The myocardial potential of proepicardial cells: From development to cardiac regeneration
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Epicardium and Myocardium Originate from a Common Cardiogenic Precursor Pool

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Abstract

During development, the epicardium, an epithelial layer that covers the heart, gives rise to a large portion of the non-myocardial cells present in the heart. The epicardium arises from a structure, called the proepicardium, which forms at the inflow of the developing heart. By epithelial to mesenchymal transformation mesenchymal cells are formed that will subsequently populate the stroma of the proepicardium and the subepicardium. Based on labeling analysis the proepicardium and part of the myocardium have been shown to be derived from a common cardiogenic precursor population. In this review we will discuss the common cardiogenic origin of proepicardial and myocardial cells, the underlying processes and factors that play a role in the separation of the lineages, and their potential role in cardiac regenerative approaches.
Introduction

Given the limited regenerative capacity of the adult heart, cardiac damage frequently progresses to congestive heart failure. Attempts to stimulate cardiac repair, have seen large efforts being made to identify and isolate cardiac progenitors. Several lines of evidence suggest that the epicardium, the non-myocardial epithelium covering the myocardium, could conceivably play a role in cardiac regeneration. In zebrafish, reactivation of the epicardium plays a critical role in the regeneration of injured myocardium. Although the mammalian heart possesses little regenerative capacity in contrast to the heart of zebrafish, its epicardium has also been suggested to be involved in myocardial regeneration.

During development, the epicardium arises from a cauliflower-like structure called the proepicardium. Around the third day of development (Hamburger Hamilton stage 16) in chicken, 9.5 days of development in mouse, and approximately 3.5 weeks (Carnegie stage 10) of human development, the proepicardium arises form splanchnic mesoderm which covers the vitelline veins at the inflow of the developing heart. Until this stage the heart lies “naked” within the pericardial cavity. The proepicardium protrudes into the pericardial cavity and contacts the myocardium of the atrioventricular canal. Subsequently, epicardial cells migrate over the heart tube to cover the myocardium. Within the proepicardium and later also in the epicardium that covers the heart tube, a subset of the epithelial cells undergoes epithelial-to-mesenchymal transformation (EMT). These mesenchymal cells provide a large portion of the non-myocardial cells of the heart, for instance cardiac fibroblasts, coronary smooth muscle cells and coronary endothelial cells. Although still an issue of debate, recent genetic labelling studies suggest that cells derived from the epicardium might contribute to the myocardial lineage, as well.

The wide variety of cells the proepicardium contributes to the heart coupled with the fact that the (pro-)epicardium and myocardium share a common cardiogenic precursor makes epicardial cells a potential source of cardiac progenitor cells and as such may serve a potential source of cells for regenerative approaches. In this review we will discuss the common origin of epicardial and myocardial cells, the processes and factors that play a role in the separation of myocardial and non-myocardial lineages, and the potential role of epicardial-derived cells as progenitor cells.

Common origin of epicardial and myocardial cells

Classic lineage tracing analyses

The heart is formed from cells of the splanchnic mesoderm. The bilateral zones of the splanchnic mesoderm, which will derive the heart, are referred to as the heart forming regions. Within these regions cells have been subdivided into two developmental fields, the first and second heart fields. This distinction should be considered a spatio-temporal separation of cells within a greater heart forming region. The lateral cells of this region, the first heart
field, are the first to exit the cell-cycle, subsequently differentiating into myocardium and giving rise to the primary heart tube. The cells located medially and caudally within the heart forming region are referred to as the second heart field (Figure 1). These cells continue to proliferate in an undifferentiated state after the formation of the linear heart tube, and are gradually added to both the inflow and outflow regions of the heart\textsuperscript{18} as it loops. At the inflow of the heart, these progenitors contribute both myocardial and non-myocardial cells. The non-myocardial component of the heart is largely derived from cells of the proepicardium which develops at the inflow of the heart. In the cardiac progenitor pool, present upstream of the heart tube before the induction of the proepicardium, a lineage decision takes place defining cells which will differentiate into cardiomyocytes and be added to the venous pole of the heart or cells which will differentiate into the proepicardial lineage.\textsuperscript{14}

An in depth study of the various cell types contributing to the inflow of the heart and the proepicardium has long been lacking. Classic lineage analyses, using vital dyes, radioactive deposits, or retroviruses, were hampered because the venous pole of the heart is difficult to approach in the living embryo owing to technical limitations. Only recently, have we been able to demonstrate the contribution of the second heart field to both the inflow
myocardium and the proepicardium, using a vital dye labelling. In this analysis a small group of splanchnic mesodermal cells, expressing the T-box transcription factor 18 (Tbx18) at the inflow region of the heart, was labelled one day before the initiation of proepicardium formation (stage 11). At the time of labelling, these cells were neither positive for myocardial markers, like cardiac Troponin I or Myosin Heavy Chain isoforms, nor showed morphological characteristics of proepicardium formation. Twenty-four hours later, the label was detected both in myocardial cells of the inflow tract and in proepicardial cells. Since, in this experiment, a small group of cells was labelled rather than a single cell, it cannot be excluded that the labelled population comprises a mixed population instead of bipotential progenitor cells. However, this would seem unlikely, as gene expression analysis of the mesoderm at the stage of labelling, i.e. prior to formation of the proepicardium, displays no heterogeneity in gene expression. Earlier cell tracing studies in which (1) quail proepicardia were transplanted into chicken embryos, (2) adenovirus-labelled chicken proepicardia were transplanted into chicken acceptors, (3) pro-epicardia were retrovirus-labelled in ovo were unable to demonstrate proepicardial contribution to the myocardium. Although cell tracing analysis revealed no contribution of proepicardial cells to the myocardium, proepicardial cells have been shown to differentiate into heart muscle cells in vitro. The two most important differences between the recent labelling experiments and the earlier analyses are the developmental stage at which the cells were labelled, and the techniques used to label the pro-epicardia. In the recent experiment, the mesoderm upstream of the forming heart tube was labelled prior to the induction and formation of the proepicardium at stage 11, whereas in the earlier experiments the proepicardium was labelled later, at stage 16-17. Second, in the studies in which proepicardia were transplanted, the donor tissue was not in direct contact with the inflow of the recipient embryo. Consequently, the physical barrier, i.e. the pericardial cavity, prevented the transplanted cells from contributing to the inflow myocardium.

The expression of Tbx18 first becomes evident in the splanchnic layer immediately upstream of the heart tube at stage 11. One day later during development, Tbx18 expression can be observed in distinct regions at the inflow of the heart, being (1) myocardial cells just cranial to the base of the proepicardium, (2) sinus horn myocardium, i.e. the myocardial sleeves covering the cardinal veins, and (3) proepicardial cells. Histological analysis of the proepicardium has shown that the proepicardial villi are sheathed in epithelial cells, and filled with mesenchymal cells and extracellular matrix. Because of the presence of genes involved in EMT, like Wt1 and Snail, in the proepicardium it is likely that during proepicardium formation epithelial cells transform into mesenchymal cells which invade

Figure 1. Initial heart formation (previous page)
A, B and C spatially illustrates early heart formation in chicken, from the ventral side. A The heart forming regions (HFR) are indicated in grey (primary heart field, Nkx2.5 lineage) and blue (second heart field, Isl1 lineage). The dotted red line indicates the area of the HFR that will express Wilms’ tumor 1(Wt1) and the transcription factor Tbx18. B The bilateral HFR adjoin in the midline by folding of the embryonic disc (hatched arrows). The red line indicates the area of the HFR that expresses Wt1 and Tbx18. C Progressive folding lengthens the heart tube in caudal direction, forms the foregut, and establishes the pericardial back wall.
the proepicardial matrix. Within the proepicardium, several genes have been reported to be heterogeneously expressed, e.g. P-Erk\textsuperscript{14}, FGF2\textsuperscript{22}, Flk\textsubscript{19}, PAR3 and ZO1,\textsuperscript{27} indicating that the proepicardium is a heterogeneous population of cells.

Taken together, a homogeneous, Tbx18-expressing mesodermal cell population upstream of the inflow of the heart, that covers the vitelline veins at stage 11, gives rise to proepicardial and inflow myocardial cells. Within the proepicardium epithelial cells transform into mesenchymal cells that will subsequently invade the proepicardial matrix.

**Genetic lineage analyses**

The development of a two-component mouse system, in which one mouse harbors a conditional reporter gene, like lacZ or GFP, and the other mouse expresses Cre (Causes recombination), has permitted genetic lineage tracing analyses to be carried out.\textsuperscript{28} The last couple of years have provided a variety of different Cre-lines that have been used to analyse the development of the inflow of the heart.

The Nkx2.5\textsubscript{IRES}-Cre, in which an IRES-Cre cassette is inserted into the 3'UTR of the Nkx2.5 gene,\textsuperscript{29} showed robust reporter gene expression in all myocardial cells and the proepicardium, but not in sinus horn myocardium.\textsuperscript{15, 24, 29} Similar results were obtained using an Nkx2.5\textsubscript{Cre} line\textsuperscript{15} or an Isl1\textsubscript{Cre} line,\textsuperscript{30, 31} in which Cre is knocked-in at the translation start sites. In situ hybridization and immunohistochemical analyses of the inflow tract have shown that, neither the proepicardium nor the sinus horn myocardium express Nkx2.5 or Isl1.\textsuperscript{15, 24, 31} These findings reveal that cells of the proepicardium and sinus horn myocardium are derived from progenitors that have at some point expressed Nkx2.5 and Isl1 during development, though not necessarily at the same time. This suggest that both proepicardium and sinus horn progenitors are derived from the second heart field, but turn off Isl1 earlier, and do not activate nkx2.5 like other second heart field progenitors. However, the relation between the pro-epicardial and sinus horn progenitors is currently unclear. Indeed, sinus horn myocardium is formed subsequent to the formation of the proepicardium, i.e. from E9.5 onwards, and, in contrast to epicardium and all other myocardium is unrecombined in Nkx2.5\textsubscript{IRES}-Cre;R26R embryos, suggesting a much briefer period of Nkx2.5 expression in these progenitors compared to the pro-epicardial progenitors.\textsuperscript{24} Consequently, the myocardium located directly cranial to the proepicardium is different form sinus horn myocardium. In the neonatal heart, the myocardium directly cranial of the proepicardium finds its destiny in the myocardium of the sinus node and right venous valve.\textsuperscript{32}

Since the insertion of Cre in the Nkx2.5\textsubscript{Cre} and Isl1\textsubscript{Cre} lines disrupts expression of functional Nkx2.5 and Isl1 protein, respectively, their role in proepicardial development could be tested. Homozygous disruption of Nkx2.5 or Isl1 results in growth retardation and cardiac malformation. In Nkx2.5\textsubscript{Cre}/\textsubscript{Cre} mice, the proepicardium fails to develop, whereas in Isl1\textsubscript{Cre}/\textsubscript{Cre} the proepicardium remains under-developed.\textsuperscript{15} These findings suggest that Nkx2.5, although not expressed in the proepicardium, is required for its formation, this not being the case for Isl1. Isl1 seems to be required for proper expansion of the cardiac progenitor pool.\textsuperscript{33}
and consequently for providing sufficient progenitors for pro-epicardial development.

Analysis of Tbx18Cre mice, revealed recombination in the forming epicardium and adult heart. Wilms' tumor 1 (Wt1)-Cre, another Cre-line of importance in (pro)epicardial development, demonstrated a similar pattern of recombination, i.e. in the epicardium and in cells of the adult heart (details of both Cre-lines will be discussed later in this review). The contribution of the Tbx18Cre and Wt1Cre lineage to the inflow myocardium has not been addressed in these studies. Wt1-expressing proepicardial cells were shown to be derived from cells that were found to belong to both the Nkx2-5 and Isl1 lineages, even though Wt1 protein was never found to be co-expressed with Isl1 nor Nkx2-5 proteins.

Taken together, labeling studies of Tbx18-expressing progenitors, together with lineage analyses using Isl1- and Nkx2-5-Cre lines, have identified a common origin of myocardial and (pro)epicardial cells.

Regulation of the separation of myocardial and non-myocardial cells
The earliest markers that identify the myocardial and pro-epicardial progenitor population are Tbx18 and Wt1. Expression of Tbx18 and Wt1 starts just before E8.0 in mouse and stage 11 in chicken. Both in mouse (E9.5) and chicken (stage 16) the proepicardium starts to become morphologically evident, as villous structures protruding into the pericardial cavity. Proepicardial villi appear on both the left and right vitelline veins. At the right side the villi expand and form the proepicardium, whereas at the left side, in chicken, the villi undergo apoptosis and disappear. The pathways regulating this left-right asymmetry are beyond the scope of this review and have been discussed elsewhere. By contrast, in mouse, the villi expand and form a proepicardium that is located medially.

The factors that induce expression of Tbx18 and Wt1 are presently unknown. However, xenotransplantation of stage 17 quail liver buds to a caudal location in chicken of various developmental stages, showed induction of Tbx18 and/or Wt1 expression in the adjacent mesoderm from stage 12 onward, indicating that secreted factors derived from the hepatic endoderm induce the expression of Tbx18 and Wt1. Similarly transplanting lung buds, as a control, did not have this effect, hinting that the forming hepatic endoderm is the probable source of factors that induce expression of Tbx18 and Wt1 in the splanchnic mesoderm. Since lung buds produce Bmp and Fgf, these growth factors are unlikely candidates to induce the expression of Tbx18 and Wt1. Although Tbx18 marks the progenitor population of proepicardial and inflow myocardial cells, its expression is not essential for the formation of the proepicardium, because functional disruption of Tbx18 does not result in aberrant proepicardial development. Wt1's involvement in EMT suggests that EMT plays an important role in the formation of proepicardial cells. Besides EMT, the regulation of cell polarity also seems to be involved in proepicardium formation, as deletion of the mammalian homolog of the Caenorhabditis elegans polarity protein, Par3, results in defective (pro)epicardial development.
The factors that regulate the division of the progenitor population into myocardium on the one hand and proepicardium on the other are largely unknown. Although Bmp and Fgf do not induce the expression of Tbx18 or WT1 in this progenitor population, they do play a role in the separation of myocardial and non-myocardial cells from this population. Exposing embryos to Bmp2, Fgf2 and/or the Mek1 inhibitor U0126 has shown that Bmp2 and Fgf2 signaling pathways cooperate in the separation and differentiation of the Tbx18-positive progenitor pool into cardiomyocytes and pro-epicardial cells. Smad-mediated Bmp-signaling directs the progenitors into the myocardial lineage. Erk-mediated Fgf2-signalling directs, on the one hand the progenitors into the pro-epicardial lineage, and on the other blunts the differentiation of the progenitors into the myocardial lineage. The inhibition of myocardial differentiation of the progenitors by Fgf2 was found to be the result of disrupting Smad-mediated Bmp-signaling by phosphorylating receptor-Smad, such that it cannot interact with common-Smad4 and thus accumulates in the cytoplasm. As a consequence the initiated Bmp signal is not conveyed to the nucleus, thereby disrupting the Bmp response (Figure 2).
Taken together, these data indicate that (an) unknown signal(s) secreted from the hepatic endoderm induces Tbx18 and Wt1 expression in the pre-cardiac progenitor population. Separation of this progenitor population into the myocardial and epicardial lineage is regulated by the cooperative interaction between Bmp and Fgf-signalling.\textsuperscript{14, 21, 36}

**Proepicardial contribution to the heart**

Xeno-transplantation studies using the quail–chick chimera technique as well as cell fate analyses using retroviral and adenoviral cell labelling have revealed that the proepicardium contributes cells to the epicardium, subepicardial mesenchyme, coronaries, cardiac fibroblasts, and to the mesenchyme of the atrioventricular valves.\textsuperscript{11-13, 38} Genetic Tbx18Cre and Wt1Cre lineage analyses confirmed the contribution of pro-epicardial cells to these populations.\textsuperscript{9, 10} In the Tbx18Cre lineage analyses, reporter expression was visible in a subset of the vascular smooth muscle cells and cardiac fibroblasts, but not in the coronary endothelium. Unexpectedly, recombination also occurred in cardiomyocytes, suggesting that they too, are derived from epicardium.\textsuperscript{9} However, the interpretation based on the Tbx18Cre lineage study was called into question by the demonstration that cardiomyocytes themselves express Tbx18, and that recombination in myocytes also occurred in mouse models in which epicardium formation was compromised.\textsuperscript{8}

From the Wt1Cre lineage analysis\textsuperscript{10} it was observed that epicardium and subepicardial mesenchyme were positive for the reporter, as well as most of the smooth muscle cells and a minority of the endothelial cells of the coronaries. This analysis also showed patches of ßGal–expressing cardiomyocytes located throughout the myocardium, which comprised approximately 10% of the total amount of cardiomyocytes. This finding was unexpected as classic labelling analysis using chicken quail chimeras had never shown an epicardial contribution to the myocardium\textsuperscript{11} and Wt1 expression has not been reported in cardiomyocytes. The epicardial origin of progenitors contributing to the myocardium was further substantiated using a tamoxifen inducible Cre (CreERT2). This Cre-enzyme becomes biologically active in the presence of tamoxifen. Upon activation of the Cre-enzyme after formation of the embryonic epicardium at E10.5 and E11.5, a very small number of reporter-positive cells could be found, compared to the analysis using the Wt1Cre line. Nevertheless, the reporter-positive cells were found in smooth muscle, endothelial and myocardial cells, pointing to an epicardial origin of these cells. Alternatively, since recombination by Cre is considered very efficient, it may point to a low level Wt1 expression, which is not reported, as yet, but sufficient to induce Cre-mediated recombination.

The observed limited contribution of epicardially-derived cells to the non-myocardial component in the formed heart, as assessed in genetic lineage analyses compared to classic labeling analyses, is puzzling. In classic lineage analyses, all cells contributing to the coronaries, including endothelium, smooth muscle cells and pericytes, as well as the majority, if not all, intermyocardial fibroblasts, and a small population of the mesenchyme in the atrioventricular valve leaflets were found to be of pro-epicardial origin.
The reason for the discrepancies between the classic and genetic lineage analyses might be related to; 1) the invasive nature of quail pro-epicardial cells used in the classic analysis 2) the onset of the genetic lineage tracing, showing the developmental history from the start of gestation onwards and 3) the shorter developmental window in which transplanted quail cells can be traced. Further studies will be required to properly analyze the epicardial-derived contribution to the (non-)myocardial component of the heart. A further question that needs addressing is whether all cells of the proepicardium are able to contribute to the different lineages at later stages or that within the proepicardium a subset of cells is already committed to a specific-lineage.

**The epicardium as a progenitor population**

The capacity of epicardial cells to contribute to a variety of cells in the adult heart, including myocardial cells, identifies the epicardial cells as a potentially interesting cell type in the search for progenitor cells. Epicardium-Derived Progenitor Cells (EDPCs), the mesenchymal cells derived from the epicardium by EMT, are suggested to function as cardiac stem cells.\(^7\) Another indication for the presence of epicardially-derived cardiac progenitor cells is the

![Figure 3. Model of addition of progenitor cells to the developing heart](image)

**A** Undifferentiated cardiac progenitors (blue) proliferate outside the heart, in the pericardial back wall and are formed by EMT. Progenitors are added to the inflow and outflow poles of the heart (black arrows) and differentiate into cardiomyocytes when passing a zone of high levels of Bmp2 (red). The proepicardium (green) develops just caudally of the zone of the highest level of Bmp2. **B** In Situ hybridization showing the pattern of expression of Bmp2, cranially of the proepicardium of a chicken embryo stage 16. **C** By-passing the zone of the highest level of Bmp2, via the formation of the proepicardium, enables the addition of cardiac progenitors (blue) to the developing heart.
capacity of mesoangioblasts, present in the adult heart to enter the cardiac lineage.\textsuperscript{39} Recently, it has been shown that during development Wt1 is required for cardiovascular progenitor cell formation through transcriptional control of Snail and E-cadherin in the epicardium.\textsuperscript{40}

The presence of progenitor cells in the heart, originating from the epicardium might indicate that these progenitors are added to the heart via the proepicardium during development. During early development, progenitors are added to the heart at both the inflow and outflow poles of the heart. These undifferentiated cardiac progenitors proliferate outside the heart,\textsuperscript{18} and are formed by the process of EMT.\textsuperscript{40} EMT has already been observed in the proepicardium.\textsuperscript{26} Undifferentiated cardiac progenitors differentiate into myocardial cells when added, via the inflow- or outflow pole, to the heart. Bmp signalling plays an important role in the addition of the undifferentiated cardiac progenitor to both poles of the heart.\textsuperscript{33} Cardiac progenitor cells proliferate in the presence of low levels of Bmp2 but differentiate into myocardial cells when passing a zone of higher concentrations of Bmp2 present at both the inflow and outflow poles of the heart.\textsuperscript{33, 41} The proepicardium develops just caudally of the zone of the highest levels of Bmp2 located at the inflow pole of the heart.\textsuperscript{14, 22} A subset of these progenitors could be added to the heart via the proepicardium, bypassing this zone and thus retaining their undifferentiated precardiac precursor state (Figure 3). In line with this, it has been shown in vitro that in the presence of Bmp2 and Fgf2 the progenitors of proepicardia remain in an undifferentiated state.\textsuperscript{14, 22} The cooperative action of Bmp and Fgf may serve to keep these progenitors, also at later stages, in an undifferentiated and proliferative state. This idea is underscored by the presence of cKit-positive progenitors\textsuperscript{3} and activated Bmp and Fgf signalling pathways in the subepicardial mesenchyme.\textsuperscript{14}

**Future questions**

The possibility that cardiac progenitor cells are added to the heart via the proepicardium raises several intriguing questions that need to be addressed; (1) Is the interaction of Bmp and Fgf signalling, that regulates the lineage separation of myocardial and epicardial cells, during the development of the proepicardium, still operational in epicardially-derived progenitors? (2) Do adult epicardial cells retain the capacity to form progenitor cells that can differentiate into myocardial cells? (3) If progenitors are added via the proepicardium, where are these progenitors “stored” in the formed heart?

The common origin of myocardial and epicardial cells, and the signals that regulate the differentiation of progenitors into myocardial and non-myocardial cells, or that regulate the maintenance of a progenitor state in proepicardial-derived cells provide challenging inroads in the analysis of the existence, differentiation and use of these potential progenitor cells.
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