

Review

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Review

Novel Insights into the Molecular Mechanisms Governing Embryonic Epicardium Formation

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Abstract: The embryonic epicardium originates from the proepicardium, an extracardiac primordium constituted by a cluster of mesothelial cells. In early embryos, the embryonic epicardium is characterized by a squamous cell epithelium resting on the myocardium surface. Subsequently, it invades the subepicardial space and thereafter the embryonic myocardium by means of epithelial-mesenchymal transition. Within the myocardium, epicardial-derived cells present multilineage potential, later differentiating into smooth muscle cells and contributing both to coronary vasculature and cardiac fibroblasts in the mature heart. Over the last decades, we have progressively increased our understanding of those cellular and molecular mechanisms driving proepicardial/embryonic epicardium formation. This study provides a state-of-the-art review of the transcriptional and emerging post-transcriptional mechanisms involved in the formation and differentiation of the embryonic epicardium.

Keywords: keyword 1; keyword 2; keyword 3 (List three to ten pertinent keywords specific to the article; yet reasonably common within the subject discipline.)

Origin of the embryonic epicardium

During cardiac development, the epicardium originates from an extracardiac primordium, the proepicardium (PE), which is constituted by a cluster of mesothelial cells located on the cephalic and ventral surfaces of the liver-*sinus venosus* limit in avian embryos [1–8], and the pericardial side of the *septum transversum* in mammalian embryos [9]. In early embryos, the epicardium acquires the form of a squamous cell epithelium that either rests directly on the surface of the myocardium or covers a subepicardial space that appears to be densely populated by mesenchymal cells [10]. It is also assumed that the epicardium is not a simple mesothelium but is, in fact, made up of discrete clusters of heterogeneous cell types, which include haematopoietic contribution from distinct origins, encased by an extracellular matrix (ECM) that anatomically resembles a stem or progenitor cell ‘niche’ [11].

It has been reported that PE originates in the periphery of the heart forming fields in the lateral plate mesoderm (LPM), as part of an early cardiac progenitor lineage [12]. A single PE bud is formed during zebrafish cardiogenesis [13], while in other fish -such as the sturgeons- bilateral primordia are formed, which subsequently converge into a single PE structure in the embryonic midline [14]. Noticeably, in mice, bilateral PE anlage is also established and it further develops similarly to sturgeons, while in chick embryos only the right-side anlage develops [15]. Interestingly, chicken PE arises both from the splanchnic layer of the LPM and the somatic mesoderm, which also contributes to the mesothelial portion of the PE that forms the typical villous protrusions [16–18]. The above observations suggest that the embryonic left-right signal might play a significant role during PE

development. Furthermore, while all proepicardial cells in a given species (e.g., in mouse, zebrafish and chick) are morphologically similar, they exhibit a distinct differentiation potential due to various marker expression [19–21]. Therefore, detailed composition of the embryonic epicardium is not well known.

After PE formation, cells translocate to the myocardial surface of the looping heart, where they adhere, migrate and proliferate to form a squamous epithelial layer: the embryonic epicardium [22]. It has been described that PE translocation to the myocardium takes place through distinct mechanisms among species, including direct contact and/or the release of free floating cell clusters (or cysts) into the pericardial cavity, or even PE cells migration from the *sinus venosus* towards the heart along the surface of the inflow tract [23–26]. After attachment to the myocardial surface, the cells start to migrate laterally, until the complete heart is enveloped by the epicardium [5]. It has been described that the epicardium surrounding the arterial pole does not originate from the PE, but from the coelomic/pericardial mesothelium at the area where the aortic sac leaves the pericardial cavity [27,28]. This cell population will contribute to the outer mesenchymal layer of the arterial pole within the pericardial cavity, contributing to the arterial epicardium formation, whereas the PE-derived epicardium will cover the myocardial outflow tract (Figure 1A).

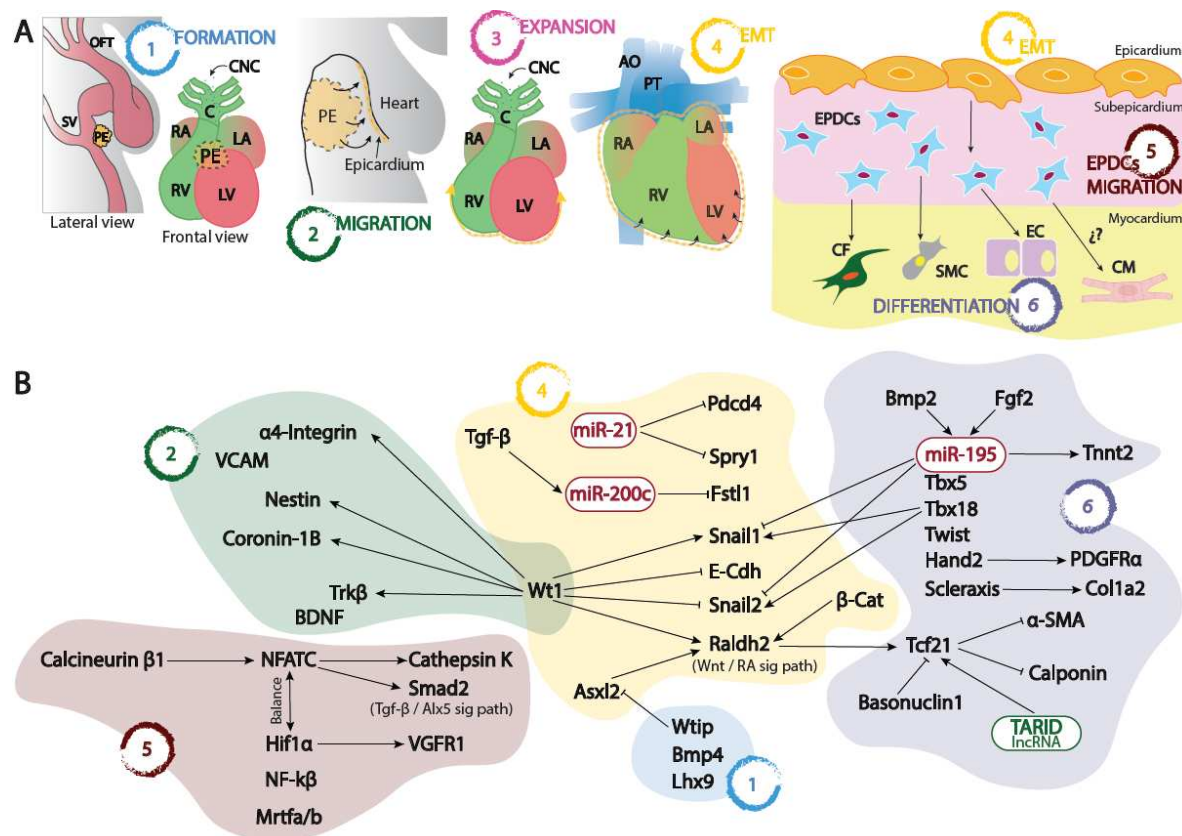


Figure 1. Panel A. Schematic representation of the proepicardium (PE) and embryonic epicardium formation since its origin at the *sinus venosus-septum transversum* (1), its migration (2) and expansion (3) into the naked myocardium, its epithelial to mesenchymal transition leading to the formation of the EPDCs (4) and finally the migration and invasion (5) of the embryonic myocardium differentiating into distinct cell types (6). Panel B. Schematic representation of the distinct transcriptional and post-transcriptional regulatory mechanisms involved in each of the distinct processes depicted in panel A. OFT, outflow tract; SV, sinus venosus; PE, proepicardium; RA, right atrium; LA, left atrium; RV, right ventricle; LV, left ventricle; CNC, cardiac neural crest, C, conus; AO, aorta; PT, pulmonary trunk; EPDCs, epicardial derived cells; CF, cardiac fibroblasts; SMC, smooth muscle cells; EC, endothelial cells; CM, cardiomyocytes; EMT, epithelial to mesenchymal transition.

Derivates of the embryonic epicardium

Once the epicardium is established, epicardial cells will be directly involved in the formation of the myocardium. A group of epithelial cells will undergo an epithelial-mesenchymal transition (EMT), giving rise to the epicardium derived cells (EPDCs), and then migrate into the matrix in the subepicardial layer to form the subepicardium [22]. The subepicardium thickness will eventually vary according to the underlying heart structure to be covered, and may vary among species. In particular, in chick embryos, the subepicardium is relatively thin in the atrial and ventricular myocardium. However, in the atrioventricular sulcus the subepicardium is thicker in order to provide those EPDCs needed for coronary formation [29,30].

From the subepicardium, mesenchymal EPDCs will form migratory processes and invade the myocardium, in a spatio-temporary regulated fashion, where several factors expressed in the underlying myocardium will define the permissiveness for EPDCs. These migrate into the underlying myocardium in a tangential pattern, and most of them are retained directly underneath the local area of the subepicardium [5,30,31].

Within the myocardium, EPDCs present multi-lineage potential, differentiating into smooth muscle cells (SMCs) and contributing to the coronary vasculature and cardiac fibroblasts (CF) of the mature heart [22,26]. Most EPDCs reach their final positions: i) around the coronary arteries as smooth muscle cells (SMCs) and adventitial fibroblasts [27,29,32–34]; ii) in the atrioventricular cushions [27,31,35]; iii) in the subendocardium of the ventricular trabeculae and atria [27,30,31]; and iv) in the ventricular myocardium as interstitial fibroblasts [31]. Other contributions of EPDCs to cardiac endothelial cells (ECs) [36–38] and cardiomyocytes (CMs) [39,40] have also been described, although this issue needs further research [41,42]. Therefore, both the *sinus venosus* and ventricular endocardium are considered major contributors to ECs [43], while EPDCs have a low contribution, if any [44]. With respect to epicardial-derived cardiomyocytes, lineage tracing studies -by using Scleraxis, WT1 and TBX18- have indicated possible epicardial-derived cardiomyocyte labeling, although their contribution is still controversial [39,41,42].

Additionally, after the embryonic epicardium has covered the developing heart, the epicardial cells will produce cytokines and growth factors in order to induce the myocardial development. In this sense, impaired embryonic epicardium development and/or cytokines and growth factor delivery results in deficient ventricular chamber maturation [45–50].

In contrast to the embryonic epicardium, the postnatal mammalian epicardium seems to be a dormant single-cell layer, since most genes involved in epicardial activation, such as WT1, Tbx18 and Raldh2 are rapidly downregulated postnatally, being scarcely detectable only during the first 3 months, in mice [51].

Transcriptional regulation of the embryonic epicardium

The development of the embryonic epicardium and its cellular derivatives is a highly complex and regulated process. In the former section it has been shown the crucial importance of the epicardial derivatives for both, the constitution of the fibrous skeleton of the heart and its vascularization. Furthermore, the epicardium and EPDCs are the origin of molecular signals towards the developing myocardium. The correct growth and compaction of the cardiac wall depend from these pathways. Thus, the precise regulation of these cellular and molecular mechanisms requires a precise orchestration of a set of transcription factors in order to activate or inhibit the genes involved in all these processes.

Transcription factors act at different levels during the development of the epicardium and its derivatives [52]. We can distinguish the development of the proepicardium and the migration of epicardial cells over the myocardium, the epithelial-mesenchymal transition (EMT) giving rise to EPDCs, the differentiation of these EPDCs mainly into fibroblasts and vascular SMCs, and the invasion of the epicardial derivatives into the myocardial wall. We will describe below the main transcription factors involved in the control of all these processes.

Proepicardial development

As we have described above, the proepicardium develops in the ventral side of the venous pole of the heart. Little is known about the molecular mechanisms governing location and development of this cluster of cells that expresses most of the characteristic epicardial genes. Liver-derived signals induce ectopic expression of proepicardial markers in chick embryos [53], and this can explain the proximity of the proepicardial bud to the developing liver. BMP4 signaling has also been proposed as a proepicardial inductor [15,54]. In *Xenopus*, the LIM homeodomain protein Lhx9 is essential for the correct position of the proepicardial organ on the *septum transversum* [55]. Its deficiency leads to proepicardial malposition and loss of attachment to the heart surface.

The hypothesis of an evolutionary origin of the proepicardium from an ancestral external glomerulus should also be considered in order to explain the mechanisms of its development [56,57]. For example, Wilms' tumor 1 interacting protein (WTIP), an interacting partner of Wilms' tumor protein (WT1) essential for the development of podocytes is also necessary for PE specification in the zebrafish heart. Overexpression of WTIP mRNA, induces ectopic expression of PE markers in the cardiac and pharyngeal arch regions [58].

Epicardial migration and epicardial EMT

The function of the Wilms tumor suppressor gene *Wt1* was originally related with the development of kidneys and gonads [59]. However, its importance for epicardial development was soon evidenced. WT1 acting as a transcriptional activator or repressor is involved in a number of genetic mechanisms leading to the transformation of the epicardium into EPDCs (reviewed in [60]). WT1 promotes the expression of integrin $\alpha 4$, a receptor for myocardial VCAM. This regulates adhesion of the epicardial cells to the myocardial wall and their migration. Other targets of WT1 are nestin (an intermediate filament) and coronin-1B (an actin-binding protein), related with cell motility and migration [61,62]. WT1 activates expression of TrkB, the receptor of BDNF, involved in coronary vascularization [63]. But the main processes activated by WT1 in the epicardium are directly related to the core mechanisms of the epithelial-mesenchymal transition. These are the Snail/E-cadherin and the Wnt/retinoic acid pathways.

WT1 is a direct transcriptional activator of Snail1 and a repressor of E-cadherin in mice [64]. Snail1 plays a pivotal role in the regulation of EMT in mammals, through repression of epithelial genes and activation of genes related with mesenchymal phenotype and cell motility. Repression of E-cadherin also leads to loss of the epithelial phenotype. However, it has been shown that Snail1 silencing in the epicardium does not impair the epicardial EMT [65], suggesting that this process can be activated by different ways. In fact, Snail2 have also been suggested as involved in the EMT of the murine epicardial cells, although this transcription factor would be activated by Tbx18 and inhibited by WT1 [66]. On the other hand, there is evidence of the importance of Snail1 in avian epicardial EMT [67]. Thus, the roles of Snail1 and Snail2 as direct executors of the epicardial EMT, repressing the epithelial phenotype and promoting a mesenchymal phenotype, need further investigation.

Retinoic acid and the canonical Wnt pathway have also been involved in the epicardial EMT. WT1 has a significant role in the retinoic acid signaling pathway since it promotes the epicardial expression of RALDH2, a key retinoic acid-synthesizing enzyme highly expressed in the epicardium [68]. Retinoic acid promotes a cytoskeletal rearrangement in epicardial cells necessary for EMT in a RhoA-dependent fashion [69]. Systemic loss of the nuclear receptor α RXR leads to malformation of the epicardium and deficient EMT [70]. Conditional deletion of this receptor in the epicardium causes a similar phenotype [47]. These authors identified a retinoid-dependent *Wnt* signaling pathway cooperating in the epicardial EMT. In fact, β -catenin is essential for this process [71]. Thus, in parallel with the role played by WT1 in the control of Snail1 and E-cadherin expression, WT1 regulates epicardial EMT through canonical Wnt, non-canonical Wnt, and retinoic acid signaling pathways [72].

The retinoic acid signaling pathway must be carefully regulated both positive and negatively for a correct epicardial and coronary vessel development [69]. The above mentioned factor WTIP, a WT1 partner expressed in the proepicardium and epicardium, can be relevant in the inhibition of this

pathway. WTIP blocks ASXL2, a chromatin factor highly expressed in the heart that promotes the retinoic acid signaling [73].

Invading the myocardium

A key event in the epicardial contribution to heart development is the invasion of the myocardial wall by the EPDCs. This is necessary in order to establish the fibrous skeleton of the heart and to organize the complex vascularization of the myocardium. The transcription factor NFATC1 becomes activated and moves to the nucleus when it is dephosphorylated by calcineurin. NFATC1 is required for myocardial invasion of EPDCs, by induction of cathepsin K expression, an enzyme that degrades extracellular matrix. Loss of NFATc in murine EPDCs causes loss of cathepsin K expression in the myocardial interstitium and embryonic death. The mutant embryos show reduced coronary vessel and fibrous matrix penetration into myocardium [74]. Conditional depletion of calcineurin b1 (a NFAT activator) in the epicardium also provokes defects in the coronary smooth muscle [75]. This study also showed a direct role for NFATc in the transcription of Smad2, another transcription factor necessary for transduction of TGF β -Alk5 signaling.

The hypoxia inducible transcription factor-1 α (HIF-1 α) seems to be a negative regulator of the myocardial invasion by EPDCs. Expression of constitutively active HIF-1 α into the embryonic avian epicardium reduced EPDCs migration into the myocardium probably through upregulation of the VEGFR1, a decoy receptor that sequesters VEGFR [76]. A balance between signals promoting and inhibiting myocardial invasion by EPDCs may be necessary for a correct patterning of the coronary vasculature.

The protein NF κ B plays also a role in the acquisition of invasive ability of epicardial cells. Epicardial cells incubated with an inhibitor of NF- κ B signaling cannot invade a collagen gel in response to TGF β 2 or BMP2. TGF β 3-null mice fail to activate the NF κ B pathway in the epicardium and show reduction of EPDCs and coronary vascularization [77].

Finally, the myocardin-related transcription factors MRTF-A and B have been related with the control of EPDCs motility and the activation of the migratory and invasive program. Conditional ablation of MRTF in the epicardium leads to a decreased migration of EPDCs and subepicardial haemorrhage due to depletion of pericytes, a cell type derived from the EPDCs in an MRTF-dependent process [78].

Differentiation of EPDCs

The EPDCs mainly contribute to fibroblasts and coronary smooth muscle. It is uncertain if different lineages of EPDCs are already established in the proepicardium or if the EPDCs are multipotent and differentiate in response to local cues [79,80]. A number of transcription factors, mainly belonging to the bHLH family, are critically involved in the control of their differentiation.

The bHLH protein TCF21 (aka epicardin, POD1, capsulin) is required for normal epicardial development and it regulates EPDCs differentiation (reviewed in [81]). TCF21 loss of function leads to premature differentiation of EPDCs. TCF21 heterodimerizes with E12, another bHLH factor, for repression of transcription. In the cardiac interstitium downregulation of TCF21 leads to differentiation of EPDCs into smooth muscle, while persistence of its expression promotes fibroblast identity. Although targets of TCF21 have not been identified, in mesenchymal cells the smooth muscle markers SM22a, calponin and α SMA are targets repressed by TCF21, suggesting a similar role in EPDCs. Interestingly, retinoic acid activates expression of TCF21, thus keeping the EPDCs in an undifferentiated state [82]. This repressor role of TCF21 on the EPDCs has also been demonstrated in *Xenopus* [83]. Loss of TCF21 function in zebrafish also reduces FGF and VEGF signaling in the heart, reducing myocardial growth [84].

The epicardial expression of TCF21 is negatively regulated by basoonuclin-1, a zinc-finger transcription factor, and this can be related with the balance in the differentiation of fibroblasts and smooth muscle cells. Loss of basoonuclin-1 in epicardium derived from human pluripotent stem cells leads to a predominance of TCF21+ cells, and a reduction of smooth muscle progenitors [85].

The T-Box transcription factor Tbx18 has also a role in maintaining the progenitor status of EPDCs, similar to that described above for TCF21. The role of Tbx18 in murine epicardial EMT is controversial, it may induce the process by activation of Snail2 [66], but other studies consider Tbx18 dispensable for epicardial EMT [86,87]. However, Tbx18 has a critical role to avoid the premature differentiation of smooth muscle cells. Tbx18-null mice show defects in the remodeling of the coronary vascular plexus and alteration in the expression of genes related with vascularisation [86,87]. Hypoxia provokes upregulation of Tbx18 in the epicardium, inducing the expression of Snail1 and enhancing EMT and motility [88]. Another T-Box factor, Tbx5, probably plays also a role in epicardial development, since its deletion in the proepicardium leads to low production of EPDCs, reduced migration into the myocardial wall, and low density of the coronary vessels [89].

Other bHLH transcription factors are involved in the origin and differentiation of EPDCs. For example, Twist1 is expressed in EPDCs of avian embryos and it promotes mesenchymal cell proliferation and migration [90]. Scleraxis is expressed in a proepicardial subdomain and during the early stages of the epicardium. The loss of function in mice leads to persistent expression of EMT markers, suggesting a role in the differentiation of EPDCs, mainly towards fibroblasts [91]. In fact, Scleraxis induces the expression of Col1a2 in adult cardiac fibroblasts [92]. Finally, Hand2 is necessary for a normal development of the epicardium, where it activates PDGFR α [93]. This receptor of PDGF, is required for epicardial EMT and differentiation of EPDCs [94].

Other transcription factors involved in epicardial development

A number of transcription factors have been recently identified as participating in different phases of epicardial development. For example, the SRY-box protein Sox9 is expressed in EPDCs and it must play some role in epicardial EMT since its overexpression rescues the defective EMT caused by PDGF receptor ablation in the epicardium [94]. This role appears to be dispensable, since Sox9 loss of function has no consequences in this process.

The epicardial deletion of GATA4 and GATA6 leads to a drastic reduction of the number of coronary endothelial cells [95]. The replacement of GATA4 by GATA6 in the systemic GATA4-null mice does not rescue the epicardial phenotype suggesting that they are not playing redundant roles in this tissue [96].

CDX1, a caudal-related family member, has recently been involved in epicardial EMT. Its expression promotes EMT, but a low-dose CDX1 is required for enhanced migration and differentiation of EPDCs into vascular smooth muscle. Both, continued high-level expression of CDX1 or CDX1 deficiency reduce the ability of EPDCs to migrate and to differentiate [97].

The kinases LATS1 and LATS2 are important regulators of cell fate. Epicardial deletion of LATS1/2 in mice embryos is lethal due to defective coronary artery remodelling. These kinases inhibit the transcriptional function of the factor YAP, a Hippo pathway effector that prevents EPDCs differentiation into fibroblasts [98]. In fact, YAP inhibition reduces proliferation in EPDCs [99]. These studies reveal the involvement of the Hippo signaling pathway in the regulation of the EPDCs differentiation.

Finally, TFEB, a member of the microphthalmia-associated transcription factor family has been involved in the negative regulation of the epicardial EMT by activation of the TGIF1 promoter. TGIF1 (thymine-guanine-interacting factor 1) is a repressor of the TGF β /SMAD signaling pathway [100]. Thus, epicardial overexpression of TFEB is lethal due to defective EMT.

Post-transcriptional control of epicardial development

Transcriptional regulation is main molecular mechanism driving cell specification and determination during embryonic development. The identification of novel players, i.e. non-coding RNAs, that modulate post-transcriptional gene regulation has added an additional layer of complexity to the understanding of the molecular mechanisms driving these morphogenetic processes. Non coding RNAs are currently classified according to their length into two subclasses, a) small non-coding RNAs (<200nt) including herein piwiRNAs, microRNAs, snoRNAs among other and long non-coding RNAs (>200nt), including herein lncRNAs and circRNAs [101,102]. microRNAs

represent the most abundant and well-studied class of small non coding RNAs. microRNAs are nuclearly encoded and transcribed, exported to the cytoplasm when maturation occurs. Mature microRNAs exert their function by base-pair complementary with target transcripts leading to RNA instability and/or translation blockage [103]. On the other hand, lncRNAs are also nuclearly transcribed but they can exert their function both within the nucleus as well as in the cytoplasm [104,105]. Finally, circular RNAs (circRNAs) are normally generated by exon-exon back-splicing and they have been found in a wide range of eukaryotic species, exerting a variety of biological functions, upon which is particularly relevant their function as microRNAs sponges [106].

Multiple evidences have demonstrated that non-coding RNAs, including particularly herein both microRNAs and lncRNAs display differential expression profiles in homeostasis and pathological conditions [107–109]. Within the cardiovascular field, multiple evidences have demonstrated tissue-specific expression in both normal and pathological conditions [110–112], as well as, during cardiac development and regeneration [113–116]. Similarly, ample evidence is available about the functional role of distinct microRNAs and lncRNAs during both cardiogenesis as well as in distinct pathophysiological conditions, such as cardiac structural and arrhythmogenic diseases [117–121]. However, in the context of PE and epicardial development, limited information is yet available.

First evidence on the functional role of microRNAs was reported by the seminal work of Singh et al. [122] demonstrating that conditional deletion of *Dicer*, a key microRNA processing exonuclease in the developing epicardium was essential for correct development of the coronary vessels in mice. Subsequently, several studies have provided additional evidences on the role of microRNAs in key epicardial-derived biological processes such as epithelial-to-mesenchymal transition (EMT) [123–125], cardiac tissue repair [126–128] and cardiomyocyte proliferation [129,130].

EMT is required for the colonization of the embryonic subepicardial space emanating from the nascent embryonic epicardial layer, leading to the formation of EPDCs. Bronnum et al. [131] identified miR-21 as a key microRNA regulating *Pdcd4* and *Spry1* and thus controlling fibrogenic EMT while more recently Pontemuzzo et al. [132] reported that Tgf- β 1 induced EMT resulted in miR-200c inhibition that, in turn, modulated *Fstl1* impacting thus on mouse epicardial cell transition.

Epicardial deployment is essential for myocardial wall growth, as indicated in previous sections. Absence or impaired development of the epicardial layer results in thin myocardium and reduced compact myocardium development [133]. Jang et al. [134] recently reported that HDAC3 regulation of miR-322 and miR-503 in the epicardial layer is essential for the modulation of two distinct growth factors, i.e. *Igf2* and *Fgf9*, that if impaired restrict myocardial growth.

Activation of the epicardial layer is required for cardiac repair in different species. Curiously, proepicardial cells, if cultured in isolation, can spontaneously generate beating cardiomyocytes, a process that is promoted by Bmp and halted by Fgf signaling [135]. Furthermore, adult epicardial cells, if primed with thymosin β 4, can differentiate into mature and fully functional integrated cardiomyocytes in adverse conditions, i.e. in myocardial infarction, yet in a very limited yield [136]. All these evidences nonetheless support the notion of a key role of the epicardial cells in cardiac regeneration. Dueñas et al. [137] demonstrated that Bmp and Fgf regulated miR-195 expression and furthermore miR-195 administration could promote PE to enhance cardiomyogenesis, a process modulated by Smurf1 and Smad3 in chicken explants. More recently, Garcia-Padilla et al. [138] reported that while Bmp and Fgf signaling could similarly modulate miR-195 expression in mouse PE explants, they failed to augment cardiomyogenesis, reporting thus species-specific differences in this microRNA modulated pathway.

Cardiomyocyte proliferation represents a key biological cornerstone to heal the broken heart. Diverse experiments in different species have reported an essential role of the epicardium promoting cardiomyocyte proliferation in several cardiac injury models [139–142]. Del Campo et al. [143] reported that epicardial-derived extracellular vesicles (EVs) loaded with cardiomyogenic reparative microRNAs, i.e. miR-30a, miR-30e, miR-27a and miR-100, were capable of inducing cardiomyocyte cell cycle reentry in a mouse model of myocardial infarction. Similarly and more recently, Zhu et al.

[144] reported that lncRNA TARID could modulate Tcf21 transcription factor function in EPDCs leading to improve cardiac function in a mouse model of myocardial infarction.

In sum these studies reported the emerging role of non-coding RNAs modulating key biological processes orchestrated by or with contribution of the epicardium. In coming years, we will witness increasing evidence of the functional role of these types as well as of other types of non-coding RNAs, including therein lncRNAs and circRNAs in epicardial development in homeostasis and disease.

Conclusions and perspectives

For a long time, it was believed that the epicardium derived from the outer layer of the embryonic heart, and so labelled as 'epimyocardium'. However, more recent studies have revealed an extracardiac origin of the epicardium - derived from a proepicardium located at the posterior end of the cardiac tube- together with evidence of an epicardial EMT process, opening a new highly productive research field. We now understand that a major portion of cardiac cells, primarily coronary smooth muscle and fibroblasts, originate from the embryonic epicardium. There is also evidence suggesting the existence of a minor EPDCs differentiation into other cell types, such as coronary endothelium. In addition to this large-scale cellular contribution, the epicardium and its mesenchymal derivatives play a crucial signaling role in myocardial growth and maturation. Therefore, the role of epicardium is fundamental for cardiac morphogenesis, implying complex molecular mechanisms of control and regulation. As described in this review, numerous signaling and regulatory pathways -both at transcriptional and post-transcriptional levels- have been uncovered in the last three decades.

Despite the present knowledge of the epicardial contribution to cardiac development, many aspects still remain unclear. In particular, it is not well known whether different derivatives from the embryonic epicardium belong to distinct cell lineages established in the proepicardium, or that, alternatively, EPDCs are in fact pluripotent [see for a recent review 145]. The signaling mechanisms between the epicardium/EPDCs and the embryonic myocardium are not fully understood as yet. Noticeably, the importance of small and long non-coding RNAs in epicardium and EPDCs development is just beginning to be fruitful. As an interesting approach, is the adult epicardium capable of recovering the potential to acquire embryonic features? What is more, would it be able to transdifferentiate or produce signals for cardiac repair?

These and other questions related to the epicardium will continue to drive research on the embryonic epicardium and its crucial roles in cardiac morphogenesis and regeneration.

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