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Wt1 is required for cardiovascular progenitor cell formation through transcriptional control of Snail and E-cadherin

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The epicardial epithelial-mesenchymal transition (EMT) is hypothesized to generate cardiovascular progenitor cells that differentiate into various cell types, including coronary smooth muscle and endothelial cells, perivascular and cardiac interstitial fibroblasts and cardiomyocytes. Here we show that an epicardial-specific knockout of the gene encoding Wilms' tumor-1 (*Wt1*) leads to a reduction in mesenchymal progenitor cells and their derivatives. We show that Wt1 is essential for repression of the epithelial phenotype in epicardial cells and during embryonic stem cell differentiation through direct transcriptional regulation of the genes encoding Snail *(Snai1)* and E-cadherin *(Cdh1)*, two of the major mediators of EMT. Some mesodermal lineages do not form in *Wt1*-null embryoid bodies, but this effect is rescued by the expression of *Snai1*, underscoring the importance of EMT in generating these differentiated cells. These new insights into the molecular mechanisms regulating cardiovascular progenitor cells and EMT will shed light on the pathogenesis of heart diseases and may help the development of cell-based therapies.

In the developing heart, very little is known about the molecular and cellular mechanisms controlling epicardial EMT and the formation of cardiovascular progenitor cells[1]. *Wt1* encodes a zinc-finger protein with a crucial role in the normal development of several organs, such as kidney, gonads, spleen and heart[2]. Coronary vascular defects in *Wt1*-mutant mice are believed to arise through defective EMT[3]. However, until now it has not been possible to determine whether Wt1 in the epicardium is directly involved in EMT, or whether EMT is essential for the formation of cardiovascular progenitor cells.

To investigate the role of Wt1 in the epicardium, we generated *Wt1* conditional knockout mice (**Supplementary Fig. 1**) that were crossed with *Wt1* knockin mice expressing green fluorescent protein (GFP) reporter ($Wt1^{GFP/+}$; ref. 4) and mice transgenic for *Gata5-Cre*[5] (see Online Methods for details of breeding). The resulting *Gata5-Cre*; $Wt1^{loxP/GFP}$ (referred to here as Cre^{+}) mice died between embryonic day 16.5 (E16.5) and E18.5 as a result of cardiovascular failure. Cre^{+} E16.5 embryos showed edema and accumulation of blood in the systemic veins (**Fig. 1a**,**b**).

We confirmed efficient deletion of *Wt1* in epicardial cells by immunohistochemical analysis of heart sections and real-time PCR analysis of FACS-sorted GFP^{+} epicardial cells isolated from Cre^{+} mice (**Supplementary Fig. 2a–c**). The gross morphology of Cre^{+} embryos was normal, but the right ventricles of some mutant embryos had thinner free walls compared to control embryos (**Fig. 1c**,**d**, arrows), whereas the left ventricles were apparently normal. Mutant embryos also showed pericardial hemorrhaging (**Fig. 1d**, asterisk). We also confirmed embryonic expression of the *Gata5-Cre* transgene in the heart. At E10.5, we detected cells expressing *Cre* in the epicardium; at E12.5, *Gata5-Cre*–derived cells were abundant within the heart[5] (**Supplementary Fig. 3** and data not shown). Optical projection tomography (OPT) revealed that the coronary arteries did not form in Cre^{+} mice (**Fig. 1e**,**f** and **Supplementary Movies 1** and **2**). We confirmed this result by staining for the adhesion molecule PECAM-1 (CD31) and α-smooth muscle actin (α-SMA), markers for endothelial and smooth muscle cells, respectively (**Fig. 1g**,**h**).

GFP^{+} epicardial cells covered the surface of the myocardium in Cre^{+} mice, confirming the integrity of this structure in the mutant mice (**Supplementary Fig. 4b**). Because epicardial EMT has a very important role in the formation of coronary vascular precursor cells, we studied the expression patterns of the major markers of EMT in the *Wt1* mutant epicardium. Expression of Snail, a key activator of EMT, was reduced in the epicardium of mutant hearts compared to controls (**Fig. 1i**,**j**). Conversely, the epithelial markers E-cadherin and cytokeratin were upregulated (**Fig. 1k–n**). Vimentin expression was also downregulated in mutant hearts (**Fig. 1m**,**n**). We also compared the levels of *Snai2* (also known as *Slug*) in Cre^{+} and Cre^{-} FACS-sorted epicardial cells by real-time PCR and confirmed that, similar to *Snai1*, *Snai2* levels were reduced by 70% in Cre^{+} compared to Cre^{-} cells.

To determine whether Wt1 has a direct and cell-autonomous role in epicardial EMT, we generated tamoxifen-inducible *Wt1*-knockout immortalized epicardial cells (Cre^{+} CoMEECs; **Fig. 2**; see Online

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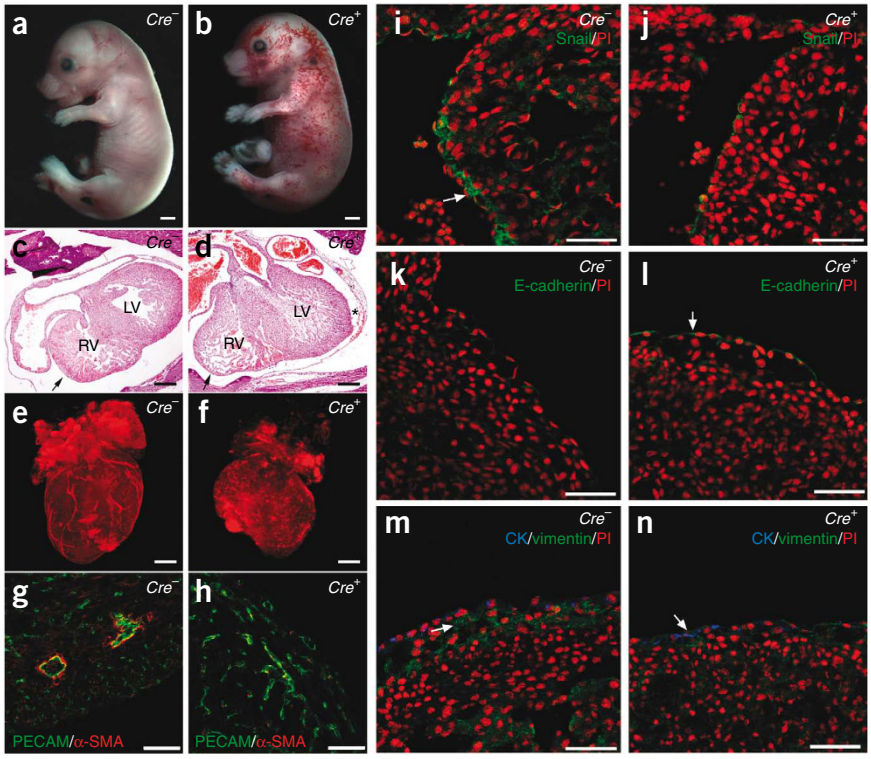

Figure 1 Heart defects in epicardial-specific *Wt1*-mutant embryos. (**a**,**b**) *Gata5-Cre*; *Wt1*$^{loxP/GFP}$ (*Cre*$^{+}$) embryos (**b**) showed edema and accumulation of blood in the systemic veins. A littermate control (*Cre*$^{-}$) is shown in **a.** Scale bars represent 100 μm. (**c**,**d**) Hematoxylin- and eosin-stained sections of *Cre*$^{-}$ and *Cre*$^{+}$ E16.5 embryos. The right ventricles (RV) of some of the mutant embryos (**d**) had thinner walls (arrows) compared to control embryos (**c**), whereas the left ventricles (LV) were apparently normal. Mutant embryos showed pericardial hemorrhage (**d**, asterisk). Scale bars represent 50 μm. (**e**,**f**) OPT images of control and mutant hearts at E16.5. Scale bars represent 50 μm. (**g**,**h**) Immunofluorescence staining for the indicated blood vessel markers. Only control embryos (**g**) showed arteries with a well-differentiated smooth muscle layer. (**i–n**) EMT markers were analyzed with antibodies to Snail (**i**,**j**), E-cadherin (**k**,**l**) and vimentin and cytokeratin (CK; **m**,**n**). Abnormal E-cadherin (**l**) and decreased Snail (**j**) and vimentin (**n**) expression was observed in epicardial cells from *Cre*$^{+}$ embryos. PI, propidium iodide. Arrows point to expression of the indicated proteins in control and mutant epicardial cells. Scale bars represent 50 μm.

Methods). CoMEECs had typical cobblestone morphology (**Fig. 2c**), highlighted by ZO-1 staining at the cellular junctions (**Fig. 2e**), and showed robust Wt1 and GFP expression (**Fig. 2d**,**f**). Loss of Wt1 after tamoxifen treatment led to a robust increase in E-cadherin expression in a dose-dependent manner (**Fig. 2g**), which was associated with downregulation of N-cadherin and α-SMA (**Fig. 2g**). RT-PCR analysis also revealed a marked downregulation of *Snai1* after *Wt1* deletion (**Fig. 2h**). Treatment of the *Cre*$^{+}$ CoMEECs with tamoxifen led not only to changes in the EMT marker pattern, but also to reduced cell migration (**Fig. 2i**). We did not observe any difference in the markers that analyzed or in the migration properties of *Cre*$^{-}$ CoMEECs after tamoxifen treatment (**Supplementary Fig. 5a**,**b**).

We next examined whether *Snai1*, one of the master regulators of EMT[6], is directly regulated by Wt1. We identified three conserved potential Wt1 binding sites in the *Snai1* genomic sequence (**Fig. 3a**). The −KTS Wt1 isoform (Lys-Thr-Ser tripeptide deleted from exon 9) functions as a transcription factor[2] and was able to activate the *Snai1* fragment containing the promoter binding site in a dose-dependent manner (**Fig. 3b**). The other fragments were insensitive to Wt1 activation (data not shown). The transcriptional activation was abolished when we mutated the functional binding site (**Fig. 3c**). Chromatin immunoprecipitation (ChIP) assays showed *in vivo* binding of Wt1 to the endogenous *Snai1* promoter but not to the intronic and 3′ UTR regions in epicardial cells (**Fig. 3d**). The endogenous *Snai1* promoter of epicardial cells was enriched in acetylated Lys9 and trimethylated Lys4 of histone H3 (H3K9ac and H3K4me3, respectively), but depleted in trimethylated Lys27 of histone H3 (H3K27me3), which is compatible with the activated state of *Snai1* in these cells (**Fig. 3d**).

Wt1 has been shown to transcriptionally activate the *Cdh1* promoter in NIH3T3 cells[7]. We examined whether Wt1 directly represses *Cdh1* in epicardial cells (**Fig. 3**). ChIP with primers flanking the previously

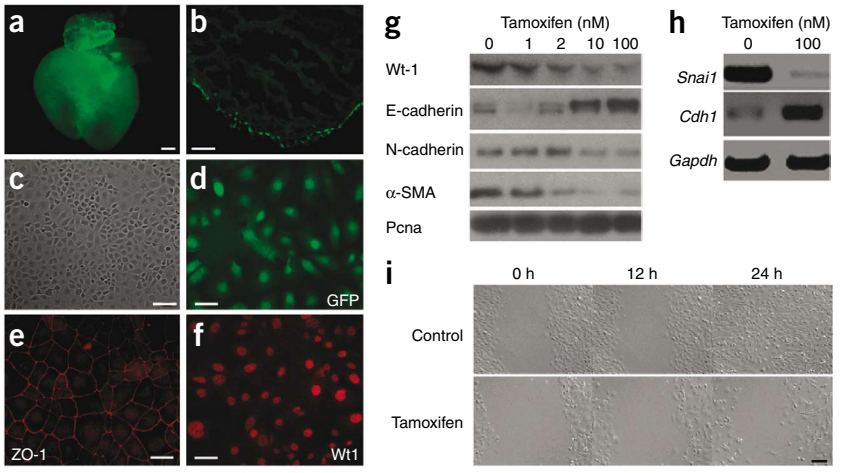

Figure 2 Wt1 expression is necessary to maintain a mesenchymal phenotype in immortalized epicardial cells. (**a**) Heart of *Wt1*$^{GFP/+}$ knockin mouse. Scale bar represents 50 μm. (**b**) Direct GFP expression analysis in heart cryosection of *Wt1*$^{GFP/+}$ knockin embryos showed GFP expression in epicardial cells. Scale bar represents 50 μm. (**c**) Phase-contrast micrograph of CoMEECs. Scale bar represents 100 μm. (**d–f**) GFP, ZO-1 and Wt1 expression in CoMEECs. Scale bars represent 50 μm. CoMEECs showed a cobblestone monolayer typical of epicardial cells (**c**,**e**). (**g**) Protein blot analysis of *Cre*$^{+}$ tamoxifen-inducible CoMEECs cultured in the presence of various concentrations of tamoxifen. Pcna, proliferating cell nuclear antigen. (**h**) RT-PCR analyses of *Snai1* and *Cdh1* in *Cre*$^{+}$ CoMEECs in the presence of tamoxifen. (**i**) The migratory behavior of *Cre*$^{+}$ CoMEECs in the presence of tamoxifen was analyzed in an *in vitro* wound model. Scale bar represents 100 μm.

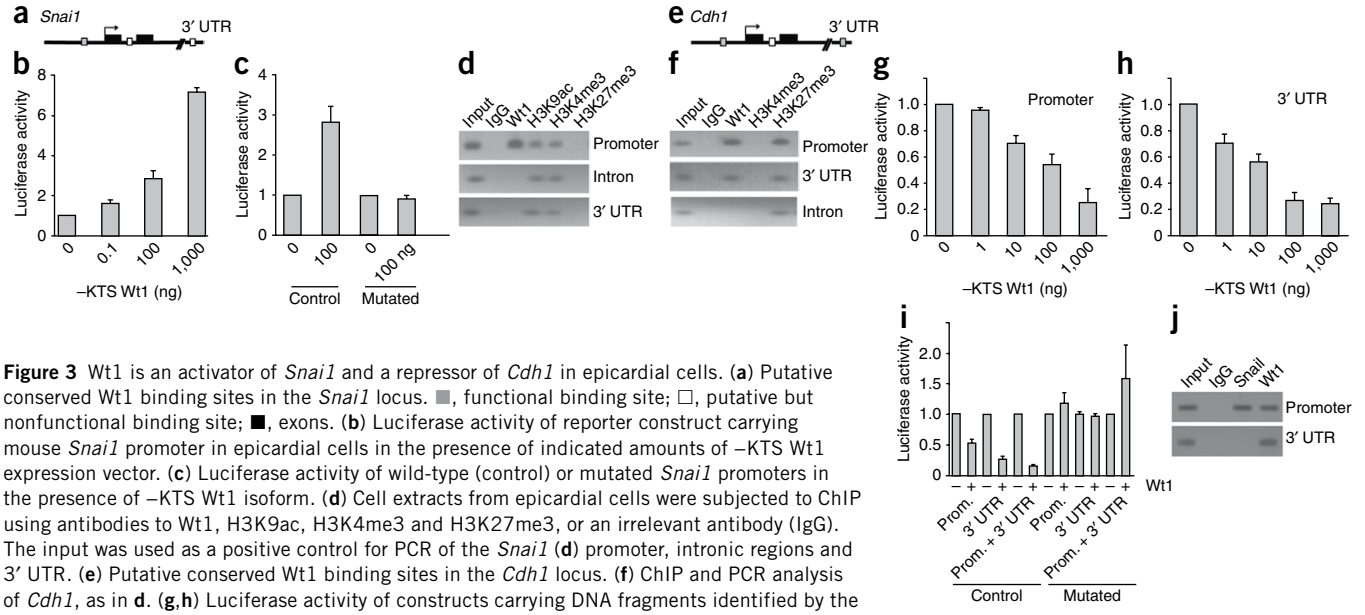

Figure 3 Wt1 is an activator of *Snai1* and a repressor of *Cdh1* in epicardial cells. (**a**) Putative conserved Wt1 binding sites in the *Snai1* locus. ■, functional binding site; □, putative but nonfunctional binding site; ■, exons. (**b**) Luciferase activity of reporter construct carrying mouse *Snai1* promoter in epicardial cells in the presence of indicated amounts of –KTS Wt1 expression vector. (**c**) Luciferase activity of wild-type (control) or mutated *Snai1* promoters in the presence of –KTS Wt1 isoform. (**d**) Cell extracts from epicardial cells were subjected to ChIP using antibodies to Wt1, H3K9ac, H3K4me3 and H3K27me3, or an irrelevant antibody (IgG). The input was used as a positive control for PCR of the *Snai1* (**d**) promoter, intronic regions and 3′ UTR. (**e**) Putative conserved Wt1 binding sites in the *Cdh1* locus. (**f**) ChIP and PCR analysis of *Cdh1*, as in **d**. (**g**,**h**) Luciferase activity of constructs carrying DNA fragments identified by the *Cdh1* ChIP assay in epicardial cells, together with various concentrations of –KTS Wt1 isoform. (**i**) Luciferase activity of control or mutated *Cdh1* constructs in the presence of 100 ng of –KTS Wt1 isoform. (**j**) ChIP assays for Snail and Wt1 at the endogenous *Cdh1* promoter in epicardial cells. All graphs show mean values ± s.e.m. from three independent experiments.

identified Wt1 binding sequence in the *Cdh1* promoter confirmed that Wt1 binds directly to the endogenous *Cdh1* promoter in epicardial cells (**Fig. 3f**). Consistent with a repressed state, the *Cdh1* promoter was depleted in H3K4me3 and enriched for H3K27me3 (**Fig. 3f**). An extended analysis of the *Cdh1* genomic sequence revealed two new conserved potential Wt1 binding sites in the intron and 3′ UTR (**Fig. 3e**). ChIP with primers flanking these regions showed *in vivo* binding of Wt1 in epicardial cells to the 3′ UTR but not to the intronic region (**Fig. 3f**). In epicardial cells, the –KTS Wt1 isoform had a repressive effect on the *Cdh1* promoter fragment containing the Wt1 binding site shown previously to be activated in NIH3T3 cells (**Fig. 3g** and data not shown) and on the 3′ UTR construct (**Fig. 3h**). The transcriptional repression was abolished when the functional binding sites were mutated (**Fig. 3i**). The +KTS isoform was able to regulate the *Snai1* and *Cdh1* constructs, but to a much lesser degree (data not shown).

Our finding that Wt1 can directly repress *Cdh1* raised the question of whether Snail is also a repressor of *Cdh1* in this system. ChIP analysis showed that Snail does interact with the *Cdh1* promoter in epicardial cells (**Fig. 3j**). Furthermore, knockdown of *Snai1* in epicardial cells with a short hairpin RNA targeting *Snai1* led to an increase in *Cdh1* promoter activity (**Supplementary Fig. 6**). These results led us to propose a model in which Wt1 promotes downregulation of *Cdh1* directly and indirectly by increasing levels of Snail.

To determine whether Wt1 regulates EMT and cardiovascular differentiation in another cellular system, we investigated Wt1 in embryonic stem cells[8]. Fluorescence analysis ($Wt1^{GFP/+}$ knockin embryonic stem cells) and protein blotting showed that Wt1 was almost undetectable in undifferentiated embryonic stem cells (day 0); Wt1 expression was first detectable on day 3 and peaked between days 9 and 11 during embryoid body differentiation (a proliferative period for undifferentiated mesenchymal cells; **Fig. 4a**,**b**). Wt1 expression declined thereafter, as various terminally differentiated cell types started appearing (data not shown).

To determine whether Wt1 is required for EMT in embryonic stem cells, we examined the expression of the principal epithelial and mesenchymal markers in wild type (control) and *Wt1*-knockout embryoid bodies (**Fig. 4c**). Control embryoid bodies expressed high levels of vimentin, α-SMA and Snail, but not E-cadherin, indicating that these cells had undergone EMT. By contrast, *Wt1*-knockout embryoid bodies maintained high levels of E-cadherin and did not induce the mesenchymal markers, reflecting their inability to undergo EMT (**Fig. 4c**). Additionally, cells derived from control

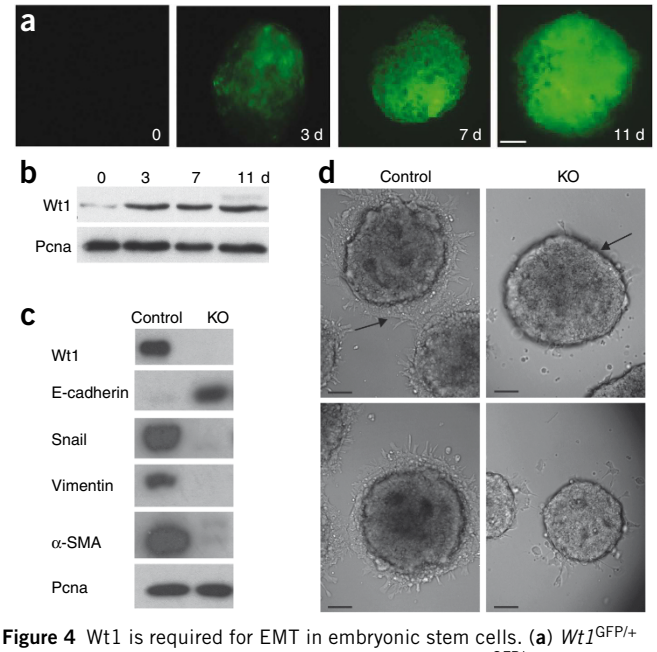

Figure 4 Wt1 is required for EMT in embryonic stem cells. (**a**) $Wt1^{GFP/+}$ expression during embryoid body differentiation of $Wt1^{GFP/+}$ knockin embryonic stem cells. Scale bar represents 50 μm. (**b**) Protein blot analysis of endogenous Wt1 protein in embryoid bodies. (**c**) Protein blot analysis of epithelial and mesenchymal protein markers in wild-type (control) and *Wt1*-knockout (KO) embryoid bodies. (**d**) Phase-contrast microscopy of control and *Wt1*-knockout embryoid body migrating cells. Arrows in **d** indicate presence of migratory cells in control embryoid bodies and absence from knockout embryoid bodies. Scale bars represent 50 μm.

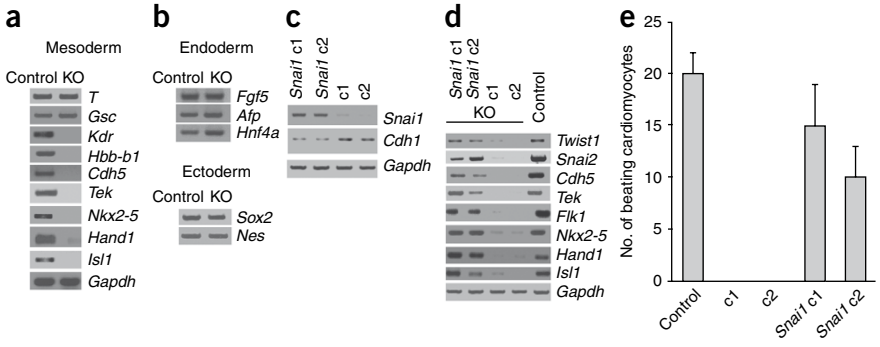

Figure 5 Wt1 is required for the formation of some mesodermal lineages in embryoid body differentiation. (**a**,**b**) RT-PCR analysis of expression of mesodermal (**a**) and endodermal and ectodermal (**b**) markers in wild-type (control) and *Wt1*-knockout (KO) embryoid bodies. (**c**) RT-PCR analysis of expression of *Snai1* and *Cdh1* in *Wt1*-knockout *Snai1*-expressing clones (*Snai1* c1 and *Snai2* c2) and *Wt1*-knockout GFP-expressing clones (c1 and c2). (**d**) RT-PCR analysis of expression of mesodermal markers in *Snai1* and GFP clones. (**e**) Numbers of beating cardiomyocytes in control and *Wt1*-knockout clones. Graph shows mean values ± s.e.m. from three independent experiments.

embryoid bodies had the capacity to migrate, whereas cells derived from *Wt1*-knockout embryoid bodies showed a clear impairment in their migration properties (**Fig. 4d**).

Because *Wt1*-knockout embryoid bodies were unable to undergo EMT, we examined whether Wt1 is required for the generation of mesodermal lineages from embryonic stem cells. We analyzed the expression patterns of the primitive streak genes encoding Brachyury (*T*) and Goosecoid (*Gsc*). Both of these genes were normally expressed in *Wt1*-knockout embryoid bodies (**Fig. 5a**). The more mature mesodermal markers, including hematopoietic (*Kdr* and *Hbb-b1*), endothelial (*Kdr*, *Tek* and *Cdh5*) and cardiac (*Kdr*, *Nkx2-5*, *Hand1* and *Isl1*) markers clearly showed reduced expression in *Wt1*-knockout embryoid bodies compared to control embryoid bodies (**Fig. 5a**). The ectodermal (*Sox2* and *Nes*) and endodermal (*Fgf5*, *Afp* and *Hnf4a*) markers showed no differences (**Fig. 5b**).

To examine whether the EMT defect in *Wt1*-knockout embryoid bodies is the principal cause of impairment in the formation of mesoderm precursors, we expressed *Snai1* in *Wt1*-knockout embryonic stem cells. *Snai1*-positive clones expressed lower levels of *Cdh1* and higher levels of *Snai2* and *Twist1* than did control GFP-expressing clones (**Fig. 5c,d**), suggesting a rescue of the EMT process. Expression of the endothelial and cardiac mesodermal markers was also rescued after *Snai1* expression (**Fig. 5d**). To confirm the presence of mature functional mesoderm, we analyzed the formation of beating cardiomyocytes. These were observed in control embryoid bodies and *Snai1*-rescued clones but were absent from the control GFP clones (**Fig. 5e**).

Our findings support a crucial role for Wt1 in the generation of mesenchymal cardiovascular progenitor cells in the epicardium and during embryonic stem cell differentiation, through direct regulation of the Snail transcription factor and E-cadherin. A model built on our principal findings is shown in **Supplementary Figure 7**. Wt1 expression is reactivated in hearts after ischemia[9], so it would be interesting to determine whether Wt1 is required for the regeneration and repair of damaged hearts through the pathways described here.

The embryonic stem cell–embryoid body model has been particularly useful in elucidating the molecular events involved in the specification of cell lineage differentiation[10,11]. Our data suggest that in *Wt1*-null embryonic stem cells, disruptions during EMT cause defects in the differentiation of some mesodermal lineages. This conclusion is based on the finding that Snail, the master regulator of EMT, rescued the *Wt1*-null phenotype. The role of Wt1 in coronary blood vessel cell formation has been shown previously[3,12] and in the present study. However, our data provide the first evidence that Wt1 is also required for the formation of cardiomyocytes, through the regulation of EMT.

It is unclear whether the role of Wt1 in embryoid bodies recapitulates the transitions in the epicardium that are required for the formation of cardiovascular progenitor cells, or whether it reflects an earlier function of the *Wt1* gene in mesoderm formation. Notably, a recently developed data set that samples the mouse transcriptome from gastrulation through organogenesis[13] revealed a massive increase in Wt1 expression during gastrulation, implicating this gene in the extensive morphological changes that occur within the embryo throughout this stage. Despite early embryonic expression of Wt1, the deficient embryo shows no overt phenotype just after gastrulation, although the formation of several mesodermal tissues is impaired. More extensive analysis will be required to evaluate whether Wt1 has an earlier role in mesoderm formation.

We have compiled evidence that Wt1 regulates the reverse process—mesenchymal-epithelial transition—in the kidney, and we have begun to dissect the molecular mechanisms involved (A.E., P.H., O.M.M.-E. and N.D.H., unpublished data). Together, all of our findings suggest that Wt1 has a major role regulating the balance between two fundamental cell states in several mesodermal tissues. In this context, it is interesting to consider the pattern of expression of Wt1 during development. The highest sites of expression are in the mesothelium, podocytes of the kidneys and Sertoli cells of the testis. All of these cells have dual epithelial and mesenchymal properties. The other major sites of expression are mesenchymal cell populations whose fates have not been determined. Further studies will be necessary to elucidate whether these cells are progenitors for particular cell types in the developing fetus.

Our findings may have relevance far beyond the cardiovascular field. For example, Wt1 and Snail are both expressed at high levels in a range of adult cancers. Expression of both genes is correlated with poor prognosis in breast cancer[14,15]. A recent study showed that introduction of *Snai1* into mammary epithelial cells converts them into cells with properties of mammary cancer stem cells[16]. We speculate that Wt1 regulates *Snai1* and EMT in various contexts and is involved in establishing cancer stem cells and tumor cells with invasive properties.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturegenetics/.

Note: Supplementary information is available on the Nature Genetics website.

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AUTHOR CONTRIBUTIONS

O.M.M.-E. designed, conducted and analyzed experiments and wrote the manuscript. L.A.L. generated *Wt1*$^{loxP/loxP}$ mice. A.E. conducted part of the promoter and ChIP experiments. J.A.G. conducted immunohistochemistry experiments. J.S. assisted with the embryonic stem experiments. V.V. conducted immunohistochemistry and flow cytometry experiments. E.H. and J.R. conducted initial experiments with immortalized epicardial cells. P.S.D. conducted blastocyst microinjection and helped with mouse maintenance. P.H. helped set up genetic crosses and is responsible for the mouse database. N.H. provided the *Wt1*$^{GFP/+}$ knockin mice. R.E.H. contributed to discussion. R.M.-C. designed experiments and cowrote the manuscript. N.D.H. obtained funding, helped with the design and analysis of experiments and cowrote the manuscript.

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ONLINE METHODS

Generation of *loxP*-flanked *Wt1* targeting construct and *Wt1*$^{loxP/loxP}$ mice. A genomic fragment 5.8 kb in length surrounding exon 1 of the mouse *Wt1* locus (representing nucleotides 104,963,917 to 104,969,768 of chromosome 2 in Ensembl release 50) was subcloned into PolyIII. An oligonucleotide containing a *loxP* site marked with a PstI restriction enzyme recognition site was cloned in the unique AatII restriction site at position 2270 within this fragment. A neomycin cassette, flanked by FLP recognition target sites and carrying a single *loxP* site (from vector p451), was targeted by bacterial recombination[17] to position 3955. This produced a targeting vector with two external homology arms of 2.2 kb and 1.9 kb and an internal homology arm of 1.7 kb (the completed targeting vector is shown in **Supplementary Fig. 1a–e**). The insert was removed from the vector backbone by digestion with NotI and purified using an Elutrap (Schleicher & Schuell). After homologous recombination in E14Tg2AIV embryonic stem cells (a gift from A. Smith) using standard techniques[18], 307 clones were screened by Southern blotting of PstI-digested genomic DNA using a 3′ probe external to the targeting vector. Of the nine clones correctly targeted at the 3′ end, subsequent screening using an internal probe showed that three correctly carried the lone 5′ *loxP* site (**Supplementary Fig. 1c**). Two of these lines were electroporated with a vector expressing FLPe recombinase to remove the neomycin cassette (**Supplementary Fig. 1d**) and subsequently with a vector expressing Cre recombinase to verify accurate excision of exon 1 (**Supplementary Fig. 1e**). Clones were reanalyzed by Southern blotting (**Supplementary Fig. 1f**), karyotyped and injected into mouse blastocysts. Germline transmission of the mutant allele was initially shown by Southern blotting, but subsequent genotyping was done by PCR using the primers *Wt1*$^{loxP/loxP}$ (**Supplementary Table 1**).

Generation of epicardial-specific *Wt1*-mutant mice. Epicardial *Wt1*-mutant mice (*Gata5-Cre*; *Wt1*$^{loxP/GFP}$) were generated by cross-breeding *Wt1*$^{loxP/loxP}$ mice with mice heterozygous for *Wt1*$^{GFP/+}$ knockin[4] and *Gata5-Cre*[5]. Transgenic mice were genotyped by PCR using the primers Gata5cre, Wt1-GFP and Wt1$^{loxP/loxP}$ (**Supplementary Table 1**). Mouse studies were conducted under guidance issued by the UK Medical Research Council and the UK Home Office.

Histology and immunohistochemistry. For histological analysis, 6-μm-thick wax sections were stained with hematoxylin and eosin. For immunostaining, antigens were retrieved by boiling samples in a pressure cooker for 4 min in TEG buffer (10 mM Tris and 0.5 mM EGTA, pH 9.0). Slides were then incubated in 50 mM NH_4Cl in PBS (pH 7.4) for 30 min and blocked in 1% BSA, 0.2% gelatin and 0.05% saponin three times for 10 min each. Samples were incubated overnight at 4 °C with antibodies to Wt1 (1:800; C19, Santa Cruz Biotechnology) or GFP (1:800; Abcam) diluted with 0.1% BSA and 0.3% Triton X-100 in PBS (pH 7.4), followed by washing with 0.1% BSA, 0.2% gelatin and 0.05% saponin.

Antibodies to the following molecules were used for standard immunohistochemistry procedures: α-SMA (1:100; clone 1A4, Sigma), E-cadherin (1:800; BD Bioscience), cytokeratin (1:200; Dako), vimentin (1:200; Dako), PECAM-1 (1:50; clone Mec13.3, BD PharMingen) and Snail[19] (1:25; gift from A. García de Herreros). Samples were incubated with the secondary antibodies and counterstained with Vectashield with DAPI or propidium iodide (Vector Laboratories).

Real-time PCR of FACS-sorted GFP$^+$ epicardial cells. The heart ventricles of *Cre*$^+$; *Wt1*$^{loxP/GFP}$ and *Cre*$^-$; *Wt1*$^{loxP/GFP}$ mice were trypsinized for 15 min at 37 °C. RNA isolated from FACS-sorted GFP$^+$ epicardial cells was reverse-transcribed using a First Strand cDNA kit for RT-PCR (Roche). Analysis of gene expression was carried out by TaqMan real-time PCR. The levels of *Wt1*, *Snai1* and *Snai2* were normalized to that of the housekeeping gene *Gapdh* (Roche). Relative values are shown as ratios of gene to *Gapdh*.

Generation of CoMEECs. To generate *Wt1*$^{loxP/GFP}$ immortalized epicardial cell lines, *Wt1*$^{GFP/+}$ mice were crossed with 'Immorto' mice carrying the *Tg(H2-K1-tsA58)* transgene (Jackson Laboratory) carrying a temperature-sensitive simian virus 40 large T antigen[20]. *Wt1*$^{GFP/+}$; *Tg(H2-K1-tsA58)*$^{+/-}$ mice were mated with *Wt1*$^{loxP/loxP}$ mice. Hearts from E11.5 *Wt1*$^{loxP/GFP}$; *Tg(H2-K1-tsA58)*$^{+/-}$ mice were placed in 24-well gelatinized dishes. After 24 h, the hearts were removed, and the epicardial monolayer of cells attached to the gelatin-coated surface was grown until confluent. Cells were propagated at 33 °C in DMEM with 10% heat-inactivated FCS and 20 ng/ml mouse gamma-interferon (PeproTech). We generated tamoxifen-inducible *Wt1*-mutant cells by transfecting *Wt1*$^{loxP/GFP}$ immortalized epicardial cells with a CAGGs-CreERT2-IRES-puroR expression construct (gift from L. Grotewold). Stable clones were generated after selection with 5 μg/ml puromycin.

Migration assays. Immortalized epicardial cells that had been cultured for 6 d in the presence or absence of 100 nM of tamoxifen were seeded in six-well culture dishes at a density of 0.5×10^6 cells per well. A wound was incised 24 h later in the central area of the confluent culture and carefully washed to remove detached cells, and fresh medium was added before a further 24 h of incubation. The images were captured using a live-cell imaging system (Zeiss Axiovert 200 fluorescence microscope).

Embryonic stem cell culture and embryoid body differentiation. *Wt1*-knockout[21], *Snai1*-rescued, *Wt1*$^{GFP/+}$ knockin and wild-type E14tg2aIV embryonic stem cells were used in this study. Briefly, undifferentiated embryonic stem cells were cultured on gelatin-coated dishes in BHK-21 medium (Glasgow minimum essential medium, GIBCO) supplemented with 10% FCS, 2 mM L-glutamine, 1 mM sodium pyruvate (Sigma), 0.1 mM nonessential amino acids (Sigma), 0.1 mM mercaptoethanol (Sigma) and leukemia inhibitory factor. Embryoid bodies were formed by culturing embryonic stem cells (1×10^6 per 9-cm bacterial dish) for the indicated number of days on nonadherent bacterial plates in medium without leukemia inhibitory factor.

For migration analysis, day 11 embryoid bodies were transferred to gelatinized plates in DMEM medium. After 6 h of migration, representative images were recorded from each type of embryoid body.

To analyze the differentiation of cardiomyocytes, embryoid bodies were transferred into gelatinized plates on day 7 of differentiation. Embryoid bodies were monitored for beating from days 1 to 7. Spontaneously contracting embryoid bodies were counted by visual inspection under a light microscope.

Generation of *Snai1* transfectant cells. The constructs expressing Myc-tagged Snail and GFP were made by cloning the coding regions of *Snai1* and *GFP* into pcDNA6.2-v5-DEST (Invitrogen). *Snai1* and *GFP* vectors (100 μg each) were electroporated into 1×10^7 *Wt1*-knockout embryonic stem cells. Stable clones were generated after selection in 10 μg/ml blasticidin. Two independent clones (c1 and c2) from each construct were analyzed during the rescue experiments.

Protein blot and RT-PCR analysis. For protein blotting, lysates from day 7–11 embryoid bodies or epicardial cells treated with tamoxifen for 6 d were analyzed using antibodies to E-cadherin (1:2,000; Transduction Laboratories), Wt1 (1:2,000; C-19, Santa Cruz Biotechnology), vimentin (1:1,000; Abcam), α-SMA (1:5,000; Sigma), proliferating cell nuclear antigen (1:10,000; Santa Cruz Biotechnology) and Snail (1:100)[19].

For gene expression analysis, total RNA was isolated from immortalized epicardial cells treated with tamoxifen for 6 d, and from day 3, 7 and 11 embryoid bodies, using an RNA purification kit (Invitrogen). Total RNA was reverse-transcribed as described above. Primers used are listed in **Supplementary Table 1.**

Luciferase assays and ChIP. ChIP-positive fragments were cloned into a pGL3 plasmid (**Supplementary Table 1**). These reporter constructs (0.1 μg) were transfected into immortalized epicardial cells in the presence of the indicated amounts of expression construct encoding the −KTS Wt1 isoform[22]. The total amount of transfected DNA was normalized to that of LacZ–expressing plasmid. *Renilla* luciferase–expressing plasmid was also cotransfected as a control for efficiency. Twenty-four hours after transfection, firefly and *Renilla* luciferase activities were measured using the Dual Luciferase Reporter Assay System (Promega). To mutate the putative Wt1 binding sequences, a QuikChange Site-Directed Mutagenesis Kit (Stratagene) was used.

ChIP assays were conducted on immortalized epicardial cells. Immunoprecipitation of the cross-linked chromatin was carried out with

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rabbit polyclonal antibodies to Wt1 (C-19; Santa Cruz Biotechnology) and Snail (Abcam). Rabbit serum served as a negative control, and a 1:10 dilution of the input sample served as a positive control. The modification status of histones at the *Snai1* and *Cdh1* promoters was checked. Immunoprecipitation of the cross-linked chromatin was carried out with antibodies to H3K9ac (Upstate Biotechnology), H3K4me3 (Abcam) and H3K27me3 (Upstate Biotechnology). The amplified DNA was separated on 2% agarose gel and visualized with ethidium bromide. Primers used are listed in **Supplementary Table 1.**

***Snai1* knockdown experiments.** Immortalized epicardial cells were cultured in the presence of *Snai1*-targeting and control short hairpin RNA lentiviral particles (sc-38399-V and sc108080, respectively; Santa Cruz Biotechnology). After 3 d of culture, cells were transfected with the *Cdh1* promoter. *Renilla* luciferase plasmid was cotransfected as a control for efficiency. Firefly and *Renilla* luciferase activities were measured as described above.

OPT scanning. Embryos were fixed in 4% paraformaldehyde. The samples were then embedded and imaged as previously described[23].

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