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EDITORIAL

A glimpse of Cre-mediated controversies in epicardial signalling

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This editorial refers to 'Epicardial function of canonical Wnt-, Hedgehog-, Fgfr1/2-, and Pdgfra-signalling' by C. Rudat *et al.*, pp. 411–421, this issue.

Among the many important aspects of cardiac development, formation of the epicardium and the underlining signalling pathways that direct the mobilization and differentiation of epicardial cells have been one of the highly pursued research subjects within the past decade. These epicardium-derived cells (EPDCs) are reported to contribute to several critical cell types which include cardiac myofibroblasts and coronary smooth muscle contributing to the formation of the coronary vasculature network within the developing embryonic heart.[1] Recent data suggesting that epicardial progenitor cells (EPCs) can contribute to newly formed cardiomyocytes in injured hearts[2,3] has drawn further attention into the mechanisms of epicardiogenesis, as the successful use of epicardial cells in cell replacement therapies will undoubtedly have a high clinical impact on treating heart disease. To achieve this goal, a better understanding of the molecular mechanism that drive epicardiogenesis will be required.

As the outermost cell layer of the vertebrate heart, the epicardium is derived from the proepicardial organ (PE), which originates from splanchnic mesenchyme within the septum transversum that is juxtaposed near the venous pole of the embryonic day 9.5 (E9.5) mouse heart (E22 for human). EPCs detach from the PE, migrate into pericardial cavity, and adhere to the myocardial surface where they rapidly spread to form a mesothelial monolayer covering the atria, atrioventricular canal, and ventricles, and in doing so, establish a primary epicardium by E10.5 in the mouse. Two days later, at E12.5, some of the primary epicardial cells undergo an epithelial to mesenchymal transition to convert into highly mobile and developmentally plastic EPDCs (*Figure 1*). These EPDCs proceed to invade the subepicardial matrix and subsequently migrate into the myocardium, where they differentiate into interstitial and perivascular fibroblasts, as well as coronary smooth muscle cells.[1,4]

Identification of the origin and nature of the cell autonomous and non-cell autonomous signals responsible for the EPDCs mobilization and differentiation was not possible before the availability of refined mouse genetic tools with the power to interrogate the functional roles of specific cell types during epicardial formation. Critical instructive signals can reside within the epicardial cells themselves, and/or from underlying cardiomyocyte, and/or endocardial cells. Cre/LoxP-based recombination technologies have allowed for both cell lineage tracing and conditional genetic gain-of-function and loss-of-function manipulation with far greater specificity than systemic transgenic approaches. Use of Cre/LoxP strategies has led to the discovery of a number of autocrine and paracrine signalling pathways important for epicardiogenesis and formation of EPDCs; however, the caveats of the Cre/LoxP recombination system have proved to be problematic in the interpretation of the generated data. The major challenge has always been the need to consider the spatiotemporal expression patterns of both the Cre driver line and the conditional alleles being investigated.[4,5] Rudat *et al.*[6] provide another provocative data set challenging our interpretations of Cre/LoxP genetic manipulations of epicardial cell biology.

Previous published findings show that canonical Wnt-,[7,8] hedgehog (HH)-,[9] fibroblast growth factor (FGF)-,[10] and platelet-derived growth factor (PDGF)[11]-mediated signalling are important in epicardial and EPDC development (*Figure 1*). A number of tissue-restricted Cre mouse lines were critical in determining the role for these signalling pathways during epicardiogenesis. These Cre drivers include $Gata5^{Cre}$,[12] $Tbx18^{Cre}$,[13] $Wt1^{GFP/Cre}$,[14] and the tamoxifen-inducible $Wt1^{CreERT2}$.[14] The importance of these signalling pathways in regulating various developmental processes and organogenesis is well established, thus there was little controversy with the idea that these near ubiquitously critical signalling pathways were also crucial in epicardial formation and EPDC differentiation. Rudat *et al.*[15,16] re-evaluated these pathways within the forming epicardium using the newly generated $Tbx18^{Cre}$ mice, and found unexpectedly that canonical Wnt-, HH-, and FGF-mediated signalling all appear to be dispensable to epicardial development.[6] These new data stand contradictory to earlier findings,[7–10,11] and may recalibrate our current understanding of the signalling mechanisms that regulate the mobilization and differentiation of EPDCs. For example, in previous studies, conditional inactivation of both FGF receptor 1 (*Fgfr1*) and FGF receptor 2 (*Fgfr2*) alleles using *Wt1Cre* resulted in fewer EPDCs within the myocardium,[10] suggesting a direct role of FGF signalling regulating EPDC cardiac myofibroblast invasion and differentiation within the embryonic myocardium. Within cardiomyocytes, *Fgfr1*- and *Fgfr2*-mediated signalling were suggested to trigger a wave

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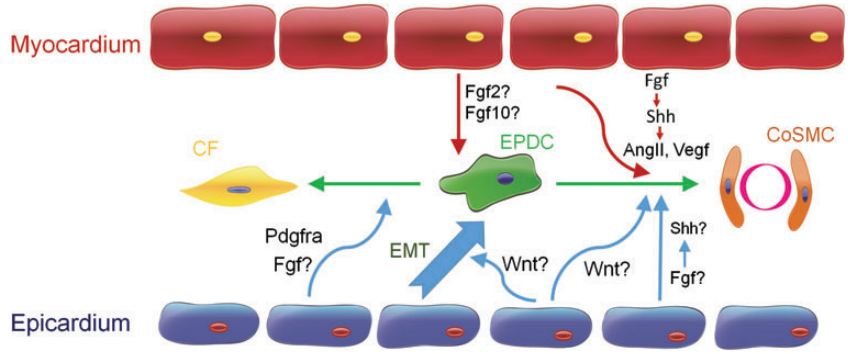

Figure 1 Schematic diagram of potential epicardial mediated signalling and development. Signalling pathways that regulate epicardium-derived cell (EPDC) fate, including EPDC differentiate into coronary vessel smooth muscle cells and cardiac fibroblast cells. CoSMC, coronary smooth muscle cells; CF, cardiac fibroblast cells.

of HH activation that was essential for coronary vessel development.[17] If Rudat's findings hold true, the influence of FGF on epicardial development may most likely be the result of a non-cell autonomous FGF and HH signalling centre residing within the underlying myocardium.

One of the obvious explanations for the differences between the finding of Rudat *et al.* and previous reports are that the different Cre-lines employed in these studies have varied characteristics in their spatiotemporal expression, which results in distinct phenotypic outcomes. The epicardial-restricted *Gata5*^{Cre} and *Wt1*$^{GFP/Cre}$ mice utilized in early studies cited above have been shown to display ectopic or leaky Cre expression in the myocardium as well as other cell lineages such as endocardial/endothelial and myocardial cells of the developing heart.[12,14] The tamoxifen-inducible *Wt1*^{CreERT2} [14] and the newly reported *Tbx18*^{Cre} [15,16] epicardial Cre mouse lines are refined epicardially restricted lines; however, Rudat *et al.* demonstrate clear differences between phenotypes derived from utilizing these two Cre drivers. A probable explanation for these discrepancies lies within the consideration of the Cre/LoxP recombination efficiency between these Cre drivers and/or the differences in genetic background and of course the influence and temporal issues with tamoxifen induction. The new *Tbx18*^{Cre} mouse line mediates clean epicardial-restricted recombination at the right ventricle side of the heart; however, on the left ventricle side, a subset of cardiomyocytes in the intraventricular septum are also Cre positive, which is consistent with a previous report.[5] Thus, Rudat's analysis focused only on the epicardial structures on the right ventricular side of the heart, which minimized the contribution of cardiomyocytes in their data sets.

Another possible explanation for the data disparity is subtle (or not so subtle) differences in the spatiotemporal expression patterns, i.e. the timing and extent of genetic deletion within the early EPCs. A good place to look for such important expression differences would be the undifferentiated mesodermal cells at the venous pole prior to the formation of PE.[18] The current collection of Cre drivers (*Gata5*, *Wt1*, and *Tbx18*) are all expressed within the mesoderm of the venous pole. Therefore, it is possible that early difference in Cre-mediated recombination within these undifferentiated mesodermal cells prior to the PE formation could have a great influence on phenotypic outcomes while revealing little difference in lineage-mapping analysis. In support this idea, both*Tbx18* and *Wt1* have been shown to express within a subset of PE cells that are distinct from PE cells that express two epicardial markers, *Scleraxis* (Scx) and *Semaphorin3D* (Sema3D),[19] suggesting a diversified heterogeneous population EPCs within PE that may not be completely encompassed by the expression of any of the existing Cre line. To move forward, additional experiments will be required to sort out whether cells within the PE are a homogenous population of EPCs or a diverse heterogeneous collection of cells that exhibit variations in Cre activity between the *Wt1*^{CreERT2} and *Tbx18*^{Cre} mouse lines.

Although the use of mouse Cre/loxP-based genetic manipulation has proved to be a powerful tool for dissecting various signalling pathways governing the programmes that orchestrate embryonic organ developmental, the work by Rudat *et al.* remind us once again the generation of data is the easy part of the scientific process, and that the interpretations of Cre-generated data are subjected to the pitfall and caveats of what we do not know regarding the reagents we all employ, thus we will undoubtedly be re-visiting the evaluation of the signalling pathways that govern formation of the epicardium, EPDCs, and their derivative cell types for some time to come.

Conflict of interest: none declared.

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