WT1 regulates epicardial epithelial to mesenchymal transition through β-catenin and retinoic acid signaling pathways

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ABSTRACT

An epithelial sheet, the epicardium, lines the surface of the heart. In the developing embryo, the epicardium expresses the transcriptional regulator Wilm's Tumor Gene 1 (Wt1). Through incompletely understood mechanisms, Wt1 inactivation derails normal heart development. We investigated mechanisms by which Wt1 regulates heart development and epicardial epithelial to mesenchymal transition (EMT). We used genetic lineage tracing approaches to track and isolate epicardium and epicardium derivatives in hearts lacking Wt1 (Wt1[KO]). Wt1[KO] hearts had diminished proliferation of compact myocardium and impaired coronary plexus formation. Wt1[KO] epicardium failed to undergo EMT. Wt1[KO] epicardium expressed reduced Lef1 and Ctnnb1 (β-catenin), key components of the canonical Wnt/β-catenin signaling pathway. Wt1[KO] epicardium expressed decreased levels of canonical Wnt downstream targets Axin2, Cyclin D1, and Cyclin D2 and exhibited decreased activity of the Batgal Wnt/β-catenin reporter transgene, suggestive of diminished canonical Wnt signaling. Hearts with epicardium-restricted Ctnnb1 loss of function resembled Wt1[KO] hearts and also failed to undergo epicardial EMT. However, Ctnnb1 inactivation did not alter WT1 expression, positioning Wt1 upstream of canonical Wnt/β-catenin signaling. Wnt5a, a prototypic non-canonical Wnt with enriched epicardial expression, and Raldh2, a key regulator of retinoic acid signaling confined to the epicardium, were also markedly downregulated in Wt1[KO] epicardium. Hearts lacking Wnt5a or Raldh2 shared phenotypic features with Wt1[KO]. Although Wt1 has been proposed to regulate EMT by repressing E-cadherin, we detected no change in E-cadherin in Wt1[KO] epicardium. Collectively, our study shows that Wt1 regulates epicardial EMT and heart development through canonical Wnt, non-canonical Wnt, and retinoic acid signaling pathways.

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Introduction

A mesothelial sheet of cells, the epicardium, covers the surface of the heart. Epicardium participates in heart development by engaging in reciprocal signaling with the subjacent tissue, and by forming mesenchymal cells through epithelial to mesenchymal transition (EMT) (Wilm et al., 2005; Winter and Gittenberger-de Groot, 2007). These mesenchymal cells contribute to cardiomyocytes, smooth muscle, fibroblast, and endothelial lineages of the developing heart (Cai et al., 2008; Merki et al., 2005; Wilm et al., 2005; Zhou et al., 2008). Epicardial inhibition in avian embryos and epicardial gene disruption in murine embryos have demonstrated that epicardium is required for normal myocardial and coronary vessel development (Gittenberger-de Groot et al., 2000; Kwee et al., 1995; Lavine et al., 2006; Lin et al., 2010; Merki et al., 2005; Yang et al., 1995; Zamora et al., 2007).

The molecular regulation of epicardial function is just beginning to be investigated. The transcriptional regulator Wilm's tumor gene 1 (Wt1) is expressed in epicardium, as well as in other mesothelia and the developing genitourinary system (Moore et al., 1998). Deletion of Wt1 caused abnormal development of multiple organs, including the heart (Ijpenberg et al., 2007; Kreidberg et al., 1993; Moore et al., 1999). In the developing heart, Wt1 expression is confined to the epicardium (Moore et al., 1999; Zhou et al., 2008). Loss of Wt1 caused embryonic lethality, peripheral edema, pericardial hemorrhage, and thinning of the myocardial wall (Kreidberg et al., 1993; Martinez-Estrada et al., 2010; Moore et al., 1999). However, the molecular mechanisms underlying this phenotype are not well understood.

In this study we investigated the effect of *Wt1* loss of function on epicardium function in the developing heart, focusing on the effect of *Wt1* deficiency on the formation of epicardium-derived cells (EPDCs) by epicardial EMT. We found that *Wt1* is required for epicardial EMT, acting upstream of canonical Wnt, non-canonical Wnt, and retinoic acid signaling pathways.

Materials and methods

An expanded Methods section is available in the Online Data Supplement.

Mice

Wt1^{GFPCre} (Zhou et al., 2008), Wt1^{CreERT2} (Zhou et al., 2008), Rosa26^{mTmG} (Muzumdar et al., 2007), Wnt5anull (Yamaguchi et al., 1999), Batgal (Maretto et al., 2003), and Ctnnb1^{flox} (Brault et al., 2001) alleles have been previously described, and mice are available from Jackson Laboratories (stock numbers 010911, 010912, 007676, 004758, 005317, and 004152, respectively). Mice were on a mixed genetic background. Epicardial cells were purified by dissociation of fetal hearts and FACS sorting, as described previously (Zhou et al., 2010). Tamoxifen was suspended in sunflower seed oil at 12 mg/ml by sonication. 0.12 mg/g body weight tamoxifen was administered to pregnant dams by gavage at E10.5. All-trans retinoic acid (ATRA; 2.5 μg/g body weight) was given to pregnant females by gavage from E10.5 to E13.5. All procedures involving mice were performed following protocols approved by the Institutional Animal Care and Use Committee.

Gene expression

RNA was isolated using the RNeasy Micro kit (Qiagen), reverse transcribed using Superscript III, and quantitated by qRTPCR with Sybr green chemistry on an ABI7300 real time PCR system. Relative gene expression was calculated using the ΔΔCt method and normalized to *Gapdh*. For linear RNA amplification and conversion to cDNA, we used the Ovation PicoSL WTA system (NuGen). Primer sequences are provided in Suppl. Table 1.

Immunohistochemistry

Sections were stained with primary antibodies listed in Suppl. Table 2 and detected with Alexa Fluor conjugated secondary antibodies (Invitrogen). Nuclei were counterstained with DAPI. In some cases, signal amplification was performed using secondary antibodies conjugated to multimerized horse radish peroxidase (SuperPicture, Invitrogen) followed by incubation with tyramide-Cy3 or tyramide-Cy5 (PerkinElmer). Where necessary, tissue GFP and RFP fluorescence from the Rosa26^{mTmG} allele were bleached by treatment with 3% H_2O_2 in methanol and illumination on a fluorescent light box from 4 h to overnight at 4 °C. Images were acquired on a Fluoview F1000 confocal microscope (Olympus). Unless otherwise noted, images are representative of at least eight fields from each of at least three separate embryos per group.

Statistics

Values are reported as mean ± SEM. The *t*-test was used to test for statistical significance between groups.

Results

Wt1 knockout cardiac phenotype

We previously generated Wt1^{CreERT2} and Wt1^{GFPCre} knockin alleles (Zhou et al., 2008). In addition to expressing CreERT2 or GFPCre fusion

proteins under control of *Wt1* regulatory elements, these alleles are protein null for WT1, as demonstrated by immunohistochemistry of Wt1$^{CreERT2/GFPCre}$ embryos (Suppl. Fig. 1). *Wt1* knockout (Wt1^{KO}) embryos died at E13.5 to E14.5, and no embryos survived to birth. E13.5 embryos showed remarkable hydrops fetalis, with cutaneous edema and an obvious pericardial effusion (Figs. 1A–B and E–F). The hearts of Wt1^{KO} embryos were smaller, appeared developmentally delayed, and exhibited a bifid apex of varying severity (Figs. 1C and G). Histological sections showed that Wt1^{KO} hearts were four chambered and had normal atrio-ventricular and ventriculo-arterial connections. However, as previously noted the myocardial wall was moderately thinned and the superior cardinal veins developed abnormally (Figs. 1D, H, and I and data not shown) (Moore et al., 1999; Norden et al., 2010). Decreased cardiomyocyte proliferation contributed to the myocardial hypoplasia, as phosphorylated histone H3 staining showed reduced proliferation in Wt1^{KO} hearts compared to littermate controls (Fig. 1J).

Consistent with previous reports (Martinez-Estrada et al., 2010; Wagner et al., 2005), Wt1^{KO} hearts exhibited markedly impaired coronary vascular development (Figs. 1K–L). Although most coronary endothelial cells do not derive from Wt1-marked epicardial progenitors (Wilm et al., 2005; Zhou et al., 2008), the coronary vascular plexus develops immediately subjacent to the epicardium, and epicardium is a rich source of angiogenic paracrine factors (Zhou et al., 2011). We hypothesized that impaired expression of angiogenic paracrine factors contributes to the observed coronary vascular defect of Wt1-deficient embryos. To test this hypothesis, we isolated Wt1-expressing epicardial cells from E13.5 Wt1$^{GFPCre/GFPCre}$ or Wt1$^{GFPCre/+}$ hearts by fluorescence activated cell sorting (FACS) for GFP (Fig. 2A). This population, which we named EPI, contained primarily epicardial cells, plus the subset of EPDCs that transiently continue to express *Wt1* or are marked as a result of perdurance of GFP. Each fetal heart yielded approximately 2000 EPI cells, from which we purified approximately 5 ng total RNA. After reverse transcription and linear RNA amplification, we measured gene expression by quantitative RTPCR (qRTPCR). In Wt1$^{GFPCre/+}$ hearts, EPI cells were highly enriched for epicardial markers *Wt1* and *Raldh2* compared to non-epicardial cells (Fig. 2B), validating the purity of the FACS-sorted cells. Next, we compared gene expression between control and mutant hearts. Wt1 was robustly detected in control EPI cells and markedly downregulated in Wt1^{KO}, while expression of the epicardial gene mesothelin (*Msln*) did not differ between control and Wt1^{KO} (Fig. 2C). These data provide technical validation of our ability to detect gene expression differences between control and mutant genotypes. We then measured EPI expression of angiogenic paracrine factors. *Vegfa* and *Angpt1* were significantly downregulated in Wt1^{KO}, while expression of other angiogenic factors such as *Fgf2* were unchanged (Fig. 2D). Together, these data suggest that deficient epicardial expression of angiogenic factors such as *Vegfa* and *Angpt1* contribute to abnormal coronary vessel development in Wt1^{KO} heart.

Wt1 is required for normal epicardial EMT

Loss of *Wt1* was previously shown to reduce the subepicardial mesenchyme, indirectly suggesting impaired epicardial EMT (Moore et al., 1999). We previously used Wt1$^{CreERT2/+}$ to show by genetic fate mapping that epicardial cells undergo EMT to generate mesenchymal cells that migrate into the myocardium (Zhou et al., 2008, 2010). To directly test the hypothesis that *Wt1* is required for epicardial EMT, we used this genetic lineage tracing system to identify epicardium derived mesenchymal cells (EPDCs) in the *Wt1* null background. The Cre-activated reporter, Rosa26^{mTmG}, expresses membrane localized red fluorescent protein (mRFP) prior to recombination, and membrane localized GFP (mGFP) following Cre recombination (Muzumdar et al., 2007). We induced recombination in Wt1$^{CreERT2/CreERT2}$ Rosa26^{mTmG} (knockout) and Wt1$^{CreERT2/+}$ Rosa26^{mTmG} (control)
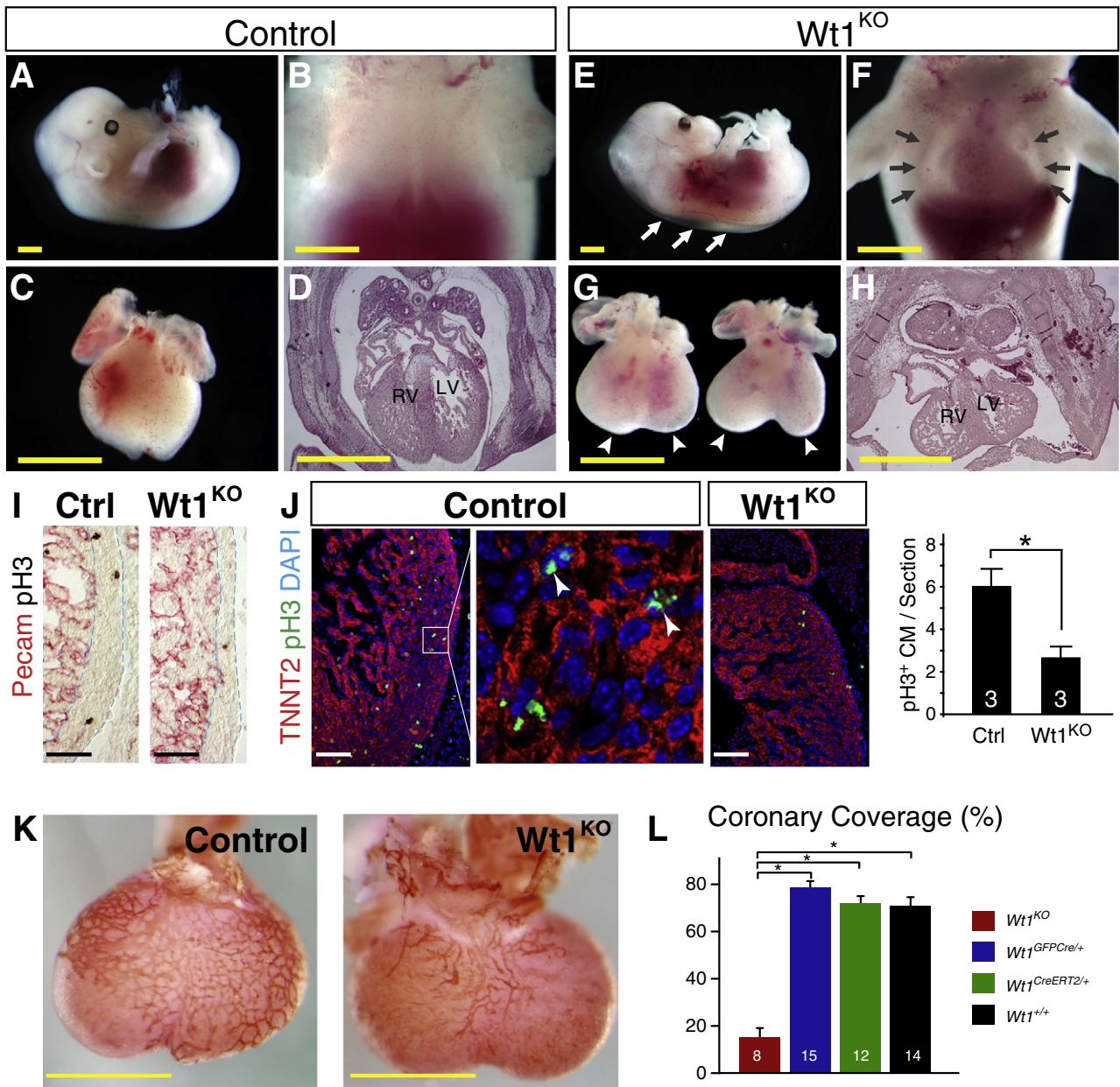
Fig. 1. Phenotype of E13.5 Wt1[KO] embryos. A–H. Compared to control (Wt1^{+/−}; A–D), Wt1[KO] (E–H) embryos displayed body wall edema (white arrows) and a pronounced pericardial effusion, evident on backlighting by a translucent chest cavity (black arrows) in which the cardiac silhouette was clearly visible. Wt1[KO] hearts were smaller, with more rounded and bifid apices (arrowheads). Ventricular myocardium was thinned. I. Thinning of Wt1[KO] compact myocardium, marked by dashed lines. Pecam staining (red) highlighted the endocardial margin of the compact myocardium. J. Reduced proliferation of cardiomyocytes (CM) in Wt1[KO], as indicated by pH3 staining. Arrowheads, pH3[+] CM nuclei. K. Deficient coronary vessel development in Wt1[KO], by whole mount Pecam staining. L. Quantitation of K. Coronary coverage was measured as the projected fraction of the dorsal surface of the ventricles covered by a vascular network. *, $P < 0.05$. Scale bars 1 mm (yellow), 100 μm (other).

embryos by treating with one dose of tamoxifen (Tam) at E10.5, a time point when *Wt1* expression in the heart is restricted to the epicardium (Zhou et al., 2008). In control embryos, this induction protocol labeled epicardium (arrowheads, Fig. 3A), as well as EPDCs within the myocardium (arrows, Fig. 3A). In *Wt1* knockout embryos, epicardium was labeled and covered most areas of the heart (arrowheads, Fig. 3B), although there were patchy areas lacking epicardium as noted previously (Moore et al., 1999). GFP^{+} cells were not observed within the Wt1[KO] myocardium, indicating that *Wt1* is required for formation of EPDCs from epicardium by EMT.

In addition to epicardium, *Wt1* marks mesothelium overlying most visceral organs, including gut mesothelium (Wilm et al., 2005). Gut mesothelium also undergoes EMT, forming smooth muscle cells that contribute to the gut vasculature (Wilm et al., 2005). Tam pulse at

E10.5 of Wt1[CreERT2/+] Rosa26[mTmG] embryos labeled gut mesenchymal cells (Fig. 3C), confirming their origin from gut mesothelium. In littermate Tam-pulsed, *Wt1*-deficient Wt1[CreERT2/CreERT2] Rosa26[mTmG] embryos, we continued to observe robust formation and labeling of gut mesenchymal cells (Fig. 3D). These data suggest that *Wt1* is required for EMT in some but not all mesothelia.

The transcription factors SNAI1 (also known as SNAIL) and SNAI2 (SLUG) regulate EMT by repressing the intercellular adhesion molecule CDH1 (E-cadherin) (Thiery and Sleeman, 2006). Recently, deficient EMT in *Wt1* knockout embryos was proposed to be due to downregulation of SNAIL and SLUG and consequent ectopic expression of E-cadherin in epicardial cells (Martinez-Estrada et al., 2010). We investigated putative upregulation of epicardial E-cadherin as a mechanism for impaired EMT in Wt1[KO] heart. We were not able to
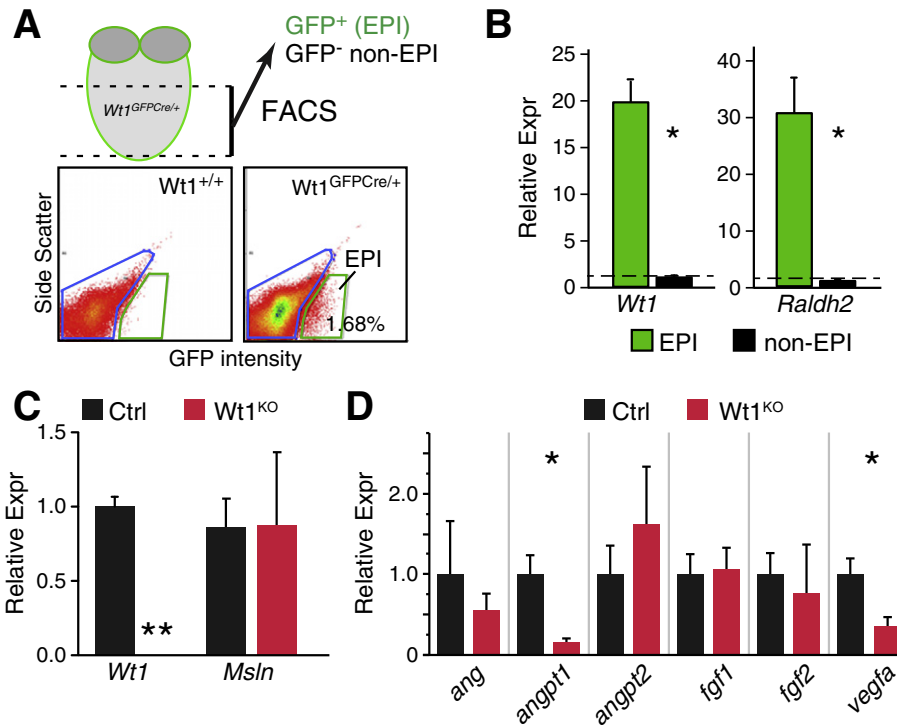
Fig. 2. FACS isolation of Wt1[KO] and control epicardial cells and measurement of epicardial expression of angiogenic factors. A. FACS isolation of WT1-expressing cells by GFP fluorescence from Wt1[GFPCre] ventricular apex. The GFP[+] population was named EPI. B. Quantitative RTPCR (qRTPCR) of EPI and non-EPI populations showed strong enrichment of epicardial marker genes in Wt1[GFPCre/+] hearts. C. *Wt1* but not *Msln* was strongly downregulated in Wt1[GFPCre/GFPCre] (KO) compared to Wt1[GFPCre/+] (Ctrl) in the EPI population. D. Downregulation of *Vegfa* and *Angpt1* in Wt1[KO] epicardium. *, $P<0.05$. **, $P<0.01$. N = 4–5 per group.
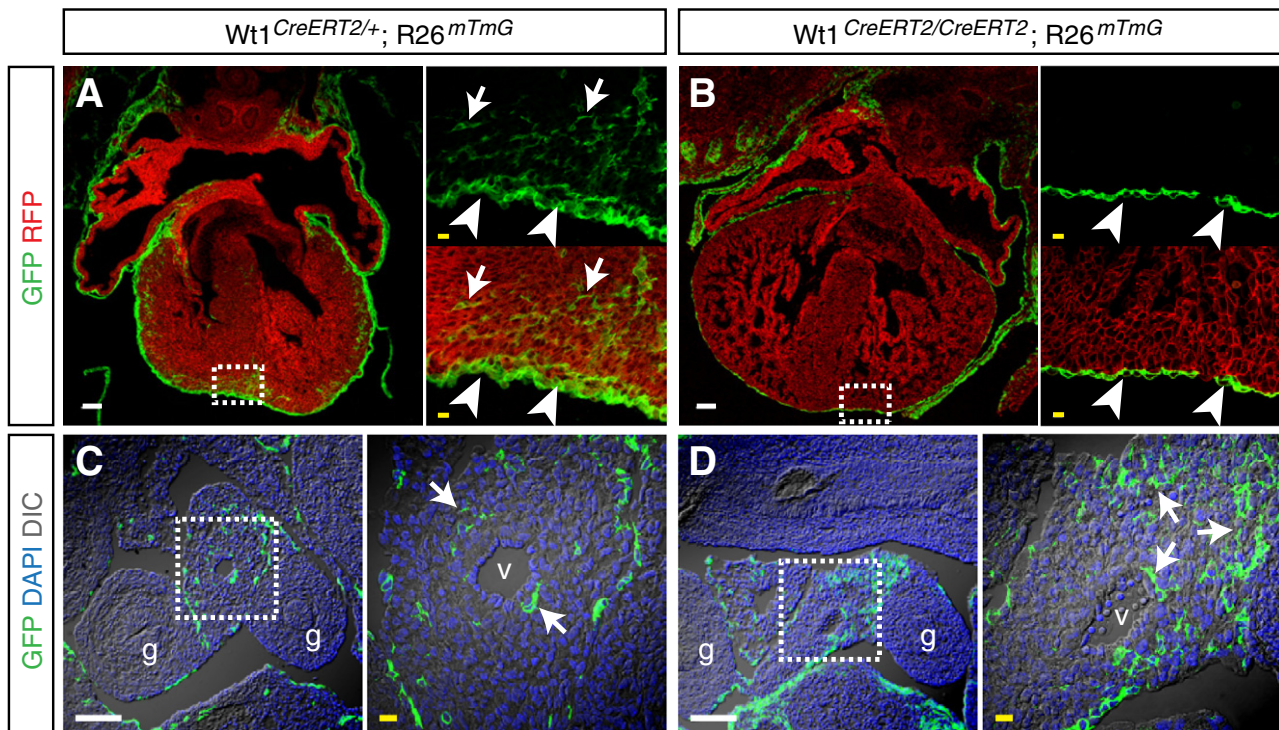
Fig. 3. Defective mesothelial EMT in Wt1[KO] heart but not gut. A–B. Cryosections of E13.5 control and Wt1[KO] hearts. Wt1[CreERT2], activated by Tam at E10.5, efficiently labeled epicardial cells (arrowheads). The membrane-localized GFP genetic lineage tracer demonstrated epicardium-derived EPDCs (arrows) within control but not Wt1[KO] myocardium. Insets show magnifications of boxed areas. C–D. Cryosections of E13.5 abdomens. Arrows indicate mesothelium-derived mesenchymal cells within developing gut mesentry. g, gut. v, mesenteric vessel. Scale bars: 100 μm (white), 10 μm (yellow).

detect E-cadherin immunoreactivity in either control or Wt1^KO epicardium (Suppl. Fig. 2A). Meanwhile, E-cadherin was robustly detected in bronchial epithelium in both groups (Suppl. Fig. 2A), excluding technical failure of E-cadherin staining. These data indicate that proposed upregulation of E-cadherin in Wt1^KO does not account for the EMT defect. We also investigated expression of SNAIL and SLUG. In control embryos, SNAIL and SLUG were expressed in a subset of subepicardial mesenchyme rather than in epicardium itself, suggesting activation of SNAIL and SLUG in these cells soon after undergoing epithelial to mesenchymal transition (Suppl. Figs. 2B–C). In *Wt1* knockout epicardium, subepicardial cells continued to stain for SNAIL and SLUG, although the number of these cells was decreased (Suppl. Figs. 2B–C). As an internal staining control, SNAIL and SLUG were detected in endocardial cushions as previously reported (Niessen et al., 2008).

To evaluate Snail, Slug, and E-cadherin expression downstream of *Wt1* using a more objective and quantitative approach, we used qRTPCR to measure transcript levels in the FACS-purified EPI population. *E-cadherin* transcript was undetectable in either control or Wt1^KO EPI, supporting our immunostaining data. *Snail* and *Slug* were reduced or tended to be reduced in Wt1^KO ($P < 0.05$ and $P = 0.12$, respectively; Suppl. Fig. 2D), consistent with the reduced number of subepicardial mesenchymal cells seen by immunostaining (Suppl. Figs. 2B–C). The qRTPCR and immunostaining data together indicate *Snail*, *Slug*, or *E-cadherin* are unlikely to be direct downstream targets of *Wt1* within the epicardium that link *Wt1* loss of function with deficient EMT.

Wt1 promotes epicardial β-catenin signaling

β-Catenin signaling is an important regulator of EMT (Thiery and Sleeman, 2006). Recently, it was reported that inactivation of floxed *Ctnnb1* (β-catenin) with Gata5-Cre, whose recombination domain includes the epicardium, resulted in a cardiac phenotype similar to Wt1^KO, including abnormal coronary vessel development and impaired expansion of the subepicardial mesenchyme suggestive of deficient epicardial EMT (Zamora et al., 2007). These data suggested an essential role of Wnt signaling in epicardial EMT. However, because of the multiple functions of β-catenin in cellular adhesion as well as Wnt signaling, the role of Wnt signaling in this process remained unclear. To gain further insights into the role of Wnt signaling in epicardial EMT, we used genetic lineage tracing to identify EPDCs in explant cultures treated with DKK1 or soluble frizzled 1 or 2 (sFRP1 or sFRP2), inhibitors of canonical Wnt signaling. In control explants, GFP⁺ EPDCs were observed in the subepicardial region and migrating into the myocardium. While sFRP1 did not consistently affect epicardial EMT, DKK1 or sFRP2 treatment abrogated GFP⁺ EPDCs in the subepicardial region and within the myocardium (Fig. 4A). These data indicate that Wnt signaling is required for epicardial EMT.

To evaluate whether or not Wnt/β-catenin signaling is downstream of Wt1, we used qRTPCR to measure expression of genes involved in this signaling pathway in FACS-purified Wt1^KO EPI. Key β-catenin signaling pathway components *Lef1* and *Ctnnb1* were downregulated in Wt1^KO ($P < 0.05$; Fig. 4B). The Wnt inhibitor *Sfrp2* tended to be upregulated but this did not reach statistical significance (Fig. 4B). We further confirmed CTNNB1 downregulation at the protein level by immunostaining (Fig. 4C). In control hearts, CTNNB1 was detected in both myocardium and epicardium. In Wt1^KO hearts, CTNNB1 expression in myocardium was preserved, but expression in epicardium was diminished (Fig. 4C). CTNNB1 activity is modulated by phosphorylation of Ser37 and Thr41, which promote its degradation, and Ser675, which enhances its nuclear accumulation and transcriptional activity. Thus, modification specific antibodies to non-phosphorylated Ser37/Thr41 or phosphorylated Ser675 detect activated subsets of CTNNB1. Immunoreactivity to these modification specific antibodies was likewise reduced in Wt1^KO (Fig. 4C), consistent with an overall decrease in CTNNB1 levels and lack of enhanced

activation of CTNNB1 that might compensate for decreased overall levels.

Downregulation of *Ctnnb1* and *Lef1* transcripts in Wt1^KO epicardium indicated that *Wt1* regulates canonical Wnt signaling. To further test this hypothesis, we asked if transcripts of downstream targets of canonical Wnt signaling were decreased in FACS-purified Wt1^KO EPI cells. Indeed, the typical Wnt downstream targets *Axin2*, *Ccnd1* (*Cyclin D1*), and *Ccnd2* (*Cyclin D2*) (Jho et al., 2002; Megason and McMahon, 2002) were downregulated in Wt1^KO EPI (Fig. 4D), consistent with decreased CTNNB1/LEF1 signaling. Moreover, activity of the Batgal transgene reporter of Wnt/β-catenin signaling (Maretto et al., 2003) was decreased in Wt1^KO epicardium compared to control (Fig. 4E).

Ctnnb1 was previously inactivated in the Gata5-Cre recombination domain (Zamora et al., 2007), which includes epicardium and additional domains within the heart. The timing and tissue distribution of recombination differs from that catalyzed by Wt1^CreERT2 embryos induced by Tam treatment at E10.5. To further analyze the function of *Ctnnb1* in epicardium, we treated Ctnnb1^flox/flox Wt1^CreERT2/+ embryos with Tam at E10.5. These embryos, referred to as Ctnnb1^EPI, exhibited selective loss of CTNNB1 immunoreactivity in the epicardium at E13.5, confirming epicardium-restricted loss of function and also confirming specificity of CTNNB1 immunostaining (Fig. 5A). Ctnnb1^EPI embryos had peripheral edema like Wt1^KO, but lacked pericardial effusion (Fig. 5B). As in Wt1^KO, E-cadherin immunoreactivity was not detected in Ctnnb1^EPI or control embryos (Suppl. Fig. 3). Examination of the Cre-activated GFP genetic lineage tracer confirmed markedly reduced epicardial EMT compared to heterozygous Ctnnb1^flox/+ Wt1^CreERT2/+ controls (Fig. 5C). We noted that control Ctnnb1^flox/+ Wt1^CreERT2/+ hearts, heterozygous for both *Wt1* and *Ctnnb1* in epicardium, had less GFP⁺ EPDCs compared to Ctnnb1^+/+ Wt1^CreERT2/+ hearts (Fig. 3A vs. Fig. 5C). We confirmed this observation by quantitating GFP⁺ EPDCs ($P = 0.03$; Suppl. Fig. 4). This difference suggested genetic interaction between *Wt1* and *Ctnnb1* in epicardium, consistent with WT1 and CTNNB1 function in the same pathway (see Discussion). WT1 immunoreactivity was unchanged in Ctnnb1^EPI compared to controls, demonstrating that epicardium formation was intact in the absence of *Ctnnb1* (Fig. 5D). Moreover, this result positioned *Ctnnb1* downstream and not upstream of *Wt1*. Overall, these data indicate that downregulation of CTNNB1 downstream of *Wt1* contributes to the Wt1^KO phenotype.

In addition to canonical Wnt signaling through CTNNB1/LEF1, Wnt signaling also proceeds through non-canonical pathways. WNT5A is a prototypical non-canonical Wnt ligand. In situ hybridization demonstrated epicardial enrichment of *Wnt5a* (Visel et al., 2004) (www.genepaint.org; Suppl. Fig. 5), and this was confirmed by qRTPCR of FACS-purified EPI cells (Fig. 6A). *Wnt5a* was previously implicated as an epicardial factor that promoted compact myocardial growth (Fraidenraich et al., 2004). In the Wt1^KO FACS-purified EPI population, *Wnt5a* expression was reduced ($P < 0.05$; Fig. 6B), indicating that *Wnt5a* is downstream of *Wt1*. *Wnt5a* knockout embryos were reported to die perinatally from outflow tract abnormalities (Schleiffarth et al., 2007). However, other defects in cardiac development in Wnt5a^−/− embryos were not previously examined. We found that Wnt5a^−/− hearts, like Wt1^KO hearts, developed thinning of the compact myocardium (Fig. 6C), suggesting that *Wnt5a* downregulation in Wt1^KO epicardium contributes to the mutant phenotype. WT1 expression was unchanged in Wnt5a^−/− heart, indicating that *Wt1* is genetically upstream and not downstream of *Wnt5a* (Fig. 6D). Likewise, CTNNB1 immunoreactivity was unchanged in Wnt5a^−/− (Fig. 6D).

Wt1 is required for normal epicardial expression of RALDH2

Retinoic acid signals are crucial for cardiac patterning and morphogenesis (Chen et al., 2002; Hochgreb et al., 2003; Lin et al., 2010; Stuckmann et al., 2003). Cardiac expression of RALDH2, the rate-limiting enzyme in retinoic acid synthesis, is confined to the epicardium (Moss

et al., 1998). Measurement of *Raldh2* expression in FACS-purified EPI cells by qRTPCR showed that it is markedly downregulated in Wt1[KO] (Fig. 7A). To further validate downregulation of RALDH2 protein in Wt1[KO] epicardium, we performed RALDH2 immunohistochemistry.

RALDH2 was markedly downregulated in Wt1[KO] epicardium (Fig. 7B, blue arrowheads). Intriguingly, RALDH2 expression in mesothelium overlying the lung was not altered (Fig. 7B, green arrowheads), suggesting that regulation of RALDH2 is distinct in different subtypes
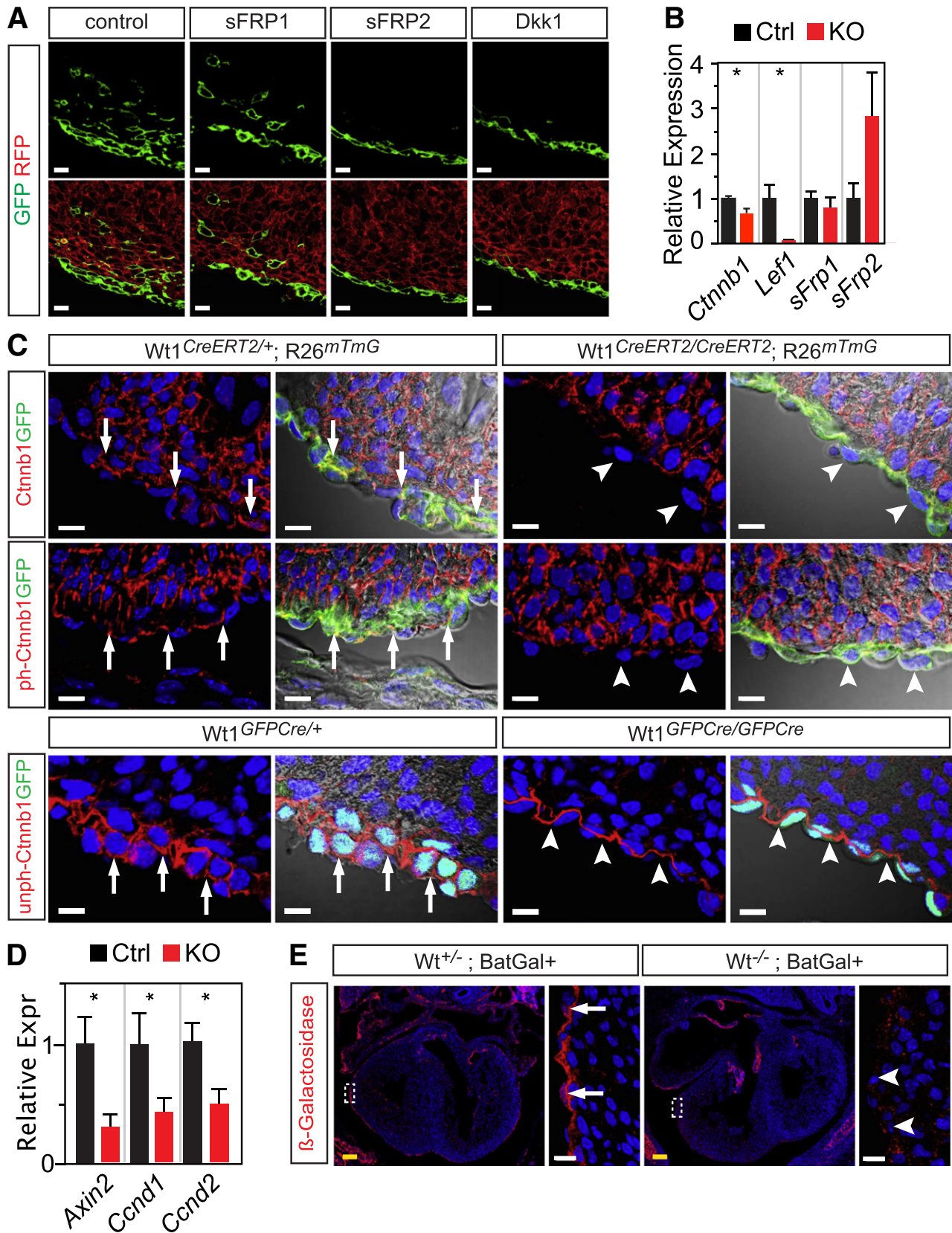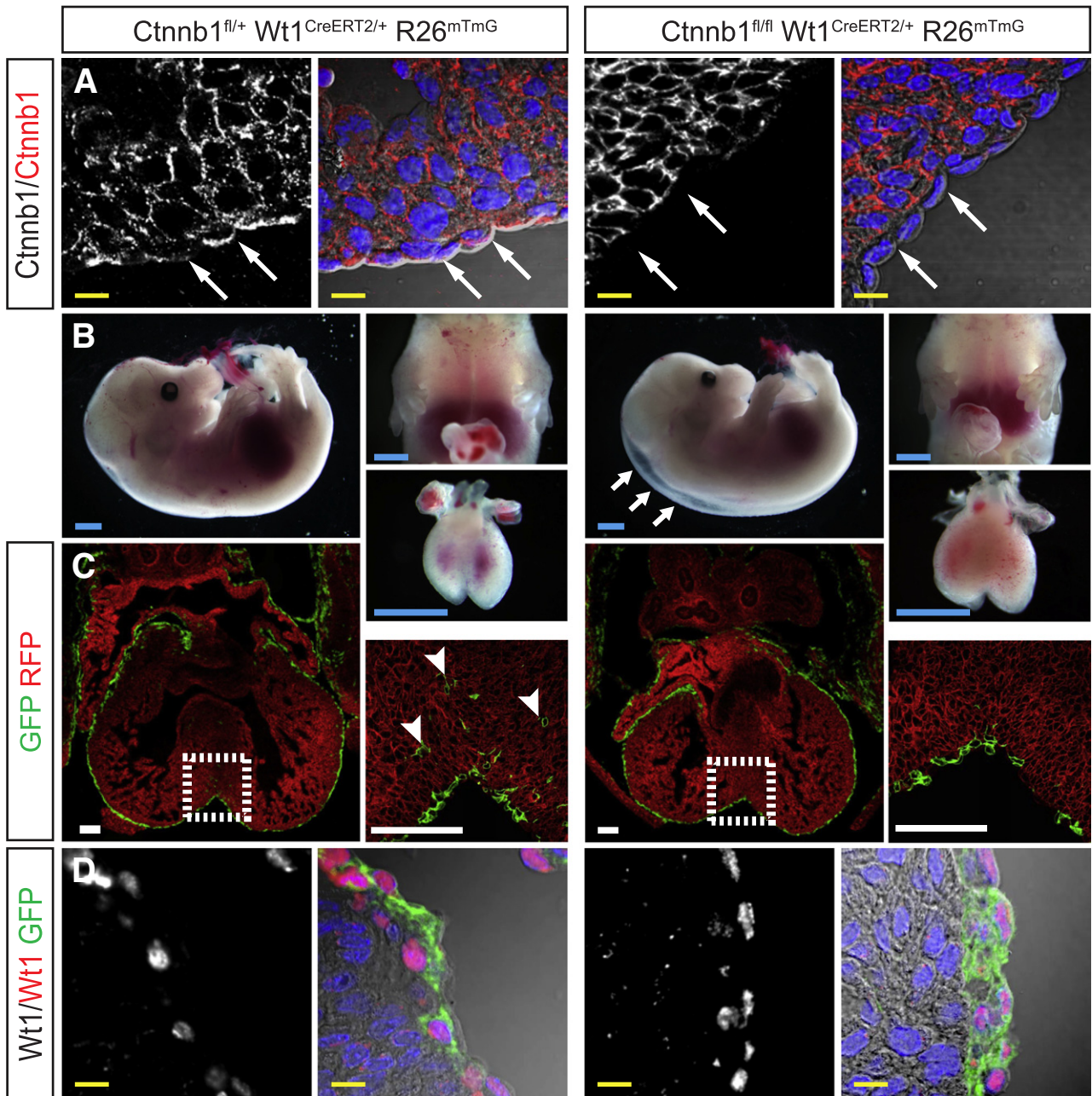
Fig. 5. Wnt/β-catenin signaling is downstream of *Wt1*. A. Loss of epicardial CTNNB1 immunoreactivity (arrows) in E13.5 Ctnnb1^{EPI} (Ctnnb1$^{flox/flox}$ Wt1$^{CreERT2/+}$) epicardium. B. Gross morphology of E13.5 Ctnnb1^{EPI} and control embryos and hearts. Arrows indicate peripheral edema. No pericardial effusion was evident. C. Deficient epicardial EMT in E13.5 Ctnnb1^{EPI}. GFP$^+$ epicardium-derived cells (arrowheads) were present within the myocardium of control (Ctnnb1$^{fl/+}$ Wt1$^{CreERT2/+}$) but not mutant hearts. D. WT1 expression was not significantly changed in E13.5 Ctnnb1^{EPI} compared to control heart. Scale bars: 1 mm (blue), 100 μm (white), 10 μm (yellow).

of mesothelium. Epicardium, marked by GFP in Wt1$^{CreERT2/+}$ Rosa26^{mTmG} embryos, was intact in regions with diminished RALDH2 immunoreactivity, excluding loss of epicardial cells as the cause (Fig. 7C). In contrast to Wt1^{KO} embryos, Ctnnb1^{EPI} and Wnt5a$^{−/−}$ hearts expressed RALDH2 normally (Figs. 7D and 6D), indicating that *Raldh2* is downstream of *Wt1* but not *β-catenin* or *Wnt5a*. Furthermore,

this result indicates the abnormality of *Raldh2* expression is specific to Wt1^{KO} and not a general property of mutants with perturbed epicardial gene expression or epicardial EMT.

Like Wt1^{KO} embryos, *Raldh2* null embryos, treated with exogenous retinoic acid to bypass early embryonic lethality, exhibited myocardial hypoplasia and coronary vessel abnormalities (Lin et al., 2010),

Fig. 4. Downregulation of Wnt/β-catenin signaling components in Wt1 loss of function. A. Wt1$^{CreERT2/+}$ Rosa26^{mTmG} hearts were pulsed with Tam for 12 h in vivo, then explanted and cultured in the presence of the indicated inhibitor of canonical Wnt signaling. DKK1 and sFRP2 consistently blocked formation of GFP$^+$ EPDCs. B. Differential expression of Wnt/β-catenin signaling components in Wt1^{KO} (Wt1$^{GFPCre/GFPCre}$) compared to Ctrl (Wt1$^{GFPCre/+}$) FACS-purified epicardial cells. *β-Catenin* (*Ctnnb1*) was measured with unamplified RNA, n = 10–12. Other genes were measured with amplified RNA, n = 4–5. C. Cryosections of E13.5 heart stained for CTNNB1, CTNNB1 phosphorylated at Ser675 (ph-CTNNB1), or CTNNB1 lacking phorphorylation at Ser37 or Thr41 (unph-CTNNB1). GFP immunostaining marked epicardium and derivatives. In controls, CTNNB1 was expressed in epicardium (arrows) and myocardium. In mutant heart, CTNNB1 was unchanged in myocardium but decreased in epicardium (arrowheads). D. Downstream targets of Wnt/β-catenin signaling were downregulated in Wt1^{KO}, consistent with decreased activity of this signaling pathway. Amplified RNA, n = 4–5. E. Activity of the Batgal transgene, which expresses LacZ in response to canonical Wnt signaling, in control and Wt1^{KO} epicardium. *, P < 0.05. Scale bars: 10 μm (white), 100 μm (yellow).
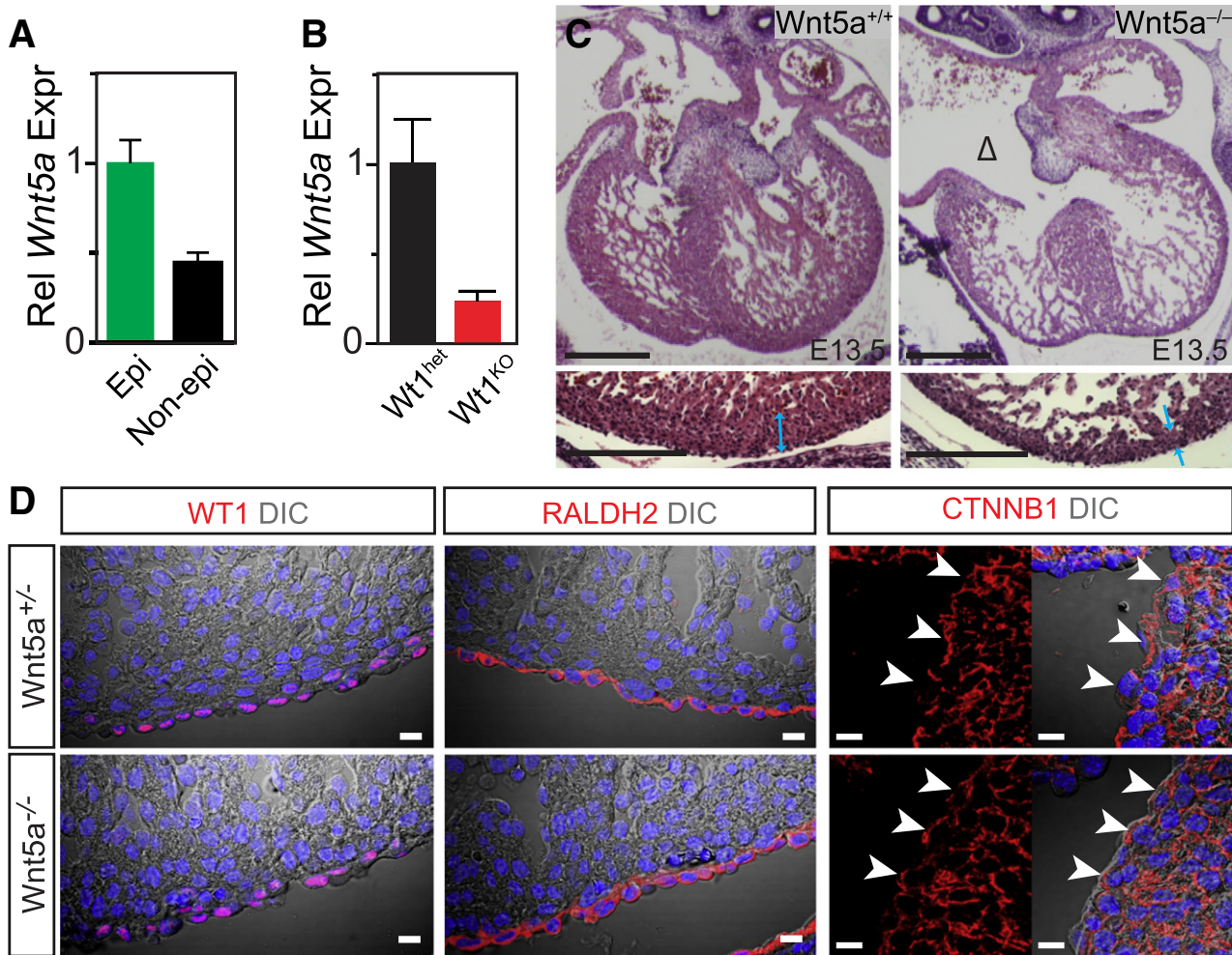
Fig. 6. Abnormal *Wnt5a* expression in Wt1[KO] epicardium. A. Enrichment of *Wnt5a* in epicardium. n = 3. *, P<0.01. B. Downregulation of *Wnt5a* in Wt1[KO] epicardium. n = 4–5. *, P = 0.03. **C**. *Wnt5a* regulates growth of compact myocardium. Bottom panels show magnifications of left ventricular free wall. *Wnt5a* mutant hearts had thin compact myocardium (blue arrows) and common atrioventricular canal (Δ). Bar = 200 μm. D. WT1, RALDH2, and CTNNB1 immunoreactivity were unchanged in Wnt5a^{−/−} epicardium. Arrowheads, CTNNB1 in epicardial cells. Bar = 10 μm.

suggesting that decreased retinoic acid signaling may contribute to defective EMT in Wt1[KO] heart. To further address whether *Raldh2* deficiency contributes to the defect in EMT observed in Wt1[KO] embryos, we asked if bypassing *Raldh2* deficiency by supplementation with all-trans retinoic acid (ATRA) restored EMT in Wt1[KO] heart. ATRA treatment of pregnant mothers from E10.5 to E13.5 partially rescued epicardial EMT (Fig. 7E). Wt1[CreERT2]-labeled EPDCs were present in ATRA-treated Wt1[KO] myocardium, but were not observed in untreated Wt1[KO] myocardium. However, the ATRA rescue was incomplete, as less EPDCs were observed in ATRA-treated WT1[KO] than in control embryos. These results indicate that abnormal retinoic acid synthesis in Wt1[KO] epicardium contributes in part to deficient epicardial EMT in Wt1[KO] heart.

Discussion

Transitions between epithelial and mesenchymal cell types are crucial for development and disease. *Wt1* has been implicated in regulating these transitions in multiple contexts, including kidney development, heart development, and cancer. However, the mechanisms by which *Wt1* regulates these changes in cellular programs are poorly understood. Here, we investigated the requirement of *Wt1* for epicardial EMT, using genetic tools to label, track, and isolate epicardial cells. Using this unambiguous approach, we confirmed

that epicardial EMT is strongly reduced in *Wt1* knockout epicardium, and we identified signaling pathways downstream of *Wt1* that mediate its effects on EMT and heart development (Fig. 8).

Recently, *Wt1* was proposed to directly regulate *Snail*, *Slug*, and *E-cadherin* in cardiac progenitors and in epicardium, so that *Wt1* loss of function putatively caused upregulation of E-cadherin and thereby inhibited epicardial EMT (Martinez-Estrada et al., 2010). We could not reproduce these results by immunostaining, nor did we find upregulation of E-cadherin transcripts in highly purified Wt1[KO] epicardial cells by quantitative real time PCR, a more objective and quantitative method. In fact, we did not detect epicardial E-cadherin expression above background by either method in control or mutant genotypes. While immunostaining and qRTPCR showed overall reduction of *Snail* and *Slug* expression, this was due to reduced number of subepicardial mesenchymal cells rather than reduced expression within epicardial cells. Thus, reduced expression of SNAIL and SLUG was a consequence of impaired EMT, rather than a mechanism through which Wt1 loss of function abrogates EMT.

On the other hand, we found that *Wt1* stimulated epicardial Wnt/β-catenin signaling. A prior study of *Ctnnb1* epicardial-restricted loss of function demonstrated that β-catenin was required for epicardial EMT (Zamora et al., 2007). Because β-catenin is essential for canonical Wnt signaling, these studies suggested that canonical Wnt signaling promotes epicardial EMT. However, β-catenin has
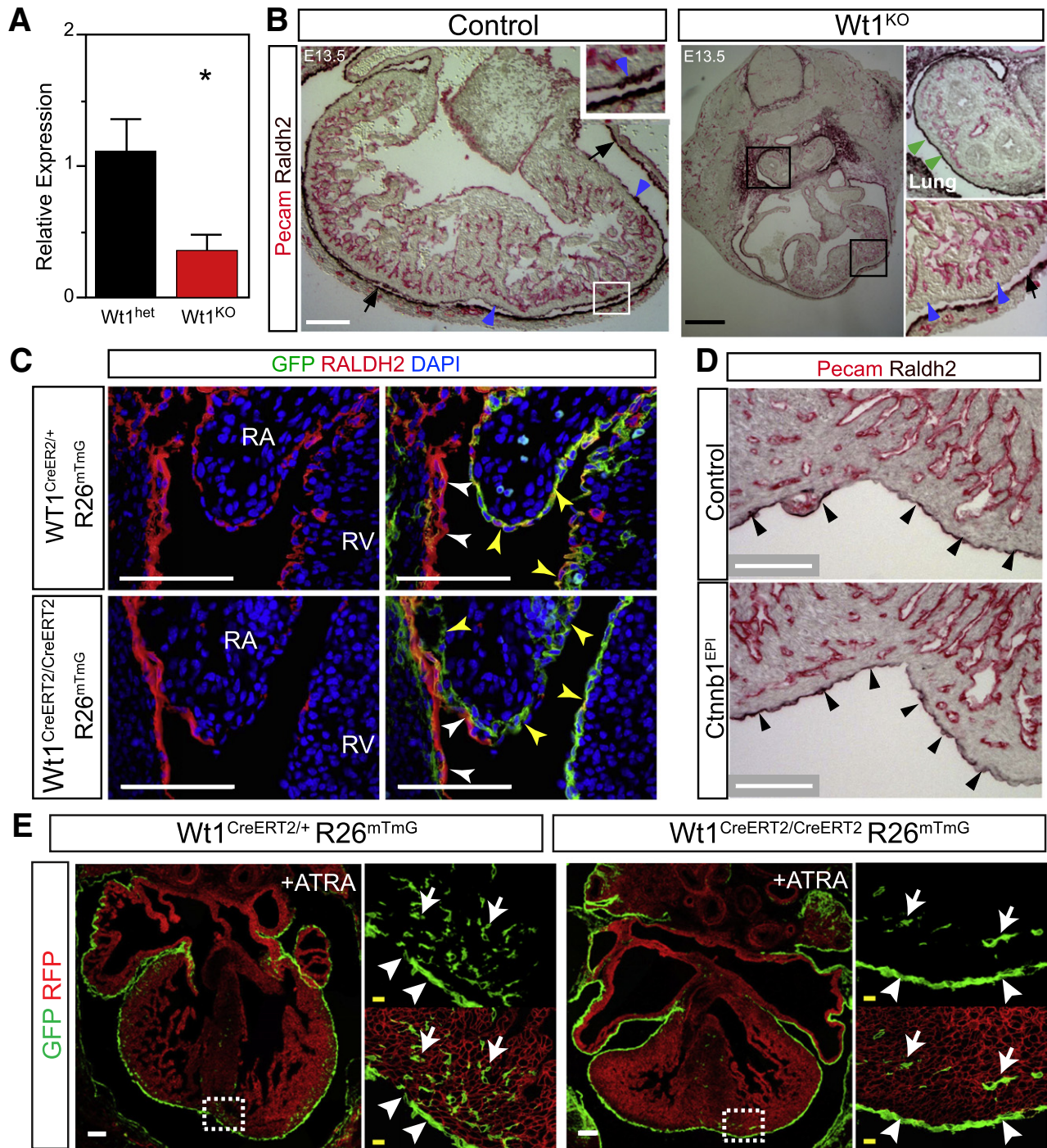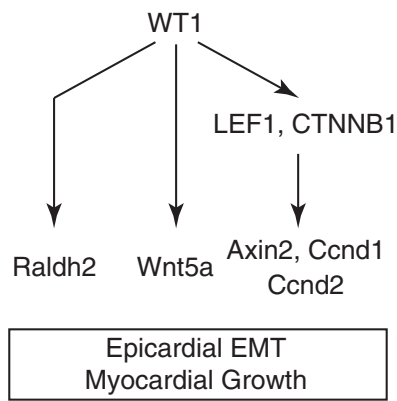
Fig. 7. Decreased epicardial expression of RALDH2 in Wt1^{KO}. A. *Raldh2* transcript level, measured by qRTPCR, was decreased in FACS purified epicardial cells from E13.5 Wt1^{KO} heart compared to control. *, P<0.05. n=4–5. B. In control embryos, epicardium expressed RALDH2 (blue arrowheads). The lining of the pericardium also expressed RALDH2 (arrows). In Wt1^{KO} embryos, epicardial RALDH2 expression was markedly downregulated (blue arrowheads), while expression in lung mesothelium (green arrowheads) and pericardium (arrows) were unchanged. C. Markedly decreased RALDH2 immunoreactivity in Wt1^{KO} heart. Co-staining for epicardium (GFP expressed from Wt1^{GFPCre}; yellow arrowheads) showed that loss of RALDH2 immunoreactivity was not due to lack of epicardium. White arrowheads, pericardium. D. RALDH2 expression was not significantly altered in Ctnnb1^{EPI}. E. ATRA supplementation between E10.5 and E13.5 partially corrected deficient epicardial EMT in Wt1^{KO} heart. Arrow, EPDC. Arrowhead, epicardial cell. EPDCs were present in ATRA-treated Wt1^{KO}, but were absent in untreated Wt1^{KO} (Fig. 2). Scale bars: 500 μm (black), 100 μm (white), 10 μm (yellow).

multiple cellular roles, including intercellular adhesion, and resolving the multiple functions of *Ctnnb1* in epicardial EMT was problematic. In addition to confirming the epicardial requirement of *Ctnnb1* in epicardial EMT, we provide two additional lines of evidence that the role of epicardial canonical Wnt signaling in epicardial EMT. First, we show epicardial activation of the Batgal transgene, a reporter of canonical Wnt signaling (Maretto et al., 2003). Second, in explants we

show that epicardial EMT was strongly inhibited by two different inhibitors of canonical Wnt signaling. Having established an important role of Wnt/β-catenin signaling in epicardial EMT, we next showed that Wt1 was a positive upstream regulator of this pathway. *Wt1* promoted epicardial expression of *Ctnnb1* and *Lef1*, essential components of the β-catenin signaling pathway. Moreover, down-regulation of common targets of β-catenin signaling (*Axin2, Ccnd1,*

WT1

LEF1, CTNNB1

Raldh2   Wnt5a   Axin2, Ccnd1
                   Ccnd2

Epicardial EMT
Myocardial Growth

Fig. 8. WT1 regulates epicardial EMT and heart development through multiple signaling pathways. WT1 promotes canonical Wnt/β-catenin signaling, as well as expression of *Wnt5a*, a non-canonical Wnt ligand, and *Raldh2*, a key regulator of retinoic acid signaling.

and *Ccnd2*) and the Batgal reporter in epicardial cells suggested decreased activity of this pathway in *Wt1*-deficient embryos. WT1 was expressed normally in epicardial *Ctnnb1* knockouts, positioning β-catenin signaling genetically downstream of *Wt1*.

Downregulation of β-catenin signaling in Wt1[KO] epicardium likely accounted for phenotypic similarity of epicardial loss of function of *Wt1* and *Ctnnb1*. Diminished EMT in embryos with compound heterozygosity for *Wt1* and *Ctnnb1* suggested a genetic interaction, consistent with *Wt1* and *Ctnnb1* function in the same pathway. However, we were unable to assess embryos with epicardium-restricted heterozygosity for *Ctnnb1* alone because Cre-expressing mice were obligate *Wt1* heterozygotes. *Wt1* heterozygosity may also contribute to the defects observed in Ctnnb1^{fl/fl} Wt1[CreERT2/+] hearts, although this limitation does not alter our conclusion that both *Wt1* and *Ctnnb1* function in a pathway essential for epicardial EMT.

Wt1 has been reported to regulate canonical Wnt signaling in both kidney development and cancer (Kim et al., 2009; Li et al., 2004). Interestingly, as we saw in epicardium, *Wt1* also stimulated expression of *Lef1* in models of kidney development (Kim et al., 2009). In this system WT1 was found to occupy LEF1 regulatory elements, suggesting direct transcriptional regulation. However, *Wt1* has largely been described to repress canonical Wnt signaling in mesenchymal cells in kidney development and cancer (Kim et al., 2009; Li et al., 2004), while our data suggests that *Wt1* stimulates Wnt/β-catenin signaling in epicardium. The differing roles of *Wt1* in these systems may reflect differing functions in epithelial cells such as epicardium, where Wt1 promotes epithelial to mesenchymal transition, compared to mesenchymal cells, such as kidney metanephric tissue and Wilm's tumor cells, where *Wt1* promotes mesenchymal to epithelial transition.

While impaired β-catenin signaling contributed to features of epicardial *Wt1* loss of function, attempts to rescue Wt1[KO] mutants by lithium chloride-induced activation of canonical Wnt signaling (Tian et al., 2010) were unsuccessful (data not shown). In part, this may have been due to toxicity of this treatment at the later gestational stage necessary for our study. This result may also have been due to *Wt1* regulation of essential epicardial functions that are independent of β-catenin.

In addition to regulating epicardial β-catenin signaling, Wt1 also regulated Wnt5a and retinoic acid signaling pathways. *Wnt5a*, a key regulator of non-canonical Wnt signaling, was enriched in epicardium, and *Wnt5a* was downregulated in Wt1[KO] epicardium. Like Wt1[KO] hearts, Wnt5a^{−/−} hearts had marked thinning of the compact myocardium, suggesting that impaired epicardial expression of *Wnt5a* contributes to myocardial thinning seen in Wt1[KO] heart.

However, epicardial-restricted inactivation of *Wnt5a* will be required to confirm the epicardial role of *Wnt5a*.

Retinoic acid signaling was another *Wt1*-dependent, β-catenin independent signaling pathway. This finding was corroborated by the recent report by Guadix et al. (2011) that *Wt1* regulates epicardial *Raldh2* expression. Retinoic acid signals are essential for cardiac morphogenesis and myocardial growth (Chen et al., 2002; Hochgreb et al., 2003; Lin et al., 2010; Stuckmann et al., 2003). Cardiac expression of the enzyme RALDH2, which catalyzes an essential step in retinoic acid synthesis, is restricted to the epicardium (Moss et al., 1998), and *Raldh2* loss of function resulted in abnormalities of cardiac growth and coronary development similar to those in WT1[KO] embryos (Lin et al., 2010). Deficient epicardial synthesis of retinoic acid contributes to the epicardial EMT defect of WT1[KO] embryos, since the EMT defect was partially rescued by retinoic acid replacement. However, the rescue was incomplete and epicardial derivatives formed by EMT were present in Raldh2^{−/−} heart (Lin et al., 2010), suggesting that EMT does not absolutely require *Raldh2*. Retinoic acid also regulates differentiation of epicardium-derived cells (Azambuja et al., 2010), and therefore the contribution of RALDH2 deficiency to the Wt1[KO] phenotype is multifactorial.

Interestingly, mesothelial requirement for *Wt1* differed between subtypes of mesothelium. For instance, *Wt1* marks mesenchymal cells derived from both epicardium (Wilm et al., 2005; Zhou et al., 2008; this study) and gut mesothelium (Wilm et al., 2005). However, *Wt1* was dispensable for gut mesothelium EMT, but was required for normal epicardium EMT. *Wt1* regulation of *Raldh2* also differed between mesothelial subtypes: *Wt1* was required for *Raldh2* expression in epicardium (this study and Guadix et al., 2011) and liver mesothelium (Ijpenberg et al., 2007), but was dispensable for *Raldh2* expression in lung mesothelium. It will be interesting to determine the mechanisms responsible for the differing roles of *Wt1* in these various types of mesothelium.

Wt1 loss of function impaired formation of the coronary vascular plexus (this study and Martinez-Estrada et al., 2010; Moore et al., 1999). *Wt1*-lineage cells make little cellular contribution to the coronary endothelium (Wilm et al., 2005; Zhou et al., 2008), indicating that *Wt1* acts cell non-autonomously. Epicardium overlies the nascent coronary vascular plexus, and we recently showed that fetal epicardium is a rich source of angiogenic factors (Zhou et al., 2011). In *Wt1* loss of function, epicardial expression of *Vegfa* and *Angpt1* were significantly diminished, indicating that abnormal expression of epicardial angiogenic factors contributes to coronary vascular defects seen in these mutant hearts.

Cardiac injury reactivates a fetal epicardial program that includes re-expression of *Wt1*, epicardial expansion via EMT, and upregulation of *Raldh2* (Zhou et al., 2011). Reactivation of WT1 and reawakening of fetal epicardial properties likely contribute to the myocardial injury response. Thus, understanding of *Wt1* regulation of epicardial function during development may improve our understanding of mechanisms that govern myocardial injury responses and may translate into strategies to therapeutically modulate these responses to improve outcome in adult heart disease.

Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.ydbio.2011.05.668.

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