Circulation Research
JOURNAL OF THE AMERICAN HEART ASSOCIATION

Genetic Cre-loxP Assessment of Epicardial Cell Fate Using Wt1-Driven Cre Alleles

Bin Zhou and William T. Pu

Circ Res. 2012;111:e276-e280
doi: 10.1161/CIRCRESAHA.112.275784
Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

Print ISSN: 0009-7330. Online ISSN: 1524-4571

Genetic Cre-loxP Assessment of Epicardial Cell Fate Using Wt1-Driven Cre Alleles

Bin Zhou, William T. Pu

Rationale: **Wt1-Cre-based tools are important reagents for studying epicardial cell fate and gene function.**

Objective: **To better describe the properties of Wt1-Cre-based tools to enhance their use in Cre-loxP-based experiments.**

Methods and Results: **In contrast to recently reported results, we show that constitutive $Wt1^{GFPCre}$ in combination with certain Cre-activated reporters can be used to trace (pro) epicardial cell fate. $Wt1^{CreERT2}$ can be efficiently induced by tamoxifen administration. We show substantial labeling of coronary endothelial cells when induction is performed at late but not early stages of heart development.**

Conclusions: **Wt1-based Cre alleles are useful tools for genetic lineage tracing of epicardial cells and mesothelium of other organs. Using these tools with proper understanding of their properties and limitations enables genetic labeling of epicardial cells and their derivatives. (*Circ Res*. 2012;111:e276-e280.)**

Key Words: cre-loxp ■ epicardium lineage ■ genetic lineage tracing ■ Wt1

Epicardium is a specialized mesothelium that is crucial for heart development. Cells from the proepicardium, an outpouching of the septum transversum, seed the surface of the developing heart at approximately embryonic day 9.5 of mouse gestation (E9.5). These cells spread as an epithelial sheet over the surface of the heart to form the epicardium.[1–3] Epicardium regulates heart development both by exchanging paracrine signals with myocardium and by undergoing epithelial to mesenchymal transition to form mesenchymal cells known as epicardium-derived cells, which differentiate into several different lineages of the developing heart. Epicardium is quiescent in the normal adult heart, but is reactivated in the setting of myocardial injury.[4,5] The developmental plasticity and paracrine activity of epicardium has raised interest in its potential applications to modulate remodeling and regeneration of the injured heart.[4,6,7]

Studies of epicardium have been facilitated by development of Cre alleles that permit selective labeling and isolation of epicardial cells and their derivatives. A number of such alleles has been reported.[8–13] Each has its own strengths and weaknesses, and none described to date are completely specific to epicardium. Nevertheless, through proper consideration of the properties of each Cre allele, it has been possible to make advances in understanding the function and fate of epicardium in heart development and adult heart disease. These Cre alleles, in combination with complementary labeling approaches in avian embryos, have shown that epicardium-derived cells migrate into the underlying myocardium to differentiate into fibroblasts and smooth muscle cells.[1,3] They also contribute to vascular endothelial cells, although quantitative studies in mammals suggest that the fraction of vascular endothelial cells that arise from epicardium is small.[10,11,14] Epicardium-derived cells also potentially contribute to cardiomyocytes,[10,11] although this result has remained controversial.[15]

Recently, Drs Rudat and Kispert[16] reported limitations of using Wt1-based Cre tools in dissecting epicardial cell fate. In this Research Commentary, we refine some of the observations made in this study and add additional data on the properties of Wt1-based Cre alleles. By understanding the properties of these tools, they can be productively used to selectively label epicardium and epicardium-derived cells in the embryonic and adult heart.

Methods

$Wt1^{GFPCre}$ (named $Wt1^{creEGFP}$ in the Rudat and Kispert study),[16] $Wt1^{CreERT2}$, $Rosa26^{fsLz}$, $Rosa26^{mTmG}$, and $Nkx2\text{-}5^{LacZ}$ were described previously.[11,17–19] Immunostaining and immunohistochemistry were performed as previously described.[20,21] For tamoxifen (Tam) induction, we dissolved Tam (Sigma, T5648) in ethanol and then emulsified it in sesame oil with sonication to a final concentration of 12.5 mg/mL. To induce Cre, 200 µL to 300 µL freshly emulsified Tam was injected by gavage at indicated time point. For neonatal CreERT2 induction, we injected 0.2 mg Tam directly into the stomach at P4. The CreERT2 induction for adult heart was described previously.[4] Mice were used in accordance with protocols approved by the Institutional Animal Care and Use Committee at Children's Hospital Boston.

The extent of Cre recombination catalyzed by $Wt1^{CreERT2}$ was quantitated in histological sections. Cells in the epicardium were scored

Original received June 20, 2012; revision received August 13, 2012; accepted August 20, 2012. In August 2012, the average time from submission to first decision for all original research papers submitted to *Circulation Research* was 11.48 days.

From the Key Laboratory of Nutrition and Metabolism, Institute for Nutritional Sciences, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai, China (B.Z.); and the Department of Cardiology, Children's Hospital Boston, Harvard, Medical School, Boston, MA (B.Z., W.T.P.)

Correspondence to Bin Zhou, 294 Taiyuan Road, Shanghai 200031, China (e-mail zhoubin@sibs.ac.cn); or William T. Pu, Enders1254, 300 Longwood Ave, Boston, MA, 02115 (e-mail: wpu@enders.tch.harvard.edu).

Circulation Research **is available at http://circres.ahajournals.org** **DOI: 10.1161/CIRCRESAHA.112.275784**

Non-standard Abbreviations and Acronyms	
EPDC	epicardium-derived cell
Tam	tamoxifen

for expression of the Cre-activated reporter in >5 images from each of 3 $Wt1^{CreERT2/+}$::$Rosa26^{mTmG}$ hearts and reproduced in a second set of embryos treated, processed, and analyzed by a different investigator.

Results and Discussion

Cre-loxP genetic fate-mapping requires that the Cre is active in progenitors but not in their descendants. The results of Cre-loxP fate-mapping are strongly influenced by level of Cre expression and by Cre-loxP reporter sensitivity. Lack of Cre reporter activity might indicate low promoter activity of the reporter (as sometimes seen in adult tissues where the reporter is driven by endogenous Rosa26), insensitive reporter gene detection, or resistance of the reporter to recombination by the level of Cre being expressed.[22] On the other hand, Cre-recombination detected by very sensitive methods and reporters may conceal important and functional differences between levels of Cre activity, such as the difference between higher levels of tissue-specific Cre activity and trace expression without interpretable biological function.

The choice of Cre-activated reporter is important when using the constitutive $Wt1^{GFPCre}$ line (Table). We observed that $Wt1^{GFPCre}$ broadly activates $Rosa26^{LacZ}$ generated by P. Soriano[23] in a pattern consistent to that reported by Rudat and Kispert[16] for $Rosa26^{mTmG}$. However, using the $Rosa26^{LacZ}$ line generated by S. Orkin,[17] we observed specific recombination in proepicardium without detectable recombination in the myocardium at E9.5 (Figure 1A–1C; representative of 3 embryos studied at that stage). In addition, strong and specific X-gal staining was observed in the developing urogenital region in $Wt1^{GFPCre/+}$; $Rosa26^{LacZ/+}$ embryos (Figure 1D). Likely, a thresholding effect inherent in Cre-loxP experiments[22] magnified differences in reporter gene recombination catalyzed by low levels of Cre activity in the early $Wt1^{GFPCre}$ embryo,[24,25] so that the more sensitive reporters became broadly recombined and hence uninformative. These data suggest that there are circumstances when $Wt1^{GFPCre}$ can be used to selectively recombine certain targets, if control experiments indicate that broad, early recombination has not occurred. Additional experiments using independent approaches, such as dye labeling, multiple independent Cre transgenes, or selective viral Cre delivery, should be performed to confirm results. However, because of the potential for $Wt1^{GFPCre}$-mediated recombination outside of the mesothelial lineage, we generally no longer favor the use of this reagent for epicardial fate-mapping or gene inactivation studies, unless it can be shown that the floxed target of interest undergoes epicardium-restricted recombination. It does remain useful as a WT1 knockout allele and for genetically marking cells that actively express WT1 with green fluorescent protein[4,6,20,21]

Inducible Cre alleles allow one to activate recombination at defined times, when the domain of Cre expression can be carefully defined. The $Wt1^{CreERT2}$ allele thereby circumvents the potential for widespread recombination in the early embryo that can occur using the constitutive allele in combination with some floxed constructs. The $Wt1^{CreERT2}$ labeling described by Rudat and Kispert[16] by Tam administration at E11.5 was less efficient than what we have observed and reported.[11,21] The reasons for this inefficiency, and for the inability to deliver Tam before E11.5, are unclear to us. We have found that strain background (outbred superior to inbred) and administration route (gavage preferable to intraperitoneal injection) influence Tam side effects and efficacy. We do observe Tam toxicity in late gestation (past ≈E16.5) and impaired delivery of fetuses, requiring reduced Tam dose, delivery by cesarian section, and fostering to surrogate mothers. The problems in Tam administration by Rudat and Kispert[16] likely contributed to their lack of detection of cardiomyocyte labeling. We find the $Wt1^{CreERT2}$ line is useful for epicardial lineage tracing and gene ablation in both embryonic and adult epicardium, and also mesothelium of other organs like liver and testis.[4,6,21,26–28] We achieve efficient labeling (70.2±10.4% of epicardial cells labeled by Tam administration at E10.5 [n=3]; Figure 2A). This degree of recombination is sufficient for most fate-mapping studies, and for many gene inactivation studies, but incomplete inactivation should be considered when interpreting results. Higher levels of recombination might be achieved with multiple Tam doses. We reproducibly observe labeling of a small subset of cardiomyocytes.[11] Using independent epicardial Cre lines ($Sema3d^{Cre}$, Scx^{Cre}, and $Wt1^{IRES\text{-}GFPCre}$), other groups have also observed cardiomyocyte labeling, although these experiments also require cautious interpretation.[9,13]

A more difficult issue appropriately raised by Rudat and Kispert[16] is whether cardiomyocytes express Wt1 (or Cre driven

Table. Combinations of Wt1-Cre and Cre-Activated Reporters

Allele	Reporter	Observations	Pitfalls
$Wt1^{GFPCre}$	Rosa26Lz-Orkin	Recombination largely confined to proepicardium at E9.5.	Low level recombination in other tissues/cells cannot be excluded.
	Rosa26Lz-Soriano	Broad recombination throughout embryo	Recombination not restricted to epicardium.
	$Rosa26^{mTmG}$	Broad recombination throughout embryo	Recombination not restricted to epicardium.
$Wt1^{CreERT2}$	Rosa26Lz-Orkin	Inefficient recombination after Tam induction	Low labeling efficiency.
	Rosa26Lz-Soriano	Moderately efficient Tam-induced recombination with one dose at E9.5-E11.5	Endothelial labeling if induced Tam treatment performed at E14.5 or P4. Late gestational Tam toxicity and failure to yield live pups.
	$Rosa26^{mTmG}$	Moderately efficient Tam-induced recombination with one dose at E9.5-E11.5	Endothelial labeling if induced Tam treatment performed at E14.5 or P4. Late gestational Tam toxicity and failure to yield live pups.

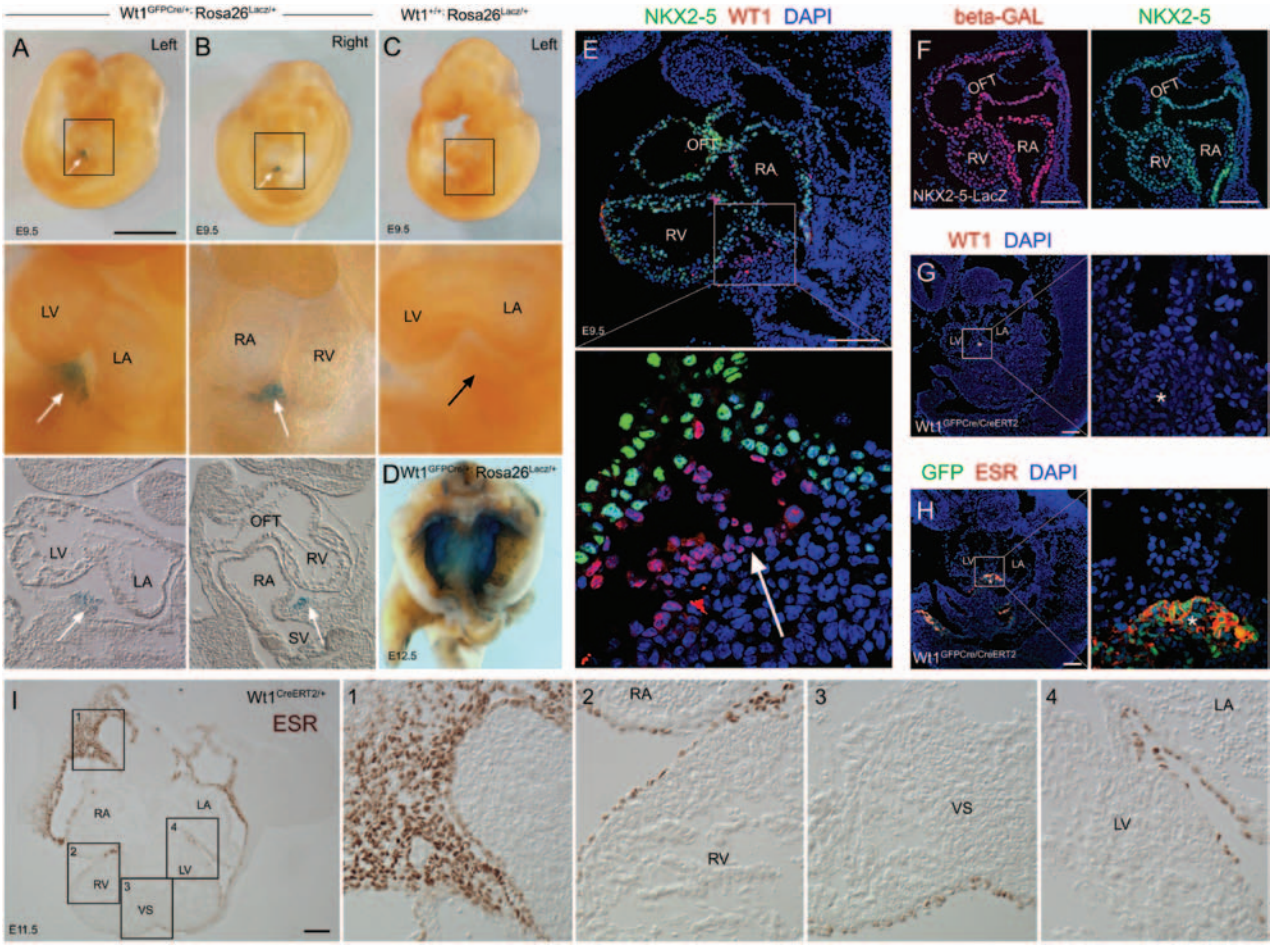

Figure 1. Wt1 expression and fate map. A–C, Wt1GFPCre specifically labeled proepicardium at E9.5 in $Wt1^{GFPCre/+}$; $Rosa26^{LacZ/+}$ embryos (**A–B**, white arrows), where $Rosa26^{LacZ/+}$ indicates the Orkin allele.[17] Black arrow in **C** indicates proepicardium in $Wt1^{+/+}$; $Rosa26^{LacZ/+}$ littermate control. **D**, Strong and specific Cre labeling of the urogenital region of an E12.5 $Wt1^{GFPCre/+}$; $Rosa26^{LacZ/+}$ embryo. **E**, WT1 was not expressed in NKX2-5^{+} cardiomyocytes. White arrow indicates proepicardium at E9.5. **F**, Specific staining of NKX2-5 antibody as shown by beta-gal expression in $Nkx2\text{-}5^{LacZ}$ embryo. **G** and **H**, Immunostaining conditions for WT1 were specific, as WT1 was not detected in $Wt1^{GFPCre/CreERT2}$ proepicardium. In contrast, both green fluorescent protein (GFP) and estrogen receptor (ESR) (contained in the CreERT2 protein) were specifically detected on proepicardium (asterisk). **I**, WT1-driven expression of CreERT2 was detected by ESR antibody in E11.5 heart. Expression was detected in epicardium but not in myocardium, as shown in 4 representative magnified regions. White bar=100 μm. Black bar=0.5 mm. LA indicates left atrium; RA, right atrium; LV, left ventricle; RV, right ventricle; OFT, outflow tract; SV, sinus venosus; DAPI, 4′,6-diamidino-2-phenylindole.

by Wt1). The correct interpretation of Cre fate-mapping results depends on clear definition of the Cre expression map. However, obtaining expression maps with the appropriate spatiotemporal resolution and sensitivity can be problematic. First, Cre fate-maps represent the accumulation of continuous Cre-mediated recombination, whereas expression maps represent expression at a discrete time point and have the potential risk of missing detection of expression in certain cell populations at other time points. Second, immunostaining or in situ hybridization has imperfect sensitivity and specificity, and their dynamic range may not match well with that of the Cre-loxP method. In examining whether cardiomyocytes express WT1, antibody constraints precluded Rudat and Kispert[16] from reaching a definitive conclusion.[16] We[11] and others[29] did not observe WT1 expression in cardiomyocytes in costaining experiments, with strict staining controls (Figure1E–1I). However, given immunostaining limitations and potential mismatch between immunostaining compared with Cre-loxP detection, we cannot absolutely exclude potential cardiomyocyte expression of WT1 in the developing heart. The basis for any Cre-loxP fate-map is clear and definitive expression map; therefore, whether epicardium contributes to cardiomyocytes requires further investigation. Additional experiments using complementary strategies that do not hinge on expression or lack of expression of Cre in a cell population of interest are needed to resolve this and similar controversies.

The origin of coronary endothelial cells remains controversial. We and others showed little contribution of Wt1 or Tbx18 lineages to coronary vessels in mouse heart.[10,11,14,30] Rudat and Kispert[16] detected WT1 expression in endothelial cells as early as E9.5, although the frequency was not reported. Tam induction of $Wt1^{CreERT2}$ at E10.5 led to rare labeling of coronary endothelial cells, suggesting that few E10.5 cells destined to become endothelial cells express Wt1-driven CreERT2. This result suggests that most coronary endothelial cells do not arise from the epicardial Wt1 lineage, and they may arise from an epicardial subpopulation not labeled by $Wt1^{CreERT2}$, as recently suggested by Katz et al or from a nonepicardial

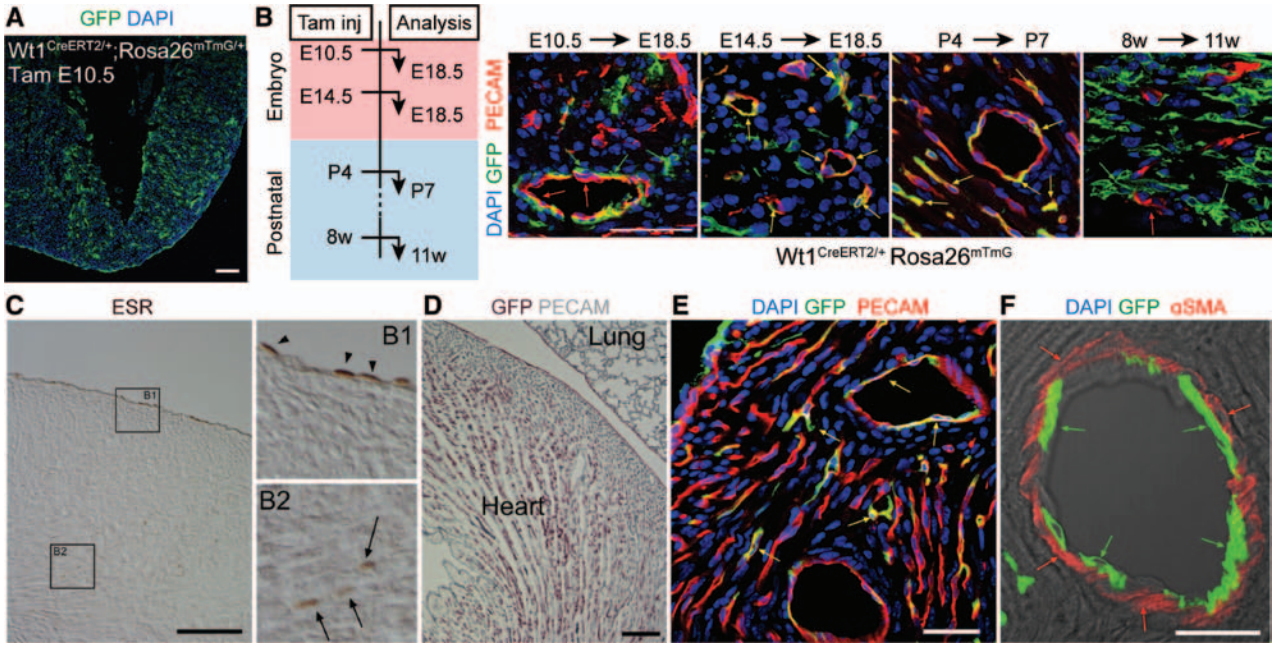

Figure 2. Wt1 and coronary endothelial cell fate. A, Efficient labeling of epicardial cells and derivatives by Tam administration at E10.5 and analysis at E18.5. **B**, Tam induction of Wt1^{CreERT2} at 4 different time points yields different coronary endothelial labeling results. Induction at E10.5 left the large majority of endothelial cells unlabeled when assessed at E18.5. Induction at E18.5 and P4 labeled a substantial fraction of coronary endothelial cells when assed at E18.5 and P7, respectively. Adult stage induction labeled very few coronary endothelial cells. Yellow arrows indicate the Wt1^{CreERT2}-labeled PECAM^{+} cells. Green arrows indicate Wt1-labeled, Pecam^{-} cell, and red arrows indicate PECAM^{+} cells not labeled by Wt1^{CreERT2}. **C**, Detection of weak Wt1^{CreERT2} expression by ESR antibody staining within the neonatal heart (black arrows). Strong expression was detected in the epicardium (black arrowheads). Black bar=100 μm. **D**, Induction of Wt1^{CreERT2} at P4 did not label a substantial fraction of endothelial cells in the lung, in contrast to heart. **E** and **F**, A substantial fraction (28%) of PECAM^{+} cells in the neonatal heart was labeled by neonatal activation of Wt1^{CreERT2}. However, coronary smooth muscle cells (α SMA+) were not labeled by neonatal Wt1^{CreERT2} activation. Yellow arrows indicate GFP^{+};PECAM^{+} cells; green arrows indicate GFP^{+};SMA^{-} cells; red arrows indicate GFP^{-};SMA^{+} cells. White bar=50 μm. GPF indicates green fluorescent protein; PECAM, platelet/endothelial cell adhesion molecule 1; SMA, smooth muscle actin alpha; ESR, estrogen receptor; DAPI, 4',6-diamidino-2-phenylindole.

source.[13,30] However, activation of Wt1^{CreERT2} at E14.5 or P4 revealed substantial labeling of endothelial cells at E18.5 and P7, respectively (Figure 2B). Consistent with expression of Wt1 in endothelial cells at these stages of development, as reported by Rudat and Kispert[16] and as shown in Figure 2C, Wt1^{CreERT2} labeling of endothelial cells at these stages likely reflected expression of Cre within these cells rather than their origin from epicardial cells. Surprisingly, Wt1^{CreERT2} did not substantially label endothelial cells in the neonatal lung (Figure 2D), suggesting stage- and tissue-specific regulation of endothelial cell Wt1 expression. Unlike the fetal epicardium,[10,11,31] neonatal epicardium did not continue to contribute to coronary smooth muscle cells (Figure 2E–2F). The large majority of endothelial cells of the adult heart do not express WT1,[4,32] and consistent with this result very few endothelial cells were labeled by adult induction of Wt1^{CreERT2} (Figure 2B).[4] In summary, the data indicate that Wt1-expressing cells at E10.5 do not substantially contribute to coronary endothelial cells. At late fetal and neonatal stages, the expression of Wt1 (and Wt1^{CreERT2}) within endothelial cells precludes using this tool at these stages to assess epicardial contribution to the endothelial lineage. Different tools or strategies are needed to address the origin of endothelial cells during this time window.[13]

One additional Cre allele useful for inducible recombination in the epicardium, Tcf21^{MerCreMer}, was recently reported.[12] Tcf21 is selectively expressed in epicardial cells and endocardial cushion mesenchyme. When induced at E10.5, this Tcf21^{MerCreMer} knock-in allele efficiently recombines the epicardium and in the atrioventricular valves. Cre-activated lineage tracer was detected in vascular smooth muscle and fibroblast lineages, but not in endothelial or cardiomyocyte lineages.[33] When induced at E14.5 or P5-7, little epicardial labeling was observed. Rather, label was predominantly found in cardiac fibroblasts but not in smooth muscle, endothelial, or cardiomyocyte lineages.[33] These data suggest that Tcf21 initially labels most epicardial cells, but later is confined to the cardiac fibroblast lineage. Thus this allele appears to be useful for lineage tracing of the epicardium before epithelial to mesenchymal transition (e.g. ≈E10.5 and before) and of a subset of cardiac fibroblasts at subsequent fetal and postnatal stages.

The Cre-loxP system is powerful but clearly has pitfalls. Detailed understanding of the technology and the properties of Cre alleles and Cre-activated reporters is essential to properly interpret Cre-based gene inactivation and lineage tracing studies. The Wt1^{GFPCre} allele has limitations, as we noted in the Jackson labs database and as pointed out by Rudat and Kispert.[16] Proper choice of Cre-activated reporters can reduce but not eliminate the problem, and as a result we no longer favor use of this allele for epicardial-specific gene inactivation or genetic fate-mapping experiments. We find that Wt1^{CreERT2} is a useful and specific tool for labeling epicardium and its derivatives. This allele can be induced by a single dose of Tam with moderately high efficiency, and has proven to be specific for epicardium and its derivatives when activated by Tam

between E9.5 and E11.5. Tam-treated embryos survive well to late gestation, but postnatal study of Tam-treated fetuses is complicated by problems in labor and delivery. Tam treatment in later gestation or in the neonatal period is complicated by recombination within coronary endothelial cells, precluding fate mapping into this cell population. As with most techniques, complementary approaches, such as selective cell labeling with dyes or with viruses, are needed to independently support lineage tracing results obtained with Wt1-based Cre tools. In some cases, resolution of controversies awaits further studies using complementary approaches that do not hinge on whether one categorizes Cre as expressed or not in a specific cellular compartment.

Sources of Funding

This work was supported by National Institutes of Health grants U01 HL100401 and R01 HL094683 (to W.T. Pu), and by the Chinese Ministry of Science and Technology grant 2012CB945102, CAS Hundred Talents Program No.874 and Innovation Program KSCX2-EW-R-09, and Shanghai PuJiang grant 11PJ1411400 (to B. Zhou).

Disclosures

None.

References

1. von Gise A, Pu WT. Endocardial and epicardial epithelial to mesenchymal transitions in heart development and disease. *Circ Res*. 2012;110:1628–1645.
2. Olivey HE, Svensson EC. Epicardial-myocardial signaling directing coronary vasculogenesis. *Circ Res*. 2010;106:818–832.
3. Pérez-Pomares JM, de la Pompa JL. Signaling during epicardium and coronary vessel development. *Circ Res*. 2011;109:1429–1442.
4. Zhou B, Honor LB, He H, et al. Adult mouse epicardium modulates myocardial injury by secreting paracrine factors. *J Clin Invest*. 2011;121:1894–1904.
5. Russell JL, Goetsch SC, Gaiano NR, Hill JA, Olson EN, Schneider JW. A dynamic notch injury response activates epicardium and contributes to fibrosis repair. *Circ Res*. 2011;108:51–59.
6. Smart N, Bollini S, Dubé KN, Vieira JM, Zhou B, Davidson S, Yellon D, Riegler J, Price AN, Lythgoe MF, Pu WT, Riley PR. De novo cardiomyocytes from within the activated adult heart after injury. *Nature*. 2011;474:640–644.
7. Zhou B, Honor LB, Ma Q, Oh JH, Lin RZ, Melero-Martin JM, von Gise A, Zhou P, Hu T, He L, Wu KH, Zhang H, Zhang Y, Pu WT. Thymosin beta 4 treatment after myocardial infarction does not reprogram epicardial cells into cardiomyocytes. *J Mol Cell Cardiol*. 2012;52:43–47.
8. Merki E, Zamora M, Raya A, Kawakami Y, Wang J, Zhang X, Burch J, Kubalak SW, Kaliman P, Izpisua Belmonte JC, Chien KR, Ruiz-Lozano P. Epicardial retinoid X receptor alpha is required for myocardial growth and coronary artery formation. *Proc Natl Acad Sci USA*. 2005;102:18455–18460.
9. Wessels A, van den Hoff MJ, Adamo RF, Phelps AL, Lockhart MM, Sauls K, Briggs LE, Norris RA, van Wijk B, Perez-Pomares JM, Dettman RW, Burch JB. Epicardially derived fibroblasts preferentially contribute to the parietal leaflets of the atrioventricular valves in the murine heart. *Dev Biol*. 2012;366:111–124.
10. Cai CL, Martin JC, Sun Y, Cui L, Wang L, Ouyang K, Yang L, Bu L, Liang X, Zhang X, Stallcup WB, Denton CP, McCulloch A, Chen J, Evans SM. A myocardial lineage derives from Tbx18 epicardial cells. *Nature*. 2008;454:104–108.
11. Zhou B, Ma Q, Rajagopal S, Wu SM, Domian I, Rivera-Feliciano J, Jiang D, von Gise A, Ikeda S, Chien KR, Pu WT. Epicardial progenitors contribute to the cardiomyocyte lineage in the developing heart. *Nature*. 2008;454:109–113.
12. Acharya A, Baek ST, Banfi S, Eskiocak B, Tallquist MD. Efficient inducible Cre-mediated recombination in Tcf21 cell lineages in the heart and kidney. *Genesis*. 2011;49:870–877.
13. Katz TC, Singh MK, Degenhardt K, Rivera-Feliciano J, Johnson RL, Epstein JA, Tabin CJ. Distinct compartments of the proepicardial organ give rise to coronary vascular endothelial cells. *Dev Cell*. 2012;22:639–650.
14. Wilm B, Ipenberg A, Hastie ND, Burch JB, Bader DM. The serosal mesothelium is a major source of smooth muscle cells of the gut vasculature. *Development*. 2005;132:5317–5328.
15. Christoffels VM, Grieskamp T, Norden J, Mommersteeg MT, Rudat C, Kispert A. Tbx18 and the fate of epicardial progenitors. *Nature*. 2009;458:E8–9; discussion E9.
16. Rudat C, Kispert A. Wt1 and epicardial fate mapping. *Circ Res*. 2012;111:165–169.
17. Mao X, Fujiwara Y, Orkin SH. Improved reporter strain for monitoring Cre recombinase-mediated DNA excisions in mice. *Proc Natl Acad Sci USA*. 1999;96:5037–5042.
18. Muzumdar MD, Tasic B, Miyamichi K, Li L, Luo L. A global double-fluorescent Cre reporter mouse. *Genesis*. 2007;45:593–605.
19. Tanaka M, Chen Z, Bartunkova S, Yamasaki N, Izumo S. The cardiac homeobox gene Csx/Nkx2.5 lies genetically upstream of multiple genes essential for heart development. *Development*. 1999;126:1269–1280.
20. Zhou B, von Gise A, Ma Q, Hu YW, Pu WT. Genetic fate mapping demonstrates contribution of epicardium-derived cells to the annulus fibrosis of the mammalian heart. *Dev Biol*. 2010;338:251–261.
21. von Gise A, Zhou B, Honor LB, Ma Q, Petryk A, Pu WT. WT1 regulates epicardial epithelial to mesenchymal transition through β-catenin and retinoic acid signaling pathways. *Dev Biol*. 2011;356:421–431.
22. Ma Q, Zhou B, Pu WT. Reassessment of Isl1 and Nkx2-5 cardiac fate maps using a Gata4-based reporter of Cre activity. *Dev Biol*. 2008;323:98–104.
23. Soriano P. Generalized lacZ expression with the ROSA26 Cre reporter strain. *Nat Genet*. 1999;21:70–71.
24. Martínez-Estrada OM, Lettice LA, Essafi A, Guadix JA, Slight J, Velecela V, Hall E, Reichmann J, Devenney PS, Hohenstein P, Hosen N, Hill RE, Muñoz-Chapuli R, Hastie ND. Wt1 is required for cardiovascular progenitor cell formation through transcriptional control of Snail and E-cadherin. *Nat Genet*. 2010;42:89–93.
25. Kreidberg JA, Natoli TA, McGinnis L, Donovan M, Biggers JD, Amstutz A. Coordinate action of Wt1 and a modifier gene supports embryonic survival in the oviduct. *Mol Reprod Dev*. 1999;52:366–375.
26. Asahina K, Zhou B, Pu WT, Tsukamoto H. Septum transversum-derived mesothelium gives rise to hepatic stellate cells and perivascular mesenchymal cells in developing mouse liver. *Hepatology*. 2011;53:983–995.
27. Baek ST, Tallquist MD. Nf1 limits epicardial derivative expansion by regulating epithelial to mesenchymal transition and proliferation. *Development*. 2012;139:2040–2049.
28. Manuylov NL, Zhou B, Ma Q, Fox SC, Pu WT, Tevosian SG. Conditional ablation of Gata4 and Fog2 genes in mice reveals their distinct roles in mammalian sexual differentiation. *Dev Biol*. 2011;353:229–241.
29. Zeng B, Ren XF, Cao F, Zhou XY, Zhang J. Developmental patterns and characteristics of epicardial cell markers Tbx18 and Wt1 in murine embryonic heart. *J Biomed Sci*. 2011;18:67.
30. Red-Horse K, Ueno H, Weissman IL, Krasnow MA. Coronary arteries form by developmental reprogramming of venous cells. *Nature*. 2010;464:549–553.
31. Dettman RW, Denetclaw W Jr, Ordahl CP, Bristow J. Common epicardial origin of coronary vascular smooth muscle, perivascular fibroblasts, and intermyocardial fibroblasts in the avian heart. *Dev Biol*. 1998;193:169–181.
32. Wagner KD, Wagner N, Bondke A, Nafz B, Flemming B, Theres H, Scholz H. The Wilms' tumor suppressor Wt1 is expressed in the coronary vasculature after myocardial infarction. *FASEB J*. 2002;16:1117–1119.
33. Acharya A, Baek ST, Huang G, Eskiocak B, Goetsch S, Sung CY, Banfi S, Sauer MF, Olsen GS, Duffield JS, Olson EN, Tallquist MD. The bHLH transcription factor Tcf21 is required for lineage-specific EMT of cardiac fibroblast progenitors. *Development*. 2012;139:2139–2149.