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Review

Epicardial Lineages

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Abstract: The epicardium is the mono-layered epithelium that covers the outer surface of the myocardium from early in cardiac development. Long thought to act merely passively to protect the myocardium from frictional forces in the pericardial cavity during the enduring contraction and expansion cycles of the heart, it is now considered to be a crucial source of cells and signals that direct myocardial growth and formation of the coronary vasculature during development and regeneration. Lineage tracing efforts in the chick, the mouse and the zebrafish unambiguously identified fibroblasts in interstitial and perivascular locations as well as coronary smooth muscle cells as the two major lineages that derive from epithelial-mesenchymal transition and subsequent differentiation from individual epicardial cells. However, controversies exist about an additional endothelial and myocardial fate of epicardial progenitor cells. Here, we review epicardial fate mapping efforts in three vertebrate model systems, describe their conceptual differences and discuss their methodological limitations to reach a consensus of the potential of (pro-)epicardial cells *in vitro* and *in vivo*.

Keywords: epicardium; epicardial lineages; EPDCs; fibroblasts; smooth muscle cells

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1. The Epicardium—A Cardiac Tissue of Extracardiac Origin

The vertebrate heart with its chamber organization and three-layered tissue design is the result of a complex morphogenetic program that progressively transforms a simple organ rudiment into a large muscular pump. Endocardium and myocardium, *i.e.* the inner and the muscular layer of the heart, are present in the simple peristaltically active tube that is established shortly after gastrulation to support the need of the rapidly growing embryo. The epicardium, the outer tissue layer, is only added once this simple tube has undergone a complicated looping process and established the chambers.

The myocardium and endocardium arise from fusion of two bilateral fields of cardiac precursor cells in the anterior lateral plate mesoderm (the first heart field), and subsequent recruitment and delayed myocardial differentiation of pharyngeal mesoderm (the second heart field) [1]. The epicardium, in contrast, derives from an outgrowth of the coelomic epithelium that is established posterior to the growing heart tube from a subpool of the anterior lateral plate mesoderm [2,3] at embryonic day (E) 8.5 in the mouse [4,5], at Hamburger and Hamilton stage (HH) 14 in the chick, and 48 h post fertilization (hpf) in the zebrafish [3]. This mesothelial outgrowth, which gains a cauliflower-like shape, was coined proepicardial organ, or more frequently, proepicardium. The proepicardium is an unpaired midline structure, but it forms bilaterally in close proximity to the two wings of the sinus venosus myocardium. The left proepicardial anlage regresses during further development in the chick, whereas both proepicardial anlagen merge in the midline of the mouse embryo [6].

While the epicardium is thought to arise exclusively from the proepicardium, the proepicardium most likely does not solely contribute to the epicardium but seems to be a mixed population of epicardial precursors and endothelial cells [7–10]. The close spatial arrangement of different precursor tissues including that of the liver, the septum transversum, and the myocardium next to the proepicardium makes the analysis of the lineage relationships at the posterior heart pole even more complicated. This, and the lack of a unique genetic label for a proepicardial character of cells may at least partly explain the discrepancies between the different lineage tracing approaches performed in the different vertebrate models. Nonetheless, the proepicardium, is a transient embryonic tissue. Proepicardial cells are transferred to the myocardium via an extracellular matrix bridge in avian embryos [11] and by finger-like protrusions contacting the heart as well as proepicardial vesicles in the murine and zebrafish heart [5]. Once proepicardial cells attach to the myocardial surface of the looped heart, they proliferate and migrate to ensheath the heart down to the base of the outflow tract, at E10.5 in murine heart development (HH25 in the chick [12]). From this point in development, the epicardium supports myocardial growth by a cocktail of paracrine factors (for review see Olivey and Svensson 2010 [13]).

Shortly later at E11.5 to E12.5 (HH20 in the chick), the first cells start to leave the continuity of the mono-layered epithelial epicardium. This process progresses from the atrio-ventricular groove to the apex of the ventricles in parallel with the outgrowth of the coronary plexus. At around E15.5 (HH27 in the chick [14]) epicardial cells are no longer mobilized under developmental conditions, and are likely to differentiate terminally. The lineages to which epicardium-derived cells (EPDCs) [15] give rise have been analyzed in the development of chick, mouse and zebrafish embryos using a number of methods with a consensus on the main fates but with diverging views of additional minor fates.

2. Proepicardial Lineage Tracing in Chick Development—Fibroblasts, Coronary Smooth Muscle Cells and Possibly Endothelial Cells

The earliest study that analyzed the fate of proepicardial cells was performed by Mikawa and Gourdie in 1996. They used retroviral gene transfer to transduce a *lacZ* reporter gene into proepicardial cells both *in ovo* as well as *in vitro* with subsequent transplantation of a proepicardial cell cluster into isochronic recipients in the latter case. Using ß-galactosidase histochemistry on sections of E18 hearts they identified coronary smooth muscle cells and coronary endothelial cells as well as perivascular connective tissue as being proepicardium-derived, demonstrating for the first time that cells of the coronary vasculature (at least partly) derive from the proepicardium. Intriguingly, immunoconfocal microscopy identified both endothelial and smooth muscle cell marker gene expression within the proepicardium, suggesting that these lineages are already present within the proepicardium and that they migrate along with future epicardial cells onto the myocardium [16].

While the presence of endothelial cells in or at the proepicardium is likely (see also below), an immigration of differentiated smooth muscle cells from the proepicardium into the myocardium has not been confirmed. However, Perez-Pomares and co-workers identified delaminating cells between the epicardial and myocardial tissue layers [14], which are mesenchymal but lack differentiation markers of the smooth muscle and endothelial cell lineages [7,17]. Further, proepicardial explant cultures that were induced by angiogenic growth factors like fibroblast growth factor 2 (FGF2) and vascular endothelial growth factor (VEGF) become mesenchymal and invade collagen matrices [17]. Collectively, these results suggested that individual epicardial cells undergo an epithelial-to-mesenchymal conversion, and only subsequently differentiate further.

Fate mapping of quail epicardial cells (HH18) transduced with ß-galactosidase encoding adenoviruses after transplantation into HH15 chick recipients revealed the contribution of these epicardium-derived mesenchymal precursor cells to coronary smooth muscle cells, perivascular and intermyocardial fibroblasts, mesenchymal cells of the atrio-ventricular cushions and some ventricular endocardial cells [17]; similar results were obtained by quail to chick transplantation of proepicardia [15,18]. However, studies of these quail-chick chimeras additionally identified endothelial cells of the coronary vessels as proepicardium-derived [7,19]. An endothelial fate of epicardial cells is controversial as Poelmann and colleagues has provided evidence that endothelial cells found in proepicardial grafts are liver-derived [12] and Dettman and colleagues have shown that epicardial grafts do not contribute to the endothelial cell lineage of the avian heart [17]. Furthermore, immunohistochemical analysis of avian proepicardia and proepicardial explants indicate that endothelial cells are present within the proepicardium of quail and chick embryos [16] and their abundance varies with the age of the proepicardial graft [20]. In the end, we would like to point out the study by Männer in 1999 who investigated the contribution of epicardial cells to the myocardial lineage of the heart. He did not detect any quail-derived myocardial cells in quail-chick chimeras [18], which is in line with missing reports of epicardium-derived cardiomyocytes in avian embryos.

In conclusion, (pro-)epicardial fate mapping has revealed that embryonic epicardial cells are a multipotent precursor population. While most of them contribute to the mature epicardium, some become mesenchymal and differentiate into vascular lineages (coronary smooth muscle cells, perivascular fibroblasts), mesenchymal cells of the atrio-ventricular cushion tissue (mitral and

triscupid valves), interstitial fibroblasts and in a minor fraction to endocardial and subendocardial cells in the chick embryo (see Table 1 for a summary). Endothelial cells of unknown origin exist in the proepicardium and may contribute to the coronary vasculature by co-migration with epicardial precursor cells. A differentiation of (pro-)epicardial cells into the endothelial cell lineage seems unlikely.

3. Epicardial Lineage Tracing in Mouse Development—An Epicardial Origin of Myocardial Cells?

As intrauterine development largely precludes or at least hinders the direct manipulation and labeling of the (pro-)epicardium in the mouse, lineage tracing of these cells has only become possible with the advent of suitable genetic labeling systems. Cre is a phage-encoded recombinase that specifically recognizes short *loxP* called DNA stretches to delete (if the two *loxP* sites are oriented in the same direction) or flip (if the *loxP* sites are reversed) the intervening sequences. If *loxP* sites are suitably placed around a reporter gene (such as *GFP* or *lacZ*), which has been introduced in a ubiquitously expressed locus such as *Rosa26*, a single cre mediated recombination event can irreversibly activate the reporter (e.g., by removing a *loxP* flanked stop cassette in front of the reporter) in all descendants of a founder cell. Hence, if *cre* is expressed under the control of an (pro-)epicardial-specific promoter, the fate of these cells can be analyzed at any time-point of development by a simple co-expression analysis of the reporter and the differentiation marker. Therefore, epicardial fate mapping in the mouse (and other species that allow efficient genetic manipulation such as the zebrafish) boils down to having a sensitive reporter line and a specific *cre* driver line at hand [21,22]. While the first task was quickly achieved, finding a suitable *cre* line turned out to be much more of a problem.

The first report on such a genetic fate mapping approach in the mouse came from Wilm and co-workers in 2005 who reported descendents of epicardial cells in the coronary vasculature (almost all smooth muscle cells, and a low degree of endothelial cells) based on a *cre* knock-in in a 280 kbp YAC of the *Wilms tumor 1* (*Wt1)* locus [23]. *Wt1* is a gene that is strongly expressed in the proepicardium and in cells of the coelomic lining including the epicardium [24]. Furthermore, a regulatory fragment of the chick *Gata5* locus mediated cre expression in the mouse epicardium and identified cardiac fibroblasts and smooth muscle cells as epicardial descendants again confirming the data obtained in the chick [25].

Two additional independent lineage tracings by *cre/loxP* technology further confirmed fibroblasts and smooth muscle cells as epicardial fates and suggested, surprisingly, that epicardial cells albeit with different patterns also differentiate into cardiomyocytes in the mouse. The study by Cai and co-workers used a *cre* knock-in allele of *T-box gene 18* (*Tbx18*) (*Tbx18*$^{tm2(cre)Sev}$) for epicardial lineage tracing and observed that cardiomyocytes of the left ventricle and the interventricular septum were positive for reporter activity [26]. Lineage tracing using a *creEGFP* knock-in allele of *Wt1* (*Wt1*$^{tm1(EGFP/cre)Wtp}$) reported a substantial contribution of epicardial cells to the myocardium of all four cardiac chambers and in the interventricular septum, constituting 7–10% of cardiomyocytes in ventricles and 18% in atria starting from E10.5 [27]. As an epicardial origin of myocardial cells was incongruent with all previous fate-mapping efforts both in the chick and the mouse, the suitability of the cre lines was quickly challenged. *Tbx18* is expressed in the proepicardium and its expression is maintained in epicardial cells until E16.5. However, *Tbx18* is also expressed endogenously in cardiomyocytes of the interventricular septum and the left ventricle *i.e.*, exactly in those regions which the study by Cai *et al.* suggested to derive from the epicardium [28]. Using an independent

cre knock-in allele of *Tbx18* (*Tbx18*$^{tm4(cre)Akis}$) Grieskamp and colleagues showed that epicardial cells overlying the right ventricular myocardium do not differentiate into cardiomyocytes but give exclusively rise to smooth muscle cells and fibroblasts [29]. More recently, it has been shown that *Wt1* is not specific for epicardial cells but is also expressed in coronary endothelial cells and possibly in cardiomyocytes [30,31]; an (pro-)epicardial origin of these cell types can therefore not be analyzed with *Wt1*-based *cre* lines. Independent of these limitations, it has recently been reported that *Wt1*$^{tm1(EGFP/cre)Wtp}$ mediates widespread ectopic expression in the early embryo including the heart, further questioning the results of earlier *Wt1*-based lineage tracing efforts [30,31]. However, similar to the chick heart, former *Wt1*-expressing EPDCs replace mesenchymal cells of the atrio-ventricular cushion tissue in a second invasion and contribute to mitral and tricuspid valves as well as to the annulus fibrosus [32,33].

Due to the mentioned limitations of cre lines based on *Tbx18* and *Wt1* regulatory elements for epicardial lineage tracing, several groups investigated the potential of other promoter elements to specifically label (pro-)epicardial cells. *Transcription factor 21* (*Tcf21*) is expressed in the proepicardium, epicardium and a subset of epicardium-derived cells that contribute to the fibroblast lineage of murine hearts. Lineage tracing of *Tcf21*-expressing epicardial cells of E10.5 hearts with an inducible *cre*-driver line (*Tcf21*^{MerCreMer}) revealed their contribution to coronary smooth muscle cells, interstitial and perivascular fibroblasts; *Tcf21*-expressing epicardial cells, however, did not account for coronary endothelial cells or cardiomyocytes [34].

In a recent study, Rinkevich and colleagues investigated the differentiation potential of mesothelial cells using a knock-in of a *creERT2-IRES-LacZ* cassette into the *mesothelin* locus (*Msln*^{Cln}), thereby providing a mesothelium-specific tool to trace the fate of epicardial cells. Although the study did not focus on the heart, the authors stated that they observed a restriction of descendants of *Msln*-expressing (*i.e.*, mesothelial) cells to the fibroblast and smooth muscle lineage of the heart during embryogenesis as well as during postnatal life of mice. A contribution of the mesothelium to endothelial cells or cardiac muscle was not detected [35].

Katz and colleagues used *cre* lines based on regulatory elements of *Semaphorin 3D* (*Sema3D*) and *Scleraxis* (*Scx*) to further delineate the molecular and cellular complexity of the proepicardium. Both genes are initially expressed in the proepicardium but in a pattern only partially overlapping with that of *Tbx18* and *Wt1* and are maintained in the epicardium for a variable length of time; importantly, none of these genes is expressed in coronary endothelial cells. Lineage tracing of *Sema3D*-expressing (pro)epicardial cells (*Sema3d*$^{tm1.1(GFP/cre)Cjt}$) revealed their contribution to coronary smooth muscle cells, interstitial fibroblasts, atrio-ventricular valves and coronary endothelial cells as well as a minor portion (0.36%) of cardiomyocytes. Endothelial cells derived from the *Sema3D* lineage appear in the sinus venosus and the coronary plexus growing into the heart after E11.5. These observations are in line with the derivation of coronary endothelial cells from the sinus venosus endothelium as described by Red-Horse and colleagues in 2010 [36]. However, it was known that endothelial cells that derive from the *Scx*-expressing proepicardial cell population (*Tg(Scx-GFP/cre)1Stzr*) emerged directly underneath the epicardium at E11, before the endothelial cells from the sinus venosus were detected by Red-Horse et al 2010. This finding indicates another route of proepicardial contribution to the endothelial cell lineage, possibly via endocardial cells [37]. Next to endothelial cells, *Scx*-expressing proepicardial cells also contribute to coronary smooth muscle cells and cardiomyocytes of the murine heart although in an unexplained pattern in the latter case.

In summary, genetic fate mappings showed that murine epicardial cells give rise to coronary smooth muscle cells, perivascular/interstitial fibroblasts and mesenchymal cells of the annulus fibrosus and atrio-ventricular valves [26,27,29,32,33]. Lineage-labeled cardiomyocytes have been observed in several studies, but their epicardial origin is controversial as myocardial recombination of the used *cre*-driver lines is likely [28,30]. Coronary endothelial cells are, as in the avian system, proepicardium-derived but their origin is distinct from the *Tbx18*-/*Wt1*-expressing epicardial cell population; further investigations are needed to determine their migration route. The fates of murine (pro-)epicardial cells are summarized in Table 1.

4. Epicardial Fates in Zebrafish Development—Fibroblasts and Smooth Muscle Cells and Nothing Else

The first researchers, that set out to investigate (pro-)epicardial fates in the zebrafish were Kikuchi and colleagues in 2011. *Tbx18*, *wt1b* and *tcf21* were tested for epicardium-specific genetic lineage tracings. Both, *tbx18* and *wt1b* regulatory elements did not drive epicardium-specific reporter gene expression, confirming the observations made in mice. The regulatory elements of *tcf21*, however, faithfully recapitulated specific epicardial *tcf21* expression. (Pro-)epicardial cells of the *tcf21*-lineage (*Tg(tcf21:DsRed2)*^{pd37}) contributed to the subepicardium and perivascular cells; lineage-labeled cardiomyocytes or endothelial cells were not identified. Tracing of epicardial cells of the zebrafish larva with an inducible *tcf21*-cre line (*Tg(tcf21:CreER)*^{pd42}) revealed that epicardial cells contributed to the adult epicardium, the ventricular subepicardium but never to the trabeculated myocardium. Perivascular cells of larger vessels and smooth muscle cells of the bulbus arteriosus but not cardiomyocytes were labeled by this technique [38]. Hence, *tcf21*-expressing (pro-)epicardial cells give rise to subepicardial mesenchyme, perivascular cells and a few smooth muscle cells of the bulbus arteriosus in the zebrafish heart.

5. *In Vitro* Studies Reveal Proepicardial and Epicardial Potentials

Given the origin of the epicardium from the proepicardium and their shared molecular characteristics, it has turned out difficult to independently assess the fate of cells in both tissues. A possible way out of this dilemma is offered by explant cultures of proepicardium and epicardium, that have been established for mouse, rat and chick embryos. As these *in vitro* systems allow tracing the fate of these cells under natural conditions (as far as they are known) but also under artificial conditions, they provide additional insight into the developmental potential of these cells.

Primary proepicardial explants recapitulated all the cell fates observed in (pro-)epicardial cells *in vivo*. Smooth muscle cells, fibroblasts as well as endothelial cells and cardiomyocytes can be differentiated from proepicardial cells of mouse and chick embryos under certain conditions [19,39,40]. However, it is technically extremely challenging to isolate pure proepicardial cells devoid of contaminating surrounding tissues such as the sinus venosus myocardium. Furthermore, Katz and colleagues have shown in their studies of proepicardium-derived endothelial cells that *Scx*-positive proepicardial cells have a higher potential to differentiate into endothelial cells than *Scx*-negative proepicardial cells [37] supporting the notion that the “proepicardium” is a heterogenous cell population to start with.

Table 1. Fate of (pro-)epicardial cells in chick, mouse and zebrafish embryos.

Method	Referenc	SE	cSMC	pCF	iCF	AVV	Endo	cEC	CM
Chick									
Dye labeling	[17]	X							
	[19]	X						X	
Retroviral labeling	[16]		X	X				X (PE)	
	[17]		X	X	X	X	X		
	[19]	X				X			
Quail-chick chimeras	[12]							-	
	[15]	X	X	X		X	X		
	[18]		X	X	X	X	X	X	-
	[19]	X	X	X		X	X	X	
PE explant	[41]		X						
	[19]							X	
	[40]		X						X
	[42]								X
	[20]							X	
Epicardial explant	[43]		X						
Mouse									
Dye labeling	[26]								X
	[44]	X			X				
Lineage-tracing									
Msln^{Cln}	[35]		X		X			-	-
Sema3d$^{tm1.1(GFP/cre)Cjt}$	[37]	X	X		X	X		X	X
Tbx18$^{tm2(cre)Sev}$	[26]		X			X		-	X
Tbx18$^{tm4(cre)Akis}$	[28]								-
	[29]	X	X		X				-
Tcf21^{MerCreMer}	[34]	X	X	X	X			-	-
Tg(Scx-GFP/cre)^{1Stzr}	[36]		X				X	X	X
Wt1$^{tm1(EGFP/cre)Wtp}$	[27]		X					X	X
	[32]					X			
Wt1$^{tm2(cre/ERT2)Wtp}$	[26]		X					X	X
	[30]		X					X*	-
	[31]							X*	
PE explant	[45]							X	
	[36]							X	
	[39]		X	X	X			X	X
Epicardial explant	[46]		X						
	[47]		X						
	[39]		X	X	X			-	-
Zebrafish	pSC								
Tg(tcf21:DsRed2)^{pd3}	[38]	X	X	X				-	-

Abbreviations: AVV: atrio-ventricular valve; CM: cardiomyocyte; cEC: coronary endothelial cell; cSMC: coronary smooth muscle cell (pSC: perivascular support cell in zebrafish); Endo: endocardium; iCF: interstitial cardiac fibroblast; pCF: perivascular cardiac fibroblast; PE: proepicardium; SE: subepicardium; * endogenous expression in case of *Wt1*.

Primary epicardial explants are derived from epithelial outgrowths of embryonic ventricles. These cells differentiate into fibroblasts and smooth muscle cells but not into cardiomyocytes and endothelial cells in culture arguing that epicardial cells have lost this potential compared to their proepicardial precursors, or that endothelial cells have been removed by this method [39].

Nonetheless, the combination of proepicardial and epicardial fate mapping efforts *in vitro* and *in vivo* points towards the character of the epicardium as a bipotential source of fibroblasts and smooth muscle cells. The proepicardium may have an additional endothelial fate but it is more likely that the proepicardium is a heterogeneous cell population to start with.

6. Mobilization and Differentiation of Epicardial Cells in The Injured Heart—Studies in Zebrafish and Mice

While it was long thought that the epicardium of the adult heart is terminally differentiated, it has now emerged that it can regain its embryonic character as a source of cells and signals for the underlying myocardium under conditions of injury. The first insight into this unexpected behavior was obtained from work in the zebrafish, a vertebrate that is able to fully regenerate its heart after loss of up to 20% of its cell mass [48]. Ventricular amputation or cryoinjury (simulating ischemic conditions after myocardial infarction in mammals) was found to activate an embryonic gene program in the epicardium of adult zebrafish [49,50]. Transcription factors like *Tbx18*, *Wt1* and *Aldh1a2* that are expressed in the embryonic but not in the adult epicardium are (re-)activated in the whole epicardium and become restricted to the epicardium covering the injury site at later stages [49,51]. Injury-activated epicardial cells start to proliferate, secrete angiogenic factors, and undergo a mesenchymal transition [52,53]. Lineage-tracing after heart apex amputation using an inducible *tcf21*^{cre} fish line (*Tg(tcf21:CreER)*^{pd42}) has revealed that epicardial cells contribute to vascular supporting cells but not to cardiomyocytes in the regenerating zebrafish heart [38]. A second study used the *wt1b:EGFP* line (*Tg(wt1b:EGFP)*^{li1}) to follow *Wt1*-expressing cells after cryoinjury. It was observed that these Wt1-positive (epicardium-derived) cells mostly contributed to interstitial cells, "myofibroblasts" and vascular supporting cells but not to endothelial cells or cardiomyocytes in the injured heart [51]. To avoid the limitations of lineage labeling, González-Rosa and colleagues studied the fate of genetically labeled cryoinjured ventricular grafts in a transplantation model. Epicardial cells mostly contributed to the fibroblast lineage but not to cardiomyocytes after a long-term follow up (60 days post-injury) [51] further strengthening the assumption that injury-activated epicardial cells as their embryonic relatives are bipotent with respect to differentiation into fibroblasts and smooth muscle cells.

Unlike zebrafish, mammals cannot replace lost cardiomyocytes but form extensive scars after cardiac injury leading to severely impaired cardiac function. Surprisingly however, epicardial reactivation and epithelial-mesenchymal transition is a feature that also occurs in mice after myocardial infarction [53]. The lineage commitment of these reactivated epicardial cells has remained controversial.

Lineage tracing studies using *Wt1*$^{tm2(cre/ERT2)Wtp2}$ [53] and *Tg(GATA5-cre)*^{1Krc} [54] lines identified interstitial fibroblasts, "myofibroblasts" and smooth muscle cells but not cardiomyocytes and endothelial cells amongst the scar invading epicardium-derived cells after myocardial infarction. In contrast, epicardial fate mapping using a BAC derived *Wt1*$^{tm1(EGFP/cre)Wtp}$ driver found contribution of epicardial cells to the fibroblast population, "myofibroblasts" and coronary endothelium and later also to the

cardiomyocyte population in an infarct model [55]. To complicate the situation even further, it was suggested from studies with the inducible *Wt1*$^{tm2(cre/ERT2)Wtp2}$ and the constitutively *Wt1*$^{tm1(EGFP/cre)Wtp}$ driver lines that treating mice with thymosin beta4 before induction of myocardial ischema accelerates and enhances the mobilization of epicardium-derived cells and induces their contribution to the cardiomyocyte lineage [56]. In a subsequent study, Zhou and co-workers used the same *Wt1*$^{tm2(cre/ERT2)Wtp2}$ line for lineage tracing and confirmed the beneficial effects of thymosin beta4 on revascularization and scar reduction, but did not find any evidence that thymosin beta4 treatment after myocardial infarction reprograms epicardial cells into cardiomyocytes [57]. As already mentioned for lineage-tracing studies during embryonic development, *Wt1* is expressed in coronary endothelial cells (and possibly in cardiomyocytes) and might be reactivated by the ischemic conditions after myocardial infarction [30,31,57,58] putting a question mark behind the usefulness of *Wt1*-based lineage tracing systems for epicardial fate mapping under injury conditions just as in the embryonic situation.

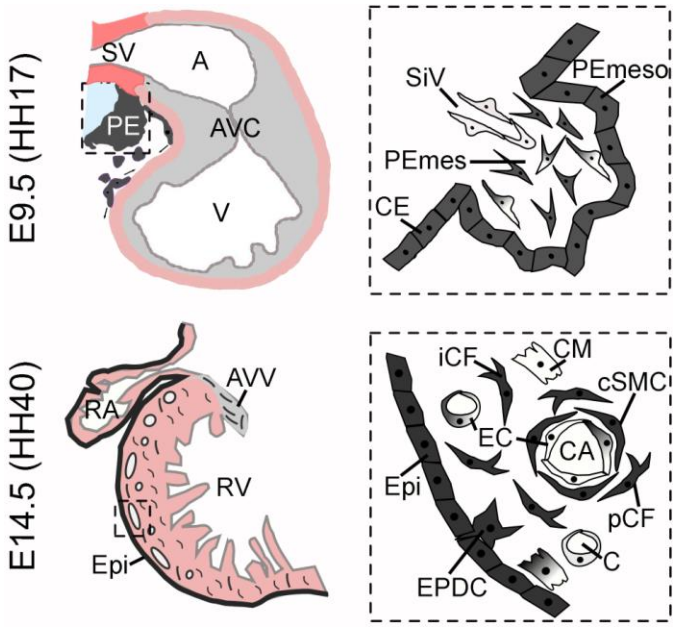
Nonetheless, we can safely state that the epicardium is reactivated after cardiac injury to participate in the wound healing response by providing (mostly) fibroblasts and smooth muscle cells to the injury site—a process highly similar in zebrafish and mice (for a summary see Table 2). A contribution to cardiomyocytes and endothelial cells cannot be excluded at this point in the mouse but is likely to be a minor one. More intriguingly, epicardial cells provide proangiogenic factors to the heart [51,53] that may improve cardiac function after injury. While the epicardial injury program is likely to be conserved in lower and higher vertebrates, the myocardial response seems fundamentally different as mammalian cardiomyocytes unlike the zebrafish do not dedifferentiate and re-enter the cell cycle. It remains to be seen whether this reflects an intrinsic difference within the cardiomyocyte or reflects the difference in a growth promoting environment in which the generation of the epicardium may also play a role. It may be noted that that the neonatal murine heart still has the ability to regenerate after ventricular resection [59].

Table 2. Fate of epicardial cells after injury.

Injury	Method	iC	CF	pSC	CM	EC
Zebrafish						
Cryoinjury (infarct model)	Transplantation [51]	X	X	X	-	-
	Lineage-tracing (*Tg(wt1b:EGFP)*^{li1}) [51]	X	X	X	-	-
Ventricular resection	Lineage-tracing (*Tg(tcf21:CreER)*^{pd42}) [38,60]			X	-	-
Mouse						
Myocardial infarction	Lineage-tracing (*Wt1*$^{tm1(EGFP/cre)Wtp}$) [55,61]	X	X	X	X	X /-
	Lineage-tracing (*Wt1*$^{tm2(cre/ERT2)Wtp}$, *Ad:Msln-Cre*) [57]	X	X	X	-	-
	Lineage-tracing (*Tg(GATA5-cre)*^{1Krc}) [54]	X		X		-

Abbreviations: CM: cardiomyocyte; EC: endothelial cell; iC: interstitial cell; CF: cardiac fibroblast; pSC: perivascular support cell (zebrafish)/coronary smooth muscle cells (mammals).

Figure 1. Scheme of (pro-)epicardial lineages. The **upper** row shows a schematic view of an E9.5 sagittally sectioned murine heart (similar to HH17 avian heart) on the left side. The proepicardium (PE) is indicated in dark grey, the chamber myocardium in light pink, the sinus venosus mesenchyme (SV) in red and the cardiac jelly and cushion mesenchyme in light grey. Note the close proximity of proepicardium, sinus venosus mesenchyme, septum transversum and liver, respectively (light blue). The right scheme represents a magnification of the proepicardium (boxed area). Coelomic epithelium (CE) and the proepicardial mesothelium (PEmeso) are indicated in dark grey and share the expression of *Tbx18* and *Wt1*. Proepicardial mesenchymal cells (PEmes) are highly diverse. *Wt1*-, *Tbx18*, *Scx*-, *Sema3D*- and *Tcf21*-expressing cells are found within the mesenchyme with varying overlapping expression patterns. Their individual descent and contribution to the heart have remained largely elusive. Sinusoid-like vessels (SiV) reach into the proepicardium and may provide endothelial cells. The **lower** row shows a scheme of the murine heart at E14.5 (resembling an avian heart at HH40). Epicardium-derived cells (EPDCs) are indicated in dark grey and invade the ventricular myocardium as well as the cushion tissue of the atrio-ventricular valves (AVV). A higher magnification of the boxed area within the right ventricle (RV) is given to indicate the fate of these cells. They mainly contribute to coronary smooth muscle cells (cSMC) and interstitial (iCF) as well as perivascular fibroblasts (pCF). An epicardial origin of endothelial cells (EC) and cardiomyocytes (CM) is unlikely (indicated by a gray-white gradient within these cells).



Abbreviations: A: atrium; AVC: atrio-ventricular canal; C: capillary; CA: coronary artery; Epi: epicardium; RA: right atrium; V: ventricle.

7. Summary and Perspectives

The last 20 years have seen a large number of studies that collectively suggest that the epicardium is a crucial source of cells for the developing and regenerating vertebrate heart. Smooth muscle cells and fibroblasts are the two main cell types that differentiate from epicardium-derived mesenchymal cells,

making the epicardium an essential tissue for the generation of the cardiac fibroskeleton and the coronary vasculature (Figure 1). It remains an open question as to how individual epicardial cells are mobilized and when they become committed to one or another fate. Although a number of reports have suggested an endothelial and myocardial differentiation of epicardial cells, this contribution—if not just a mere technical artefact—is definitely a very minor one both in development and regeneration. Nonetheless, efforts to find suitable cocktails of factors that can reprogram epicardial cells to one of these fates may be worthwhile to pursue in the future.

While the epicardium derives from the proepicardium, the latter may present a more complex pool of cells that comprises epicardial precursors as well as endothelial and myocardial cells. However, we do need a more elaborate panel of markers that distinguish the proepicardium from surrounding tissue and/or describe its lineage segregation to better state its role as a cell source in development.

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