20 minutes at room temperature. The remaining steps follow the LightShift Chemiluminescent EMSA Kit protocol (Pierce).

RNA extraction and semi-quantitative PCR

Total RNA from immortalised epicardial cells was extracted using RNeasy Mini Kit (Qiagen) and reverse-transcripted using oligo dT18 (First Strand cDNA Synthesis Kit/AMV, ROCHE). β-Actin was amplified as reference gene.

Quantitative PCR

Real-time quantitative PCR reactions were performed in an ABI7500 (Applied Biosystems, Carlsbad, CA, USA) and carried out using specific TaqMan Gene Expression Assays, using Eukaryotic 18s rRNA (reference 4319413E from Applied Biosystems) as a reference gene.

Data analysis involved a relative quantification with $2^{-\Delta Cq}$ method [modified from Yuan et al. (Yuan et al., 2006)]; included $2^{-\Delta Cq}$ Std Err values between 1-8.5%, followed by an unpaired *t*-test with $n \geq 3$ ($P<0.05$).

For qPCR of FACS-sorted GFP$^+$ epicardial cells, hearts of $Wt1^{GFP/+}$ and $Wt1^{GFP/-}$ mice were dissected and the ventricles trypsinised for 15 minutes at 37°C in a temperature controlled shaker at 1000 rpm. Analysis of gene expression was carried out by Taqman real-time PCR (Roche) using *Gapdh* as reference gene. qPCR on embryonic epicardial immortalised cells was performed using the following references from Applied Biosystems: RXRα, Mm00441182_m1; PDGFRβ, Mm01262489_m1; PDGFRα, Mm01211694_m1.

RESULTS AND DISCUSSION

To investigate whether Wt1 plays a role in the regulation of *Raldh2* expression in the embryonic epicardium, we analysed Raldh2 protein expression in the heart of *Wt1*-null embryos. The epicardium/EPDC of *Wt1*-null mice express other epicardial markers normally (cytokeratin, Fig. 1A,B), but display a clear decrease in Raldh2 protein (compare Fig. 1C,E with 1D,F). Strikingly, Raldh2 protein expression in *Wt1*-null atrial epicardium is not as affected as in the ventricles. This singular feature of the Wt1-null cardiac phenotype could depend on the impact of different segment-specific signalling properties of the embryonic myocardium (Mjaatvedt et al., 1987) in the epicardial transcriptome, rather than on chamber-related intrinsic/autonomous epicardial programs. This hypothesis is partially supported by the loss of cardiac inflow epicardial Raldh2 expression in *Wt1*-mutant mice (Norden et al., 2010). By using the *Wt1* GFP KI mice (Hosen et al., 2007), we have been able to select GFP$^+$ epicardial cells in control and KO mice. qPCR analysis of FACS-sorted GFP$^+$ epicardial cells confirmed the lack of Wt1 expression in *Wt1*-null cells (see Fig. S1 in the supplementary material) and indicated a clear downregulation of *Raldh2* levels in the mutant epicardium

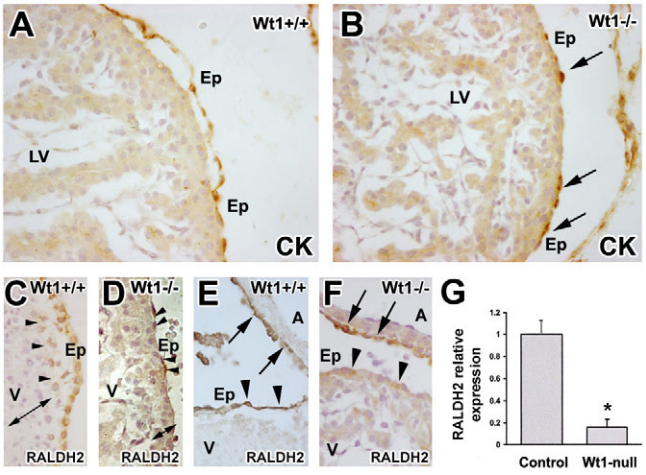

Fig. 1. Wt1 deletion is associated with downregulation of Raldh2 expression in the epicardium. (**A**,**B**) Cytokeratin (CK) immunoreactivity in the left ventricle (LV) of E11.5 control (A) and *Wt1*-null (B) mouse embryos. The epicardium (Ep) is discontinuous in mutant embryos, but present epicardial cells normally express CK (arrows in B). (**C**,**D**) Raldh2 immunoreactivity is conspicuous in wild-type ventricular (V) epicardium (Ep) and epicardially derived cells (C, arrowheads), but clearly reduced in *Wt1*-null mice (D, arrowheads). The thickness of compact ventricular myocardium is reduced in *Wt1*-null mice (C,D, double-headed arrows). (**E**,**F**). Raldh2 expression in the atrioventricular (A, atrium; V, ventricle) region of wild-type (E) and *Wt1*-null (F) mice. Atrial immunoreactivity is not significantly reduced (E,F, arrows) when compared with the decay of protein levels in the ventricles of *Wt1*-null mice (arrowheads). (**G**) *Raldh2* qPCR in control and *Wt1*-null GFP-sorted E11.5 epicardial cells (*$P<0.05$, 10 d.o.f.). Data are mean+s.e.m.

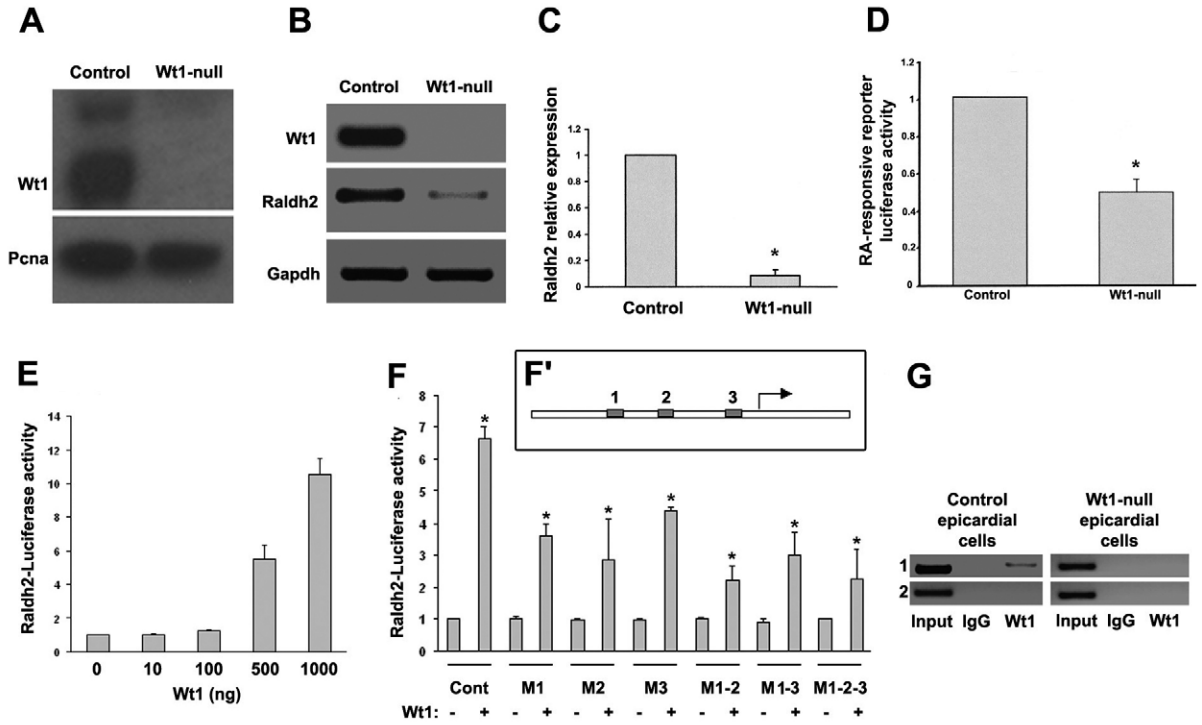

Fig. 2. Wt1 is an activator of *Raldh2* in epicardial cells. (**A**) Wt1 western blot in control and *Wt1*-null immortalised epicardial cells. (**B**,**C**) *Raldh2* semi-qPCR (B) and qPCR (C) in control and *Wt1*-null immortalised epicardial cells. (**D**) RA-luciferase responsive reporter activity assay in control and *Wt1*-null immortalised epicardial cells. (**E**) The –KTS Wt1 isoform activates a *Raldh2* promoter fragment containing the putative binding sites in a dose-dependent manner. (**F**,**F'**) *Raldh2* promoter-luciferase mutagenesis assay. Reporter constructs, carrying single, double or triple mutations (see the schematic representation of the putative conserved Wt1-binding sites in the *Raldh2* promoter, F′) were transiently co-transfected into epicardial cells with –KTS Wt1 expression vector. (**G**) ChIP assay in control and Wt1-null immortalised epicardial cells. Binding of Wt1 protein to the *Raldh2* promoter is amplified with primers for Wt1 putative binding sites to *Raldh2* promoter (1) or primers for regions upstream these sites (2). All asterisks indicate statistical significance (*P<0.05, 10 d.o.f.). Data are mean+s.e.m.

(Fig. 1G). These first results, together with Wt1/Raldh2 protein overlapping spatiotemporal patterns, set the main and primary objective of our study, i.e. to examine whether Raldh2 embryonic epicardial expression is regulated by Wt1.

To initiate a molecular analysis of Wt1-*Raldh2* relationship, we generated *Wt1* KO immortalised epicardial cells. FACS analysis demonstrated GFP expression in control and *Wt1* KO epicardial cells (data not shown), while western blot analysis showed Wt1 expression only in control cells (Fig. 2A). We also checked *Raldh2* expression using semi-qPCR (Fig. 2B) and qPCR (Fig. 2C) in both control and *Wt1*-null immortalised epicardial cells, and found similar levels of downregulation as in the vivo epicardium. A RA-luciferase responsive reporter assay showed a significantly lower signal in mutant immortalised epicardial cells (Fig. 2D), indicating that *Raldh2* downregulation in absence of *Wt1* expression involves an actual reduction of RA synthesis.

Despite the importance of RA/Raldh2 in epicardium and heart development, very little is known about the transcriptional regulation of epicardial *Raldh2*. The strong reduction of *Raldh2* mRNA levels in both freshly isolated and cultured *Wt1* KO epicardial cells prompted us to analyse whether Wt1 could be a direct transcriptional regulator of the *Raldh2* gene. We identified three putative conserved Wt1-binding sites in the *Raldh2* promoter that were confirmed to interact in vitro with Wt1 protein by using an EMSA (see Figs S2 and S3 in the supplementary material). The –KTS Wt1 isoform was able to activate the *Raldh2* promoter fragment containing the putative binding sites in a dose-dependent manner (Fig. 2E). Then, we generated a series of reporter constructs that carried individual, double or triple mutations in the binding sites (Fig. 2F′). Constructs with a single mutation (M1, M2 or M3) became less sensitive to –KTS Wt1 activation when compared with the control group. Double mutation (M1-2; M1-3) reduced Wt1 activation by 70%; similar results were obtained for the triple mutant (M1-2-3) (Fig. 2F). Thus, Raldh2 is activated via Wt1 sites in its promoter. Chromatin-IP (Fig. 2G) confirmed binding of Wt1 to the endogenous *Raldh2* promoter in epicardial cells.

RA activity has multiple effects on a variety of signalling pathways. As we have shown that Wt1-null epicardial RA levels are significantly reduced, a secondary objective of our study was to evaluate whether this RA decrease actually affected RA-related receptors known to be important for epicardial and EPDC development such as RXRα, PDGFRα and PDGFRβ. Wt1, which is expressed in all epicardial and EPDC during the first days of epicardial embryonic development (Fig. 3A-C), colocalises with these three receptors in the epicardium and EPDC (Fig. 3D-F). In vivo mRNA expression of these molecules in $Wt1^{GFP}$-sorted E11.5 epicardial cells was confirmed by qPCR (see Fig. 4).

RXRα is expressed in epicardial cells and its signalling is crucial to epicardial and myocardial development (Chen et al., 1998; Chen et al., 2002; Merki et al., 2005). This, together with the similarities reported for the cardiac phenotype of the *Wt1*-null and the *Rxra*-null mouse embryos (Kreidberg et al., 1993; Kastner et al., 1994), led us to study RXRα epicardial expression in Wt1-null embryos. RXRα-positive epicardial cells are infrequent in control and Wt1-

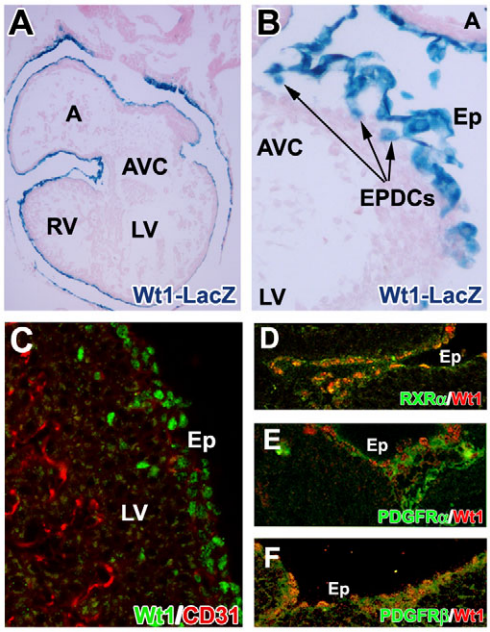

Fig. 3. Mouse embryonic epicardial cells express RA-related receptors. (**A**,**B**) E11.5 *Wt1-lacZ* mouse heart. At this stage, all epicardial and epicardially derived cells express Wt1, as shown by the reporter activity (blue). (**C**) Wt1 protein expression in E11 epicardium is confirmed by immunohistochemistry. Protein colocalisation of Wt1 with RXRα, PDGFRα and PDGFRβ is illustrated in D-F. Abbreviations: A, atrium; AVC, atrioventricular canal/cushions; Ep, epicardium; EPDCs, epicardially derived cells; LV, left ventricle; RV, right ventricle.

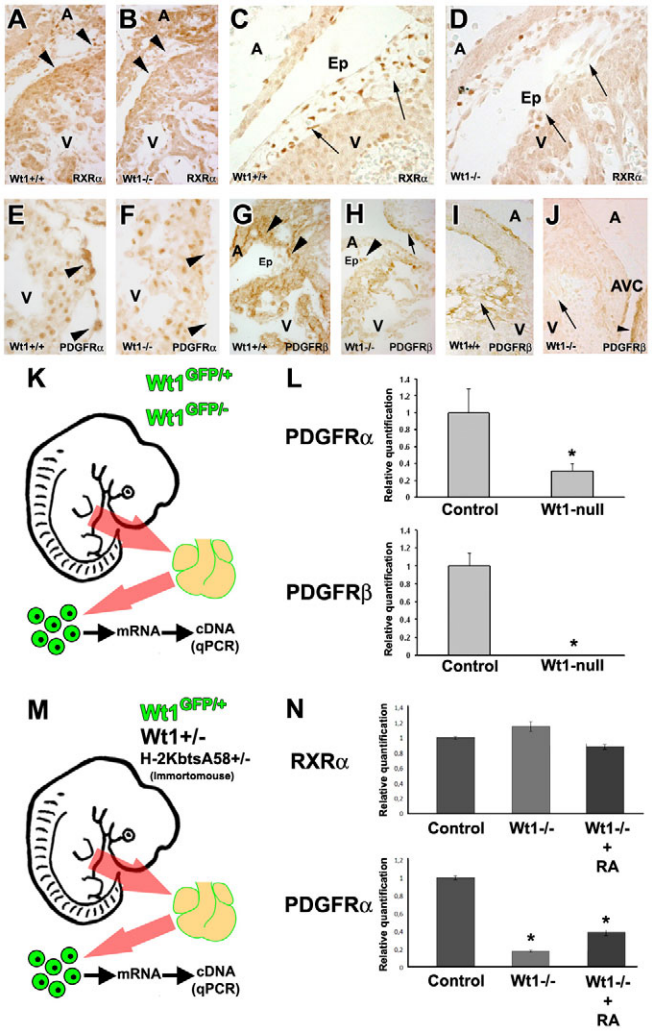

Fig. 4. Status of RA-related receptors in Wt1-null epicardial cells. (**A-D**) RXRα immunoreactivity in E11.5 wild-type (A) and Wt1-null (B) embryonic epicardium is not significantly different (arrowheads); differences in RXRα epicardial immunoreactivity in E13.5 wild-type (C) and Wt1-null (D) embryos are evident (arrows). (**E**,**F**) PDGFRα immunoreactivity in the ventricular epicardium of E11.5 Wt1-null embryos (F, arrowheads) is reduced when compared with stage-matched wild types (E, arrowheads). (**G-J**) PDGFRβ immunoreactivity is reduced in the ventricular (H, arrowhead) but not atrial epicardium (H, arrow) of E11.5 and E13.5 *Wt1*-null embryos (J, arrow) when compared with E11.5 (G, arrowheads) and E13.5 (I, arrow) wild types. The Wt1-negative cells of endocardial cushions are PDGFRβ positive (J, arrowhead). (**K**) Procedure for the characterisation of embryonic epicardial cells. *Wt1*$^{GFP/+}$ and *Wt1*$^{GFP/-}$ hearts were dissected, GFP-positive epicardial cells sorted, mRNA extracted and analysed by qPCR (**L**). (**M**) Procedure for the isolation and qPCR analysis of control (*Wt1*$^{GFP/+}$) and *Wt1*-null (*Wt1*$^{GFP/-}$) immortalised epicardial cells. (**N**) qPCR analysis of *Rxra*, *Pdgfra*. RA treatment (1 μM RA for 24 hours) of mutant cells did not affect *Rxra* levels but did increase *Pdgfra* levels in *Wt1*-null cells. All asterisks indicate statistical significance (P<0.05, 10 d.o.f.). Data are mean+s.e.m. Abbreviations: A, atrium; Ep, epicardium; V, ventricle.

null embryos (E11.5). Immunohistochemistry did not record any significant changes between these two groups at this developmental stage (Fig. 4A,B). This was further confirmed by qPCR on E11.5 control and Wt1-deficient epicardial immortalised cells (Fig. 4N). However, the number of RXRα-positive cells in the epicardium/EPDC of *Wt1*-null embryos, when compared with stage-matched controls, is reduced at later stages (E13.5) (Fig. 4C,D). This could relate to the defective epicardial epithelial-mesenchymal transition of Wt1 mutants (Moore et al., 1999; Martínez-Estrada et al., 2010). Interestingly, epicardial cells also express *Rara*, although at lower levels in control than in *Wt1*-null epicardial cells (see Fig. S4 in the supplementary material). This finding is in accordance with the previously reported active inhibition of RARα by Wt1 (Goodyer et al., 1995).

PDGF signalling through PDGF receptors α and β, both expressed in the epicardium (Lu et al., 2001; Guadix et al., 2006), is known to be important for epicardial/EPDC proliferation and migration (Kang et al., 2008; Mellgren et al., 2008). We found a much reduced immunoreactivity for PDGFRα (Fig. 4E,F) and PDGFRβ receptors in E11.5 (Fig. 4G,H) and E13.5 (Fig. 4I,J) Wt1-null embryos. qPCR in control (Wt1^{GFP+}) and Wt1-null (Wt1^{GFP+}) sorted E11.5 epicardial cells also revealed a significant reduction in the expression of *Pdgfra* and *Pdgfrb* in mutant versus control cells (Fig. 4L). qPCR analysis on control and Wt1-null epicardial immortalised cells confirmed these results for *Pdgfra* (Fig. 4N). However, *Pdgfrb* levels in *Wt1*-null epicardial immortalised cells seemed to be extremely variable, and therefore immortalised *Wt1*-null cells were not used as a model to study the transcriptional activity of this specific gene. Remarkably, the low expression levels of *Pdgfra* in *Wt1*-null immortalised epicardial cells could be partially rescued by RA treatment (Fig. 4N). Such stimulation of the expression of *Pdgf/Pdgfr* by RA, inducing cell proliferation, has been reported in other systems (Tsukamoto et al., 1994; Liebeskind et al., 2000), thus suggesting that Wt1 function in the regulation of *Pdgfra* is mediated in part by RA signalling. More extensive analysis will be required to delineate the molecular mechanisms underlying *Pdgfra* and *Pdgfrb* downregulation in *Wt1*-null epicardium.

In summary, the connection between Wt1 and RA suggested by our results provides new and crucial information on the regulation of epicardial development, opening new avenues on the research of the cell-autonomous and non-autonomous functions of Wt1 during cardiac embryogenesis.

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Competing interests statement
The authors declare no competing financial interests.

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References

Bayha, E., Jørgensen, M. C., Serup, P. and Grapin-Botton, A. (2009). Retinoic acid signalling organizes endodermal organ specification along the entire antero-posterior axis. *PLoS One* **4**, e5845.
Chen, J., Kubalak, S. W. and Chien, K. R. (1998). Ventricular muscle-restricted targeting of the RXRalpha gene reveals a non-cell-autonomous requirement in cardiac chamber morphogenesis. *Development* **125**, 1943-1949.
Chen, T. H., Chang, T. C., Kang, J. O., Choudhary, B., Makita, T., Tran, C. M., Burch, J. B., Eid, H. and Sucov, H. M. (2002). Epicardial induction of fetal cardiomyocyte proliferation via a retinoic acid-inducible trophic factor. *Dev. Biol.* **250**, 198-207.
Dettman, R. W., Denetclaw, W., Jr, Ordahl, C. P. and Bristow, J. (1998). Common epicardial origin of coronary vascular smooth muscle, perivascular fibroblasts, and intermyocardial fibroblasts in the avian heart. *Dev. Biol.* **193**, 169-181.
Duester, G. (2008). Retinoic acid synthesis and signalling during early organogenesis. *Cell* **134**, 921-931.
Goodyer, P., Dehbi, M., Torban, E., Bruening, W. and Pelletier, J. (1995). Repression of the retinoic acid receptor-alpha gene by the Wilms' tumor suppressor gene product, wt1. *Oncogene* **10**, 1125-1129.
Guadix, J. A., Carmona, R., Muñoz-Chápuli, R. and Pérez-Pomares, J. M. (2006). In vivo and in vitro analysis of the vasculogenic potential of avian proepicardial and epicardial cells. *Dev. Dyn.* **235**, 1014-1026.
Hochgreb, T., Linhares, V. L., Menezes, D. C., Sampaio, A. C., Yan, C. Y., Cardoso, W. V., Rosenthal, N. and Xavier-Neto, J. (2003). A caudorostral wave of RALDH2 conveys anteroposterior information to the cardiac field. *Development* **130**, 5363-5374.
Hosen, N., Shirakata, T., Nishida, S., Yanagihara, M., Tsuboi, A., Kawakami, M., Oji, Y., Oka, Y., Okabe, M., Tan, B. et al. (2007). The Wilms' tumor gene WT1-GFP knock-in mouse reveals the dynamic regulation of WT1 expression in normal and leukemic hematopoiesis. *Leukemia* **21**, 1783-1791.
IJpenberg, A., Pérez-Pomares, J. M., Guadix, J. A., Carmona, R., Portillo-Sánchez, V., Macías, D., Hohenstein, P., Miles, C. M., Hastie, N. D. and Muñoz-Chápuli, R. (2007). Wt1 and retinoic acid signalling are essential for stellate cell development and liver morphogenesis. *Dev. Biol.* **312**, 157-170.
Jat, P. S., Noble, M. D., Ataliotis, P., Tanaka, Y., Yannoutsos, N., Larsen, L. and Kioussis, D. (1991). Direct derivation of conditionally immortal cell lines from an H-2Kb-tsA58 transgenic mouse. *Proc. Natl. Acad. Sci. USA* **88**, 5096-5100.
Kang, J., Gu, Y., Li, P., Johnson, B. L., Sucov, H. M. and Thomas, P. S. (2008). PDGF-A as an epicardial mitogen during heart development. *Dev. Dyn.* **237**, 692-701.
Kang, J. O. and Sucov, H. M. (2005). Convergent proliferative response and divergent morphogenic pathways induced by epicardial and endocardial signalling in fetal heart development. *Mech. Dev.* **122**, 57-65.
Kastner, P., Grondona, J. M., Mark, M., Gansmuller, A., LeMeur, M., Decimo, D., Vonesch, J. L., Dollé, P. and Chambon, P. (1994). Genetic analysis of RXR alpha developmental function, convergence of RXR and RAR signalling pathways in heart and eye morphogenesis. *Cell* **78**, 987-1003.
Kreidberg, J. A., Sariola, H., Loring, J. M., Maeda, M., Pelletier, J., Housman, D. and Jaenisch, R. (1993). WT-1 is required for early kidney development. *Cell* **74**, 679-691.
Landerholm, T. E., Dong, X. R., Lu, J., Belaguli, N. S., Schwartz, R. J. and Majesky, M. W. (1999). A role for serum response factor in coronary smooth muscle differentiation from proepicardial cells. *Development* **126**, 2053-2062.
Lavine, K. J., Yu, K., White, A. C., Zhang, X., Smith, C., Partanen, J. and Ornitz, D. M. (2005). Endocardial and epicardial derived FGF signals regulate myocardial proliferation and differentiation in vivo. *Dev. Cell* **8**, 85-95.
Liebeskind, A., Srinivasan, S., Kaetzel, D. and Bruce, M. (2000). Retinoic acid stimulates immature lung fibroblast growth via a PDGF-mediated autocrine mechanism. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **279**, 81-90.
Lu, J., Landerholm, T. E., Wei, J. S., Dong, X.-R., Wu, S.-P., Liu, X., Nagata, K., Inagaki, M. and Majesky, M. W. (2001). Coronary smooth muscle differentiation from proepicardial cells requires RhoA-mediated actin reorganization and p160 Rho-kinase activity. *Dev. Biol.* **240**, 404-418.
Martínez-Estrada, O. M., Lettice, L. A., Essafi, A., Guadix, J. A., Slight, J., Velecela, V., Hall, E., Reichmann, J., Devenney, P. S., Hohenstein, P. et al. (2010). Wt1 is required for cardiovascular progenitor cell formation through transcriptional control of Snail and E-cadherin. *Nat. Genet.* **42**, 89-93.
Mellgren, A. M., Smith, C. L., Olsen, G. S., Eskiocak, B., Zhou, B., Kazi, M. N., Ruiz, F. R., Pu, W. T. and Tallquist, M. D. (2008). Platelet-derived growth factor receptor beta signalling is required for efficient epicardial cell migration and development of two distinct coronary vascular smooth muscle cell populations. *Circ. Res.* **103**, 1393-1401.
Merki, E., Zamora, M., Raya, A., Kawakami, Y., Wang, J., Zhang, X., Burch, J., Kubalak, S. W., Kaliman, P., Belmonte, J. C. et al. (2005). Epicardial retinoid X receptor alpha is required for myocardial growth and coronary artery formation. *Proc. Natl. Acad. Sci. USA* **102**, 18455-18460.
Mjaatvedt, C. H., Lepera, R. C. and Markwald, R. R. (1987). Myocardial specificity for initiating endothelial-mesenchyma transition by an in vivo adheron-like complex. *Dev. Biol.* **136**, 118-128.
Moore, A. W., McInnes, L., Kreidberg, J., Hastie, N. D. and Schedl, A. (1999). YAC complementation shows a requirement for Wt1 in the development of epicardium, adrenal gland and throughout nephrogenesis. *Development* **126**, 1845-1857.
Moss, J. B., Xavier-Neto, J., Shapiro, M. D., Nayeem, S. M., McCaffery, P., Dräger, U. C. and Rosenthal, N. (1998). Dynamic patterns of retinoic acid synthesis and response in the developing mammalian heart. *Dev. Biol.* **199**, 55-71.
Niederreither, K., McCaffery, P., Dräger, U. C., Chambon, P. and Dollé, P. (1997). Restricted expression and retinoic acid-induced downregulation of the retinaldehyde dehydrogenase type 2 (RALDH-2) gene during mouse development. *Mech. Dev.* **62**, 67-78.
Niederreither, K., Vermot, J., Messaddeq, N., Schuhbaur, B., Chambon, P. and Dollé, P. (2001). Embryonic retinoic acid synthesis is essential for heart morphogenesis in the Mouse. *Development* **128**, 1019-1031.
Niederreither, K., Fraulob, V., Garnier, J. M., Chambon, P. and Dollé, P. (2002). Differential expression of retinoic acid-synthesizing (RALDH) enzymes during fetal development and organ differentiation in the mouse. *Mech. Dev.* **110**, 165-171.
Norden, J., Grieskamp, T., Lausch, E., van Wijk, B., van den Hoff, M. J., Englert, C., Petry, M., Mommersteeg, M. T., Christoffels, V. M., Niederreither, K. et al. (2010). Wt1 and retinoic acid signalling in the subcoelomic mesenchyme control the development of the pleuropericardial membranes and the sinus horns. *Circ. Res.* **106**, 1212-1220.
Pérez-Pomares, J. M., Macías, D., García-Garrido, L. and Muñoz-Chápuli, R. (1998). The origin of the subepicardial mesenchyme in the avian embryo: an immunohistochemical and quail-chick chimaera study. *Dev. Biol.* **200**, 57-68.
Pérez-Pomares, J. M., Phelps, A., Sedmerova, M., Carmona, R., González-Iriarte, M., Muñoz-Chápuli, R. and Wessels, A. (2002). Experimental studies on the spatiotemporal expression of Wt1 and RALDH2 in the embryonic avian heart: a model for the regulation of myocardial and valvuloseptal development by epicardially-derived cells (EPDCs). *Dev. Biol.* **247**, 307-326.
Sinning, A. (1998). Role of vitamin A in the formation of congenital heart defects. *Anat. Rec.* **253**, 147-153.
Sucov, H. M., Dyson, E., Gumeringer, C. L. and Price, R. M. (1994). RXRa mutant mice establish a genetic basis for vitamin A signalling in heart morphogenesis. *Genes Dev.* **8**, 1007-1018.
Tomanek, R. J., Zheng, W., Peters, K. G., Lin, P., Holifield, J. S. and Suvarna, P. R. (2001). Multiple growth factors regulate coronary embryonic vasculogenesis. *Dev. Dyn.* **221**, 265-273.
Tomanek, R. J., Hansen, H. K. and Christensen, L. P. (2008). Temporally expressed PDGF and FGF-2 regulate embryonic coronary artery formation and growth. *Arterioscler. Thromb. Vasc. Biol.* **28**, 1237-1243.
Tsukamoto, T., Matsui, T., Takaishi, T., Ito, M., Fukase, M. and Chihara, K. (1994). Retinoic acid differentially affects platelet-derived growth factor and epidermal growth factor-regulated cell growth of mouse osteoblast-like cells. *Cell Growth Differ.* **5**, 207-212.
Wessels, A. and Pérez-Pomares, J. M. (2004). The epicardium and epicardially-derived cells (EPDCs) as cardiac stem cells. *Anat. Rec.* **276**, 43-57.
Xavier-Neto, J., Shapiro, M. D., Houghton, L. and Rosenthal, N. (2000). Sequential programs of retinoic acid synthesis in the myocardial and epicardial layers of the developing avian heart. *Dev. Biol.* **219**, 129-141.
Yuan, J. S., Reed, A., Chen, F. and Stewart, C. N., Jr (2006). Statistical analysis of real-time PCR data. *BMC Bioinformatics* **7**, 85.