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...
as previously described (Pérez-Pomares et al., 2002) using anti-PDGFRα, anti-PDGFRβ and anti-RXRG 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<sup>GF/F</sup>) and Wt1-null mice (Wt1<sup>GF/GFP</sup>) 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′-AAAGCTACTCTCCAGGCTATCTGGAGT-3′; reverse, 5′-AAGAACATGATCTCCGCTGGAAAGTGGC-3′. PCR product was digested with NheI and BglII 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). Western blotting

Western blots were performed as described previously (Martínez-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′-CTGGGGCGGGCCCCAAAGCGGTGGGCG-3′; Mut2. 5′- AAGAGAGGAAAACCCGGTACCTGGCC-3′; Mut3. 5′-ACAGGAGGCAAAAAACGGGACGC-3′. All mutations were verified by sequencing.

**ChIP assay**

Chromatin immunoprecipitation (ChIP) assays were performed on immortalised epicardial cells (Martinez-Estrada et al., 2010) using an EZChIP (Upstate Biotechnology) according to the manufacturer’s protocols. The primer sequences used were: positive forward, GCCTGAGAACGATCACCAGAC; positive reverse, GAGGCTGTCGG-TGGTTGGGCC; negative forward, AGCTACCTCACCATTCT; and negative reverse: GCCCTAAGTGACCGAGCAAG.

**Electrophoretic mobility shift assay**

Electrophoretic mobility shift assay was performed using biotin 3′-end labeled 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 RNaseasy Mini Kit (Qiagen) and reverse-transcribed 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<sup>−ΔΔCq</sup> method [modified from Yuan et al. (Yuan et al., 2006)]; included 2<sup>−ΔΔCq</sup> Std Err values between 1-8.5%, followed by an unpaired t-test with >3 (%P<0.05).

For qPCR of FACS-sorted GFP<sup>+</sup> epicardial cells, hearts of Wt1<sup>GF/-</sup> and Wt1<sup>GF/GFP</sup> 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<sup>+</sup> epicardial cells in control and KO mice. qPCR analysis of FACS-sorted GFP<sup>+</sup> 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 epically 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, 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.
Wt1 activates Raldh2 in embryonic epicardium

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.

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 Wt1GFP-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-
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; Martinez-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 PDGFRα 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. 4L) Wt1-null embryos. qPCR in control (Wt1GFP+ ) and Wt1-null (Wt1GFP− ) sorted E11.5 epicardial cells also revealed a significant reduction in the expression of Pdgfra and Pdgrfβ 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, Pdgrfβ 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/Pdgrf 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 Pdgrfβ downregulation in Wt1-null epicardium.

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 (E) is reduced when compared with stage-matched wild types (F, 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. WT1GFP+ and WT1GFP− hearts were dissected, GFP-positive epicardial cells sorted, mRNA extracted and analysed by qPCR. (L) Procedure for the isolation and qPCR analysis of control (WT1GFP− ) and Wt1-null (WT1GFP+ ) immortalised epicardial cells. (M) Procedure for the isolation and qPCR analysis of control (WT1GFP− ) and Wt1-null (WT1GFP+ ) immortalised epicardial cells. (N) qPCR analysis of Rara, Pdgfra. RA treatment (1 μM RA for 24 hours) of mutant cells did not affect Rara 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.
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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