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

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Abstract

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 *Wt1* leads to a reduction of mesenchymal progenitor cells and their derivatives. We demonstrate that *Wt1* is essential for repression of the epithelial phenotype in epicardial cells and during Embryonic Stem (ES) cell differentiation, through direct transcriptional regulation of *Snail* (*Snail*) and *E-cadherin* (*Cdh1*), two of the major mediators of EMT. Some mesodermal lineages fail to form in *Wt1* null embryoid bodies but this effect is rescued by the expression of *Snail*, underlining 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.

Keywords

EMT; *Wt1*; epicardium; progenitor cells; *Snail*; stem cells

In the developing heart, very little is known about the molecular and cellular mechanisms controlling epicardial EMT and the formation of cardiovascular progenitor cells¹. *Wt1* encodes a zinc finger protein, which plays a critical role in the normal development of several organs such as kidney, gonads, spleen and heart². Coronary vascular defects in *Wt1* mutant mice are conjectured to arise through defective EMT³. However, until now it has not

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Author contribution

O.M.M.E. designed, performed and analyzed experiments and wrote the manuscript, L.A.L. generated *Wt1^{loxP/loxP}* mice, A.E. performed part of the promoter and ChIP experiments. J.A.G. performed immunohistochemistry experiments. J.S. assisted with the ES experiments, V.V. performed immunohistochemistry and FACS sorting experiments, E.H. and J.R. performed initial experiments with immortalized epicardial cells, P.S.D. performed the blastocyst microinjection and helped with the mice 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 co-wrote the manuscript, N.D.H. obtained the funding, help with the design and analysis of experiments and co-wrote the manuscript.

The authors declare no conflict of interest.

been possible to test if *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*-GFP knockin mice⁴ and transgenic *Gata5-Cre* mice (See Online Methods for details of breeding). The resulting *Wt1* mutants die between embryonic day 16.5 (E16.5) and embryonic day 18.5 (E18.5) due to cardiovascular failure. Embryos at E16.5 display edema and accumulation of blood in the systemic veins (Fig. 1a, b). Efficient deletion of *Wt1* in epicardial cells was confirmed using immunohistochemistry on heart sections and real time-PCR analysis on FACS-sorted GFP⁺ epicardial cells isolated from *Gata5-Cre⁺/Wt1^{loxP/gfp}* (*Cre⁺*) mice (Supplementary Fig. 2a-c). The gross morphology of *Cre⁺* embryos is normal. However the right ventricle (RV) of some mutant embryos (Fig. 1d) showed a thinner free wall as compared to the control (Fig. 1c) (arrows), while the left ventricle (LV) was apparently normal. Mutant embryos also display pericardial haemorrhaging (* in Fig. 1d).

We confirmed the embryonic expression of the *Gata5-Cre* transgene in the heart. At E10.5 we detected cells expressing *Cre* in the epicardium, while at E12.5 *Gata5-Cre* derived cells were abundant within the heart (Supplementary Fig. 3 and data not shown) 5.

Optical projection tomography (OPT) showed that the coronary arteries failed to form in *Wt1* mutant mice (Fig. 1e, f and Supplementary movies 1, 2). This result was confirmed by staining for platelet/endothelial cell adhesion molecule-1 (PECAM-1) and α -smooth muscle actin (α -SMA), markers for endothelial and smooth muscle cells respectively (Fig. 1g, h).

The presence of GFP⁺ epicardial cells covering the surface of the myocardium in *Cre⁺* mice demonstrated the integrity of this structure in mutant mice (Supplementary Fig. 4b). Since epicardial EMT plays 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. We found that E-cadherin and Cytokeratin (CK), epithelial markers, were upregulated in mutant epicardial cells in comparison with control hearts (Fig. 1k-n). Conversely, the expression of mesenchymal markers, Snail (Fig. 1i, j) and Vimentin was reduced (Fig. 1m, n). We also compared the levels of *Slug* (*Snai2*) in *Cre⁺* and *Cre⁻* FACS-sorted epicardial cells by real time-PCR and confirmed that similar to *Snail* (*Snai1*), *Snai2* levels were reduced by 70% in *Cre⁺* in comparison with *Cre⁻* cells (data not shown).

To determine whether *Wt1* plays a direct and cell autonomous role in epicardial EMT we generated tamoxifen inducible *Wt1* KO immortalised epicardial cells (*Cre⁺* CoMEEC) Fig. 2c-f (See Online Methods). Loss of *Wt1* after tamoxifen treatment leads to a robust increase in E-cadherin expression in a dose dependent manner (Fig. 2g) which correlates with a downregulation in the levels of N-cadherin and α -SMA (Fig 2g). RT-PCR analysis also showed that there was a striking downregulation of *Snai1* expression after *Wt1* deletion (Fig. 2h). Not only does treatment of the cells with tamoxifen lead to changes in the EMT marker pattern, but the cells also display decreasing cell migration (Fig. 2i). We have not observed any difference in the markers that were analysed or in the migration properties of *Cre⁻* CoMEEC after tamoxifen treatment (Supplementary Fig. 5 a, b).

We examined whether or not the *Snai1* gene, one of the master regulators of EMT 6, could be directly regulated by *Wt1*. We identified three conserved potential *Wt1* binding sites in the *Snai1* genomic sequence schematically represented in Fig. 3a. The -KTS *Wt1* isoform, which functions as a transcription factor 2, is able to activate the *Snai1* fragment containing the promoter binding site in a dose dependent manner (Fig. 3b), while the rest of the fragments were insensitive to *Wt1* activation (data not shown). The transcriptional activation was abolished when the functional binding site was mutated (Fig. 3c). In addition,

Chromatin immunoprecipitation (ChIP) demonstrated the *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 is enriched in acetylated histones H3 and trimethylated K4 of histone H3, but depleted in trimethylated K27, which is compatible with the activated state of the *Snai1* gene in these cells (Fig. 3d).

Wt1 has been shown to transcriptionally activate the *E-cadherin* (*Cdh1*) promoter in NIH3T3 cells 7. However, we also decided to explore whether Wt1 might directly repress *Cdh1* in epicardial cells. ChIP with primers flanking the previously 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 is depleted in trimethyl-K4 of histone H3 and enriched for trimethylated K27 (Fig. 3f). An extended analysis of the *Cdh1* genomic sequence revealed two new conserved potential Wt1 binding sites in the intron and in the 3'UTR (Fig. 3e). ChIP with primers flanking these regions demonstrated the *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 does exert 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 the *Cdh1* gene raises 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 the knockdown of *Snai1* in epicardial cells with a shRNA-*Snai1* leads to an increase in *Cdh1* promoter activity (Supplementary Fig. 6). These results lead us to propose a model in which Wt1 can promote downregulation of *Cdh1* directly and indirectly through increasing the levels of Snail.

We wanted to determine whether Wt1 might regulate EMT and cardiovascular differentiation in another cellular system, so we chose to investigate ES cells 8. Wt1 expression analysis, detected by either fluorescence (Wt1-GFP Knockin ES cells) or Western blots, showed that Wt1 was almost undetectable in undifferentiated ES cells (day 0), while expression was first detectable at 3 days and peaked between days 9 and 11 during embryoid bodies (EB) differentiation (Fig. 4a, b) (a proliferative period for undifferentiated mesenchymal cells). Thereafter, Wt1 expression declined, as various terminally-differentiated cell types started appearing (data not shown).

To determine whether Wt1 is required for EMT in ES cell we examined the expression of the principal epithelial and mesenchymal markers in wild type (control EB) versus *Wt1* KO (KO EB) EB (Fig. 4c). Control EB express high levels of Vimentin, α -SMA and Snail but not E-cadherin, indicating that these cells have undergone EMT. By contrast, *Wt1* KO EB maintain high levels of E-cadherin expression and fail to induce the mesenchymal markers, reflecting their failure to undergo EMT (Fig. 4c). Additionally, control EB derived cells possess the capacity to migrate; whereas *Wt1* KO EB derived cells have a clear impairment in their migration properties (Fig. 4d).

Since *Wt1* KO EB failed to undergo EMT, we asked whether Wt1 is required for the generation of mesodermal lineages from ES cells. We analysed the expression patterns of the primitive streak genes *Brachyury* (*T*) and *Goosecoid* (*Gsc*). Both of these genes were normally expressed in *Wt1* KO EB (Fig. 5a). The analysis of the more mature mesodermal markers, haematopoietic (Flk1 (*Kdr*) and *Hbb-b1*), endothelial (*Kdr*, *Tie2* (*Tek*) and *Ve-cadherin* (*Cdh5*)) and cardiac markers (*Kdr*, *Nkx2.5*, *Hand1* and *Isl1*) demonstrated a clear

reduction in their expression levels in *Wt1* KO EB (Fig. 5a). The ectodermal (*Sox2*, *Nestin* (*Nes*)) and endodermal markers (*Fgf5*, *Afp* and *Hnf4*) were also analysed and no differences were found (Fig. 5b).

To test whether the EMT defect in *Wt1* KO EB could be the principal cause of impairment in the formation of mesoderm precursors, we expressed *Snail* in *Wt1* KO ES cells. Snail positive clones (*Snai1c1* and *Snai1c2*) expressed reduced levels of *Cdh1* and higher levels of *Snail2* and *Twist1*, relative to control GFP clones (c1 and c2) (Fig. 5c, d), suggesting a rescue of the EMT process. We also observed that the levels of expression of the endothelial and cardiac mesodermal markers were rescued after *Snail* expression (Fig. 5d). To measure the presence of mature functional mesoderm, we analysed the formation of beating cardiomyocytes. These were observed in control EB and *Snail* rescue clones but were absent in the control *GFP* clones (c1 and c2) (Fig. 5e).

Our new findings support a crucial role for *Wt1* in the generation of mesenchymal cardiovascular progenitor formation in the epicardium and during ES cell differentiation, through the direct regulation of the Snail transcription factor and E-cadherin. A model built on our principal findings is shown in Supplementary Fig. 7. It has been shown previously that *Wt1* expression is reactivated in hearts following ischaemia⁹. It will be interesting to determine whether *Wt1* is required for the regeneration and repair of damaged hearts through the pathways described here.

The ES cell/EB 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 ES cells, disruptions during EMT causes 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 demonstrated previously^{3, 12} and in the present study. However, this is the first evidence that *Wt1* is also required for the formation of cardiomyocytes, through the regulation of EMT.

It is not clear whether the role of *Wt1* in EB recapitulates the transitions in the epicardium that are required for the formation of cardiovascular progenitor cells, or whether the role of *Wt1* in EB reflects an earlier function of the *Wt1* gene in mesoderm formation. Interestingly, Mitiku and Baker have recently developed a dataset that samples the mouse transcriptome from gastrulation through organogenesis¹³. Surprisingly, there was a massive increase in the levels of *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 displays 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* may have an earlier role in mesoderm formation.

Intriguingly in recent unpublished studies we have consolidated the evidence that *Wt1* regulates the reverse process, mesenchymal-epithelial transition (MET), in the kidney and started to dissect the molecular mechanisms involved. Together, all of our findings suggest that *Wt1* plays a major role regulating the balance between two fundamental cell states in several mesodermal tissues. In this context it is fascinating 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 display dual epithelial/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 a much broader relevance than for the cardiovascular field alone. *Wt1* and *Snai1* 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. Very recently it was shown that introduction of *Snai1* into mammary epithelial cells converts them into cells with properties of mammary cancer stem cells 16. We speculate that *Wt1* may regulate *Snai1* and EMT, which may be involved in establishing cancer stem cells and tumour cells with invasive properties.

Methods

Generation of the loxP flanked *Wt1* targeting construct and *Wt1*^{loxP/loxP} mice

A genomic fragment 5.8kb in length surrounding exon 1 of the murine *Wt1* locus (representing nucleotides 104963917 to 104969768 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 Frt sites and carrying in addition 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 2.2kb and 1.9 kb and an internal homology arm of 1.7kb; 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 and Schuell). After homologous recombination in E14Tg2AIV ES cells (a kind gift from Austin 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 9 clones correctly targeted at the 3' end, subsequent screening using an internal probe demonstrated that 3 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 re-analysed by Southern blotting (Supplementary Fig. 1f), karyotyped and injected into mouse blastocysts. Initially, germ-line transmission of the mutant allele was demonstrated by Southern blotting but subsequent genotyping has been done by PCR using the primers *Wt1*^{loxP/loxP} (Supplementary Table I).

Generation of epicardial specific *Wt1* mutant mice

Epicardial *Wt1* mutant mice (*Gata5-Cre*⁺/*Wt1*^{loxP/gfp}) were generated by cross breeding *Wt1*^{loxP/loxP} with mice that are 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).

Histology and Immunohistochemistry

For histological analysis, wax sections of 6- μ m thickness were stained with H&E. For immunostaining, antigens were retrieved by boiling samples in a pressure cooker for 4 minutes in TEG-buffer pH 9 (10 mM Tris, 0.5mM EGTA, pH 9.0). Slides were then incubated in 50 mM NH₄Cl/PBS pH 7.4 for 30 min and blocked in 1% BSA, 0.2% gelatine, and 0.05% saponin (3 times, 10 min each). Samples were then incubated overnight at 4°C with the following antibodies: *Wt1* 1:800 (C19, Santa Cruz); GFP 1:800 (abcam) diluted in 0.1% BSA, 0.3% Triton x-100/ PBS pH7.4; followed by washing with 0.1% BSA, 0.2% gelatine, 0.05% saponin.

The following antibodies were analysed using standard Immunohistochemistry procedure: Anti α -SMA 1:100 (Clone 1A4 Sigma); E-cadherin 1:800 (BD Bioscience); Cytokeratin 1: 200 (Dako); Vimentin 1: 200 (Dako) ; PECAM 1:50 (clone Mec 13.3) and Snail19 1:25. 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 trypsinised for 15 minutes at 37°C. RNA isolated from FACS-sorted GFP⁺ epicardial cells was reverse-transcribed using first strand cDNA kit for RT-PCR (Roche). Analysis of gene expression was carried out by Taqman real-time PCR. The expression levels of *Wt1*, *Snail* and *Slug* were normalized to the housekeeping gene *gapdh* (Roche). Relative values were shown as the ratio gene/Gapdh levels.

Generation of *Wt1*^{loxP/gfp} immorto Epicardial cells (CoMEEC)

To generate *Wt1*^{loxP/gfp} immortalized epicardial cell lines *Wt1*^{gfp/+} mice were crossed with the H-2KbtsA58 “immorto” mice carrying a temperature-sensitive simian virus 40 (SV40) large T antigen 20. *Wt1*^{gfp/+} /Immorto^{+/-} were mated with *Wt1*^{loxP/loxP} mice. Hearts from E11.5 *Wt1*^{loxP/gfp}/Immorto^{+/-} mice were placed on 24 well gelatinized dishes. After 24 hrs, 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, 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-puro R expression construct (kind gift of Dr. Lars Grotewold). Stable clones were generated after selection with 5 μ g/ml puromycin.

Migration assays

Immortalised epicardial cells that had been cultured for 6 days in presence or absence of 100nM of tamoxifen were seeded in 6-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, carefully washed to remove detached cells and fresh medium added before being incubated for further 24 h. The images were captured using a live cell imaging system: Zeiss Axiovert 200 fluorescence microscope.

ES cell culture and EB differentiation

Wt1 KO ES cells 21, *Snail* rescue ES cells, *Wt1*-GFP knockin ES cells and wild-type E14tg2aIV ES cells were used in this study. Briefly, undifferentiated ES cells were cultured on gelatin-coated dishes in BHK-21 medium (Glasgow MEM, GIBCO) supplemented with 10% FCS, 2 mM L-glutamine, 1 mM sodium pyruvate (SIGMA), 0.1 mM non essential amino acids (SIGMA), 0.1mM mercapthoethanol (SIGMA) and leukaemia inhibitory factor (LIF). EB were formed by culturing ES cells (1×10^6 per 9cm bacterial dishes) for the indicated number of days on non-adherent bacterial plates in medium without LIF.

For migration analysis, day 11 EB were transferred to gelatinized plates in DMEM medium and after 6 hours of migration representative images were recorded from each type of EB.

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

Generation of Snail transfectant cells

The myc-Snail and GFP constructs were made by cloning the coding region of *Snail* and *GFP* into pcDNA6.2-v5-DEST (Invitrogen). 100 µg of *Snail* and *GFP* vectors were electroporated into 1×10^7 *Wt1* KO ES cells. Stable clones were generated after selection in 10 µg/ml blasticidin. Two independent clones from each construct were analyzed during the rescue experiments: *Snail*; Snail-C1, Snail-C2; GFP; C1 and C2.

Western Blot (WB) and RT-PCR analysis

For WB, lysates from 7- 11 day EB or six day treated epicardial cells were analyzed using antibodies against E-cadherin (1:2000, Transduction Laboratories), Wt1 (C-19, 1:2000, Santa Cruz Biotechnology), Vimentin (1:1000, ABCAM), α -SMA (1:5000, SIGMA), Pcn α (1:10000, Santa Cruz) and Snail (1:100) 19.

For gene expression analysis, total RNA was isolated from six days tamoxifen treated immortalised epicardial cells and EB (3, 7 and 11 days) 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 immortalised epicardial cells, in the presence of indicated amounts of expression construct encoding –KTS Wt1 isoform 22. The total amount of transfected DNA was normalized with lacZ plasmid. Renilla plasmid was also cotransfected as a control for efficiency. 24 hours after transfection, firefly luciferase 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 performed on immortalised epicardial cells. Immunoprecipitations of the cross-linked chromatin were carried out with Wt1 (rabbit polyclonal antibody, C-19, Santa Cruz Biotechnology) and Snail (rabbit polyclonal antibody, abcam). Rabbit serum served as a negative control and a 1:10 dilution of the ‘input sample’ as positive control. The modification status of histones at the *Snail* and *Cdh1* promoter was checked. Immunoprecipitations of the cross-linked chromatin were carried out with the following antibodies anti-acetyl-histone H3 (abcam), anti-H3 (tri methyl K4, abcam) and anti- H3 (tri methyl K27) (upstate). The amplified DNA was separated on 2% agarose gel and visualized with ethidium bromide. Primers used are listed in Table 1.

Snail knockdown experiments

Immortalised epicardial cells were cultured in the presence of Snail and control shRNA lentiviral particle (sc-38399-V and sc108080; Santa Cruz Biotechnology). After three days of culture Snail and control infected cells were transfected with the *Cdh1* promoter. Renilla plasmid was also cotransfected as a control for efficiency. The luciferase and renilla activities were measured as described above.

OPT scanning

Embryos were fixed in 4% paraformaldehyde. The samples were then bleached in H₂O₂ and paraformaldehyde at 4°C. Samples were mounted in 1% Low Melting Point agarose, dehydrated in methanol and then cleared in 1 Benzyl Alcohol: 2 Benzyl Benzoate. The sample was imaged using fluorescence channels 488nm excitation light and imaged using a 520LP filter. The images were reconstructed using in-house software designed as part of the

Edinburgh mouse atlas project. Bioptronics 3001 OPT Scanner software was used to generate the 3D embryos image. An MIP (Maximum Image Projection) digital filter was used to increase the over all contrast of the image.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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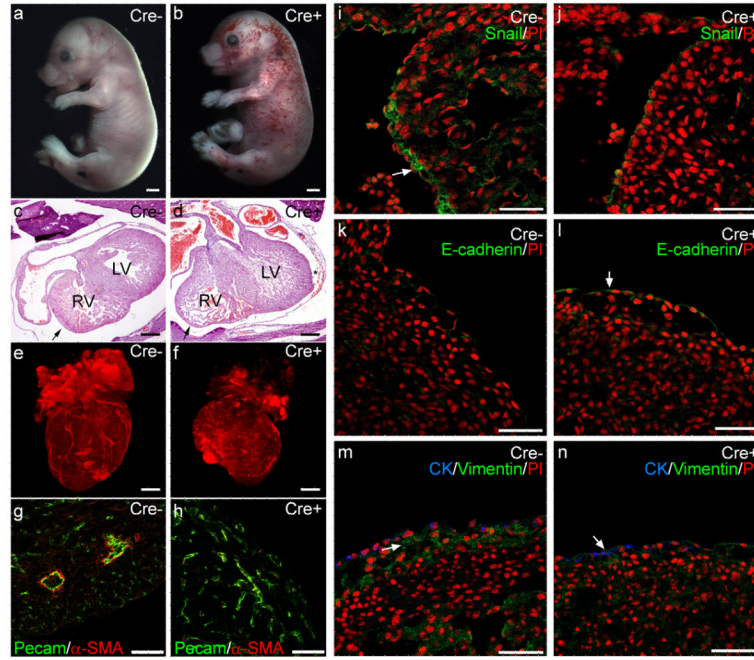


Figure 1.

Heart defects in epicardial-specific *Wt1* mutant embryos. (b) *Gata5-Cre*⁺/*Wt1*^{loxP/gfp} (*Cre*⁺) embryos display edema and accumulation of blood in the systemic veins; a littermate control (*Cre*⁻) is shown in panel (a). Scale bars represent 100 μ m. (c, d) Haematoxylin and eosin-stained sections of *Cre*⁻ and *Cre*⁺ E16.5 embryos. The right ventricle (RV) of some the mutant embryos (d) shows a thinner wall (arrows) as compared to the control (c), while the left ventricle (LV) is apparently normal. Mutant embryos show pericardial haemorrhage (* in d). Scale bars represent 50 μ m. (e, f) OPT image of control and mutant hearts at E16.5. Scale bars represent 50 μ m. (g, h) Immunofluorescence staining for the indicated blood vessel markers. Only control embryos show arteries with a well differentiated smooth muscle layer (g). Analysis of EMT markers with antibodies against: *Snail* (i, j), *E-cadherin* (k, l), *Vimentin* and *Cytokeratin* (m, n). Abnormal *E-cadherin* (l) and decreased *Snail* (j) and *Vimentin* (n) expression is observed in epicardial cells from *Cre*⁺ embryos. Scale bars represent 50 μ m.

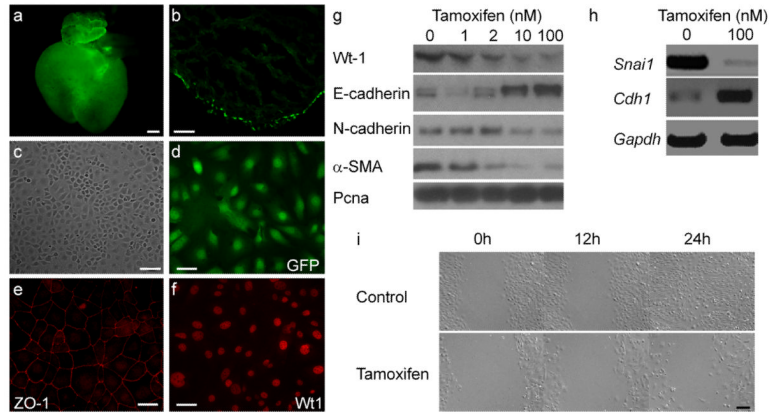
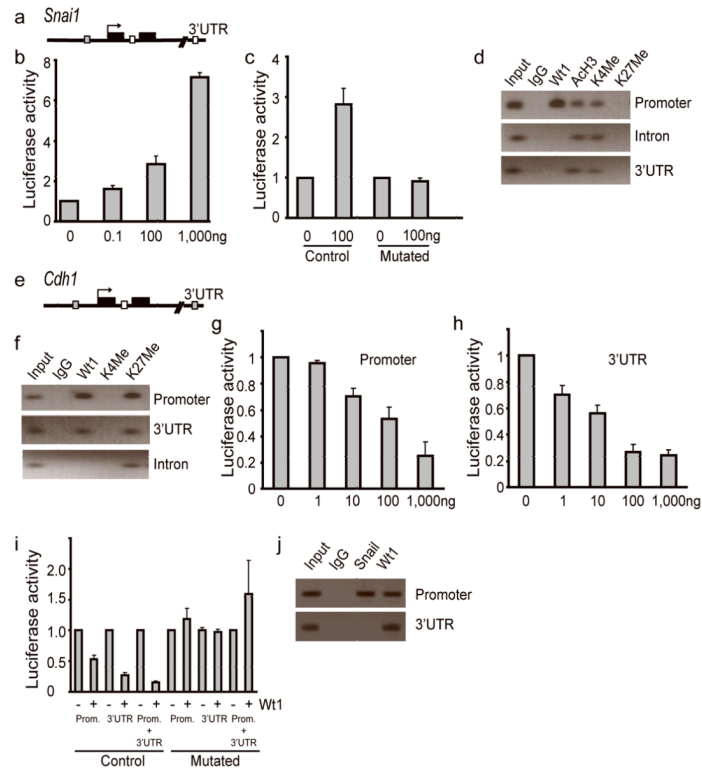


Figure 2.

Wt1 expression is necessary to maintain a mesenchymal phenotype in immortalised epicardial cells. (a) Heart of Wt1-GFP knockin mouse. Scale bar represents 50 μm . (b) Direct GFP expression on heart cryosection of Wt1-GFP knockin embryos shows GFP expression in epicardial cells. Scale bar represents 50 μm . (c) Phase-contrast micrograph of Wt1 conditional KO immortalised mouse epicardial cells (CoMEEC). Scale bar represents 100 μm . (d-f) GFP, ZO-1 and Wt1 expression in CoMEEC. Scale bars represent 50 μm . CoMEEC displayed a cobblestone monolayer typical of epicardial cells (c, e). (g) Western blot analysis was conducted in *Cre*⁺ tamoxifen inducible CoMEEC cultured in the presence of different concentrations of tamoxifen. (h) RT-PCR analyses of *Snai1* and *Cdh1* expression in *Cre*⁺ CoMEEC in presence of tamoxifen. (i) The migratory behaviour of *Cre*⁺ CoMEEC in 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, e) Schematic representation of the putative conserved Wt1 binding sites (■, □) in the *Snai1* and *Cdh1* loci, ■ (functional binding site), □ (putative but non functional binding site) and ■ (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, f) Cell extracts from epicardial cells were chromatin immunoprecipitated (ChIP), using antibodies against Wt1, anti-acetyl-histone H3 (ACh3), anti-H3 tri methyl K4 (K4Me) and anti- H3 tri methyl K27 (K27Me) or an irrelevant antibody (IgG). The input was used as a positive control for PCR of the *Snai1* (d) and *Cdh1* (f) promoters, intronic regions and 3'UTRs. (g, h) Luciferase activity of constructs carrying DNA fragments identified by the *Cdh1* ChIP assay in epicardial cells, together with different concentrations of -KTS Wt1 isoform. (i) Luciferase activity of Control or mutated *Cdh1* constructs in the presence of -KTS Wt1 isoform (100ng). (j) ChIP assays of Snail and Wt1 at the endogenous *Cdh1* promoter in epicardial cells. The graphs represent the mean values \pm s.e.m from three independent experiments.

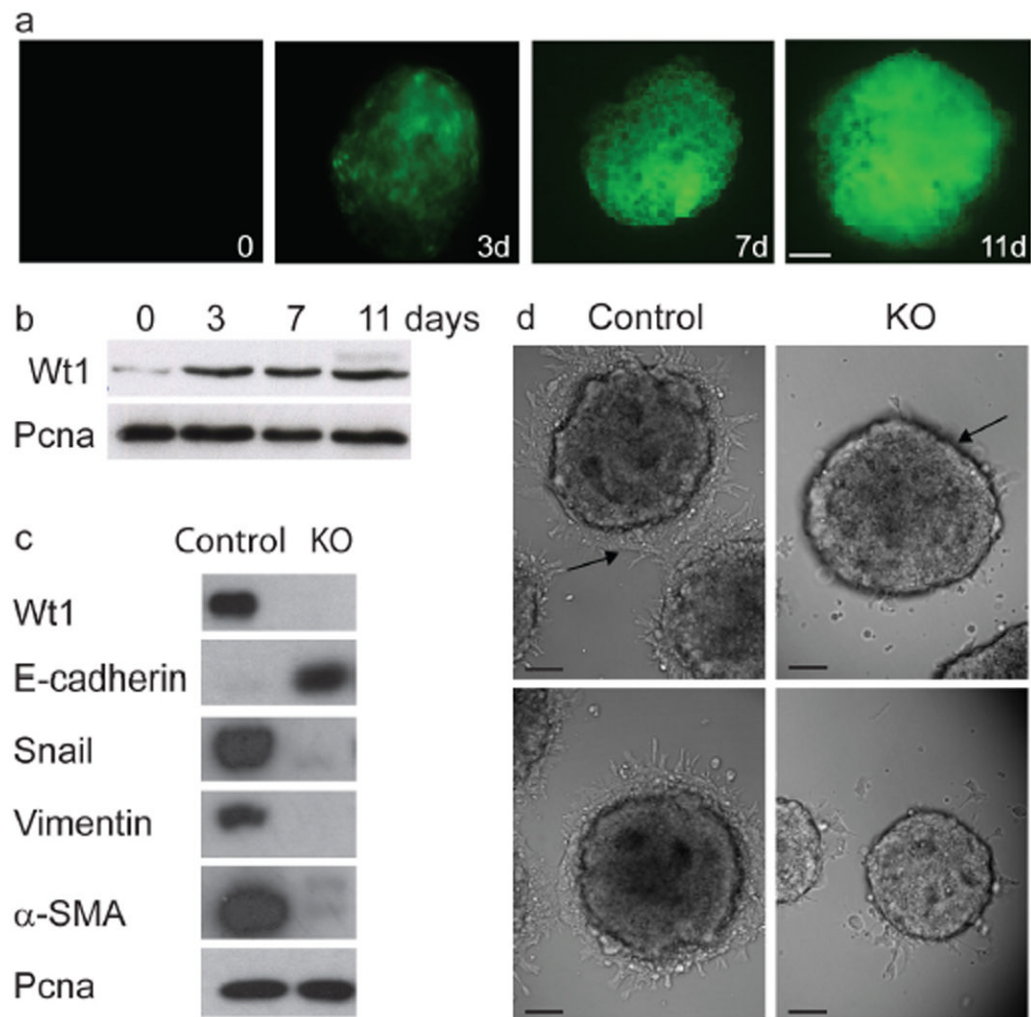
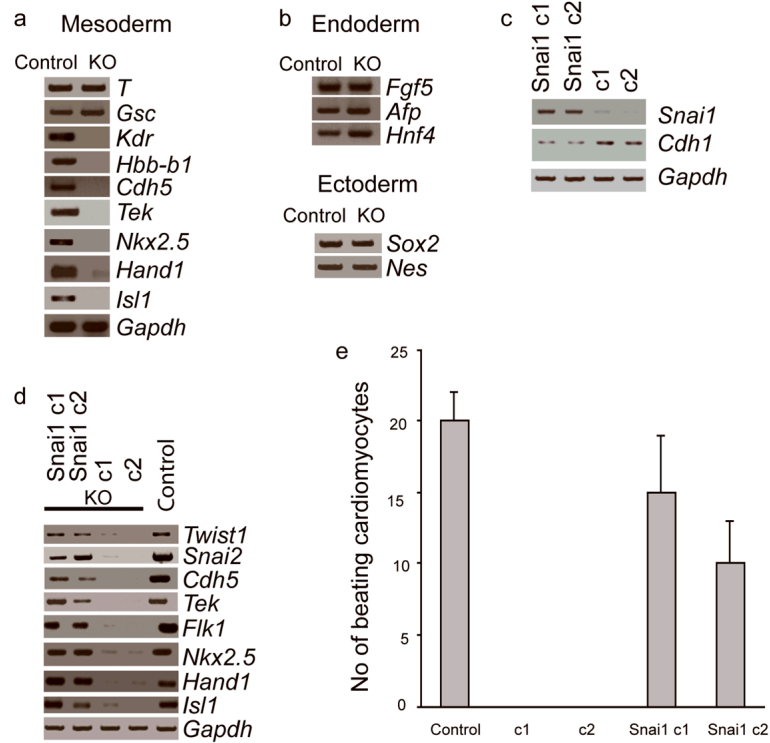


Figure 4. Wt1 is required for EMT in ES cells. (a) Wt1-GFP expression during EB differentiation of Wt1-GFP knockin ES cells. Scale bar represents 50 μm (b) Western Blot (WB) analysis of the endogenous Wt1 protein in EB. (c) WB of epithelial and mesenchymal protein markers in wild type EB (control) and *Wt1* KO EB (KO). (d) Phase-contrast microscopy of control and *Wt1* KO EB migrating cells. Scale bars represent 50 μm .

**Figure 5.**

Wt1 is required for the formation of some mesodermal lineage in EB differentiation. RT-PCR analysis for expression of mesodermal (a), endodermal and ectodermal markers (b) in wild type EB (control) and *Wt1* KO EB (KO). (c) RT-PCR analysis for expression of *Snai1* and *Cdh1* in *Wt1* KO *Snai1* expressing clones (snailc1 and snailc2) versus *Wt1* KO GFP expressing clones (c1 and c2). (d) RT-PCR analysis for expression of mesodermal markers in *Snai1* clones (Snai1c1 and Snai1c2) versus GFP clones (c1 and c2). (e) Quantification of the number of beating cardiomyocytes in control, and *Wt1* KO clones (c1, c2 and Snai1c1, Snai1c2). The graph represents the mean values \pm s.e.m from three independent experiments.