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Review article

Origin and fates of the proepicardium

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ABSTRACT

The embryonic heart initially consists of only two cell layers, the endocardium and the myocardium. The epicardium, which forms an epithelial layer on the surface of the heart, is derived from a cluster of mesothelial cells developing at the base of the venous inflow tract of the early embryonic heart. This cell cluster is termed the proepicardium and gives rise not only to the epicardium but also to epicardium-derived cells. These cells populate the myocardial wall and differentiate into smooth muscle cells and fibroblasts, while the contribution to the vascular endothelial lineage is uncertain. In this review we will discuss the signaling molecules involved in recruiting mesodermal cells to undergo proepicardium formation and guide these cells to the myocardial surface. Marker genes which are suitable to follow these cells during proepicardium formation and cell migration will be introduced. We will address whether the proepicardium consists of a homogenous cell population or whether different cell lineages are present. Finally the role of the epicardium as a source for cardiac stem cells and its importance in cardiac regeneration, in particular in the zebrafish and mouse model systems is discussed.

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INTRODUCTION

The outermost layer of the heart is the epicardium. The early embryonic heart lacks an epicardium and consists only of two cell layers, the endocardium and the myocardium. The epicardium and the epicardium derived cells (EPDC), which are found in the mature heart, such as cardiac fibroblast or cells of the coronary vasculature are not present at the tubular heart stage. These cell lineages are derived from a cluster of cells, which is known as the proepicardium (PE) [1,2]. The term proepicardial serosa, which sometimes is also used, characterizes the whole region of the pericardial wall harboring the cauliflower-like PE cell cluster and also includes the wall of the sinus venosus with its endothelium. The PE is a transitory cell cluster, which develops at the base of the venous inflow tract in all vertebrate hearts (Fig. 1). It develops at the junction between the forming sinus venosus myocardium and the posterior undifferentiated lateral plate mesenchyme. The PE develops in close proximity of the heart and liver and inductive interactions between these organ anlagen and the PE have been proposed [3,4]. The PE is first morphologically identified as cuboidal cells that form on the sinus horns when tubular heart formation has been completed and cardiac looping is well underway [5]. This stage in development is reached in the chick embryo at Hamburger-Hamilton (HH) stage 14, which is equal to about three days of incubation.

In its mature form the PE in the chick has been described to have a cauliflower or grape-like appearance due to the formation of multiple villi. It consists of an outgrowth of cuboidal mesothelial cells overlying an internal cluster of mesenchymal cells, which is embedded in an extensive amount of extracellular matrix [6]. Underneath is a stratified cuboidal cell layer, which is adjacent to the endocardial layer of the sinus horns [6]. Between HH stage 14 and 17, the PE grows in size and villous projections extend from the PE towards the dorsal aspect of the inner curvature of the heart tube. Ultimately the PE makes contact to the heart at the AV junction and forms a tissue bridge. PE cells will migrate onto the heart and spread over the surface forming a single squamous epithelium, which is termed the epicardium. Underneath the epicardium an extracellular-rich subepicardial mesenchyme forms. Some of this mesenchyme is believed to be a direct descendent of the proepicardial mesenchyme [6]. Myocardial signals induce epicardial cells to undergo an epithelial-to-mesenchymal transformation (EMT) and either populate the subepicardial mesenchyme or migrate into the myocardial wall.

In this paper we are reviewing the current status of our understanding of the formation of the PE using data that has accumulated in recent years mainly through work in the chick and mouse embryo and some recent work in zebrafish. We will discuss the different signaling pathways that have been implicated in PE formation and the mechanisms of cellular transfer of PE cells to the heart, for which different strategies are found in different vertebrates. An important issue is whether the PE consists of a homogeneous population of cells or harbors multiple cell lineages. Moreover it is a debated issue,

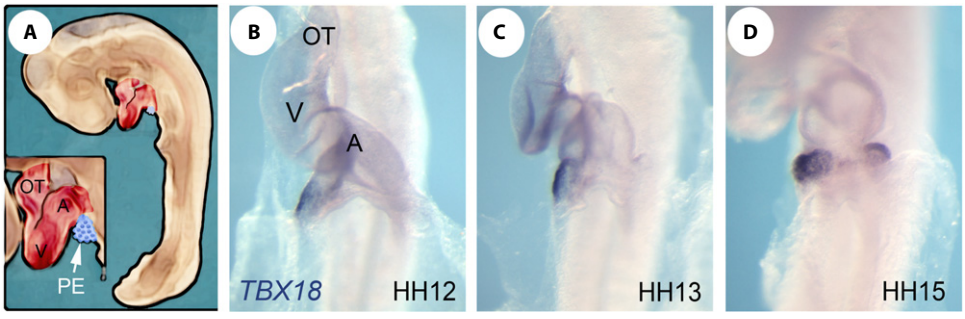

Figure. 1 Proepicardial marker gene expression. (A) The PE develops at the venous pole of the early heart tube (red) as a cluster of villous protrusions (blue). (B–D) In the chicken embryo, proepicardial marker gene expression represented by *TBX18* can first be detected at stage HH 11–12 (B), which is approximately three stages before the onset of villous outgrowth on the right sinus horn. (C) Expression persists during the entire period of PE formation. Note the asymmetric expression being unilaterally present on the right sinus horn between HH stages 11–14. (D) By HH stage 15 also on the left side a much smaller expression domain is observed. A (atrium), OT (outflow tract) V (ventricle). Panel B–D are taken from: Schlueter, J and Brand, T. (2009). A right-sided pathway involving FGF8/Snai1 controls asymmetric development of the proepicardium in the chick embryo. Proc Natl Acad Sci USA 106:7485–7490.

whether there are contributions of proepicardial cells to the myocardial and endothelial cell lineages and we will discuss the evidence. Other important aspects of epicardial biology such as coronary artery formation have recently been extensively discussed [7,8], and in the interest of brevity; we will not touch on this topic in this review. In the last section of this review, we will discuss the current understanding of the significance of the epicardium for myocardial regeneration, which has been predominantly studied in the zebrafish model, however recently also first studies in the mouse have been reported.

STRATEGIES OF EPICARDIUM FORMATION AMONG VERTEBRATES

The occurrence of a distinctive cluster of PE cells that secondarily colonize the heart is an intriguing feature of cardiac development and is found in all vertebrates. Interestingly, across the different vertebrate classes PE formation displays similarities but also some significant differences, most likely due to species-specific morphological peculiarities, including asymmetrical development and the process of cell transfer to the heart.

PE formation in the chick embryo

For many years, the chick embryo has served as a model organism of PE formation and epicardial development and thus in this respect it is one of the best-studied organism so far. This model organism has provided insight into many basic developmental steps during PE formation and epicardialization of the embryonic heart [9,10]. In the chick, the PE starts to form on the right sinus horn around HH stage 14 as mesothelial cells that cluster and form protruding villi [2,11]. On the left side this proepicardial outgrowth is severely retarded (Fig. 2) [5,12,13].

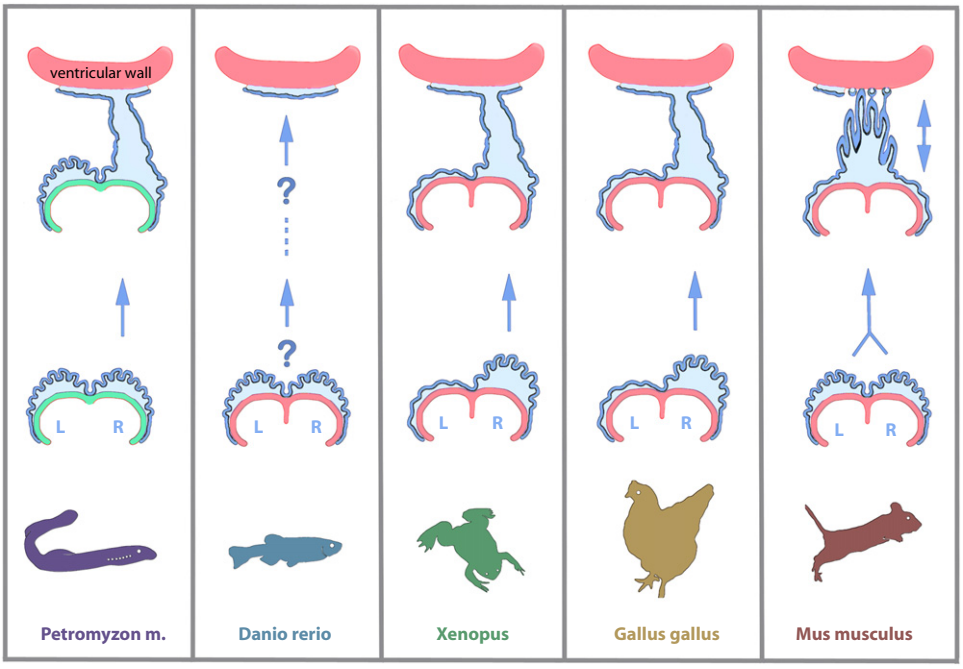

Figure. 2 Asymmetrical PE development and mechanisms of cell transfer. The PE is formed as an asymmetrical outgrowth of mesothelial cells (blue) on the bilateral sinus venosus (red) in different vertebrates like Xenopus and chick, which ultimately forms a tissue bridge to establish contact to the surface of the ventricular wall and to colonize the heart. This morphological feature remains unclear in the zebrafish whereas in the lamprey Petromyzon only the right of initially two bilateral PE anlagen forms a transient tissue bridge, which arises from the coelomic wall (green). PE development in the mouse appears to be symmetrical with two bilateral anlagen fusing on the midline. After massive outgrowth, the cells attach to the ventricle and are subsequently pulled off by the contracting heart. Thereby cell clusters attach to the myocardial surface and form islands of epicardium, which subsequently coalesce to establish a confluent epicardial sheet.

Recently, a right-sided pathway was identified that is responsible for asymmetric PE development [14]. This pathway involves *Fgf8*,[1] which is expressed on the right side of Hensen's node [15]. FGF8 is able to induce the transcriptional repressor *Snai1* in the right lateral plate mesoderm, which has the ability to suppress the expression of the left-side determinant *Pitx2* [16]. Gain- and loss-of-function modulations of the right-sided *Fgf8/Snai1* pathway and the left-sided *Nodal/Pitx2* pathway lead to the conclusion that the PE in the chick is induced by *Fgf8/Snai1* on the right side rather than being repressed by *Nodal/Pitx2* on the left side [14]. Further support for the model of a unilateral, right-sided PE identity stems from the observation that chick embryos with a surgically-induced *cardia bifida* display two ventricles but only the right ventricle is covered by an epicardium whereas the left ventricle remains uncovered [14].

After induction on the right sinus, the PE cells proliferate rapidly and finally attach to the dorsal side of the ventricle. The PE cells migrate onto the heart via a matrix-rich tissue bridge, and cover the ventricle and the atria but only parts of the outflow tract with an epicardial layer [2]. The distal part of the outflow tract known as the *truncus arteriosus* is covered by mesothelial cells that are derived from the pericardial wall connected to the arterial pole of the heart tube [2,17].

The fate of proepicardial cells was first studied with a replication defective virus establishing that coronary vascular smooth muscle and endothelial cells as well as fibroblasts are derivatives of the PE [18]. This differentiation potential of proepicardial cells was subsequently confirmed by quail-chick chimera analysis [19–21]. However there is also data suggesting that in particular the endothelium may not be derived from the PE but from the neighboring liver [22]. The controversy surrounding this still remains and the different sources that have been proposed for coronary endothelium are discussed later in this paper.

PE formation in the mouse heart

In the mouse the PE starts to form at incubation day 8.5 [5,23]. Some of the basic processes described in the chick embryo could in principle be verified in other model organisms, although it is still a matter of debate, whether mammals in particular are using different mechanisms to transfer proepicardial cells onto the heart. It was generally proposed that in the mouse, proepicardial villi are not connected to the ventricle via an extracellular matrix bridge but are released as free floating vesicles, which pass the pericardial cavity establishing epicardial islands on the surface of the heart [5,24–28]. This principle was however recently challenged by a detailed descriptive analysis performed by Rodgers et al. in which they were able to show that murine proepicardial vesicles are indeed in direct contact with the myocardium (Fig. 2) [23]. However, free vesicles were also observed, in particular in the region of the atrioventricular *sulcus*. These findings resulted in a model in which proepicardial villous projections attach to the heart, passively elongate by retraction of the beating heart tube, ultimately collapse and leave a vesicular patchwork on the myocardial surface [23]. Furthermore, data from rat embryos also confirm a matrix-rich connection between the PE and myocardium, which might be similar to that found in the chick [29]. A more significant difference between mouse and chick is the bilateral formation of the murine PE anlagen on the *sinus venosus*, which subsequently fuse ventrally at the midline before the cells colonize the ventricle [5]. The right-sided *Fgf8/Snai1* pathway that was proposed in the chick for induction of the PE however, is not deployed in a similar fashion in the mouse. This might also be reflected by the different strategies of left-right asymmetry generation in the mouse and chick [30]. In the chick, FGF8 is asymmetrically expressed on the right side and acts as a determinant of the right side [14,15]. In the mouse, the role of *Fgf8* in L–R axis determination is not fully understood [31]. *Fgf8* is not asymmetrically expressed but is thought to act as a left side determinant, possibly through its involvement in cilia formation in the node [32]. The mechanisms of PE induction in the mouse remain unknown to a large extent, but there is an increasing amount of data available on the origin and the differentiation potential of the epicardial lineage, which will be discussed in more detail later.

[1] The nomenclature for genes and proteins differs between species. In order to facilitate the reading of this text, the authors have decided to harmonize the nomenclature and applied the rules that are used for the mouse for each gene and protein mentioned in this manuscript.

PE formation in lower vertebrates

Strikingly, compared to the chick embryo, the PE in lower vertebrate embryos shares similar morphological features. The PE in *Xenopus* can be first found at stage 41, equivalent to approximately 3 days of embryonic development [33]. It is strictly formed on the right side as a cluster of mesothelial cells that colonizes the ventricle via a distinct and persisting tissue-bridge (Fig. 2) [33]. Asymmetric right-sided tissue bridge formation is also described in a related amphibian species, the axolotl [34]. In the zebrafish, the PE is first seen at 2 days post-fertilization [35]. Presently, the morphology of the PE and the spatial relation to the inflow tract and the venous system remains poorly understood, although few initial studies describe a villous outgrowth and propose a release of floating vesicles as a tissue bridge has not been observed [35,36]. In addition, proepicardial primordia visualized by marker gene expression have been described as being bilateral, but it is unclear whether both primordia contribute to the epicardium.

The sturgeon is another, yet more primitive fish species in which proepicardial development has been investigated in more detail. Around four days post hatching (dph) the PE can be observed as a bilateral cluster of cells between the *sinus venosus* and the ducts of Cuvier [37]. These two clusters fuse at the midline and subsequently attach to the heart. Interestingly, different strategies are used to colonize the different regions of the heart. The conus myocardium is covered by a cohesive proepicardial epithelium. In contrast, free-floating vesicles apparently colonize the ventricle. However, the possibility for a mechanism similar to the one that was proposed for the formation of the murine epicardium by Rodgers et al. described above, should not be excluded. In accordance to higher vertebrates, the bulbus epicardium appears to be derived from mesothelium of the pericardial wall. The tissue bridges that are formed during development persist and connect the coronary vascular plexus and the *sinus venosus* and some are even found to carry cardiac innervation.

One of the most primitive organisms studied to date is the lamprey *Petromyzon marinus*. The lamprey is a jawless fish-like organism, which together with the hagfish forms a primitive sister group (agnathans) to all other vertebrates (gnathostomes). The lamprey develops two bilateral PE anlagen around 17 days post fertilization (dpf) but only the right cell cluster forms a transient tissue bridge to transfer cells onto the heart (Fig. 2) [38]. In later stages, after the epicardium has formed, the two cell clusters give rise to the pronephric external glomeruli, which become highly vascularized. The relation to the pronephros might be a primitive feature represented in agnathans and potentially might reveal the evolutionary origin of proepicardial cells. Higher vertebrates have undergone various morphological rearrangements, for instance the enlargement of the liver and the incorporation of the sinus venosus into the inflow tract of the heart, which has caused the PE to be brought closer in spatial relationship to the liver primordium. The proposed connection between the pronephros and the PE in lower vertebrates might resonate in the expression of several proepicardial marker genes in the intermediate mesoderm, which gives rise to the nephric system in higher vertebrates.

MARKER GENES TO STUDY PE AND EPICARDIUM FORMATION

Initially the PE was mainly studied by morphological methods [2,39,40]. However in recent years several different marker genes were employed that are suitable to follow epicardial cells during various steps of proepicardium and epicardium formation. In the majority of studies, the transcription factors TBX18, WT1, and TCF21, the competence factor for nodal-like signaling factors CFC, and the retinoic acid synthesizing enzyme RALDH2 have been used as markers. Although none of these markers are uniquely expressed in the PE, the combinatorial expression of these markers is specific to the PE.

Tbx18 encodes a member of the T-box family of transcription factors, of which several members are expressed in the heart [41]. *Tbx18* expression is associated with the cardiac venous pole [42]. Expression is found in the PE, in the epicardium, and both in mesenchymal progenitors as well as in differentiated myocardium of the *sinus venosus* region [43]. During the time of PE formation *Tbx18* expression is confined to the PE and thus can be used as a reliable PE marker gene (Fig. 1) [12].

Wt1 encodes a zinc-finger transcription factor, which is important for kidney development, but loss of function of *Wt1* also results in impaired formation of the epicardium due to premature differentiation and reduced EMT resulting in poor development of the coronary arteries and hypoplasia of the embryonic ventricle [44]. *Wt1* prevents the precocious differentiation of epicardial cells by modulating cell adhesion and by maintaining high levels of *Aldh1a2* (*Raldh2*) expression [45–47]. *Wt1* expression is maintained in the epicardium, in the subepicardial

mesenchyme as well as in migratory EPDC [48]. After epicardial cells reach their final destination and start differentiation, *Wt1* is down regulated [49]. *Wt1* serves as an excellent marker of early PE formation being strongly expressed in the PE of the chick embryo by HH stage 13 [12].

The basic helix-loop helix gene *Tcf21* (epicardin/capsulin/ *Pod1*) is specifically expressed in the chick and mouse PE, epicardium and in the pericardium [50]. It is believed to act as a repressor of cell differentiation [51]. Null mutants die at birth from multiple organ defects that include lung hypoplasia, asplenism, and renal dysplasia [52–55]. Inspite of the specific expression pattern of *Tcf21* in the epicardium, no epicardial phenotype was noted in the null mutant, although a hemopericardium was observed in the neonate, which possibly is related to defective coronary vessel maturation.

The EGF–CFC family encodes proteins required as co-factors needed to present at the plasma membrane for Nodal and related members of the TGFβ superfamily to bind to the receptor [56]. *Tbx18* and *Cfc* are the earliest expressed marker genes for PE development [12]. Expression is maintained after PE formation and is also found in the fully formed epicardium [57]. Currently it is not known, whether PE expression of *Cfc* is unique to avian embryos or whether EGF–CFC protein family members in other species are also expressed in the PE. CFC might be required for a yet to be characterized Nodal-like signal involved in PE induction, however it is also possible that EGF–CFC proteins function in a Nodal-independent context [58–62].

Aldh1a2 has also served as a marker gene to identify proepicardial and epicardial cells [63–65]. It is one of the rate-limiting enzymes of retinoic acid biosynthesis and is expressed in the PE and in the pericardium [66]. Retinoic acid functions as a survival factor for PE cells, since increased apoptosis in PE cells and an impaired epicardialization of the myocardium was observed in the *Rxra* receptor null mutant [67]. Recent data suggest that retinoic acid is also involved in setting the timing of smooth muscle cell differentiation after EPDC have migrated into the myocardial wall [68].

Several additional genes that are specifically expressed in the epicardium have been recently identified in a dedicated screen to identify genes associated with epicardium formation and cell differentiation [69]. These include genes known to be expressed in epithelia such as basonuclin, dermokine, and glycoprotein M6A [69]. It remains to be determined whether these novel marker genes might be suitable to distinguish different cell lineages present in the PE and in the epicardium (see below).

SIGNALING MOLECULES AND TRANSCRIPTION FACTORS INVOLVED IN PE FORMATION

The heart is formed from bilateral heart fields present in the splanchnic layer of the anterior lateral plate. Within this area two different populations of cells are distinguished, the primary or first heart field cells, which will form the initial tubular heart and the second heart field from which cells are derived that extend the tubular heart both at the venous and arterial pole and give rise to the right ventricle and outflow tract [70–72]. The first heart field cells are positioned more laterally while the cells of the second heart field are located more medially and caudally. At the venous pole it is thought that the progenitor cells of the second heart field contribute to both myocardial and PE lineages. Experimental evidence for this model is derived from DiI labeling of splanchnic mesodermal cells at the inflow tract region of the heart at HH stage 11. Cells that were labeled gave rise to myocardium of the inflow tract and the PE [73]. As the labeling technique employed in this experiment labeled a group of cells rather than single cells it cannot distinguish between bipotential precursors or a mixed population having distinct cell fate. Further support for this model comes from the observation that myocardial cells that have recently entered the heart at the inflow tract display co-expression of both mesothelial and myocardial marker proteins [74].

Genetic fate mapping experiments in the mouse, also give support to the concept of a common origin of inflow tract myocardium and PE. Mouse lines that express Cre recombinase under the control of *Nkx2.5*, *Isl1*, *Hand1*, and *Msp1* all showed labeling of both the myocardium and the PE [75–80]. Since none of these genes are expressed in the PE suggests that labeling of these cells is due to an expression before these cells actually enter the PE. All of this cited work is compatible with a model in which inflow tract myocardium and at least a subset of PE cells have a common origin and are probably derived from a common precursor in the splanchnic mesoderm that enters the heart from the second heart field.

Loss of function mutations in cardiac transcription factors affect PE formation

Interestingly, *Nkx2.5* but not *Isl1* is required for normal PE development. Loss of *Nkx2.5* resulted in a severe reduction of WT1 labeled PE cells. However since heart development is severely disrupted in *Nkx2.5* null mutants, a secondary effect cannot currently be ruled out [78]. Another important cardiac transcription factor that is expressed in the precardiac mesoderm but also in the PE is *Gata4*. Loss of *Gata4* results in a complete loss of PE formation [81]. However due to the broad expression of *Gata4* in the myocardium as well as in the forming liver endoderm, the loss of PE formation could be due to a cell autonomous function or secondary to aberrant development in these surrounding tissues. Loss of *Hand2* using a *Hand1*-Cre line did not affect PE formation, but nonetheless resulted in an epicardial phenotype characterized by defective epicardialization and a failure to form coronary arteries [82]. A more severe phenotype was observed when the function of *Hand2* was analyzed in zebrafish embryos. In *Hand2* mutants a complete absence of the PE was observed [35]. However, since the heart was severely affected in this mutant, it is possible that the observed loss of PE formation is secondary to the myocardial defects.

In conclusion the loss of several myocardial transcription factors severely affects PE development. This either can be interpreted as further support for a common origin of the PE and the venous myocardium, but could also be indicative of an inductive relationship between the two tissues. However the loss or ectopic positioning of the PE could be secondary to aberrant inflow tract formation.

The liver bud endoderm might act as a PE inducer

The PE develops in close proximity to the liver bud endoderm [1,6,9,40]. Interestingly, when a donor quail liver bud is implanted in the posterior-lateral regions of host chick embryo, the expression of some PE marker genes can be induced in host mesodermal cells ectopically at the site of implantation [4]. This work suggests a potential role of liver bud endoderm-derived paracrine signals in PE induction (Fig. 3). Since the PE is closely apposed not only to the liver but also to the sinoatrial myocardium, both of these tissues have been proposed to play a role as inducers of PE development [9]. Since induction of ectopic PE marker gene expression can be triggered by a liver implant alone without co-implantation of myocardial tissue, this suggests that one or more

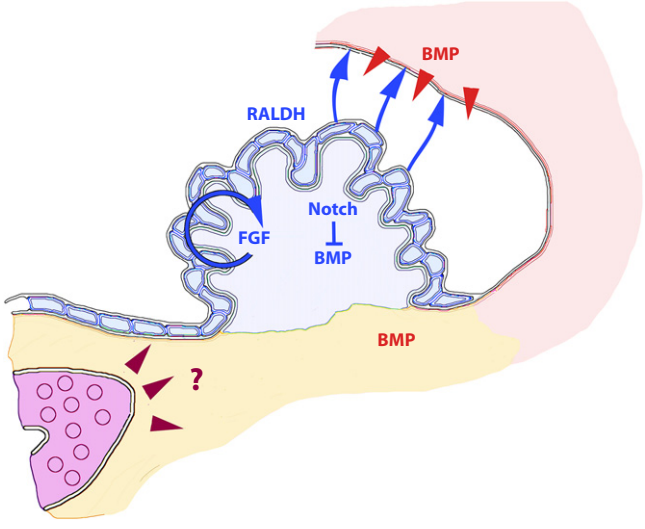

Figure. 3 Signaling molecules involved in PE specification and secondary tissue bridge formation. The liver (pink structure) secretes presently unknown PE inducing molecules. BMP is expressed in the sinus horn myocardium and weakly in the PE, and is required to maintain PE-specific gene expression. Several elements of the Notch signaling pathway are expressed in the PE. Notch suppresses BMP expression in the PE and thereby preventing *trans*-differentiation of PE cells into cardiac myocytes. FGFs are expressed in the PE and are responsible for cell survival and stimulating cell proliferation. Raldh2 is expressed early during PE formation, however no specific function during PE formation has been established for retinoic acid. BMP is also expressed in the AV canal myocardium and act as a chemoattractant to establish secondary tissue bridge formation.

liver-derived signal(s) is sufficient for the induction of some PE marker genes. A stringent test for the model of liver-derived signals as inducers for PE formation is to study this in a genetic model with impaired liver bud formation. While such studies have not yet been performed in chick or mice, in zebrafish the liver primordium seems not to be required for PE development [35]. PE specification still occurs in two zebrafish mutants, in which hepatoblast specification is significantly delayed or completely absent. These findings are consistent with the fact that zebrafish PE is not located close to the liver when it arises and suggests that the role of the liver as a source for PE-inducing factors is not evolutionary conserved.

Bmp2 and 4 are involved in PE formation

In the chick embryo both, *Bmp2* and *Bmp4* are expressed in the venous pole myocardium (*Bmp2*) or in the PE itself (*Bmp4*) (Fig. 3) [12,74]. Additionally some other BMPs (*Bmp5*, -7, and -10) are also expressed in the inflow tract myocardium [74]. In vitro and in vivo data suggest that BMP signaling is required to maintain PE marker gene expression [12]. Significantly *Bmp4* is expressed in the PE itself and by blocking BMP signaling in PE explant cultures leads to a loss of PE marker gene expression, which suggests an important autocrine function of BMP for maintaining PE identity [12]. The role of BMP formation appears to be evolutionary conserved. In the mouse, *Bmp2* expression is present in the venous pole myocardium, while *Bmp4* is expressed in the PE [83]. Moreover, proepicardial expression of *Gata4* expression is BMP-dependent [84]. In zebrafish embryos with impaired BMP signaling *Tbx18* and *Tcf21* gene expression was lost [35]. Interestingly, gain of function studies using an inducible *Bmp2* expression system resulted in induction of ectopic *Tbx18* expression, but not *Tcf21*, suggesting that BMP signaling alone was insufficient to completely commit cells to a proepicardial fate. Overexpression of *Tcf21* could also induce *Tbx18*, but this required BMP signaling input, consistent with a model in which at least two signals (of which one is BMP) are required for proepicardial specification.

BMP signals and cell fate decisions at the venous pole

BMP signaling might also be involved in the decision whether lateral plate mesoderm will enter the myocardial or proepicardial cell lineage. Two independent studies observed that cells in proepicardial explant cultures undergo cardiac myocyte differentiation when BMP is added to the culture medium [12,74]. It therefore has been proposed that the level of BMP signaling needs to be precisely controlled and that the BMP level is involved in cell fate decisions at the venous pole [12]. If BMP levels exceed a certain threshold epicardial marker genes are lost, while myocardial marker genes are induced. Consistent with this observation made in PE explants cultured in serum-free medium was the observation made in a mutant with impaired Notch signaling [85]. In this mutant ectopic proepicardial expression of *Bmp2* and enhanced levels of P-SMAD1/5/8 were described which resulted in ectopic myocardium formation within the mesenchymal core of the PE. This suggests that Notch signaling might be upstream of *Bmp2* preventing it of being expressed in the PE. Although several elements of the Notch signaling pathway are expressed in the mouse PE [83], there is no evidence for the essential requirement of Notch signaling other then preventing ectopic *Bmp2* expression in the PE (Fig. 3) [83,86]. It is likely that Notch also has an important secondary role in coronary artery formation later during development. At this stage Notch probably prevents precocious smooth muscle differentiation by regulating TGFβ and PDGF signaling [86] and is also involved in arterial endothelium commitment and differentiation [83].

FGF signals control proliferation and villous outgrowth of the PE

Several FGF genes are expressed in the inflow tract myocardium and in the PE itself [12,74]. *Fgf8* is expressed in the inflow tract myocardium, while *Fgf2* and *Fgf10* are present in the PE. Torlopp et al. observed an important role of FGF signals in promoting PE cell precursor expansion leading to villous outgrowth of the PE (Fig. 3) [13]. These in vivo data were corroborated by experiments using explant cultures demonstrating a massive proliferative response after adding bFGF to PE explant cultures, while loss of endogenous FGF signaling resulted in a loss of cell proliferation and induced apoptosis [13]. Interestingly, the authors found no evidence for a role of FGF signaling for proepicardial recruitment since PE marker gene expression was not affected by loss of FGF signaling. In contrast, van den Hoff and co-workers recently proposed a model in which FGF signals through an Erk-mediated FGF2 signaling pathway would drive lateral plate mesoderm cells into the direction of

PE formation, while preventing them from being recruited into the myocardial lineage [73]. Although this model appears plausible and consistent with a common origin of myocardial and PE progenitors, it is mainly based on explant culture data and global FGF pathway inhibition, which at least in principle could have unspecific side effects. Interestingly recent analysis of an *Fgf3*/ *Fgf10* double null mutants in mice revealed normal proepicardial specification, while epicardium formation was affected, which is consistent with a role of *Fgf10* for proepicardial cell proliferation but not supportive of a role in proepicardial specification [87].

Wnt signals in PE formation

The role of Wnt signals in the context of proepicardium formation has thus far only been poorly studied. Several Wnt pathway genes are expressed in the proepicardium including *Wnt2a* and *Wnt5b*, the Frizzled receptors *Fzd1*, *Fzd7*, *Frzb*, *Dkk1*, *Dkk2* and *Wif1* [88]. There is evidence that *Wif1* appears to be involved in allocating lateral plate mesoderm precursor cells to enter either the PE or myocardial lineage [88]. Moreover in the double mutant of the Wnt antagonists *Dkk1* and *Dkk2*, a hypercellular proepicardium has been described [89], suggesting that Wnt signaling plays an important, but not yet fully defined role in cell fate allocation at the venous pole. Wnt signaling continues to be important at later stages and has been implicated in the communication between myocardium and epicardium [90] and in the maturation of the epicardium and recruitment of epicardial cells to the smooth muscle lineage [91]. *Wnt5a* appears to be downstream of *Wt1* and has been genetically implicated in the growth control of the ventricular wall [45]. At present the full complement of inducing factors that are involved in specifying PE formation have not yet been fully elucidated.

FACTORS REGULATING MYOCARDIAL-EPICARDIAL INTERACTION

After the PE has developed, it extends villous protrusions that attach to the atrioventricular (AV) junction on the inner curvature of the looping heart by HH stage 18. Attachment at the AV junction is guided by strands of extracellular matrix spanning the pericardial coelomic cavity between the PE and myocardial surfaces [6,29]. In addition to a directional guidance for correctly positioning the tissue bridges towards the lesser curvature, the extracellular matrix bridge may either store or concentrate signaling molecules that direct PE cells to the heart. One of the guidance cues appears to be BMP2 [92] (Fig. 3). The AV myocardium shows high-level expression of *Bmp2* and to a lesser extent also *Bmp5*, an expression pattern that is evolutionary conserved and is also found in mouse and zebrafish. Myocardial colonization of PE cells is blocked by Noggin implantation and is ectopically targeted to other myocardial territories by ectopic BMP expression [92]. In the chick, the transcription factor *Tbx5* is expressed in the PE and retrovirus-mediated overexpression of human *Tbx5* as well as antisense-mediated knockdown of chick *Tbx5* diminishes PE-derived cell transfer to the heart, and their incorporation into the nascent epicardium and coronary vasculature [93]. These findings suggest that *Tbx5* controls the migratory activity of PE cells.

Genetic evidence suggests that myocardium-PE interaction in the mouse requires precisely regulated cell–cell interaction. The molecules involved in this interaction are α_4-integrin (*Itga4*), which is expressed in PE cells, and *Vcam1*, which is expressed in myocardial cells. Loss of function mutations of either gene gives rise to defects in attachment of the PE cells to the heart [27,94,95]. Thus, more active processes such as binding of PE cells to extracellular matrix and cell–cell adhesion may be involved in translocation of PE cells to the heart.

The planar cell polarity pathway is also required for the cell transfer of PE cells to the myocardium. Loss of *Par3* in mice leads to impaired cell transfer [28]. PAR3 forms a conserved protein complex with PAR6 and aPKC, and this complex is crucial for establishing epithelial cell polarity by regulating junctional structures. PE formation is normal in the *Par3* mutant suggesting that the PCP pathway is not important for villous outgrowth but is required to generate cell polarity in the PE cells and make them competent to interact with the myocardium. Genetic evidence in zebrafish also suggests an important function of the PCP pathway for PE formation. In the case of loss of function experiments targeting two PCP genes, this was found to result in aberrant PE formation [36]. PCP is also thought to be involved in the decision of whether a cell will remain part of the epicardium or undergo EMT and migrate into the myocardial wall [96].

EPICARDIUM FORMATION AND PATTERNING OF THE MYOCARDIUM

Epicardium formation begins upon contact of PE cells with the myocardium. In the chick embryo the initial contact of the PE villi with the myocardium occur at HH stage 16, giving rise to the PE tissue bridge [1,11,39,97,98]. After contact has been established, the tips of PE villi open up, allowing PE cells to spread out to form the epicardial epithelial sheet on the surface of the myocardium with its original apical surface facing to the pericardial coelomic cavity [6]. In the mouse, epicardial sheet formation begins when PE cells attach to the myocardial surface at 9dpc [24]. PE cells flatten out forming epithelial islands on the surface of the myocardium, which fuse to generate a confluent epicardial layer at 11 days post coitum (dpc).

Paracrine signals originating from the developing epicardium play a critical role for myocardial growth during heart development [99–101]. The early myocardium consists of an outer, highly mitotic, compact zone and an inner, trabecular zone with lower mitotic activity. Both microsurgical and genetic inhibition of epicardium formation gives rise to a decrease in myocyte proliferation, accounting for a thin-walled compact myocardium [2,44,94,95,99,102–106]. Genetic data in the mouse identified a non-cell autonomous role for epicardium-derived retinoic acid and erythropoietin (EPO) signaling, which are both involved in compact layer expansion [103,104,107]. Similar conclusions have been obtained for the chick heart [108]. Recent data in the mouse have further modified this model of growth control of compact layer expansion. Novel data suggests that retinoic acid signaling is not actually active in the epicardium but in the liver, stimulating in this tissue the secretion of EPO, which binds to the epicardial EPO receptor, resulting in the secretion of Igf2, which acts as a ligand involved in compact layer expansion [109,110]. In addition fibroblast growth factor (FGF) receptor-mediated signaling is important for myocyte proliferation in the developing heart [100,101,111,112], whereby retinoic acid signaling induces epicardial FGF secretion [90,113]. In summary, a complex interplay between the liver, the epicardium and the myocardium is essential for myocyte proliferation in the compact myocardium.

EPICARDIAL LINEAGES, ORIGIN AND FATE OF PE CELLS

Retroviral mediated single cell labeling in the chick embryo provided first evidence that epicardial cells produce coronary vascular smooth muscle cells, cardiac fibroblasts and endothelial cells [114]. Subsequent lineage studies on individual PE cells gave further support to the fact that all coronary vascular cell types arise from the PE and begin to enter the developing heart along with the growing epicardium [18]. The retroviral cell lineage data have in principal been confirmed by chick/quail chimera studies [20,22,115] and with implantation of adenovirally-tagged proepicardial cells [19]. The work by Poelmann et al. however questioned proepicardial origin of the endothelium and suggested it to be derived from the neighboring liver [22] (Fig. 4). At present the embryonic origin of the endothelial cell lineage has not been fully elucidated.

Recent work in the mouse suggests that the PE in this species possibly does not contribute to the endothelial cell lineage. Red-Horse et al. show that coronary vessels, including coronary arteries, actually derive from the *sinus venosus* endothelium [116]. Using tissue recombination experiments and clonal analysis these authors have ruled out any contribution of proepicardial cells to the coronary vasculature. Based on their data the authors proposed a model by which endothelial cells sprouting from the sinus venosus endothelium dedifferentiate loosing their venous identity as they extend from the *sinus venosus* and subsequently redifferentiate and remodel into coronary arteries, capillaries and veins depending on the tissue environment (Fig. 4). Support for this model comes from data using genetic lineage tracing systems which have shown that EPDCs give rise to fibroblasts and coronary smooth muscle cells but not to endothelial cells (*Tbx18*-Cre) and that only a minority of endothelial cells are EPDC-derived (*Wt1*-Cre) [117–119]. In contrast to these findings, in another report *Wt1*-Cre mediated epicardium-specific ablation of *Notch1* resulted in a complete loss of arterial vascular endothelial cells, while the venous endothelium displayed only minor defects, which suggests that possibly the venous and arterial endothelial cells might have different embryonic origins [83]. Although the origin of the vascular endothelium remains controversial, it is evident from current data that when the PE has fully formed in the chick embryo, cells with vascular differentiation potential are present [120]. It is however possible that in particular *Tbx18* expressing mesothelial cells do not contribute to this lineage. It has been proposed that cells with a vascular potential migrate into the PE from another organ such as the liver [22] (Fig. 4). Cells that enter the PE might potentially turn on *Wt1* expression and thereby are affected in *Wt1*-Cre mediated gene ablation

experiments. Differences in the results of fate mapping studies between chicks and mice might be explained by the fact that in mice only the fate of the PE has been mapped, while in chicks the fate of the proepicardial serosa has been mapped [20].

Two independent studies observed that proepicardial explants are able to undergo cardiac myocyte differentiation in vitro [12,74]. Not all PE-derived cells are able to differentiate into cardiac myocytes, only a small subpopulation of cells are converted. This suggests that some cells within the PE are not fully committed to a mesothelial cell fate, the most likely candidates are the mesenchymal cells in the core of the PE (Fig. 4). As previously pointed out, the mesoderm that borders the PE as well as the sinus myocardium likely has a bi-potential fate to either become cardiac myocytes or participate in PE formation.

Quail-chick chimeras have provided no evidence for the existence of PE-derived cardiac myocytes [20]. Likewise retroviral cell labeling was unable to reveal any contribution of PE cells to the cardiac myocyte cell population [18]. However, recent fate map studies in the mouse embryo employing *Wt1-Cre* or *Tbx18-Cre* suggested a significant contribution of EPDCs to the myocardial cell lineage [118,119]. In the case of *Tbx18*, an alternative explanation for this finding has recently been provided. A subpopulation of cardiac myocytes which are present in the left ventricle display endogenous expression of *Tbx18* [121]. Thus, the cardiac myocyte labeling by *Tbx18*-Cre was erroneously interpreted as evidence for PE-derived cardiac myocytes in the mouse heart. Genetic fate mapping in the zebrafish heart have established that EPDCs do not contribute to the myocardial cell lineage during embryonic development or cardiac regeneration [122]. Interestingly, *Tbx18* and *Wt1* were judged to be unsuitable for genetic fate mapping of EPDCs since regulatory sequences from both genes drive expression in epicardial and non-epicardial tissue including cardiac myocytes. Thus caution should be taken with the interpretation of lineage-tracing data in the mouse.

While the proepicardial origin of cardiac fibroblast and smooth muscle cells is undisputed, it is unclear whether both cell types are generated form the same precursor. Recent evidence in the mouse demonstrated a specific effect on the fibroblast population after ablating *Pdgfra*, suggesting that both cell types have different signaling requirements [123]. This is consistent with the cell labeling experiments in the chick embryo, which suggested an early separation of both cell lineages [18].

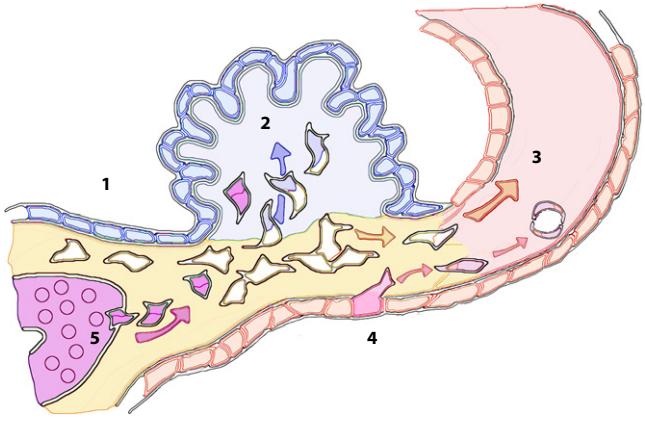

Figure. 4 Model of proepicardial cell lineages. **(1) The PE is mainly derived from pericardial mesothelium (blue) and underlying mesenchymal cells of the sinus venosus (yellow). (2) This pericardial mesothelium forms villous protrusions, which are generated by the proliferation of mesothelial cells at the base of the PE, recruitment of sinus mesenchyme and the accumulation of extracellular matrix proteins in the PE core. This combination of processes generates the characteristic cauliflower PE morphology. The sinus mesenchyme, which is recruited to the PE can also become myocardium, therefore some of these mesenchymal cells might still retain the ability to switch back to the myocardial lineage after BMP stimulation or loss of Notch signaling. (3) The myocardium of the inflow tract (red) continuously grows by proliferation and recruitment of sinus mesenchyme thereby elongating the heart tube. (4) The endothelium of the inflow tract partially dedifferentiates and invades the myocardium to form coronary veins, which connect the epicardium-derived coronary arterial vessels with the inflow tract of the heart. (5) The liver primordium might be the source for the coronary endothelium, which enters the PE and is thereby transferred to the heart to generate the arterial vasculature.**

THE EPICARDIUM AND CARDIAC REGENERATION

Embryonic hearts and neonatal mouse hearts up to 7 days of postnatal development retain the ability to regenerate in response to injury [124,125]. However, the adult mammalian myocardium is unable to substitute contractile tissue, which is lost after an ischemic insult. In contrast, hearts of lower vertebrates such as newts and zebrafish retain this ability even into adulthood [126–129]. Shortly after surgical resection of parts of the zebrafish heart, a fibrin clot forms which subsequently is substituted by myocardial cells that fully reconstitute the ventricle within two months. Significant to this review, there is evidence for rapid activation of epicardial cells in the zebrafish heart in response to ventricular wounding [130]. While the epicardium of the non-injured heart does not express any of the established marker genes of the embryonic epicardium such as *Tbx18*, *Wt1*, or *Aldh1a2*, these genes are rapidly reactivated after injury. Moreover there is a rapid activation of cell proliferation and subsequently also migration towards the injured area. A similar epicardial response is also seen when surgical resection of ventricular tissue is substituted by cryoinjury [131–133]. While a material contribution of epicardial cells to the ventricular myocardium has now been excluded [122,134,135], the epicardium is essential for vascularizing the regenerating tissue mass in the zebrafish heart.

Growth factor signaling in the regenerating zebrafish heart

In order to recruit epicardial cells into the injured area, *Fgf17b* is upregulated in the myocardium, while the activated epicardium expresses *Fgfr2* and *Fgfr4* [130]. Blocking immigration of epicardial cells by overexpression of a dominant negative FGF receptor prevents the completion of regeneration. Thus, despite the fact that epicardial cells do not differentiate into cardiac myocytes, they are essential for myocardial regeneration. Another mitogen involved in epicardial recruitment during cardiac regeneration in the zebrafish heart is *Pdgfb* which is produced by thrombocytes that initially close the wound after ventricular resection, while the *Pdgfrb* receptor is expressed in the activated epicardium [136]. Pharmacological blockade of PDGFRb receptor signaling blocks recruitment of EPDC in the injured area and prevents coronary artery formation. These findings in the adult zebrafish correspond well with the essential role of the platelet derived growth factor (PDGF) for coronary smooth muscle formation in the developing murine heart [137]. Epicardial-specific deletion of both *Pdgfra* and *Pdgfrb* resulted in hearts that lacked both cardiac fibroblasts and coronary vascular smooth muscle cells [123]. Interestingly, loss of *Pdgfra* resulted in a specific disruption of cardiac fibroblast development, while vascular smooth muscle cell development was not affected, suggesting a cell-type specific function for *Pdgfra*. *Aldh1a2* encoding the rate-limiting enzyme in RA production is expressed in response to ventricular wounding in the zebrafish heart [138]. Both, gain- and loss-of-function experiments reveal a permissive role in myocardial regeneration. This is reminiscent of the situation in the embryonic myocardium in the mouse where *Aldh1a2* has a non-cell autonomous function to promote myocardial chamber growth [103].

Epicardial activation in response to myocardial infarction in the mammalian heart

The exciting observations that have been made in the regenerating zebrafish heart have provoked research into the role of the epicardium in the adult mammalian heart. In particular it has been analyzed whether epicardial activation in response to injury is at all present in the mammalian heart. Indeed *Aldh1a2* is weakly activated in the epicardium of the mouse heart after myocardial infarction (MI) [138]. Likewise *Wt1* is re-expressed in the infarcted rat heart and found in coronary endothelial and smooth muscle cells in the border zone [139]. Since *Wt1* is regulated by Hif1α, the activation in the border zone may reflect the ischemic environment rather than being an indication of epicardial activation and cell recruitment [140]. With the help of a tamoxifen inducible Cre-ERT1 fusion protein under the control of the *Wt1* promoter, the fate of epicardial cells after MI was followed [141]. MI induces a massive induction of epicardial marker genes, and also induces EMT, which results in a thickening of the epicardial cell layer. Adult EPDCs do not migrate into the myocardial wall and do not differentiate into endothelial cells or cardiac myocytes but form fibroblasts, myofibroblasts, smooth muscle cells, and pericytes. These cells also secrete a cocktail of proangiogenic growth factors, which might be exploited to ameliorate the outcome of a MI. Another recent study employing a sensitive fluorescent Notch reporter gene identified EPDCs that are activated in response to occlusion of the left anterior descending (LAD) coronary artery or pressure overload [142]. Consistent with the *Wt1* reporter, the fate of Notch activated epicardial cells was also to differentiate predominantly into cardiac fibroblasts. Thus taken together there is evidence for an important role of epicardial cells in the healing process after MI but like in the zebrafish heart, no contribution to the myocardium was demonstrated by any of these studies.

Thymosin $\beta 4$ stimulate invasion of epicardium-derived cells into the ventricular wall resulting into de novo myocardium formation

The subepicardial mesenchyme might constitute a niche for stem cells in the heart [143,144]. It has long been postulated that resident myocardial progenitor cells might originate from EPDC, however it has been difficult to prove their myocardial potential. Very recent data suggests that if sufficient numbers of EPDCs are activated in response to myocardial wounding, some of these cells are able to differentiate into cardiac myocytes. Thymosin $\beta 4$ is a G-actin monomer binding protein implicated in actin filament remodeling and is expressed in the epicardium of the zebrafish heart after ventricular resection [145]. Loss of Thymosin $\beta 4$ (*Tmsb4x*) in the mouse results in impaired coronary artery formation [146]. Application of Thymosin $\beta 4$ to myocardial explants in vitro stimulates proliferation and migration of vasculogenic cells, while in vivo it induces thickening of the epicardium and neovascularization resulting in improved survival after MI [147,148]. A combination of MI and Thymosin $\beta 4$ treatment results in massive activation of epicardial cells which start to migrate from the epicardium into the surviving myocardium populating the uninjured myocardial wall and the border zone of the infarct. Significantly, some of these cells are able to *trans* differentiate into a functional myocardium [149]. Since a large fraction of the EPDCs however were not found to differentiate into cardiac myocytes, this leaves room for further pharmacological interventions in order to improve myocardial repopulation of an infarcted area.

Apart from regeneration in response to wounding, there is also evidence for a direct interaction of epicardial cells and cardiac myocytes in the adult heart. Thus in co-culture of both cell types, cardiac myocytes display a higher level of differentiation [142,150,151]. It is thought that some of these effects are possibly due to the secretion of endothelin by epicardial cells [152]. In this respect one needs to also mention telocytes, a recently identified population of cells, which are believed to be epicardium-derived and possibly might have an important role in cardiac homeostasis [153,154]. Thus, learning more about the role of adult epicardial cells in cardiac homeostasis and in the context of cardiac disease such as MI, hypertrophy and heart failure will be an important avenue in order to evaluate the clinical potential of this fascinating cell population [155].

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