Brief UltraRapid Communication

Wt1 and Epicardial Fate Mapping

Carsten Rudat, Andreas Kispert

Rationale: The embryonic epicardium is a crucial cell source of the cardiac fibrous skeleton as well as of the coronary system. Genetic lineage tracing systems based on Wt1 regulatory sequences provided evidence that epicardium-derived cells also adopt a myocardial fate in the mouse.

Objective: To define the adequacy of Wt1-based lineage tracing systems for epicardial fate mapping.

Methods and Results: Using in situ hybridization analysis and immunofluorescence on tissue sections, we detected endogenous expression of Wt1 mRNA and Wt1 protein in the proepicardium and epicardium and also in endothelial cells throughout cardiogenesis. Expression analysis of a sensitive GFP reporter showed that recombination mediated by cre recombinase in the Wt1creEGFP line occurs randomly and sporadically in all cells of the embryo. Recombination in cardiomyocytes was found in the linear heart tube before establishment of a (pro)epicardium. In contrast, the tamoxifen-inducible Wt1creERT2 mouse line mediated poor and variable recombination in the epicardium. Recombination in cardiomyocytes was not detected in this case.

Conclusions: Frequently used Wt1 based cre-mediated lineage tracing systems are not suitable for epicardial fate mapping because of endogenous endothelial expression of Wt1, ectopic recombination (Wt1creEGFP), and poor recombination efficiency (Wt1creERT2) in the developing heart. We conclude that claims of a cardiomyocyte fate of epicardial cells in the mouse are not substantiated. (Circ Res. 2012;111:165-169.)

Key Words: cardiomyocyte  ■  epicardium  ■  lineage tracing  ■  Wt1

The outer lining of the mature vertebrate heart, the epicardium, develops after cardiac looping from a cluster of mesothelial cells of the septum transversum. Groups of cells detach from this proepicardium, adhere to the myocardium, and spread out to form a continuous epithelial layer.1 Cell lineage tracings performed by retroviral reporter constructs and chimera analysis in the chick have shown that a subset of epicardial cells undergoes an epithelial–mesenchymal transition differentiates within the subepicardial space and myocardium into interstitial and perivascular fibroblasts and smooth muscle cells (SMCs) of the coronary arteries.2–5 Some reports also suggested an additional coronary endothelial fate of epicardium-derived cells in birds.6,7 Two independent lineage tracings by cre/loxP technology confirmed fibroblasts and SMCs as epicardial derivatives but suggested an additional myocardial fate of epicardial cells in the mouse. Using a cre knock-in allele of the T-box gene 18 (Tbx18), cardiomyocytes of the left ventricle and the interventricular septum were recognized as epicardial descendants,8 and lineage tracing with two cre knock-in alleles of the Wilms tumor 1 gene (Wt1; Wt1creEGFP, Wt1creERT2) detected a substantial contribution of epicardial cells to the myocardium of the chambers and the interventricular septum starting from embryonic day (E) 10.5.9 Furthermore, using the same two Wt1cre lines, a myocardial differentiation of epicardial cells after myocardial infarction subsequent to pretreatment with thymosin β4 was demonstrated.10 However, other cre/loxP-based epicardial lineage tracing efforts did not find evidence for a myocardial fate of epicardial cells in the mouse.11–13 In addition, it was recently reported that Tbx18 exhibits endogenous expression not only in the proepicardium and epicardium but also in cardiomyocytes of the left ventricle and the interventricular septum starting at E10.5, ie, exactly in the regions claimed to be home of epicardium-derived cardiomyocytes in the study by Cai et al.14

Given the enormous medical interest in a potential myocardial fate of epicardial cells in mammals, we wished to reevaluate the adequacy of previously used Wt1-based cre/loxP lineage tracing systems. We provide evidence by expression and reporter analyses that an endothelial and myocardial fate of epicardial cells cannot be demonstrated in the mouse using this genetic tool.
Methods

Mice and Genotyping

Mice with a knock-in of the cre recombinase gene in the Wt1 locus \((\text{Wt1}^{\text{m1(EGFP/cre)Wtp}})\) or \(\text{Wt1}^{\text{creEGFP}}\), mice with a knock-in of the tamoxifen inducible cre-recombinase in the Wt1 locus \((\text{Wt1}^{\text{m2(cre/ERT2)Wtp}})\) or \(\text{Wt1}^{\text{creERT2}}\), and the double fluorescent cre reporter line \((\text{Gt}(\text{ROSA})26Sor^{\text{tm4(ACTB-tdTomato,-EGFP)Luo}})\) were obtained from the Jackson Laboratory (Bar Harbor, ME). All mice were maintained on an outbred (NMRI) background. Animal care was in accordance with national and institutional guidelines. Detailed Methods are provided in the Online Supplement.

Results

**Wt1 Expression in the Heart Is Not Restricted to Epicardial Cells**

Genetic lineage tracing by the cre/loxP technology relies on the fact that cre is expressed only in the precursor tissue but not in the differentiated cell type for which contribution should be tested. For Wt1-based epicardial fate mapping systems, it requires that Wt1 is expressed in the (pro)epicardium but not in any other cell type of the developing and mature heart. Cardiac expression of Wt1 has been reported for the (pro)epicardium, but also for cells of unknown nature in the subepicardium and the interventricular septum, prompting us to reinvestigate Wt1 expression during cardiac development (Figure 1).

In situ hybridization analysis on embryo sections did not detect Wt1 mRNA in E8.5 hearts (Online Figure 1). At E9.5, strong Wt1 expression was found in the proepicardium and in the few epicardial cells that have settled onto the myocardium. At E14.5, the entire epicardial layer was positive for Wt1, as were individual cells in the interventricular septum and in the subepicardium and myocardium of the ventricles (Figure 1A, B).

Cardiac expression of Wt1 protein was not found in E8.5 embryos by immunofluorescence analysis (Online Figure 1). At E9.5, high levels of Wt1 protein were confined to nuclei of the proepicardium and the first epicardial cells in the sulcus region. Weak Wt1 expression was occasionally observed in nuclei of the myocardium and endocardium (Figure 1C). At E14.5, all epicardial cells were positive for nuclear Wt1 protein, whereas in the interventricular septum the subepicardium and myocardium of the ventricles individual Wt1-positive cells were detected. Expression in the myocardium occurred mostly in tight association with luminal spaces (Figure 1D). Coexpression of Wt1 and the endothelial marker isolectin B4 at E9.5 and E14.5 confirmed endogenous expression of Wt1 in endothelial cells of the endocardium and/or of the coronary vasculature (Figure 1E–G). Because of technical constraints (antibodies against Wt1 and the two well-established nuclear myocardial marker proteins Nkx2.5/Prox1 were all raised in rabbits), it was not possible to unambiguously demonstrate that Wt1+ cells are myocardial in the E9.5 myocardium and the E14.5 subepicardium (open triangles in Figure 1E, F) are myocardial in nature. Thus, Wt1 mRNA and Wt1 protein expression are restricted to the (pro)epicardium in the developing heart; they are also found in differentiated endothelial cells. A weak myocardial expression at E9.5 cannot be excluded at this point.

**Wt1\textsuperscript{creEGFP} Mediates Sporadic and Ectopic Recombination in the Heart and the Rest of the Embryo**

Although endogenous expression of Wt1 in endothelial cells of the developing heart does not allow a Wt1-based lineage tracing system to claim an epicardial origin of this cell type, fibroblasts, SMCs, and (possibly) cardiomyocytes still may be analyzed for such a descent using this genetic tool. However, this requires that the cre activity pattern recapitulates endogenous expression of Wt1 in a faithful manner. To stringently test if the Wt1\textsuperscript{creEGFP} line that was previously used for this approach fulfills this criterion, we performed a careful analysis of the recombination activity of this line.
during cardiogenesis using the Rosa26<sup>mTmG</sup> mouse line as a sensitive reporter. In this reporter line, recombination is easily visualized by bright membrane-bound green fluorescent protein (GFP) expression replacing a membrane-bound red fluorescent protein, and anti-GFP immunofluorescence analysis on sections additionally allows reliable cellular resolution of cre recombination events.15

To our surprise, Wt1<sup>cre</sup>EGFP<sup>+/-</sup> Rosa26<sup>mTmG</sup> embryos exhibited highly variable patterns of green fluorescence in the entire embryo at E8.5 when neither Wt1 mRNA nor Wt1 protein was detected (Figure 2A–C, Online Figure I). GFP expression analysis on embryo sections confirmed the stochastic presence and random distribution of GFP-positive cells in the heart at this stage. Recombination occurred in cardiomyocytes and endocardial cells, as shown by coexpression with the myocardial marker Nkx2.5 and the endothelial marker isoelectin B4, respectively (Figure 2D–F, Online Figure II). At E9.5, GFP expression did not match endogenous Wt1 expression but was again highly variable in all embryonic tissues (Online Figures I and III). GFP-positive cardiomyocytes and endocardial cells were randomly distributed throughout the heart and also were found in regions where the epicardium had not yet settled (Online Figure III). Analysis of E12.5 and E18.5 embryos revealed a similar variability of GFP expression in all embryonic tissues; variable numbers of cardiomyocytes and endothelial cells were positive for GFP in the heart. At E18.5, we detected additional recombination in the heart in a fraction of peristin-expressing fibroblasts and Acta2-expressing SMCs (Online Figures IV and V). We conclude that Wt1<sup>cre</sup>EGFP-mediated reporter activity does not reflect endogenous expression of Wt1, but occurs randomly and sporadically in all embryonic tissues including the myocardium, endocardium, and vessels of the heart.

Wt1<sup>creERT2</sup> Recombines Inefficiently in the Epicardium and Its Cellular Derivatives

Wt1<sup>creERT2</sup> represents a second Wt1 allele that was used for epicardial fate mapping as well as for “epicardium”-specific gene deletion experiments.10,20,21 This allele mediates expression of a fusion protein of cre with a variant of the estrogen receptor that allows activation of cre activity by administration of tamoxifen in a temporally controlled fashion.9 Our initial tests revealed that injection of high doses of tamoxifen before E11.5 results in embryonic lethality. Injection of 4 mg of tamoxifen in preripartitionally to pregnant dams at E11.5 led to earliest and highest recombination in our experience, although this procedure resulted in premature delivery at E16.5.

Analysis of GFP expression in Wt1<sup>creERT2</sup> Rosa26<sup>mTmG</sup> hearts at E16.5 revealed low and highly variable levels of recombination in the heart. Epicardial recombination was incomplete, and limited GFP expression was detectable throughout the ventricular compact myocardium and the interventricular septum (Figure 3A–C). Double immunofluorescence analysis for GFP and MF20 failed to detect recombination within cardiomyocytes, whereas cells double-positive for GFP and isoelectin B4 (endothelial cells) and GFP and Acta2 (SMCs) revealed rare recombination events in both cell types (Figure 3D–F). Thus, the Wt1<sup>creERT2</sup> line is inadequate to follow the cellular descendents of the (pro)epicardial entity. The lack of recombination in cardiomyocytes indicates that these cells are not epicardium-derived or, alternatively, that Wt1-expressing cardiomyocytes are not present after
E10.5, even though the low recombination efficiency may conceal some rare events.

Discussion

Our results show that Wt1 expression in the heart is not restricted to the epicardium and that available Wt1cre knock-in lines do not faithfully recapitulate endogenous expression of Wt1 (Wt1creEGFP) and recombine poorly (Wt1creERT2), respectively.

Expression analyses of Wt1 on the level of mRNA and protein reported on subepicardial expression of the gene/protein in addition to strong (pro)epicardial expression during cardiac development. It was suggested that expression occurs in epicardium-derived cells and, hence, that epicardial cells maintain Wt1 expression after they have left the epicardial continuity and become mesenchymal.16–18 Our expression mapping efforts in this species.23 Those investigators to exclude this gene from epicardial fate Wt1 also was reported in the zebrafish heart, which prompted transduction actually occur in cardiomyocytes at this stage. Endogenous expression of Wt1 in the endothelium/endocardium, and possibly the myocardium in the developing heart clearly excludes Wt1-based cre lines to trace an epicardial contribution to myocardial, endothelial, and endothelium derived cells in the mouse heart. Nopemicardial expression of Wt1 also was reported in the zebrafish heart, which prompted those investigators to exclude this gene from epicardial fate mapping efforts in this species.23

Based on the strong epicardial expression of Wt1, two cre lines have been developed to trace and manipulate epicardial cells and their descendants. Our present study clearly has shown that the Wt1creEGFP line mediates sporadic and highly variable recombination in the heart as well as in the rest of the embryo at stages when a (pro)epicardium is not present in the embryo, and in a pattern that is incompatible with endogenous Wt1 expression. The underlying reason for this random activation of cre activity in this line remains unclear. We have excluded the presence of a neo cassette, which might interfere with proper transcriptional activation of cre. At present, we assume that integration of the targeting vector was imprecise or incorrect placing cre under different regulatory elements. In contrast, recombination efficiency obtained with the tamoxifen-inducible Wt1creERT2 line in epicardial cells was extremely low, allowing for following the fate of a small set of epicardium-derived cells only. Furthermore, epicardial fate analysis was restricted to stages E11.5 onward because injection of tamoxifen at earlier stages resulted in embryonic death, as reported in other studies.24,25

We conclude that an epicardial origin of myocardial and endothelial cells in the heart cannot be deduced using Wt1-based cre/loxP technology. Our data additionally call for cautious interpretation and reinvestigation of data obtained by conditional gene deletion experiments using Wt1-based cre lines.

Sources of Funding

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Disclosures

None.

References

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**Novelty and Significance**

**What Is Known?**

- The embryonic epicardium is a source of trophic signals that affects the myocardium. It also is a source of cells that form the coronary vasculature and the fibrous skeleton of the heart.
- During cardiac development, WT1 is expressed in the proepicardium and epicardium.
- Genetic lineage tracings based on WT1 regulatory sequences have provided suggestive evidence that epicardium-derived cells also adopt a myocardial fate in the mouse.

**What New Information Does This Article Contribute?**

- WT1 expression is not restricted to epicardial cells. During cardiac development, the protein also is expressed in endothelial and endocardial cells and, weakly, in cardiomyocytes.
- The WT1creEGFP line mediates sporadic and highly variable recombination in all cardiac cell types, including cardiomyocytes, as well as ectopic recombination at extracardiac sites.
- WT1creERT2-mediated recombination in the epicardium is incomplete and highly variable but does not occur in late cardiomyocytes.

The epicardium is an epithelial monolayer that covers and mechanically protects cardiac muscle. During embryogenesis, the epicardium also acts as a signaling center that promotes myocardial growth and coronary plexus formation, and is a source of cells for the fibrous skeleton of the heart, smooth muscle cells, and fibroblasts of coronary vessels. Although these functions have been confirmed for all vertebrates, a genetic (cre/loxP-mediated) lineage tracing study based on regulatory elements of the epicardially expressed WT1 gene have suggested that in the mouse, epicardial cells also substantially contribute to the myocardium. This finding is of high clinical relevance because it may open new avenues for deriving cardiomyocytes for myocardial cell therapy. Our study shows that WT1 expression not only is restricted to the epicardium but also is expressed in differentiated cells types of the heart, namely endocardial cells and cardiomyocytes. Also, we provide evidence that the WT1creEGFP line that was used for epicardial fate mapping before mediates ectopic and sporadic recombination in cardiomyocytes as well as in other cell types in which WT1 is not expressed. In addition, the inducible WT1creERT2 line recombines poorly in the epicardium, making it difficult to follow all the descendants of this tissue. Thus, cardiomyocyte fate of epicardial cells could not be substantiated using this approach.
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Wt1 and epicardial fate mapping

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Mice and genotyping

Mice with a knock-in of the cre recombinase gene in the Wt1 locus (Wt1\textsuperscript{tm1(EGFP/cre)Wtp}, synonym: Wt1\textsuperscript{creEGFP}\textsuperscript{1}, mice with a knock-in of the tamoxifen inducible cre-recombinase in the Wt1 locus (Wt1\textsuperscript{tm2(cre/ERT2)Wtp}, synonym: Wt1\textsuperscript{creERT2}\textsuperscript{1}) and the double fluorescent cre reporter line (Gt(ROSA)26So\textsuperscript{tm4(ACCTB-tdTomato,-EGFP)Luo\textsuperscript{J}}, synonym: Rosa26\textsuperscript{mTmG}\textsuperscript{2}) were obtained from the Jackson Lab and maintained on an outbred (NMRI) background. The fate of epicardial cells was analyzed in Wt1\textsuperscript{creEGFP/+};Rosa26\textsuperscript{mTmG/+} and Wt1\textsuperscript{creERT2/+};Rosa26\textsuperscript{mTmG/+} embryos. These were obtained from matings of Wt1\textsuperscript{creEGFP/+} and Wt1\textsuperscript{creERT2/+} males, respectively, with Rosa26\textsuperscript{mTmG/mTmG} females. In the latter case, Tamoxifen (Sigma) was dissolved in ethanol at 100 mg/ml and then emulsified in corn oil (Sigma) to a final concentration of 12.5 mg/ml. 4 mg of Tamoxifen were intraperitoneally injected into mice at gestation day 11.5. Embryos for Wt1 expression analysis were obtained from matings of NMRI wildtype mice.

For timed matings, vaginal plugs were checked in the morning after mating and noon was designated as embryonic day (E) 0.5. Female mice were sacrificed by cervical dislocation. Embryos were harvested in PBS, decapitated, fixed in 4% paraformaldehyde overnight and stored in 100% methanol at -20°C before further use. Genomic DNA prepared from yolk sacs or tail biopsies was used for genotyping by PCR (protocols are available upon request). H. Hedrich, state head of the animal facility, approved the care of animals and experiments at Medizinische Hochschule Hannover.

In situ hybridization analysis

In situ hybridization analysis of 10-µm paraffine sections with digoxigenin-labeled antisense riboprobes followed published protocols.\textsuperscript{3} Details of used probes are available upon request.

Immunohistochemistry

For immunohistochemistry on 4-µm paraffine sections rabbit polyclonal antibody against Wt1 (1:100, CA1026, Calbiochem), rabbit polyclonal antibody against GFP (1:200, sc-8334, Santa Cruz), mouse monoclonal antibody against GFP (1:200, #814460001, Roche), mouse monoclonal antibody against Acta2 (1:200, C6198, Sigma), mouse monoclonal antibody against Acta2 (1:200, F3777, Sigma), Fluorescein-labeled GSL I – isolectin B4 (1:100, FL-1101, VectorLabs), mouse monoclonal antibody against MF 20 (1:200, Hybridoma Bank University of Iowa), rabbit polyclonal antibody against Nkx2.5 (1:200, ab35842, Abcam), rabbit polyclonal against Periostin (1:200, ab14041, Abcam) were used as primary antibodies.
Biotinylated goat-anti-rabbit (Dianova), Alexa488 goat-anti-rabbit (Invitrogen), Alexa488 donkey-anti-mouse (Invitrogen A21202), biotinylated goat-anti-mouse (Dianova 115-067-003), Alexa-Fluor555 goat-anti-mouse (Invitrogen A-21424) and Alexa-Fluor555 goat-anti-rabbit (Invitrogen A-21428) were used in a dilution of 1:400 as secondary antibodies. Nuclei were stained with 4’,6-diamidino-2-phenylindol (DAPI) (Roth).

For antigen retrieval all sections were boiled for 3 min in antigen unmasking solution (H-3300, Vector Laboratories Inc) in a pressure cooker. Signal amplification was performed using the Tyramide Signal Amplification (TSA) system from Perkin-Elmer (NEL702001KT, Perkin Elmer LAS). For simultaneous detection of GFP and differentiation markers primary and secondary antibodies, respectively, were applied at the same time.

References

Online Figure I. Comparative analysis of endogenous expression of Wt1 mRNA and Wt1 protein and Wt1\textsuperscript{creEGFP} mediated recombination in E8.5 and E9.5 embryos. A and D, \textit{in situ} hybridization analysis of Wt1 mRNA; B and C, E and F, immunofluorescence analysis of Wt1 (in red, B and E) and GFP protein expression (in green, C and F) on sagittal sections of Wt1\textsuperscript{creEGFP/+};Rosa26\textsuperscript{mTmG/+} and Rosa26\textsuperscript{mTmG/+} control embryos at E8.5 and E9.5 as indicated. At E8.5, neither Wt1 mRNA nor Wt1 protein is detected whereas sporadic GFP expression is found throughout the embryo. At E9.5, Wt1 mRNA and Wt1 protein is found in the proepicardium, the forming epicardium (arrow in D and E) and the urogenital ridge (arrowhead in E). The GFP expression is highly variable throughout the heart and the whole embryo. a, atrium; hd, head; hf, head folds; lv, left ventricle; pe, proepicardium; s, somite; v, primitive ventricle.
Online Figure II. *Wt1*creEGFP-mediated recombination in E8.5 embryos. Analysis of the recombination activity in *Wt1*creEGFP/+;*Rosa26*tmG/+ embryos and *Rosa26*tmG/+ control mice at E8.5. A and B, immunofluorescence analysis of GFP expression on heart sections. C through F, coimmunofluorescence analysis of expression of the lineage marker GFP, the myocardial marker Nkx2.5, and the endothelial marker IB4 on heart sections, magnified regions are indicated by white rectangles. Nuclei are counter-stained with 4',6-diamidino-2-phenylindol. v, ventricle.
Online Figure III. *Wt1*<sup>creEGFP</sup>-mediated recombination in E9.5 embryos. Analysis of the recombination activity in *Wt1*<sup>creEGFP/+;Rosa26<sup>mTmG/+</sup> embryos and *Rosa26<sup>mTmG/+</sup>* control mice at E9.5. A through C, epifluorescence analysis of GFP and RFP expression in whole embryos. D through L, (co-) immunofluorescence analysis of GFP, the myocardial marker Nkx2.5, and the endothelial marker IB4 on heart sections as indicated. Magnified regions are indicated by white rectangles. Arrows point to the epicardium. Arrowheads mark GFP<sup>−</sup>IB4<sup>+</sup> cells in the endocardium. Open triangles point to GFP<sup>−</sup>IB4<sup>+</sup> cells in the myocardium. Nuclei are counterstained with 4',6-diamidino-2-phenylindol. a, atrium; lv, left ventricle; pe, proepicardium.
**Online Figure IV.** *Wt1\textsuperscript{creEGFP} mediated recombination in E12.5 hearts.* Analysis of the cre recombination activity by GFP expression in E12.5 hearts of *Wt1\textsuperscript{creEGFP},Rosa26\textsuperscript{mTmG/+}* and *Rosa26\textsuperscript{mTmG/+}* control embryos. A through F, (Co-)immunofluorescence analysis of GFP (in green, membranes) and the myocardial marker Nkx2.5 (in red, nuclei) on transverse heart sections as indicated. D through F show higher magnification images of the right ventricle. White arrows point to the epicardium. la, left atrium; lv, left ventricle; ra, right atrium; rv, right ventricle.
Online Figure V. Wt1\textsuperscript{CreEGFP}\textsuperscript{-mediated recombination in E18.5 embryos and hearts.} Analysis of cre recombination activity in Wt1\textsuperscript{CreEGFP};Rosa26\textsuperscript{mTmG/+} embryos and Rosa26\textsuperscript{mTmG/+} control mice. A through F, epifluorescence analysis of GFP and RFP expression in embryonic trunks (A through C) and hearts (D through F). G through U, (co-)immunofluorescence analysis of GFP (in green, membranes) and the myocardial marker MF20, the endothelial marker IB4, the SMC marker Acta2, and the fibroblast marker Postn (all in red) on sections of whole hearts as indicated. J through U show higher magnification images of the right ventricle. fl, fore limb; hl, hind limb; la, left atrium; lv, left ventricle; ra, right atrium; rv, right ventricle.