

## REVIEW

# The ontogeny, activation and function of the epicardium during heart development and regeneration

Filipa C. Simões and Paul R. Riley\*

## ABSTRACT

The epicardium plays a key role during cardiac development, homeostasis and repair, and has thus emerged as a potential target in the treatment of cardiovascular disease. However, therapeutically manipulating the epicardium and epicardium-derived cells (EPDCs) requires insights into their developmental origin and the mechanisms driving their activation, recruitment and contribution to both the embryonic and adult injured heart. In recent years, studies of various model systems have provided us with a deeper understanding of the microenvironment in which EPDCs reside and emerge into, of the crosstalk between the multitude of cardiovascular cell types that influence the epicardium, and of the genetic programmes that orchestrate epicardial cell behaviour. Here, we review these discoveries and discuss how technological advances could further enhance our knowledge of epicardium-based repair mechanisms and ultimately influence potential therapeutic outcomes in cardiovascular regenerative medicine.

**KEY WORDS:** Epicardium, Epicardium-derived cells, Heterogeneity, Lineage derivatives, Migration, Recruitment, Cardiovascular regeneration

## Introduction

The epicardium is a mesothelial cell sheet that covers the surface of the heart and, together with the myocardium and the endocardium, forms the wall of the heart (Fig. 1). The epicardium exhibits extensive developmental plasticity and gives rise to a population of mesenchymal cells known as epicardium-derived cells (EPDCs) that are crucial for heart development and regeneration (reviewed by Riley, 2012). However, although its existence has been known for more than 100 years (Kurkiewicz, 1909; Manasek, 1969), the heterogeneity and fate of the epicardium both in the context of heart development and following disease/injury remains a major area of focus (Riley, 2012), and, despite many studies across different model organisms, there is still uncertainty with respect to the complete lineage potential of the epicardium and EPDCs within the forming heart.

Recent interest in the epicardium has focused on its capacity to act as a source of mitogenic signals that nurture muscle and vascular growth during heart development (Pérez-Pomares and de la Pompa, 2011). This capacity has been further realised in studies of the adult heart, which have shown that the ordinarily dormant epicardium can be reactivated in response to injury (e.g. following myocardial infarction, MI) to contribute new coronary vascular cells and to signal to maintain the survived heart muscle (Lepilina et al., 2006;

Smart et al., 2007; Wang et al., 2015; Wei et al., 2015; Zhou et al., 2011). The activated adult epicardium is characterised by embryonic molecular programmes; consequently, restoring developmental plasticity to adult EPDCs has emerged as a therapeutic approach to optimise wound healing and enable tissue regeneration in the adult mammalian heart.

This Review presents a historical overview of the epicardium and highlights the most recent data on the developmental origins and lineage potential of EPDCs. We explore the signalling cues, and genetic and epigenetic changes that influence the activation, guided migration and recruitment of EPDCs during development and following cardiac injury. We draw on key work from avian, zebrafish and mouse models, and also focus on recent human studies, which establish the basis for the expansion of clinically relevant EPDCs in the setting of cardiovascular disease. In this context, we discuss how recent technical advances could enhance our understanding of the biology of epicardial cells as the basis for translation from discovery science to clinical application.

## Ontogeny of the embryonic epicardium

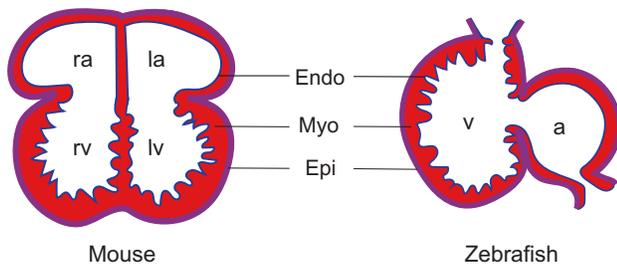
During embryonic development, numerous distinct cardiovascular cell types closely interact to build and maintain a fully functional heart. The embryonic epicardium derives from an extracardiac structure called the proepicardium (PE), which is an outgrowth of coelomic cells located dorsal to the developing heart tube (Fig. 2), between the sinus venosus and the liver (Männer et al., 2001). This transient structure is highly conserved among vertebrates, including human, mouse, chick, frog and zebrafish (Hirakow, 1992; Jahr et al., 2008; Komiyama et al., 1987; Männer, 1992; Serluca, 2008; Viragh and Challice, 1981). By contrast, the epicardium is absent in invertebrates but its primordium is found in lampreys, which belong to a basal group of vertebrates, raising questions about the evolution of the PE and the epicardium (see Box 1).

In both zebrafish and mouse, the PE appears as bilateral clusters around 48 h post-fertilisation and embryonic day 8.5, respectively, while in chick and *Xenopus* only a single (right-sided) PE develops around Hamburger-Hamilton stages 14 and stage 41, respectively (Jahr et al., 2008; Schulte et al., 2007; Serluca, 2008). This difference may be related to the distinct migration of PE cells as they colonise the heart tube and form the epicardium. In chick and frog embryos, a coherent sheet of cells forms between the PE and the surface of the heart allowing transfer of cells (Jahr et al., 2008; Männer, 1992; Nahirney et al., 2003). This unilateral tissue bridge is relatively stable but is not observed in mouse and zebrafish embryos, where instead PE cell clusters migrate in an apparently stochastic manner and either contact the beating heart and adhere to its surface, or are released into the pericardial cavity and float towards the myocardium (Peralta et al., 2013; Rodgers et al., 2008). After PE cell transfer, the attached cells collapse and proliferate, forming a single epicardial layer enveloping the heart (Nahirney et al., 2003; Peralta et al., 2013).

Department of Physiology, Anatomy and Genetics, University of Oxford, Sherrington Building, South Parks Road, Oxford OX1 3PT, UK.

\*Author for correspondence (paul.riley@dpag.ox.ac.uk)

 F.C.S., 0000-0003-2956-0311; P.R.R., 0000-0002-9862-7332



**Fig. 1. The epicardium in the context of the heart.** Schematics of the mouse and zebrafish heart illustrate the close anatomical proximity of the epicardium (Epi, purple) relative to the myocardium (Myo, red) and the endocardium (Endo, blue). These three layers form a four-chambered heart in the case of mice, with two trabecular ventricles (rv, right ventricle; lv, left ventricle) and two atria (ra, right atrium; la, left atrium), while the zebrafish heart comprises only one trabecular ventricle (v) and one atrium (a).

### Heterogeneity and cell fate potential within the developing epicardium

Once the epicardium covers the developing heart tube, a subset of epicardial cells undergoes epithelial-to-mesenchymal transition (EMT), giving rise to EPDCs (von Gise and Pu, 2012). These delaminating cells invade the subepicardial compartment and colonise the underlying myocardium to nurture further growth of the developing heart muscle and coronaries, by acting as an important source of cardiomyocyte and vascular mitogens (Pérez-Pomares and de la Pompa, 2011). As an example, it has been reported that erythropoietin (EPO) from the developing liver binds to the epicardial EPO receptor, activating production of Igf2 in the epicardium (Brade et al., 2011; Wu et al., 1999). This liver-epicardium crosstalk induces cardiomyocyte proliferation in the developing myocardium (Li et al., 2011).

In addition to providing signals, EPDCs can directly give rise to many of the different cell types that form the developing heart. A number of genes have been shown to be expressed in the PE, epicardium and EPDCs, including scleraxis (*Scx*), semaphorin 3D (*Sema3d*), *Wt1* (Wilms tumour 1), *Tcf21* (TF 21) and *Tbx18* (T-box factor 18), and these have been used to develop tools to track the fate of these cells. Cre-based lineage-tracing studies in mice, for example, have suggested a contribution from the PE to adult cardiac-resident mesenchymal stem cells (Chong et al., 2011) and the coronary endothelium (Cano et al., 2016; Katz et al., 2012). EPDCs have been shown to give rise to endothelial cells (ECs) within the ventricular free wall in both mouse and chick embryos (Dettman et al., 1998; Mikawa and Fischman, 1992; Pérez-Pomares et al., 1998, 2002; Zhou et al., 2008a). However, questions around the specificity of the transplanted chick grafts and the mouse WT1 Cre-driver line used in these studies call into question the extent of this contribution (Duim et al., 2015; Poelmann et al., 1993; Rudat and Kispert, 2012). Studies have also reported an epicardial contribution to vascular smooth muscle cells (SMCs), which are necessary for vascular support and proper coronary formation, and cardiac fibroblasts (CFs) in chick (Dettman et al., 1998; Männer, 1999; Mikawa and Gourdie, 1996; Pérez-Pomares et al., 1997), mouse (Acharya et al., 2012; Swonger et al., 2016; Wessels et al., 2012; Wu et al., 2013; Zhou et al., 2010) and zebrafish embryos (Kikuchi et al., 2011a), revealing evolutionarily conserved developmental potential of EPDCs. More recent clonal analyses and lineage-tracing studies have shown that SMCs in the mouse arise from epicardial-derived pericytes, which associate with microvessels, and that these are also present in the adult heart (Volz et al., 2015). In mice, the binary cell fate decision between

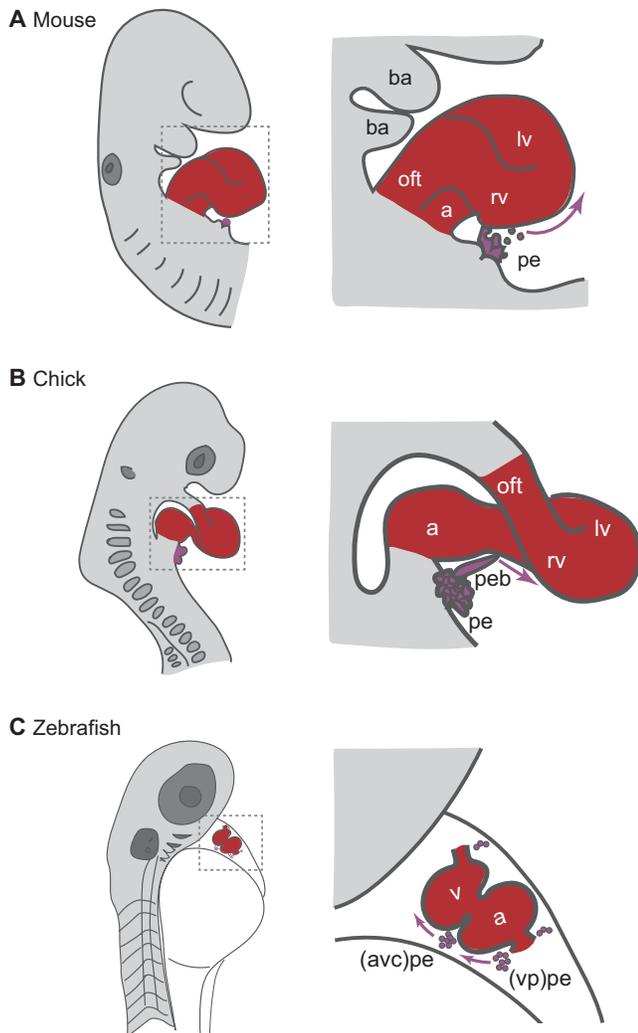
SMCs and CFs seems to be controlled by TCF21; embryonic EPDCs adopt a fibroblastic fate when TCF21 is expressed in epicardial cells, at the expense of SMC specification (Acharya et al., 2012; Braitsch et al., 2012). This cell fate decision appears to take place in pre-migratory EPDCs, before they undergo EMT and leave the epicardial sheet (Acharya et al., 2012; Braitsch et al., 2012), suggesting that the epicardium is a functionally heterogeneous epithelium.

A much less consensual view on EPDC fate exists with respect to putative differentiation into cardiomyocytes (Cai et al., 2008; del Monte et al., 2011; Guadix et al., 2006; Ruiz-Villalba et al., 2013; Zhou et al., 2008a). Fate-mapping studies using TBX18- and WT1-Cre lines in mice revealed an apparent epicardial contribution to cardiomyocytes in the interventricular septum and left ventricle of the developing heart (Cai et al., 2008; Zhou et al., 2008a). However, endogenous expression of both Cre-drivers, TBX18 and WT1, within developing cardiomyocytes and the non-recapitulation of endogenous WT1 expression by the constitutively active WT1-Cre line employed (Christoffels et al., 2009; Rudat and Kispert, 2012) has meant that the potential epicardial contribution to the myocardial lineage remains controversial.

In addition to the epicardial-derived cell types mentioned above, recent findings suggest that EPDCs can contribute to adipose tissue (Chau et al., 2014; Liu et al., 2014; Yamaguchi et al., 2015). Genetic lineage tracing has revealed that WT1- and TBX18-derived EPDCs can give rise to fat cells of the atrial-ventricular groove of the developing mouse heart (Liu et al., 2014; Yamaguchi et al., 2015). The generation of this novel epicardial-derived cell type seems to be dependent on activation of the peroxisome proliferator activated receptor gamma (PPAR $\gamma$ ) pathway, at the time EPDCs start delaminating from the epithelial layer (Yamaguchi et al., 2015). Interestingly, it has been shown that visceral fat derives from WT1-expressing mesothelial cells that originate in the lateral plate mesoderm (Chau et al., 2014), which also acts as the source of PE cells (Zhou et al., 2008b).

It is clear that EPDCs have the potential to give rise to multiple cardiovascular cell types. However, whether pre-migratory EPDCs share a common multipotent progenitor, or are functionally heterogeneous within the epicardium is unclear. Analyses of the expression of TCF21, WT1 and TBX18 seem to suggest the latter, as these functionally relevant epicardial genes are restricted to subsets of epicardial cells in the developing mouse and chick heart (Braitsch et al., 2012). Such apparent epicardial heterogeneity may arise from the PE (Katz et al., 2012; Plavicki et al., 2014); indeed, studies in mice have shown that expression of the transcription factor scleraxis (*Scx*) and the chemokine semaphorin 3D (*Sema3d*) in the PE only partially overlaps with that of WT1 and TBX18 (Katz et al., 2012). The potential of EPDCs to give rise to a multitude of functionally distinct cell populations might also reflect the fact that a small proportion of the epicardium itself is derived from other sources. Indeed, although the majority of the epicardium arises from the PE, non-PE derived CD45<sup>+</sup> haematopoietic cells have been shown to contribute to the developing mouse epicardium (Balmer et al., 2014). Furthermore, in chick and zebrafish embryos, epicardial cells covering the outflow tract seem to originate from the cephalic pericardial mesothelial cells and not the PE, suggesting conserved alternate sources (Peralta et al., 2013; Pérez-Pomares et al., 2003).

Taken together, these findings suggest that the heterogeneity attributed to both the origin and cellular composition of the PE, and its derivative epicardium, almost certainly influences the multiple cell fates of EPDCs during heart development. How this heterogeneity is first established and the implications of the different sources of EPDCs during remain poorly understood.



**Fig. 2. An overview of epicardial development across species.**

(A-C) Schematics of heart development in the mouse (A), chick (B) and zebrafish (C), with the pericardial cavity (boxed) enlarged in the right-hand images. (A) At around embryonic day 8.5, mouse proepicardium (pe) vesicles, located close to the sinus venosus, either contact the beating heart and adhere to its surface or are released into the pericardial cavity and float towards the myocardium. (B) At Hamburger-Hamilton stage 14, the PE in the chick embryo appears as a structure that forms close to the sinus venosus. The PE migrates onto the myocardium by formation of a cellular bridge (the proepicardial bridge, peb) that adheres to the heart surface. (C) In a zebrafish embryo at 48 h post fertilisation, the first PE cell vesicles attach to the myocardial surface. In the two-chambered heart, the PE arises from a cluster next to the atrioventricular canal [the (avc)pe], one close to the venous pole [the (vp)pe], and from a third source close to the arterial pole (not labelled). Heartbeat-derived fluid flow drives the attachment of these vesicles to the myocardium. After PE cell transfer in all cases (A-C), the attached cells collapse, proliferate and migrate (purple arrows), forming a single epicardial layer enveloping the heart (see Fig. 1). a, atria; ba, branchial arch; lv, left ventricle; oft, outflow tract; rv, right ventricle; v, ventricle.

Further technical advances in lineage tracing and unbiased characterisation of the genetic and epigenetic programmes regulating EPDCs are needed to obtain a more comprehensive view of their origin, fate and potential.

### The role of the epicardium during heart regeneration

The capacity and extent of regeneration in the adult heart appears to be species-specific and dependent on a number of factors, including

the type of insult and timing of injury. Myocardial infarction (MI), for example, is an extreme case of heart injury that disrupts tissue homeostasis. Following the build-up of plaque inside the coronary arteries, known as atherosclerosis, a thrombotic clot is formed, leading to deprivation of circulating blood and oxygen supply to regions of the heart muscle. This acute episode culminates in sudden death of cardiomyocytes in the infarcted heart, which rapidly triggers innate immune pathways responsible for the clearance of dead/dying cells, remodelling of extracellular matrix and the activation of collagen-producing myofibroblasts. As a result, dead cardiomyocytes are replaced with a fibrotic scar, which is essential to prevent cardiac rupture. The scar is non-contractile, so results in over-compensation by the heart, via hypertrophy of survived muscle and pathological remodelling (wall thinning and chamber dilation), which ultimately leads to heart failure (Epelman et al., 2015). Adult mammals, including humans, fail to regenerate the lost tissue post-MI. By contrast, the neonatal mouse can regenerate its heart after MI up to 7 days after birth (Porrello et al., 2011), while zebrafish maintain a remarkable ability to fully repair cardiac muscle lost by injury into adulthood (Chablais et al., 2011; González-Rosa et al., 2011; Porrello et al., 2011; Poss et al., 2002). In a recent case study of a newborn child who suffered an MI, due to coronary artery occlusion *in utero*, within hours after birth, the authors reported complete recovery in cardiac function resulting in hospital discharge after just one and a half months. Remarkably the infant had no apparent structural heart abnormalities and had complete tissue restoration (Haubner et al., 2016), suggesting that regenerative capacity in the early neonatal period is conserved across mammals.

These various studies highlight that the heart harbours an endogenous regenerative capacity. While genetic fate mapping studies in neonatal mice and adult zebrafish have shown that the major source of cardiomyocytes in the regenerating heart is via pre-existing cardiomyocytes that re-enter the cell cycle (Haubner et al., 2012; Jopling et al., 2010; Kikuchi et al., 2010; Porrello et al., 2011), a number of studies have shown that the epicardium is also implicated in the regenerative process. In mouse, the adult epicardium is thought to assume a quiescent state under homeostatic conditions: the embryonic epicardial genes that are expressed during development (including *Wt1*, *Tcf21* and *Tbx18*) are downregulated in the adult epithelial layer (Smart et al., 2011), which shows very little proliferation (Wu et al., 2010) and no signs of EMT (Zhou and Pu, 2011). In zebrafish, when the epicardium is ablated (by genetically targeting expression of bacterial nitroreductase in a tissue-specific manner), the result is a loss of regenerative capacity (Wang et al., 2015). This suggests the adult epicardium becomes reactivated and is functional in response to altered (patho-) physiological conditions. Such an idea was previously put forward by a study showing that under rapid growth conditions, zebrafish epicardial cells reactivate expression of embryonic genes and supplement the adult ventricular wall in an FGF-dependent manner (Wills et al., 2008). As such, restoring developmental potential to the epicardium appears to be crucial to ensure a robust response to injury and to facilitate heart regeneration. Indeed, a hallmark of the immediate response to cardiac injury, in both regenerative and non-regenerative model organisms, is the upregulation of embryonic epicardial genes and a reactivation of epicardial cells (Lepilina et al., 2006; Porrello et al., 2011; Ruiz-Villalba et al., 2015; Smart et al., 2007, 2011; van Wijk et al., 2012; Zhou et al., 2011).

In zebrafish, epicardial cell proliferation and migration to the site of injury is also essential for heart regeneration. The wound area is initially covered by proliferating epicardial cells, which migrate and

**Box 1. The evolutionary origins of the PE and epicardium**

Studies on the evolutionary origin of the PE suggest that this structure derives from a pair of pronephric external glomeruli (PEG), found in even the most primitive vertebrate lineage represented by the lamprey (Pombal et al., 2008). Throughout evolution, the disappearance of the most anterior part of the pronephros, combined with expansion of the cardiac inflow tract and the liver, could account for the new association of the primitive PEG/PE with the sinus venosus/liver in higher vertebrates (Pombal et al., 2008). The evolutionary conservation of primitive PEG/PE cell transfer to the myocardium might reflect the importance of these cells during heart evolution. The embryonic heart primarily lacks vasculature, which limits the thickness of the myocardial wall and its performance. The idea that glomerular cells, which have high vasculogenic potential, are transferred to the heart to supply it with blood vessel progenitors corroborates the hypothesis that this might represent the first evolutionary step that allows complete myocardial vascularisation (Pombal et al., 2008). This, in turn, would have enabled the heart to increase its size, thickness and performance, as occurs in higher vertebrates.

The many genes commonly expressed by the PE and kidney also support their evolutionary and developmental relationship. The transcription factors WT1 (Wilms tumour 1), TCF21 (TF 21, also known as epicardin/capsulin) and TBX18 (T-box factor 18) are highly expressed in both tissues and, when depleted, lead to a myriad of defects in cardiac and renal development (Airik et al., 2006; Buckler et al., 1991; Christoffels et al., 2006; Cui et al., 2003; Hidai et al., 1998; Kraus et al., 2001; Moore et al., 1999; Quaggin et al., 1999; Robb et al., 1998). The identification of the PE as akin to the primitive PEG highlights the importance of drawing parallels between the heart and the kidney genetic programmes, and could provide new insights into epicardial function during cardiac development and disease.

regenerate from the base of the heart to the apex (Wang et al., 2015). These cells strongly express extracellular matrix components such as fibronectin, periostin and collagens (González-Rosa et al., 2012; Marro et al., 2016; Wang et al., 2013), which act as a 'guiding scaffold' that is necessary not only for pre-existing cardiomyocytes to re-enter the cell cycle, but also for their correct migration into the regenerating area (Wang et al., 2013, 2015). A recent study examined the cellular dynamics taking place in the epicardium during regeneration (Cao et al., 2017). This analysis showed that the regenerating zebrafish epicardium is composed of different cells that, depending on their size and ploidy state, vary in their capacity to cover the regenerating surface of the heart. The leading cells, forming the front of the regenerating tissue, are large and polyploidy, and are followed by smaller, diploid cells. Increased mechanical tension at the front of the regenerating surface seems to lead to a failure in cytokinesis and the consequent polyploidy of 'leader' cells, which migrate faster and have a greater capacity for surface coverage. These cells are short-lived and die after directing regeneration (Cao et al., 2017). This study suggests that by having larger cells at the forefront of the regenerating surface, the healing process can be accelerated. However, it is not clear how tension triggers the cytokinetic failure in the first place and how this might relate to ploidy status in the mammalian system.

The epicardium also acts as a source of cardiomyocyte and vascular mitogens during regeneration, analogous to its role during development (Masters and Riley, 2014). For example, Raldh2, a retinoic acid (RA) synthesising enzyme, is active within the zebrafish epicardium and is necessary for cardiomyocyte proliferation after heart injury (Kikuchi et al., 2011b), whereas in adult mammals, epicardial follistatin-like 1 (*Fstll1*) improves cardiomyocyte cell cycle re-entry and division after MI (Wei et al., 2015).

A number of lineage-tracing and functional studies have revealed that, in addition to migrating and modulating the behaviour of other cell types during heart regeneration, epicardial cells and EPDCs can directly give rise to cells that are involved in regenerating the heart tissue. In zebrafish, for example, studies have shown that EPDCs directly contribute to myofibroblasts and perivascular cells in the regenerating heart (González-Rosa et al., 2012; Kikuchi et al., 2011a). Similarly, upon MI in the mouse, WT1-derived cells give rise to mesenchymal cells that express markers of fibroblast, myofibroblast and SMC lineages; however, these accumulate in the sub-epicardial region and do not migrate into the myocardial layer (Zhou et al., 2011; Zhou and Pu, 2011), limiting their paracrine effect and progenitor cell potential during wound healing. Following cardiac pressure overload, fibroblast accumulation seems to result from the proliferation of tissue-resident fibroblasts rather than from epicardial EMT (Moore-Morris et al., 2014). A recent study performed lineage-tracing analyses with four additional Cre-expressing mouse lines, revealing that the majority of myofibroblasts in the post-injured heart arise from TCF21<sup>+</sup> tissue-resident fibroblasts (Kanisicak et al., 2016). Interestingly, priming the epicardium with the G-actin monomer-binding peptide thymosin  $\beta$ 4 (T $\beta$ 4) induces the reactivation of embryonic genes, EPDC migration, differentiation and signalling in response to injury in the adult mouse heart (Bock-Marquette et al., 2009; Smart et al., 2007, 2010). In addition to this paracrine effect, T $\beta$ 4 priming of the adult epicardium prior to MI allows the generation of new blood vessels (Smart et al., 2007, 2010) and a small number of cardiomyocytes (Smart et al., 2011). It has been reported that epicardial cells can also contribute to fat cells upon injury of the adult mouse heart (Liu et al., 2014) in a manner analogous to their role in development, although it is not clear whether this mechanism is exclusive to mice or conserved across other vertebrates. In humans, embryonic ventricular epicardial cells express PPAR $\gamma$ , which is required for the adoption of an adipocyte fate *in vivo*, suggesting there may be some degree of conservation (Yamaguchi et al., 2015).

Together, these findings highlight that the epicardium and EPDCs play multiple roles in the adult heart in response to injury. These varied roles could be a reflection of the heterogeneous composition of the adult epicardium and EPDCs (Bollini et al., 2014; Cao et al., 2016), so a greater understanding of the gene regulatory circuits driving epicardial cell behaviour and fate decisions in a post-injury setting is necessary to harness their full regenerative potential. In particular, it is still unclear whether the turnover of EPDCs in the adult during homeostasis and repair (Kikuchi et al., 2011a; Wang et al., 2015; Wills et al., 2008) is exclusively driven by embryonic epicardial cells, or whether an adult-specific reservoir of progenitor cells may also contribute. Despite this, it seems clear that cardiac healing recapitulates ontogeny whereby, first and foremost, the crosstalk between the epicardium and the underlying myocardium is necessary for maintaining tissue homeostasis. Damage to either of these lineages impedes such interplay, leading to loss of homeostasis and re-adaptation through either regenerative or scar-based wound healing. As a consequence, either normal physiology is re-established (e.g. in the case of regenerative models such as adult zebrafish and neonatal mice) or fibrosis and cardiac remodelling ensues (e.g. in the non-regenerative setting, such as in adult mice and humans), resulting in altered function and progression to maladaptive heart failure.

**Mechanisms of epicardial activation and migration during development and regeneration**

The studies presented above highlight that the activation and migration of epicardial cells is essential both for normal heart development and in the context of heart regeneration. Below, we

review what is currently known about the cellular and molecular mechanisms that drive epicardial induction, activation and migration, focussing first on the developing heart and then exploring whether similar mechanisms are at play during heart repair.

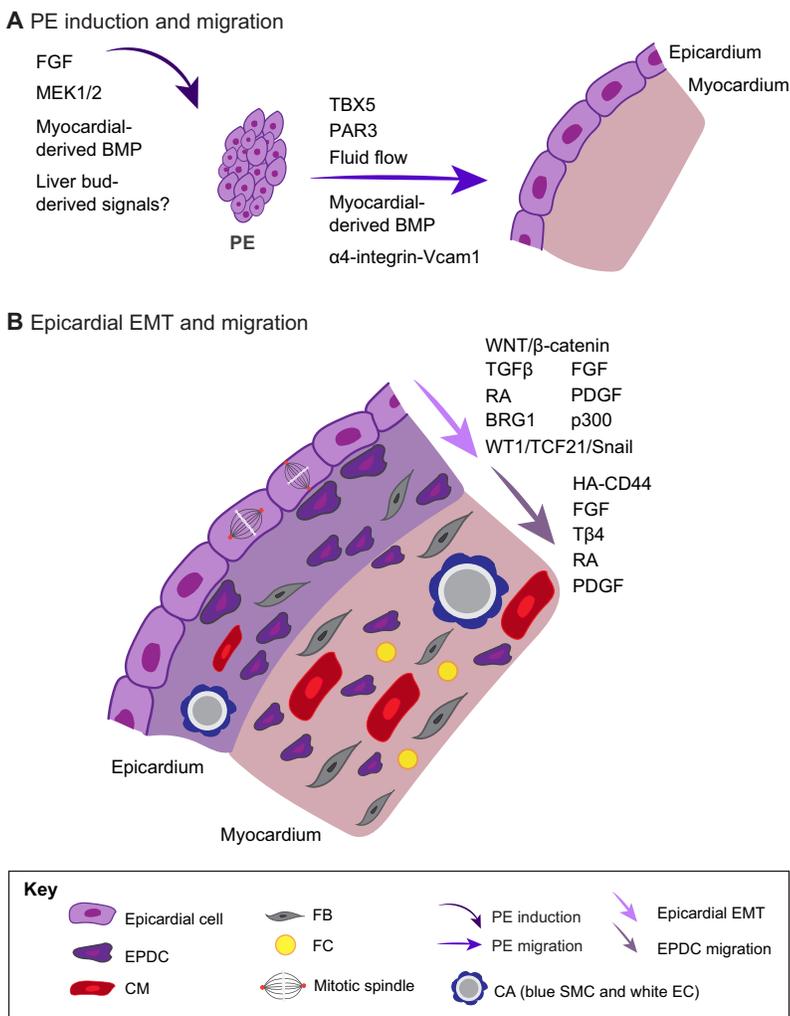
**PE induction and migration onto the myocardium during development**

In the mouse, PE progenitors are thought to arise during early development from the same Nkx2.5- and Islet 1-expressing precardiac mesoderm field that gives rise to other cardiac precursor cells in the anterior lateral plate mesoderm (Zhou et al., 2008b). In the chick, fate-mapping studies suggest that PE progenitors are located adjacent to the posterior end of the cardiogenic domain (Maya-Ramos et al., 2013). The separation of the PE lineage from the precardiac mesoderm is driven by FGF-MEK1/2 signalling (van Wijk et al., 2009). Inductive signals from the liver anlagen and the myocardium are thought to be involved in the initial formation of the PE (Fig. 3A). Myocardial-derived BMP signalling seems to be necessary for PE specification in zebrafish, mouse and chick embryos (Kruithof et al., 2006; Liu and Stainier, 2010; Schlueter et al., 2006; van Wijk et al., 2009), whereas the liver bud has been shown to induce expression of the proepicardial markers TBX18, WT1 and TCF21 in the chick embryo (Ishii et al., 2007). However, the requirement of liver-derived signals for PE specification may not be evolutionarily conserved, as zebrafish

mutants with disrupted liver formation still develop a functional PE (Liu and Stainier, 2010). After PE formation, migration and attachment to the myocardium is guided by high-level expression of BMP ligands in the atrioventricular junction of the looping heart (Ishii et al., 2010), by the T-box transcription factor TBX5 (Hatcher et al., 2004) and by the apico-basal polarity pathway protein PAR3 (Hirose et al., 2006). Additionally, it has been shown that adhesion molecules modulate the epicardial–myocardial interaction during the migration process. In particular  $\alpha$ 4-integrin, which is expressed in PE cells, and myocardial-expressed Vcam1 seem to be required to stabilise adhesion of the PE to the myocardium during epicardial enveloping of the heart (Kwee et al., 1995; Sengbusch et al., 2002; Yang et al., 1995). An elegant study has revealed the requirement of heartbeat-derived fluid flow for the appropriate migration of PE cells and for the attachment of cell clusters onto the myocardial surface of the zebrafish developing heart tube (Peralta et al., 2013). It would be interesting to elucidate whether such fluid flow functions by acting on downstream shear force regulators, such as the Klf2 (Dekker et al., 2002), or whether it triggers cilia-dependent mechanosensing mechanisms, such as those that drive left-right asymmetry (Shinohara and Hamada, 2017).

**Epicardial EMT and EPDC migration during development**

Once the epicardium is fully formed, a subset of epicardial cells undergo EMT and migrate into the subepicardial space (Fig. 3B).



**Fig. 3. Cellular and molecular interactions during epicardial development.** (A) A number of signals, many of which arise from nearby tissues, are necessary for PE induction and migration onto the myocardium. (B) Once the epicardium has fully enveloped the myocardium, a subset of epicardial cells undergoes EMT and migrates into the subepicardial space, a process that is dependent on epicardial cell division and spindle orientation. These epicardium-derived cells (EPDCs) give rise to fibroblasts (FBs, grey), vascular smooth muscle cells (SMCs, blue) and endothelial cells (ECs, white) forming the coronary arteries (CAs), fat cells (FCs, yellow) and potentially cardiomyocytes (CMs, red); see the main text for a detailed discussion of the developmental potential of EPDCs. Crosstalk between the migrating EPDCs and the myocardium, which is mediated by a number of factors, is essential for the development of a fully functional heart.

This transformation is dependent on epicardial cell division and spindle orientation, which seem to determine whether a cell remains part of the epicardium or undergoes EMT and migrates into the myocardium (Wu et al., 2010). Epicardial cell divisions in which the mitotic spindle is perpendicular to the basement membrane result in one cell remaining in the epicardium while the other cell exits the epicardial layer and undergoes EMT, in a  $\beta$ -catenin-dependent manner (Wu et al., 2010). However, it is not clear whether subpopulations of epicardial cells are specified early in development to acquire competence for EMT or if all epicardial cells have the potential to undergo EMT but only some become appropriately activated based on locally restricted signals. It has been shown that TGF $\beta$  superfamily members promote epicardial EMT and, as these are located in both the epicardium and myocardium, this suggests reciprocal signalling between the adjacent lineages (Compton et al., 2007; Molin et al., 2003; Morabito et al., 2001). TGF $\beta$ 2 has also been shown to upregulate Has2, the enzyme that synthesises hyaluronic acid (HA), which is an important component of the sub-epicardial extracellular matrix and together with its receptor CD44 is essential for inducing EPDC migration (Craig et al., 2010). FGF signals also regulate epicardial EMT and EPDC migration (Morabito et al., 2001; Pennisi and Mikawa, 2009; Vega-Hernandez et al., 2011). Specifically, myocardial FGF10 signals to FGFR1 and FGFR2b receptors to promote epicardial EMT and EPDC motility (Vega-Hernandez et al., 2011). In addition, EPDC migration in mice was shown to be dependent on the release of T $\beta$ 4 (Smart et al., 2007), RA (Merki et al., 2005) and platelet-derived growth factor (PDGF) from the developing myocardium (Bax et al., 2010; Mellgren et al., 2008; Smith et al., 2011). The proper spatiotemporal orchestration of all these signals is essential for epicardial cells to upregulate transcriptional activators of EMT, downregulate intercellular adhesion, lose apical polarity, degrade extracellular matrix and acquire a mesenchymal (migratory) phenotype.

At the transcriptional level, factors such as WT1, TCF21 and Snai1 have been shown to be important players during epicardial EMT (Acharya et al., 2012; Martínez-Estrada et al., 2010; von Gise et al., 2011). The transcription factor Nfatc1 and MRTFs (myocardin-related transcription factors) are also crucial regulators of EPDC-migration into the underlying myocardium (Combs et al., 2011; Trembley et al., 2015). A recent study elucidated an epigenetic mechanism by which regulation of the *Wt1* locus via the BRG1-SWI/SNF chromatin remodelling complex controls epicardial EMT (Vieira et al., 2017). The epigenetic regulator p300 has also been shown to be required for epicardial EMT; however, it is not clear if this acetyl-transferase acts in epicardial cells or non-cell autonomously (Shikama et al., 2003).

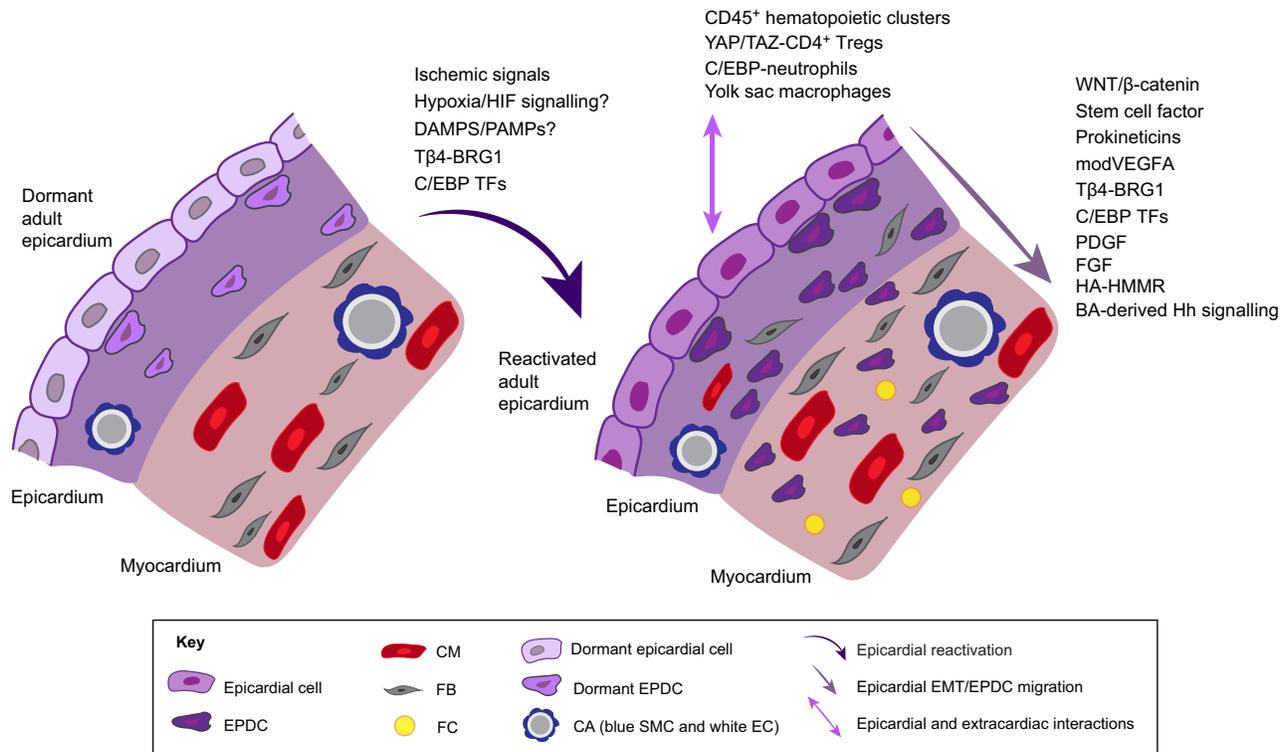
#### **Epicardial activation and migration during repair: the embryo revisited?**

A remarkable feature of the upregulation of embryonic epicardial genes following cardiac injury is that, despite the localised insult, the response is pan-epicardial, occurring rapidly after injury as a signature event across species (González-Rosa et al., 2011; Lepilina et al., 2006; Porrello et al., 2011; Smart et al., 2007). The mechanism(s) behind such an organ-wide response remains unclear (Fig. 4). In mice undergoing transverse aortic constriction to induce chronic hypertrophy and fibrosis, there is no obvious activation of WT1<sup>+</sup> cells within the epicardium (Moore-Morris et al., 2014), suggesting that acute ischemic injury (following MI) is required for activation. Ischaemia is mainly mediated by hypoxia and HIF1 $\alpha$ /2 $\alpha$  signalling, and it has been shown that hypoxia is capable of directly

inducing WT1 expression in the rat heart, through binding of HIF1 $\alpha$  to the *Wt1* promoter (Wagner et al., 2003). Therefore, hypoxia and HIF signalling may work as key regulators that trigger epicardial cell reactivation after cardiac damage. In the mouse, the transition from a hypoxic to a normoxic environment after birth has been implicated in regulating the cardiomyocyte cell cycle exit and hence reducing cardiac regenerative capacity upon injury (Puente et al., 2014). Such a transition coincides with the downregulation of WT1 expression in the epicardium after birth. Interestingly, fate mapping of hypoxic cells in the mouse heart identified a rare population of cycling cardiomyocytes that contribute to new cardiomyocyte formation in the adult heart (Kimura et al., 2015). The precise role of hypoxia in epicardial reactivation remains to be determined, although it should be noted that the intact adult epicardium has been described as a hypoxic ‘niche’ that houses a glycolytically distinct progenitor population (Kocabas et al., 2012).

Links between the epicardium and the immune system have also been implicated in the response to injury. After tissue injury, cells release damage-associated molecular pattern molecules (DAMPs) as endogenous danger signals that alert the innate immune system to trauma, ischemia and microbial invasion. In contrast, pathogen-associated molecular pattern molecules (PAMPs), which are derived from microorganisms, are recognised by pattern recognition receptor-expressing cells of the innate immune system as well as many epithelial cells (Bianchi, 2007; Lotze et al., 2007; Matzinger, 1994; Rubartelli and Lotze, 2007). Either DAMPs and/or PAMPs may provide a link between the epicardium and the immune system to underpin the organ-wide activation of epicardial genes. CCAAT/enhancer binding protein (C/EBP) transcription factors, which have been identified as crucial regulators of epicardial gene expression during development, are also activated after cardiac injury and may play a role in promoting inflammatory responses following injury (Huang et al., 2012). The disruption of C/EBP signalling in the adult epicardium reduces injury-induced neutrophil infiltration and improves cardiac function, revealing a previously unappreciated role for the epicardium in regulating the inflammatory response after injury. A more recent study described an unexpected role for Hippo signalling within the epicardium in recruiting a specific subset of suppressive CD4<sup>+</sup> regulatory T-cell (Tregs) to the injured myocardium (Ramjee et al., 2017). Specifically, epicardial loss of YAP or TAZ, which are two core Hippo pathway effectors, and the associated decrease in IFN- $\gamma$ , results in pericardial inflammation and myocardial fibrosis, with fewer suppressive Tregs in the injured myocardium. This work reveals how epicardial activation can modulate an adaptive immune response through paracrine mechanisms, although the precise mechanisms by which the epicardium regulates immune cell recruitment following injury remain unclear. It is known that, during development, macrophages are dependent on the epicardium for their recruitment to the foetal heart, such that yolk sac macrophages are seemingly recruited to a niche within the epicardium in a process that is dependent on embryonic expression of WT1 (Stevens et al., 2016). A different study, focussing on the adult epicardium, reported the presence of epicardial-associated CD45<sup>+</sup> hematopoietic cell clusters that respond dynamically to cardiac injury (Balmer et al., 2014). It will be interesting to further investigate whether and how adult epicardial-specific cues are able to recruit and/or retain circulating monocytes, macrophages and other immune cell types upon MI.

Despite the redeployment of embryonic signals after MI to promote epicardial EMT (Aisagbonhi et al., 2011; Duan et al., 2012), the mobilisation of adult EPDCs in mice is limited and cells



**Fig. 4. Signals and factors involved in adult epicardial activation and migration.** The adult epicardium assumes a quiescent state (left) but, upon injury, dormant epicardial cells are reactivated (right) by upstream signals and undergo EMT. Many factors have been shown to be involved in driving and enhancing adult epicardial EMT and EPDC migration into the myocardium. The migrating progenitors have the potential to differentiate into fibroblasts (FBs, grey), vascular smooth muscle cells (SMCs, blue) and endothelial cells (ECs, white) of the coronary arteries (CAs), fat cells (FCs, yellow) and cardiomyocytes (CMs, red); see the main text for a detailed discussion of the potential of EPDCs in the adult. Novel interactions between the epicardium and the immune system are also starting to be unravelled.

tend to remain within the surface of the heart (van Wijk et al., 2012). Such restricted EMT and migratory potential, however, can be amplified in response to distinct biological stimuli, such as stem cell factor (Xiang et al., 2014), prokineticins (Urayama et al., 2008), modified-RNA encoding for VEGFA protein (Zangi et al., 2013) and Tβ4 (Smart et al., 2007). A mechanism explaining how Tβ4 can reactivate the adult epicardium was recently reported, demonstrating that BRG1 physically interacts with Tβ4 not only at the *Wt1* locus, but also at other epicardial loci, including *Raldh2*, *Tbx18* and *Tcf21*, thus establishing an embryonic gene programme underpinning the activation and migration of adult EPDCs (Vieira et al., 2017). A similar common regulatory mechanism responsible for activating various epicardial-related genes has also been found in C/EBP binding-dependent regulatory regions of *Wt1* and *Raldh2* (Huang et al., 2012).

In contrast to the mammalian epicardium, zebrafish adult epicardial cells become highly proliferative and invade the underlying tissue upon injury (González-Rosa et al., 2011; Lepilina et al., 2006). FGF and PDGF ligands are secreted by cardiomyocytes and thrombocytes, respectively, and the activated epicardium expresses responsive receptors, which induce a downstream cascade of signals driving epicardial EMT and EPDC mobilisation (Kim et al., 2010; Lepilina et al., 2006). Using a proteomic approach, it was revealed that HA and its receptor hyaluronan-mediated motility receptor (Hmnr) are required for epicardial EMT during zebrafish heart regeneration (Missinato et al., 2015). In this context, decreased production of HA does not affect epicardial reactivation after injury but impairs epicardial EMT and EPDC migration into the injured area, resulting in failed heart regeneration. A further study revealed an unexpected role for the

outflow tract or bulbus arteriosus (BA) in epicardial cell migration (Wang et al., 2015). This study showed that, after nitroreductase-specific targeting of the epicardial layer, the epicardium rapidly regenerates through proliferation and migration of the surviving cells. The BA seems to drive this coordinated and directed movement of the regenerating cells, guiding them from the base of the ventricle to its apex, in a manner that is dependent upon short-range Sonic hedgehog (Shh) signalling acting on the migrating cells, and is necessary and sufficient for epicardial regeneration. By elegantly reproducing epicardial regeneration *ex vivo*, it was further demonstrated that Shh can substitute for the influence of the outflow tract structure, highlighting the importance of understanding how tissue interactions and signals are established during the regenerative process and how they are required for mobilising epicardial cells upon injury.

**The human epicardium and human EPDCs**

A key step towards exploiting the full therapeutic benefit of resident epicardial cells is translating their potential into the human setting. A number of *ex vivo* studies have isolated and characterised human primary epicardial cells derived from right atrial appendages, taken from individuals undergoing right coronary artery bypass (Bax et al., 2011; Clunie-O'Connor et al., 2015; Moerkamp et al., 2016; van Tuyn et al., 2007). Such atrial epicardial cells may differ from their ventricular counterparts, with the latter being potentially more relevant following MI, given the localisation of injury to the ventricular wall; however, they do still express WT1 and TBX18, and undergo EMT (Bax et al., 2011; van Tuyn et al., 2007). A recent characterisation of the human embryonic and foetal epicardium using three-dimensional optical projection tomography (OPT)

revealed that the epicardium is formed by Carnegie stage (CS) 14, significantly earlier than previously thought (Risebro et al., 2015). This study also showed that the embryonic and foetal ventricular, but not atrial, epicardium is a multi-layered epithelium, not only challenging the existing paradigm of the ventricle being enclosed by an epithelial monolayer, but also revealing what could be functionally relevant differences between the epicardium surrounding different chambers of the heart. Interestingly, the ventricular and atrial epicardium are organised differently, and while explants of ventricular epicardium undergo EMT spontaneously, the atrial epicardium retains an epithelial morphology when explanted (Risebro et al., 2015). Whether such differences are of significance with regard to the potential of the epicardium to give rise to different cardiovascular lineages is yet to be assessed. Recently, a contribution of the human embryonic epicardium to the coronary vasculature was proposed (Tomanek, 2016). These findings, by their very nature, are relatively descriptive and, consequently, further experimental validation and the development of new models are required to study human epicardial development. In this regard, recent studies have successfully differentiated epicardial-like cells from human embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) (Guadix et al., 2017; Iyer et al., 2015; Witty et al., 2014), providing alternative models for investigating the human epicardium. These studies revealed expression of key epicardial factors in epithelial-like cells after a BMP-, RA- and WNT-dependent differentiation process, and reported TGF $\beta$ -induced epicardial EMT, which culminated with smooth muscle and fibroblast cell-like derivatives (Guadix et al., 2017; Iyer et al., 2015; Witty et al., 2014). Interestingly, when human epicardial-like cells were xenotransplanted into chick embryos, via the yolk sac vasculature, they were capable of responding to long-range migratory cues/signals to colonise the host heart, and differentiate and integrate within the developing coronary vessels (Iyer et al., 2015).

Taken together, studies on the human epicardium largely support the expression of epicardial markers and EMT regulation that has been reported in various animal models and specifically in the mouse. However, these studies also reveal key differences; most notably, the human developing epicardium comprises a multi-layered epithelium, as opposed to the monolayer observed in the mouse. Whether such a difference is retained in the postnatal heart remains to be determined, as well as the potential associated differences in the epicardial-derived lineages. Another major difference between the human and mouse epicardium is the higher accumulation of adipose tissue in the human heart. In line with this observation, it has been suggested that the human adult epicardium may have a higher tendency to give rise to fat cells, an epicardial derivative reported in the mouse (Liu et al., 2014). As the origin of adipose tissue may have implications in the human response to cardiac injury and disease, it will be important to understand the relevance of such differences to advance our understanding of human heart remodelling after MI.

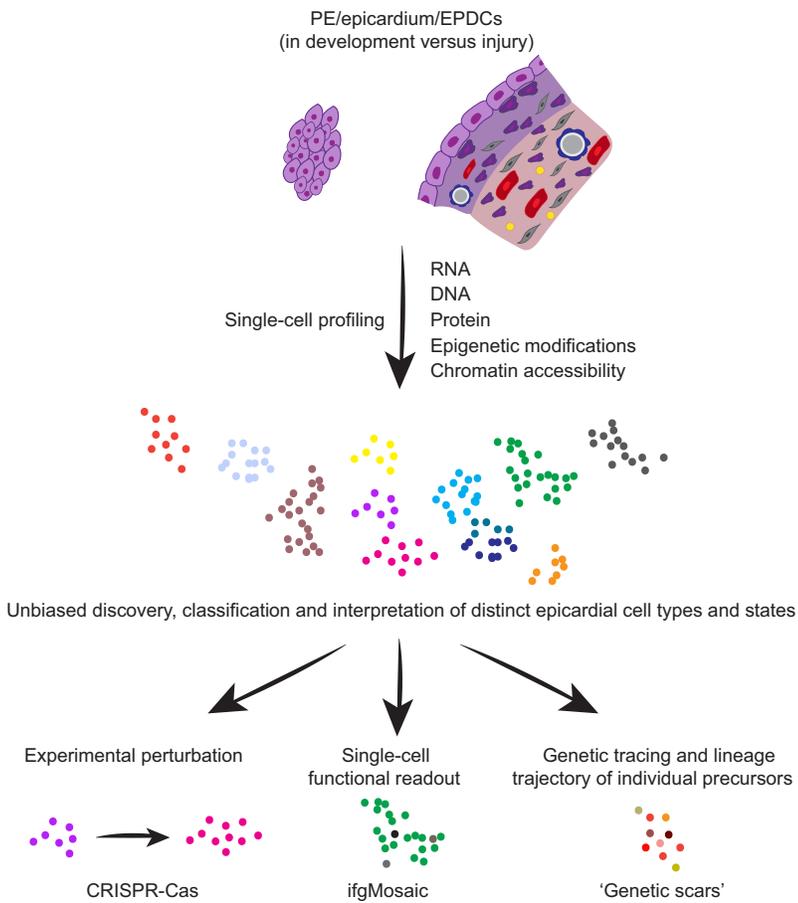
#### **New strategies to further dissect epicardial cell biology**

Although it is clear that we have made much progress in understanding the underlying biology of the epicardium and its derivatives, there still remain a number of unanswered questions and challenges, in particular with regard to deciphering cell heterogeneity and cell fate potential within the developing and adult epicardium. Moving forward to an unbiased understanding of epicardial cell populations and their lineage relationships within the heart, we must be able to unambiguously identify the source cells and their descendants.

At present, findings on the extent of EPDC contribution to cardiomyocyte and endothelial cell lineages have relied heavily on Cre-based fate mapping analyses (Acharya et al., 2011; Katz et al., 2012; Kikuchi et al., 2011a). However, these approaches have limitations and may confound interpretation of results due to problems with activation of the Cre drivers in lineage derivatives and mosaic or ectopic expression of reporters (Davis et al., 2012). Inconsistencies regarding the full extent of EPDC-fate and lineage potential may also be explained by limited use of confirmatory epicardial markers in these studies, with some being not entirely specific for epicardial cells (Christoffels et al., 2009; Rudat and Kispert, 2012) or failing to label the entire epicardial cell pool. Enhanced and more selective genetic tracing approaches could thus improve analyses of cell lineage and fate decision. A recent study has presented an interesting alternative to overcome the technical hurdle of non-specific Cre-mediated recombination; here, dual recombinases, Cre and Dre, were used to allow for exclusion of pre-existing, non-targeted cells also expressing Cre (He et al., 2017).

Current knowledge on the epicardium and EPDCs has also often failed to account for their apparently high degree of lineage heterogeneity, in terms of their developmental origins and cellular identity. In addition, the activation and migration states of EPDCs are driven by the integration of cues encountered in a spatiotemporal manner within a dynamic microenvironment. New experimental approaches that integrate these concepts may enable a better understanding of EPDC biology (Fig. 5). Until recently, most genomic profiling studies have analysed whole cell populations or, at best, subpopulations. However, recent technological advances have enabled genome-wide profiling of RNA (Mass et al., 2016; Picelli et al., 2013; Tang et al., 2009), DNA (Leung et al., 2015; Zong et al., 2012), protein (Bandura et al., 2009; Bodenmiller et al., 2012), epigenetic modifications (Farlik et al., 2015; Rotem et al., 2015; Smallwood et al., 2014) and chromatin accessibility (Buenrostro et al., 2015; Cusanovich et al., 2015) at single-cell resolution. The scale and precision of such studies offer an opportunity to build a systematic understanding of the genetic and epigenetic control of epicardial identity, refining the molecular characterisation of cellular subtypes and inferred functional output in an unbiased manner. Single-cell sequencing combined with spatial transcriptomics should also assist in elucidating how epicardial cells are organised into a multicellular layer, how the epicardium/EPDCs relate to each other in space and time, and through which molecules their function may vary during cardiac development, homeostasis and upon injury.

Even at the single cell-level, correlative changes in gene expression do not imply causation and, to move beyond phenomenology, the predictive modelling of gene regulation must be challenged by experimental perturbation approaches. Modern methods based on DNA and RNA targeting via CRISPR-Cas technology (Abudayyeh et al., 2017; Cong et al., 2013; East-Seletsky et al., 2016; Jinek et al., 2012; O'Connell et al., 2014) can be combined with single-cell genomics to interrogate epicardial function at unprecedented scale and resolution. However, one of the difficulties limiting our understanding of biological processes is the inability to distinguish such functional readouts at the single-cell level. A recent study using an inducible, fluorescent and functional genetic mosaic (ifgMosaic) analysis enables the monitoring of cell subpopulations with different genotypes in the same microenvironment (Pontes-Quero et al., 2017). This system could facilitate the interrogation of multiple and combinatorial gene function with high temporal and cellular resolution, and specifically help define cell-autonomous versus non-cell-autonomous function within epicardial subpopulations.



**Fig. 5. High-resolution technologies can enhance our knowledge of epicardial identity, lineage and function.** Single cell-based approaches, combined with cutting-edge technologies (e.g. genomic, transcriptomic and proteomic profiling, and analyses of epigenetic modifications and chromatin accessibility), can be used to functionally assess the identity of an epicardial cell over time. In addition, recently developed tools to perturb gene expression/function and trace cell lineages could be used to shed light on the main unanswered questions in epicardial cell biology. Ultimately, this knowledge will influence the development of epicardial cell-based therapies to regenerate the injured heart.

Finally, although it is clear that the epicardium as a whole is multipotent, it remains unknown whether this is true at the single-cell level, or whether the epicardium might in fact represent a collection of multiple fate-restricted cells. Recent developments in single-cell profiling of distinct cell types and (sub-) lineages could prove instrumental in addressing epicardial fate specification and determination. By CRISPR/Cas9-inducing a large variety of indels, cells and their progeny can become uniquely, irreversibly and incrementally marked over time, allowing unbiased genetic tracing and fate mapping of individual precursors (McKenna et al., 2016; Raj et al., 2017 preprint; Spanjaard et al., 2017 preprint). Adopting such experimental strategies could provide significant insight into individual epicardial progenitor cell fate and lineage potential.

### Conclusions

The central role of the epicardium in providing cardiovascular derivatives and mitogenic signals during heart development and regeneration makes it a potential therapeutic target. Understanding the different sources of epicardial cells and the biology of distinct subpopulations in the forming heart should provide important insights into developmental plasticity and how epicardial lineage heterogeneity can result in altered cell fate and function. This in turn should enable realisation of the full regenerative capacity of the adult reactivated epicardium and provide insight into how to harness and extend this capacity to adult mammals, including humans. Beyond increased basic understanding, the application of recently described cell models, combined with *ex vivo* approaches, to study human epicardium represent viable approaches to potential drug discovery (Clunie-O'Connor et al., 2015; Guadix et al., 2017; Iyer et al., 2015; Witty et al., 2014).

Despite significant advances in understanding epicardial origin and activation, many challenges remain. These include identifying the mechanisms underlying epicardial (re)activation and understanding not only how this process is initiated, but also how it terminates to reduce oncogenic risk. For example, we currently do not know the full capacity of activated epicardial cells and whether they are all fated to differentiate, either during development or in response to injury: a subset may proliferate and undergo EMT but may remain undifferentiated, and the fate of such cells, in either setting, remains to be determined. Another unknown concerns the origin of the epicardial cells that become reactivated upon cardiac injury: it is not fully understood whether embryonic-derived epicardial cells are capable of proliferation throughout life and, as such, are the sole contributors to the adult epicardium, or if reactivation is restricted to progenitors residing exclusively in the adult heart. Furthermore, potential differences or functional divergence between the human epicardial response and that described for model organisms, including zebrafish, chicken and mouse, remain to be elucidated. The initiation of the Human Cell Atlas project (Regev et al., 2017), with the promise of sequencing and mapping all the cells in the human body, may reveal human epicardial cell types and identify functionally relevant subpopulations. The subsequent understanding of their lineage relationships and interactions through development into adulthood could form the basis for future extrapolation to disease. Single-cell genomics has opened up a new frontier for understanding developmental and cell biology in general, and when applied to the epicardium should facilitate the translation of basic insights into epicardial biology into the development of cell-based therapies to regenerate the injured heart.

## Competing interests

P.R.R. is co-founder and equity holder in OxStem Cardio, an Oxford University spin-out that seeks to exploit therapeutic strategies stimulating endogenous repair in cardiovascular regenerative medicine.

## Funding

The authors' research is funded by the British Heart Foundation (RG/13/9/303269; CH/11/1/28798; RE/13/1/30181); Britain Israel Research and Academic Exchange Partnership (BIRAX) (13BX14PRET); and the Leducq Foundation (14CVD04).

## References

- Abudayyeh, O. O., Gootenberg, J. S., Essletzbichler, P., Han, S., Joung, J., Belanto, J. J., Verdine, V., Cox, D. B. T., Kellner, M. J., Regev, A. et al. (2017). RNA targeting with CRISPR-Cas13. *Nature* **550**, 280-284.
- Acharya, A., Baek, S. T., Banfi, S., Eskiocak, B. and Tallquist, M. D. (2011). Efficient inducible Cre-mediated recombination in Tcf21 cell lineages in the heart and kidney. *Genesis* **49**, 870-877.
- Acharya, A., Baek, S. T., Huang, G., Eskiocak, B., Goetsch, S., Sung, C. Y., Banfi, S., Sauer, M. F., Olsen, G. S., Duffield, J. S. et al. (2012). The bHLH transcription factor Tcf21 is required for lineage-specific EMT of cardiac fibroblast progenitors. *Development* **139**, 2139-2149.
- Airik, R., Bussen, M., Singh, M. K., Petry, M. and Kispert, A. (2006). Tbx18 regulates the development of the ureteral mesenchyme. *J. Clin. Invest.* **116**, 663-674.
- Aisagbonhi, O., Rai, M., Ryzhov, S., Atria, N., Feoktistov, I. and Hatzopoulos, A. K. (2011). Experimental myocardial infarction triggers canonical Wnt signaling and endothelial-to-mesenchymal transition. *Dis. Model. Mech.* **4**, 469-483.
- Balmer, G. M., Bollini, S., Dubé, K. N., Martínez-Barbera, J. P., Williams, O. and Riley, P. R. (2014). Dynamic haematopoietic cell contribution to the developing and adult epicardium. *Nat. Commun.* **5**, 4054.
- Bandura, D. R., Baranov, V. I., Ornatsky, O. I., Antonov, A., Kinach, R., Lou, X., Pavlov, S., Vorobiev, S., Dick, J. E. and Tanner, S. D. (2009). Mass cytometry: technique for real time single cell multitarget immunoassay based on inductively coupled plasma time-of-flight mass spectrometry. *Anal. Chem.* **81**, 6813-6822.
- Bax, N. A. M., Bleyl, S. B., Gallini, R., Wisse, L. J., Hunter, J., Van Oorschot, A. A. M., Mahtab, E. A. F., Lie-Venema, H., Goumans, M.-J., Betshtoltz, C. et al. (2010). Cardiac malformations in Pdgfra mutant embryos are associated with increased expression of WT1 and Nkx2.5 in the second heart field. *Dev. Dyn.* **239**, 2307-2317.
- Bax, N. A., Pijnappels, D. A., van Oorschot, A. A., Winter, E. M., de Vries, A. A., van Tuyn, J., Braun, J., Maas, S., Schaliq, M. J., Atsma, D. E. et al. (2011). Epithelial-to-mesenchymal transformation alters electrical conductivity of human epicardial cells. *J. Cell. Mol. Med.* **15**, 2675-2683.
- Bianchi, M. E. (2007). DAMPs, PAMPs and alarmins: all we need to know about danger. *J. Leukoc. Biol.* **81**, 1-5.
- Bock-Marquette, I., Shrivastava, S., Pipes, G. C. T., Thatcher, J. E., Blystone, A., Shelton, J. M., Galindo, C. L., Melegh, B., Srivastava, D., Olson, E. N. et al. (2009). Thymosin beta4 mediated PKC activation is essential to initiate the embryonic coronary developmental program and epicardial progenitor cell activation in adult mice in vivo. *J. Mol. Cell. Cardiol.* **46**, 728-738.
- Bodenmiller, B., Zunder, E. R., Finck, R., Chen, T. J., Savig, E. S., Bruggner, R. V., Simonds, E. F., Bendall, S. C., Sachs, K., Krutzik, P. O. et al. (2012). Multiplexed mass cytometry profiling of cellular states perturbed by small-molecule regulators. *Nat. Biotechnol.* **30**, 858-867.
- Bollini, S., Vieira, J. M. N., Howard, S., Dubé, K. N., Balmer, G. M., Smart, N. and Riley, P. R. (2014). Re-activated adult epicardial progenitor cells are a heterogeneous population molecularly distinct from their embryonic counterparts. *Stem Cells Dev.* **23**, 1719-1730.
- Brade, T., Kumar, S., Cunningham, T. J., Chatzi, C., Zhao, X., Cavallero, S., Li, P., Sucov, H. M., Ruiz-Lozano, P. and Duester, G. (2011). Retinoic acid stimulates myocardial expansion by induction of hepatic erythropoietin which activates epicardial Igf2. *Development* **138**, 139-148.
- Braitsch, C. M., Combs, M. D., Quaggin, S. E. and Yutzey, K. E. (2012). Pod1/Tcf21 is regulated by retinoic acid signaling and inhibits differentiation of epicardium-derived cells into smooth muscle in the developing heart. *Dev. Biol.* **368**, 345-357.
- Buckler, A. J., Pelletier, J., Haber, D. A., Glaser, T. and Housman, D. E. (1991). Isolation, characterization, and expression of the murine Wilms' tumor gene (WT1) during kidney development. *Mol. Cell. Biol.* **11**, 1707-1712.
- Buenrostro, J. D., Wu, B., Litzenburger, U. M., Ruff, D., Gonzales, M. L., Snyder, M. P., Chang, H. Y. and Greenleaf, W. J. (2015). Single-cell chromatin accessibility reveals principles of regulatory variation. *Nature* **523**, 486-490.
- Cai, C.-L., Martin, J. C., Sun, Y., Cui, L., Wang, L., Ouyang, K., Yang, L., Bu, L., Liang, X., Zhang, X. et al. (2008). A myocardial lineage derives from Tbx18 epicardial cells. *Nature* **454**, 104-108.
- Cano, E., Carmona, R., Ruiz-Villalba, A., Rojas, A., Chau, Y.-Y., Wagner, K. D., Wagner, N., Hastie, N. D., Muñoz-Chápuli, R. and Pérez-Pomares, J. M. (2016). Extracardiac septum transversum/proepicardial endothelial cells pattern embryonic coronary arterio-venous connections. *Proc. Natl. Acad. Sci. USA* **113**, 656-661.
- Cao, J., Navis, A., Cox, B. D., Dickson, A. L., Gemberling, M., Karra, R., Bagnat, M. and Poss, K. D. (2016). Single epicardial cell transcriptome sequencing identifies Caveolin 1 as an essential factor in zebrafish heart regeneration. *Development* **143**, 232-243.
- Cao, J., Wang, J., Jackman, C. P., Cox, A. H., Trembley, M. A., Balowski, J. J., Cox, B. D., De Simone, A., Dickson, A. L., Di Talia, S. et al. (2017). Tension creates an endoreplication wavefront that leads regeneration of epicardial tissue. *Dev. Cell* **42**, 600-615.e4.
- Chablais, F., Veit, J., Rainer, G. and Jaźwińska, A. (2011). The zebrafish heart regenerates after cryoinjury-induced myocardial infarction. *BMC Dev. Biol.* **11**, 21.
- Chau, Y.-Y., Bandiera, R., Serrels, A., Martínez-Estrada, O. M., Qing, W., Lee, M., Slight, J., Thornburn, A., Berry, R., McHaffie, S. et al. (2014). Visceral and subcutaneous fat have different origins and evidence supports a mesothelial source. *Nat. Cell Biol.* **16**, 367-375.
- Chong, J. H., Chandrakanthan, V., Xaymardan, M., Asli, N. S., Li, J., Ahmed, I., Hefferan, C., Menon, M. K., Scarlett, C. J., Rashidianfar, A. et al. (2011). Adult cardiac-resident MSC-like stem cells with a proepicardial origin. *Cell Stem Cell* **9**, 527-540.
- Christoffels, V. M., Mommersteeg, M. T., Trowe, M. O., Prall, O. W., de Gier-de Vries, C., Soufan, A. T., Bussen, M., Schuster-Gossler, K., Harvey, R. P., Moorman, A. F. et al. (2006). Formation of the venous pole of the heart from an Nkx2-5-negative precursor population requires Tbx18. *Circ. Res.* **98**, 1555-1563.
- Christoffels, V. M., Grieskamp, T., Norden, J., Mommersteeg, M. T., Rudat, C. and Kispert, A. (2009). Tbx18 and the fate of epicardial progenitors. *Nature* **458**, E8-E9; discussion E9-10.
- Clunie-O'Connor, C., Smits, A. M., Antoniadis, C., Russell, A. J., Yellon, D. M., Goumans, M. J. and Riley, P. R. (2015). The derivation of primary human epicardium-derived cells. *Curr. Protoc. Stem Cell Biol.* **35**, 2C.5.1-2C.5.12.
- Combs, M. D., Braitsch, C. M., Lange, A. W., James, J. F. and Yutzey, K. E. (2011). NFATC1 promotes epicardium-derived cell invasion into myocardium. *Development* **138**, 1747-1757.
- Compton, L. A., Potash, D. A., Brown, C. B. and Barnett, J. V. (2007). Coronary vessel development is dependent on the type III transforming growth factor beta receptor. *Circ. Res.* **101**, 784-791.
- Cong, L., Ran, F. A., Cox, D., Lin, S., Barretto, R., Habib, N., Hsu, P. D., Wu, X., Jiang, W., Marraffini, L. A. et al. (2013). Multiplex genome engineering using CRISPR/Cas systems. *Science* **339**, 819-823.
- Craig, E. A., Austin, A. F., Vaillancourt, R. R., Barnett, J. V. and Camenisch, T. D. (2010). TGFbeta2-mediated production of hyaluronan is important for the induction of epicardial cell differentiation and invasion. *Exp. Cell Res.* **316**, 3397-3405.
- Cui, S., Schwartz, L. and Quaggin, S. E. (2003). Pod1 is required in stromal cells for glomerulogenesis. *Dev. Dyn.* **226**, 512-522.
- Cusanovich, D. A., Daza, R., Adey, A., Pliner, H. A., Christiansen, L., Gunderson, K. L., Steemers, F. J., Trapnell, C. and Shendure, J. (2015). Multiplex single cell profiling of chromatin accessibility by combinatorial cellular indexing. *Science* **348**, 910-914.
- Davis, J., Maillet, M., Miano, J. M. and Molkentin, J. D. (2012). Lost in transgenesis: a user's guide for genetically manipulating the mouse in cardiac research. *Circ. Res.* **111**, 761-777.
- Dekker, R. J., van Soest, S., Fontijn, R. D., Salamanca, S., de Groot, P. G., VanBavel, E., Pannekoek, H. and Horrevoets, A. J. (2002). Prolonged fluid shear stress induces a distinct set of endothelial cell genes, most specifically lung Kruppel-like factor (KLF2). *Blood* **100**, 1689-1698.
- del Monte, G., Casanova, J. C., Guadix, J. A., MacGrogan, D., Burch, J. B. E., Pérez-Pomares, J. M. and de la Pompa, J. L. (2011). Differential Notch signaling in the epicardium is required for cardiac inflow development and coronary vessel morphogenesis. *Circ. Res.* **108**, 824-836.
- Dettman, R. W., Denetclaw, W., Jr, Ordahl, C. P. and Bristow, J. (1998). Common epicardial origin of coronary vascular smooth muscle, perivascular fibroblasts, and intermyocardial fibroblasts in the avian heart. *Dev. Biol.* **193**, 169-181.
- Duan, J., Gherghe, C., Liu, D., Hamlett, E., Srikantha, L., Rodgers, L., Regan, J. N., Rojas, M., Willis, M., Leask, A. et al. (2012). Wnt1/betacatenin injury response activates the epicardium and cardiac fibroblasts to promote cardiac repair. *EMBO J.* **31**, 429-442.
- Duim, S. N., Kurakula, K., Goumans, M.-J. and Kruthof, B. P. T. (2015). Cardiac endothelial cells express Wilms' tumor-1: Wt1 expression in the developing, adult and infarcted heart. *J. Mol. Cell. Cardiol.* **81**, 127-135.
- East-Seletsky, A., O'Connell, M. R., Knight, S. C., Burstein, D., Cate, J. H. D., Tjian, R. and Doudna, J. A. (2016). Two distinct RNase activities of CRISPR-C2c2 enable guide-RNA processing and RNA detection. *Nature* **538**, 270-273.
- Epelman, S., Liu, P. P. and Mann, D. L. (2015). Role of innate and adaptive immune mechanisms in cardiac injury and repair. *Nat. Rev. Immunol.* **15**, 117-129.
- Farlik, M., Sheffield, N. C., Nuzzo, A., Datlinger, P., Schönegger, A., Klughammer, J. and Bock, C. (2015). Single-cell DNA methylome sequencing and bioinformatic inference of epigenomic cell-state dynamics. *Cell Rep.* **10**, 1386-1397.
- González-Rosa, J. M., Martin, V., Peralta, M., Torres, M. and Mercader, N. (2011). Extensive scar formation and regression during heart regeneration after cryoinjury in zebrafish. *Development* **138**, 1663-1674.

- González-Rosa, J. M., Peralta, M. and Mercader, N. (2012). Pan-epicardial lineage tracing reveals that epicardium derived cells give rise to myofibroblasts and perivascular cells during zebrafish heart regeneration. *Dev. Biol.* **370**, 173-186.
- Guadix, J. A., Carmona, R., Muñoz-Chápuli, R. and Pérez-Pomares, J. M. (2006). In vivo and in vitro analysis of the vasculogenic potential of avian proepicardial and epicardial cells. *Dev. Dyn.* **235**, 1014-1026.
- Guadix, J. A., Orlova, V. V., Giacomelli, E., Bellin, M., Ribeiro, M. C., Mummery, C. L., Pérez-Pomares, J. M. and Passier, R. (2017). Human pluripotent stem cell differentiation into functional epicardial progenitor cells. *Stem Cell Reports* **9**, 1754-1764.
- Hatcher, C. J., Diman, N. Y. S.-G., Kim, M.-S., Pennisi, D., Song, Y., Goldstein, M. M., Mikawa, T. and Basson, C. T. (2004). A role for Tbx5 in proepicardial cell migration during cardiogenesis. *Physiol. Genomics* **18**, 129-140.
- Haubner, B. J., Adamowicz-Brice, M., Khadayate, S., Tiefenthaler, V., Metzler, B., Aitman, T. and Penninger, J. M. (2012). Complete cardiac regeneration in a mouse model of myocardial infarction. *Aging* **4**, 966-977.
- Haubner, B. J., Schneider, J., Schweigmann, U., Schuetz, T., Dichtl, W., Velik-Salchner, C., Stein, J.-I. and Penninger, J. M. (2016). Functional recovery of a human neonatal heart after severe myocardial infarction. *Circ. Res.* **118**, 216-221.
- He, L., Li, Y., Li, Y., Pu, W., Huang, X., Tian, X., Wang, Y., Zhang, H., Liu, Q., Zhang, L. et al. (2017). Enhancing the precision of genetic lineage tracing using dual recombinases. *Nat. Med.* **23**, 1488-1498.
- Hidai, H., Bardales, R., Goodwin, R., Quertermous, T. and Quertermous, E. E. (1998). Cloning of capsulin, a basic helix-loop-helix factor expressed in progenitor cells of the pericardium and the coronary arteries. *Mech. Dev.* **73**, 33-43.
- Hirakow, R. (1992). Epicardial formation in staged human embryos. *Kaibogaku Zasshi* **67**, 616-622.
- Hirose, T., Karasawa, M., Sugitani, Y., Fujisawa, M., Akimoto, K., Ohno, S. and Noda, T. (2006). PAR3 is essential for cyst-mediated epicardial development by establishing apical cortical domains. *Development* **133**, 1389-1398.
- Huang, G. N., Thatcher, J. E., McAnally, J., Kong, Y., Qi, X., Tan, W., DiMaio, J. M., Amatruda, J. F., Gerard, R. D., Hill, J. A. et al. (2012). C/EBP transcription factors mediate epicardial activation during heart development and injury. *Science* **338**, 1599-1603.
- Ishii, Y., Langberg, J. D., Hurtado, R., Lee, S. and Mikawa, T. (2007). Induction of proepicardial marker gene expression by the liver bud. *Development* **134**, 3627-3637.
- Ishii, Y., Garriock, R. J., Navetta, A. M., Coughlin, L. E. and Mikawa, T. (2010). BMP signals promote proepicardial protrusion necessary for recruitment of coronary vessel and epicardial progenitors to the heart. *Dev. Cell* **19**, 307-316.
- Iyer, D., Gambardella, L., Bernard, W. G., Serrano, F., Mascetti, V. L., Pedersen, R. A., Talasila, A. and Sinha, S. (2015). Robust derivation of epicardium and its differentiated smooth muscle cell progeny from human pluripotent stem cells. *Development* **142**, 1528-1541.
- Jahr, M., Schlueter, J., Brand, T. and Männer, J. (2008). Development of the proepicardium in *Xenopus laevis*. *Dev. Dyn.* **237**, 3088-3096.
- Jinek, M., Chylinski, K., Fonfara, I., Hauer, M., Doudna, J. A. and Charpentier, E. (2012). A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science* **337**, 816-821.
- Jopling, C., Sleep, E., Raya, M., Martí, M., Raya, A. and Izpisua Belmonte, J. C. I. (2010). Zebrafish heart regeneration occurs by cardiomyocyte dedifferentiation and proliferation. *Nature* **464**, 606-609.
- Kanisicak, O., Khalil, H., Ivey, M. J., Karch, J., Maliken, B. D., Correll, R. N., Brody, M. J., Lin, S.-C. J., Aronow, B. J., Tallquist, M. D. et al. (2016). Genetic lineage tracing defines myofibroblast origin and function in the injured heart. *Nat. Commun.* **7**, 12260.
- Katz, T. C., Singh, M. K., Degenhardt, K., Rivera-Feliciano, J., Johnson, R. L., Epstein, J. A. and Tabin, C. J. (2012). Distinct compartments of the proepicardial organ give rise to coronary vascular endothelial cells. *Dev. Cell* **22**, 639-650.
- Kikuchi, K., Holdway, J. E., Werdich, A. A., Anderson, R. M., Fang, Y., Egnaczyk, G. F., Evans, T., Macrae, C. A., Stainier, D. Y. R. and Poss, K. D. (2010). Primary contribution to zebrafish heart regeneration by *gata4*<sup>+</sup> cardiomyocytes. *Nature* **464**, 601-605.
- Kikuchi, K., Gupta, V., Wang, J., Holdway, J. E., Wills, A. A., Fang, Y. and Poss, K. D. (2011a). *tcf21*<sup>+</sup> epicardial cells adopt non-myocardial fates during zebrafish heart development and regeneration. *Development* **138**, 2895-2902.
- Kikuchi, K., Holdway, J. E., Major, R. J., Blum, N., Dahn, R. D., Begemann, G. and Poss, K. D. (2011b). Retinoic acid production by endocardium and epicardium is an injury response essential for zebrafish heart regeneration. *Dev. Cell* **20**, 397-404.
- Kim, J., Wu, Q., Zhang, Y., Wiens, K. M., Huang, Y., Rubin, N., Shimada, H., Handin, R. I., Chao, M. Y., Tuan, T.-L. et al. (2010). PDGF signaling is required for epicardial function and blood vessel formation in regenerating zebrafish hearts. *Proc. Natl. Acad. Sci. USA* **107**, 17206-17210.
- Kimura, W., Xiao, F., Canseco, D. C., Muralidhar, S., Thet, S., Zhang, H. M., Abderrahman, Y., Chen, R., Garcia, J. A., Shelton, J. M. et al. (2015). Hypoxia fate mapping identifies cycling cardiomyocytes in the adult heart. *Nature* **523**, 226-230.
- Kocabas, F., Mahmoud, A. I., Susic, D., Porrello, E. R., Chen, R., Garcia, J. A., DeBerardinis, R. J. and Sadek, H. A. (2012). The hypoxic epicardial and subepicardial microenvironment. *J. Cardiovasc. Transl. Res.* **5**, 654-665.
- Komiyama, M., Ito, K. and Shimada, Y. (1987). Origin and development of the epicardium in the mouse embryo. *Anat. Embryol.* **176**, 183-189.
- Kraus, F., Haenig, B. and Kispert, A. (2001). Cloning and expression analysis of the mouse T-box gene *Tbx18*. *Mech. Dev.* **100**, 83-86.
- Kruithof, B. P. T., van Wijk, B., Somi, S., Kruithof-de Julio, M., Pérez Pomares, J. M., Weesie, F., Wessels, A., Moorman, A. F. and van den Hoff, M. J. (2006). BMP and FGF regulate the differentiation of multipotential pericardial mesoderm into the myocardial or epicardial lineage. *Dev. Biol.* **295**, 507-522.
- Kurkiewicz, T. (1909). O histogenezie mięśnia sercowego zwierząt kregowych. *Bull. Int. Acad. Sci. Cracovie*, 148-191.
- Kwee, L., Baldwin, H. S., Shen, H. M., Stewart, C. L., Buck, C., Buck, C. A. and Labow, M. A. (1995). Defective development of the embryonic and extraembryonic circulatory systems in vascular cell adhesion molecule (VCAM-1) deficient mice. *Development* **121**, 489-503.
- Lepilina, A., Coon, A. N., Kikuchi, K., Holdway, J. E., Roberts, R. W., Burns, C. G. and Poss, K. D. (2006). A dynamic epicardial injury response supports progenitor cell activity during zebrafish heart regeneration. *Cell* **127**, 607-619.
- Leung, M. L., Wang, Y., Waters, J. and Navin, N. E. (2015). SNES: single nucleus exome sequencing. *Genome Biol.* **16**, 55.
- Li, P., Cavallero, S., Gu, Y., Chen, T. H. P., Hughes, J., Hassan, A. B., Bruning, J. C., Pashmforoush, M. and Sucov, H. M. (2011). IGF signaling directs ventricular cardiomyocyte proliferation during embryonic heart development. *Development* **138**, 1795-1805.
- Liu, J. and Stainier, D. Y. R. (2010). *Tbx5* and *Bmp* signaling are essential for proepicardium specification in zebrafish. *Circ. Res.* **106**, 1818-1828.
- Liu, Q., Huang, X., Oh, J.-H., Lin, R.-Z., Duan, S., Yu, Y., Yang, R., Qiu, J., Melero-Martin, J. M., Pu, W. T. et al. (2014). Epicardium-to-fat transition in injured heart. *Cell Res.* **24**, 1367-1369.
- Lotze, M. T., Zeh, H. J., Rubartelli, A., Sparvero, L. J., Amoscato, A. A., Washburn, N. R., Devera, M. E., Liang, X., Tör, M. and Billiar, T. (2007). The grateful dead: damage-associated molecular pattern molecules and reduction/oxidation regulate immunity. *Immunol. Rev.* **220**, 60-81.
- Manasek, F. J. (1969). Embryonic development of the heart. II. Formation of the epicardium. *J. Embryol. Exp. Morphol.* **22**, 333-348.
- Männer, J. (1992). The development of pericardial villi in the chick embryo. *Anat. Embryol.* **186**, 379-385.
- Männer, J. (1999). Does the subepicardial mesenchyme contribute to the myocardium of the chick embryo heart? A quail-chick chimera study tracing the fate of the epicardial primordium. *Anat. Rec.* **255**, 212-226.
- Männer, J., Pérez-Pomares, J. M., Macías, D. and Muñoz-Chápuli, R. (2001). The origin, formation and developmental significance of the epicardium: a review. *Cells Tissues Organs* **169**, 89-103.
- Marro, J., Pfefferli, C., de Preux Charles, A.-S., Bise, T. and Jaźwińska, A. (2016). Collagen XII contributes to epicardial and connective tissues in the zebrafish heart during ontogenesis and regeneration. *PLoS ONE* **11**, e0165497.
- Martínez-Estrada, O. M., Lettice, L. A., Essafi, A., Guadix, J. A., Slight, J., Veleda, V., Hall, E., Reichmann, J., Devenney, P. S., Hohenstein, P. et al. (2010). *Wt1* is required for cardiovascular progenitor cell formation through transcriptional control of *Snail* and *E-cadherin*. *Nat. Genet.* **42**, 89-93.
- Mass, E., Ballesteros, I., Farlik, M., Halbritter, F., Gunther, P., Crozet, L., Jacome-Galarza, C. E., Handler, K., Klughammer, J., Kobayashi, Y. et al. (2016). Specification of tissue-resident macrophages during organogenesis. *Science* **353**, aaf4238.
- Masters, M. and Riley, P. R. (2014). The epicardium signals the way towards heart regeneration. *Stem Cell Res.* **13**, 683-692.
- Matzinger, P. (1994). Tolerance, danger, and the extended family. *Annu. Rev. Immunol.* **12**, 991-1045.
- Maya-Ramos, L., Cleland, J., Bressan, M. and Mikawa, T. (2013). Induction of the proepicardium. *J. Dev. Biol.* **1**, 82-91.
- McKenna, A., Findlay, G. M., Gagnon, J. A., Horwitz, M. S., Schier, A. F. and Shendure, J. (2016). Whole-organism lineage tracing by combinatorial and cumulative genome editing. *Science* **353**, aaf7907.
- Mellgren, A. M., Smith, C. L., Olsen, G. S., Eskiocak, B., Zhou, B., Kazi, M. N., Ruiz, F. R., Pu, W. T. and Tallquist, M. D. (2008). Platelet-derived growth factor receptor beta signaling is required for efficient epicardial cell migration and development of two distinct coronary vascular smooth muscle cell populations. *Circ. Res.* **103**, 1393-1401.
- Merki, E., Zamora, M., Raya, A., Kawakami, Y., Wang, J., Zhang, X., Burch, J., Kubalak, S. W., Kaliman, P., Izpisua Belmonte, J. C. et al. (2005). Epicardial retinoid X receptor alpha is required for myocardial growth and coronary artery formation. *Proc. Natl. Acad. Sci. USA* **102**, 18455-18460.
- Mikawa, T. and Fischman, D. A. (1992). Retroviral analysis of cardiac morphogenesis: discontinuous formation of coronary vessels. *Proc. Natl. Acad. Sci. USA* **89**, 9504-9508.

- Mikawa, T. and Gourdie, R. G.** (1996). Pericardial mesoderm generates a population of coronary smooth muscle cells migrating into the heart along with ingrowth of the epicardial organ. *Dev. Biol.* **174**, 221-232.
- Missinato, M. A., Tobita, K., Romano, N., Carroll, J. A. and Tsang, M.** (2015). Extracellular component hyaluronic acid and its receptor Hmhr are required for epicardial EMT during heart regeneration. *Cardiovasc. Res.* **107**, 487-498.
- Moerkamp, A. T., Lodder, K., van Herwaarden, T., Dronkers, E., Dingenouts, C. K. E., Tengström, F. C., van Brakel, T. J., Goumans, M.-J. and Smits, A. M.** (2016). Human fetal and adult epicardial-derived cells: a novel model to study their activation. *Stem Cell Res. Ther.* **7**, 174.
- Molin, D. G. M., Bartram, U., Van der Heiden, K., Van Iperen, L., Speer, C. P., Hierck, B. P., Poelmann, R. E. and Gittenberger-de-Groot, A. C.** (2003). Expression patterns of Tgfbeta1-3 associate with myocardialisation of the outflow tract and the development of the epicardium and the fibrous heart skeleton. *Dev. Dyn.* **227**, 431-444.
- Moore, A. W., McInnes, L., Kreidberg, J., Hastie, N. D. and Schedl, A.** (1999). YAC complementation shows a requirement for Wt1 in the development of epicardium, adrenal gland and throughout nephrogenesis. *Development* **126**, 1845-1857.
- Moore-Morris, T., Guimarães-Camboa, N., Banerjee, I., Zambon, A. C., Kisseleva, T., Velayoudon, A., Stallcup, W. B., Gu, Y., Dalton, N. D., Cedeno, M. et al.** (2014). Resident fibroblast lineages mediate pressure overload-induced cardiac fibrosis. *J. Clin. Invest.* **124**, 2921-2934.
- Morabito, C. J., Dettman, R. W., Kattan, J., Collier, J. M. and Bristow, J.** (2001). Positive and negative regulation of epicardial-mesenchymal transformation during avian heart development. *Dev. Biol.* **234**, 204-215.
- Nahirney, P. C., Mikawa, T. and Fischman, D. A.** (2003). Evidence for an extracellular matrix bridge guiding proepicardial cell migration to the myocardium of chick embryos. *Dev. Dyn.* **227**, 511-523.
- O'Connell, M. R., Oakes, B. L., Sternberg, S. H., East-Seletsky, A., Kaplan, M. and Doudna, J. A.** (2014). Programmable RNA recognition and cleavage by CRISPR/Cas9. *Nature* **516**, 263-266.
- Pennisi, D. J. and Mikawa, T.** (2009). FGFR-1 is required by epicardium-derived cells for myocardial invasion and correct coronary vascular lineage differentiation. *Dev. Biol.* **328**, 148-159.
- Peralta, M., Steed, E., Harlepp, S., González-Rosa, J. M., Monduc, F., Ariza-Cosano, A., Cortés, A., Rayón, T., Gómez-Skarmeta, J.-L., Zapata, A. et al.** (2013). Heartbeat-driven pericardial fluid forces contribute to epicardium morphogenesis. *Curr. Biol.* **23**, 1726-1735.
- Pérez-Pomares, J. M. and de la Pompa, J. L.** (2011). Signaling during epicardium and coronary vessel development. *Circ. Res.* **109**, 1429-1442.
- Pérez-Pomares, J. M., Macías, D., García-Garrido, L. and Muñoz-Chápuli, R.** (1997). Contribution of the primitive epicardium to the subepicardial mesenchyme in hamster and chick embryos. *Dev. Dyn.* **210**, 96-105.
- Pérez-Pomares, J. M., Macías, D., García-Garrido, L. and Muñoz-Chápuli, R.** (1998). The origin of the subepicardial mesenchyme in the avian embryo: an immunohistochemical and quail-chick chimera study. *Dev. Biol.* **200**, 57-68.
- Pérez-Pomares, J. M., Carmona, R., González-Iriarte, M., Atencia, G., Wessels, A. and Muñoz-Chápuli, R.** (2002). Origin of coronary endothelial cells from epicardial mesothelium in avian embryos. *Int. J. Dev. Biol.* **46**, 1005-1013.
- Pérez-Pomares, J. M., Phelps, A., Sedmerova, M. and Wessels, A.** (2003). Epicardial-like cells on the distal arterial end of the cardiac outflow tract do not derive from the proepicardium but are derivatives of the cephalic pericardium. *Dev. Dyn.* **227**, 56-68.
- Picelli, S., Björklund, A. K., Faridani, O. R., Sagasser, S., Winberg, G. and Sandberg, R.** (2013). Smart-seq2 for sensitive full-length transcriptome profiling in single cells. *Nat. Methods* **10**, 1096-1098.
- Plavicki, J. S., Hofsteen, P., Yue, M. S., Lanham, K. A., Peterson, R. E. and Heideman, W.** (2014). Multiple modes of proepicardial cell migration require heartbeat. *BMC Dev. Biol.* **14**, 18.
- Poelmann, R. E., Gittenberger-de Groot, A. C., Mentink, M. M., Bokenkamp, R. and Hogers, B.** (1993). Development of the cardiac coronary vascular endothelium, studied with antiendothelial antibodies, in chicken-quail chimeras. *Circ. Res.* **73**, 559-568.
- Pombal, M. A., Carmona, R., Megías, M., Ruiz, A., Pérez-Pomares, J. M. and Muñoz-Chápuli, R.** (2008). Epicardial development in lamprey supports an evolutionary origin of the vertebrate epicardium from an ancestral pronephric external glomerulus. *Evol. Dev.* **10**, 210-216.
- Pontes-Quero, S., Heredia, L., Casquero-Garcia, V., Fernandez-Chacon, M., Luo, W., Hermoso, A., Bansal, M., Garcia-Gonzalez, I., Sanchez-Munoz, M. S., Perea, J. R. et al.** (2017). Dual ifgMosaic: a versatile method for multispectral and combinatorial mosaic gene-function analysis. *Cell* **170**, 800-814.e18.
- Porrello, E. R., Mahmoud, A. I., Simpson, E., Hill, J. A., Richardson, J. A., Olson, E. N. and Sadek, H. A.** (2011). Transient regenerative potential of the neonatal mouse heart. *Science* **331**, 1078-1080.
- Poss, K. D., Wilson, L. G. and Keating, M. T.** (2002). Heart regeneration in zebrafish. *Science* **298**, 2188-2190.
- Puente, B. N., Kimura, W., Muralidhar, S. A., Moon, J., Amatruda, J. F., Phelps, K. L., Grinsfelder, D., Rothermel, B. A., Chen, R., Garcia, J. A. et al.** (2014). The oxygen-rich postnatal environment induces cardiomyocyte cell-cycle arrest through DNA damage response. *Cell* **157**, 565-579.
- Quaggin, S. E., Schwartz, L., Cui, S., Igarashi, P., Deimling, J., Post, M. and Rossant, J.** (1999). The basic-helix-loop-helix protein pod1 is critically important for kidney and lung organogenesis. *Development* **126**, 5771-5783.
- Raj, B., Wagner, D. E., McKenna, A., Pandey, S., Klein, A. M., Shendure, J., Gagnon, J. A. and Schier, A. F.** (2017). Simultaneous single-cell profiling of lineages and cell types in the vertebrate brain by scGESTALT. *bioRxiv*, 205534.
- Ramjee, V., Li, D., Manderfield, L. J., Liu, F., Engleka, K. A., Aghajanian, H., Rodell, C. B., Lu, W., Ho, V., Wang, T. et al.** (2017). Epicardial YAP/TAZ orchestrate an immunosuppressive response following myocardial infarction. *J. Clin. Invest.* **127**, 899-911.
- Regev, A., Teichmann, S. A., Lander, E. S., Amit, I., Benoist, C., Birney, E., Bodenmiller, B., Campbell, P., Carninci, P., Clatworthy, M. et al.** (2017). The Human Cell Atlas. *Elife* **6**, e27041.
- Riley, P. R.** (2012). An epicardial floor plan for building and rebuilding the mammalian heart. *Curr. Top. Dev. Biol.* **100**, 233-251.
- Risebro, C. A., Vieira, J. M., Klotz, L. and Riley, P. R.** (2015). Characterisation of the human embryonic and foetal epicardium during heart development. *Development* **142**, 3630-3636.
- Robb, L., Mifsud, L., Hartley, L., Biben, C., Copeland, N. G., Gilbert, D. J., Jenkins, N. A. and Harvey, R. P.** (1998). epicardin: a novel basic helix-loop-helix transcription factor gene expressed in epicardium, branchial arch myoblasts, and mesenchyme of developing lung, gut, kidney, and gonads. *Dev. Dyn.* **213**, 105-113.
- Rodgers, L. S., Lalani, S., Runyan, R. B. and Camenisch, T. D.** (2008). Differential growth and multicellular villi direct proepicardial translocation to the developing mouse heart. *Dev. Dyn.* **237**, 145-152.
- Rotem, A., Ram, O., Shores, N., Sperling, R. A., Goren, A., Weitz, D. A. and Bernstein, B. E.** (2015). Single-cell ChIP-seq reveals cell subpopulations defined by chromatin state. *Nat. Biotechnol.* **33**, 1165-1172.
- Rubartelli, A. and Lotze, M. T.** (2007). Inside, outside, upside down: damage-associated molecular-pattern molecules (DAMPs) and redox. *Trends Immunol.* **28**, 429-436.
- Rudat, C. and Kispert, A.** (2012). Wt1 and epicardial fate mapping. *Circ. Res.* **111**, 165-169.
- Ruiz-Villalba, A., Ziogas, A., Ehrbar, M. and Pérez-Pomares, J. M.** (2013). Characterization of epicardial-derived cardiac interstitial cells: differentiation and mobilization of heart fibroblast progenitors. *PLoS ONE* **8**, e53694.
- Ruiz-Villalba, A., Simón, A. M., Pogontke, C., Castillo, M. I., Abizanda, G., Pelacho, B., Sánchez-Domínguez, R., Segovia, J. C., Prósper, F. and Pérez-Pomares, J. M.** (2015). Interacting resident epicardium-derived fibroblasts and recruited bone marrow cells form myocardial infarction scar. *J. Am. Coll. Cardiol.* **65**, 2057-2066.
- Schlueter, J., Männer, J. and Brand, T.** (2006). BMP is an important regulator of proepicardial identity in the chick embryo. *Dev. Biol.* **295**, 546-558.
- Schulte, I., Schlueter, J., Abu-Issa, R., Brand, T. and Männer, J.** (2007). Morphological and molecular left-right asymmetries in the development of the proepicardium: a comparative analysis on mouse and chick embryos. *Dev. Dyn.* **236**, 684-695.
- Sengbusch, J. K., He, W., Pinco, K. A. and Yang, J. T.** (2002). Dual functions of [alpha]4[beta]1 integrin in epicardial development: initial migration and long-term attachment. *J. Cell Biol.* **157**, 873-882.
- Serluca, F. C.** (2008). Development of the proepicardial organ in the zebrafish. *Dev. Biol.* **315**, 18-27.
- Shikama, N., Lutz, W., Kretzschmar, R., Sauter, N., Roth, J. F., Marino, S., Wittwer, J., Scheidweiler, A. and Eckner, R.** (2003). Essential function of p300 acetyltransferase activity in heart, lung and small intestine formation. *EMBO J.* **22**, 5175-5185.
- Shinohara, K. and Hamada, H.** (2017). Cilia in left-right symmetry breaking. *Cold Spring Harb. Perspect. Biol.* **9**, a028282.
- Smallwood, S. A., Lee, H. J., Angermueller, C., Krueger, F., Saadeh, H., Peat, J., Andrews, S. R., Stegle, O., Reik, W. and Kelsey, G.** (2014). Single-cell genome-wide bisulfite sequencing for assessing epigenetic heterogeneity. *Nat. Methods* **11**, 817-820.
- Smart, N., Risebro, C. A., Melville, A. A. D., Moses, K., Schwartz, R. J., Chien, K. R. and Riley, P. R.** (2007). Thymosin beta4 induces adult epicardial progenitor mobilization and neovascularization. *Nature* **445**, 177-182.
- Smart, N., Risebro, C. A., Clark, J. E., Ehler, E., Miquero, L., Rossdeutsch, A., Marber, M. S. and Riley, P. R.** (2010). Thymosin beta4 facilitates epicardial neovascularization of the injured adult heart. *Ann. N. Y. Acad. Sci.* **1194**, 97-104.
- Smart, N., Bollini, S., Dubé, K. N., Vieira, J. M., Zhou, B., Davidson, S., Yellon, D., Riegler, J., Price, A. N., Lythgoe, M. F. et al.** (2011). De novo cardiomyocytes from within the activated adult heart after injury. *Nature* **474**, 640-644.
- Smith, C. L., Baek, S. T., Sung, C. Y. and Tallquist, M. D.** (2011). Epicardial-derived cell epithelial-to-mesenchymal transition and fate specification require PDGF receptor signaling. *Circ. Res.* **108**, e15-e26.
- Spanjaard, B., Hu, B., Mitic, N. and Junker, J. P.** (2017). Massively parallel single cell lineage tracing using CRISPR/Cas9 induced genetic scars. *bioRxiv*, 205971.

- Stevens, S. M., von Gise, A., VanDusen, N., Zhou, B. and Pu, W. T.** (2016). Epicardium is required for cardiac seeding by yolk sac macrophages, precursors of resident macrophages of the adult heart. *Dev. Biol.* **413**, 153-159.
- Swonger, J. M., Liu, J. S., Ivey, M. J. and Tallquist, M. D.** (2016). Genetic tools for identifying and manipulating fibroblasts in the mouse. *Differentiation* **92**, 66-83.
- Tang, F., Barbacioru, C., Wang, Y., Nordman, E., Lee, C., Xu, N., Wang, X., Bodeau, J., Tuch, B. B., Siddiqui, A. et al.** (2009). mRNA-Seq whole-transcriptome analysis of a single cell. *Nat. Methods* **6**, 377-382.
- Tomanek, R. J.** (2016). Developmental progression of the coronary vasculature in human embryos and fetuses. *Anat. Rec.* **299**, 25-41.
- Trembley, M. A., Velasquez, L. S., de Mesy Bentley, K. L. and Small, E. M.** (2015). Myocardin-related transcription factors control the motility of epicardium-derived cells and the maturation of coronary vessels. *Development* **142**, 21-30.
- Urayama, K., Guillini, C., Turkeri, G., Takir, S., Kurose, H., Messaddeq, N., Dierich, A. and Nebigil, C. G.** (2008). Prokineticin receptor-1 induces neovascularization and epicardial-derived progenitor cell differentiation. *Arterioscler. Thromb. Vasc. Biol.* **28**, 841-849.
- van Tuyn, J., Atsma, D. E., Winter, E. M., van der Velde-van Dijke, I., Pijnappels, D. A., Bax, N. A., Knaan-Shanzer, S., Gittenberger-de Groot, A. C., Poelmann, R. E., van der Laarse, A. et al.** (2007). Epicardial cells of human adults can undergo an epithelial-to-mesenchymal transition and obtain characteristics of smooth muscle cells in vitro. *Stem Cells* **25**, 271-278.
- van Wijk, B., van den Berg, G., Abu-Issa, R., Barnett, P., van der Velden, S., Schmidt, M., Ruijter, J. M., Kirby, M. L., Moorman, A. F. M. and van den Hoff, M. J. B.** (2009). Epicardium and myocardium separate from a common precursor pool by crosstalk between bone morphogenetic protein- and fibroblast growth factor-signaling pathways. *Circ. Res.* **105**, 431-441.
- van Wijk, B., Gunst, Q. D., Moorman, A. F. M. and van den Hoff, M. J. B.** (2012). Cardiac regeneration from activated epicardium. *PLoS ONE* **7**, e44692.
- Vega-Hernandez, M., Kovacs, A., De Langhe, S. and Ornitz, D. M.** (2011). FGF10/FGFR2b signaling is essential for cardiac fibroblast development and growth of the myocardium. *Development* **138**, 3331-3340.
- Vieira, J. M., Howard, S., Villa Del Campo, C., Bollini, S., Dubé, K. N., Masters, M., Barnette, D. N., Rohling, M., Sun, X., Hankins, L. E. et al.** (2017). BRG1-SWI/SNF-dependent regulation of the Wt1 transcriptional landscape mediates epicardial activity during heart development and disease. *Nat. Commun.* **8**, 16034.
- Viragh, S. and Challice, C. E.** (1981). The origin of the epicardium and the embryonic myocardial circulation in the mouse. *Anat. Rec.* **201**, 157-168.
- Volz, K. S., Jacobs, A. H., Chen, H. I., Poduri, A., McKay, A. S., Riordan, D. P., Kofler, N., Kitajewski, J., Weissman, I. and Red-Horse, K.** (2015). Pericytes are progenitors for coronary artery smooth muscle. *Elife* **4**, e10036.
- von Gise, A. and Pu, W. T.** (2012). Endocardial and epicardial epithelial to mesenchymal transitions in heart development and disease. *Circ. Res.* **110**, 1628-1645.
- von Gise, A., Zhou, B., Honor, L. B., Ma, Q., Petryk, A. and Pu, W. T.** (2011). Wt1 regulates epicardial epithelial to mesenchymal transition through beta-catenin and retinoic acid signaling pathways. *Dev. Biol.* **356**, 421-431.
- Wagner, K. D., Wagner, N., Wellmann, S., Schley, G., Bondke, A., Theres, H. and Scholz, H.** (2003). Oxygen-regulated expression of the Wilms' tumor suppressor Wt1 involves hypoxia-inducible factor-1 (HIF-1). *FASEB J.* **17**, 1364-1366.
- Wang, J., Karra, R., Dickson, A. L. and Poss, K. D.** (2013). Fibronectin is deposited by injury-activated epicardial cells and is necessary for zebrafish heart regeneration. *Dev. Biol.* **382**, 427-435.
- Wang, J., Cao, J., Dickson, A. L. and Poss, K. D.** (2015). Epicardial regeneration is guided by cardiac outflow tract and Hedgehog signalling. *Nature* **522**, 226-230.
- Wei, K., Serpooshan, V., Hurtado, C., Diez-Cuñado, M., Zhao, M., Maruyama, S., Zhu, W., Fajardo, G., Noseda, M., Nakamura, K. et al.** (2015). Epicardial FSTL1 reconstitution regenerates the adult mammalian heart. *Nature* **525**, 479-485.
- Wessels, A., van den Hoff, M. J. B., Adamo, R. F., Phelps, A. L., Lockhart, M. M., Sauls, K., Briggs, L. E., Norris, R. A., van Wijk, B., Pérez-Pomares, J. M. et al.** (2012). Epicardially derived fibroblasts preferentially contribute to the parietal leaflets of the atrioventricular valves in the murine heart. *Dev. Biol.* **366**, 111-124.
- Wills, A. A., Holdway, J. E., Major, R. J. and Poss, K. D.** (2008). Regulated addition of new myocardial and epicardial cells fosters homeostatic cardiac growth and maintenance in adult zebrafish. *Development* **135**, 183-192.
- Witty, A. D., Mihic, A., Tam, R. Y., Fisher, S. A., Mikryukov, A., Shoichet, M. S., Li, R.-K., Kattman, S. J. and Keller, G.** (2014). Generation of the epicardial lineage from human pluripotent stem cells. *Nat. Biotechnol.* **32**, 1026-1035.
- Wu, H., Lee, S. H., Gao, J., Liu, X. and Iruela-Arispe, M. L.** (1999). Inactivation of erythropoietin leads to defects in cardiac morphogenesis. *Development* **126**, 3597-3605.
- Wu, M., Smith, C. L., Hall, J. A., Lee, I., Luby-Phelps, K. and Tallquist, M. D.** (2010). Epicardial spindle orientation controls cell entry into the myocardium. *Dev. Cell* **19**, 114-125.
- Wu, S.-P., Dong, X.-R., Regan, J. N., Su, C. and Majesky, M. W.** (2013). Tbx18 regulates development of the epicardium and coronary vessels. *Dev. Biol.* **383**, 307-320.
- Xiang, F.-L., Liu, Y., Lu, X., Jones, D. L. and Feng, Q.** (2014). Cardiac-specific overexpression of human stem cell factor promotes epicardial activation and arteriogenesis after myocardial infarction. *Circ. Heart Fail.* **7**, 831-842.
- Yamaguchi, Y., Cavallero, S., Patterson, M., Shen, H., Xu, J., Kumar, S. R. and Sucov, H. M.** (2015). Adipogenesis and epicardial adipose tissue: a novel fate of the epicardium induced by mesenchymal transformation and PPARgamma activation. *Proc. Natl. Acad. Sci. USA* **112**, 2070-2075.
- Yang, J. T., Rayburn, H. and Hynes, R. O.** (1995). Cell adhesion events mediated by alpha 4 integrins are essential in placental and cardiac development. *Development* **121**, 549-560.
- Zangi, L., Lui, K. O., von Gise, A., Ma, Q., Ebina, W., Ptaszek, L. M., Später, D., Xu, H., Tabebordbar, M., Gorbato, R. et al.** (2013). Modified mRNA directs the fate of heart progenitor cells and induces vascular regeneration after myocardial infarction. *Nat. Biotechnol.* **31**, 898-907.
- Zhou, B. and Pu, W. T.** (2011). Epicardial epithelial-to-mesenchymal transition in injured heart. *J. Cell. Mol. Med.* **15**, 2781-2783.
- Zhou, B., Ma, Q., Rajagopal, S., Wu, S. M., Domian, I., Rivera-Feliciano, J., Jiang, D., von Gise, A., Ikeda, S., Chien, K. R. et al.** (2008a). Epicardial progenitors contribute to the cardiomyocyte lineage in the developing heart. *Nature* **454**, 109-113.
- Zhou, B., von Gise, A., Ma, Q., Rivera-Feliciano, J. and Pu, W. T.** (2008b). Nkx2-5- and Isl1-expressing cardiac progenitors contribute to proepicardium. *Biochem. Biophys. Res. Commun.* **375**, 450-453.
- Zhou, B., von Gise, A., Ma, Q., Hu, Y. W. and Pu, W. T.** (2010). Genetic fate mapping demonstrates contribution of epicardium-derived cells to the annulus fibrosus of the mammalian heart. *Dev. Biol.* **338**, 251-261.
- Zhou, B., Honor, L. B., He, H., Ma, Q., Oh, J.-H., Butterfield, C., Lin, R.-Z., Melero-Martin, J. M., Dolmatova, E., Duffy, H. S. et al.** (2011). Adult mouse epicardium modulates myocardial injury by secreting paracrine factors. *J. Clin. Invest.* **121**, 1894-1904.
- Zong, C., Lu, S., Chapman, A. R. and Xie, X. S.** (2012). Genome-wide detection of single-nucleotide and copy-number variations of a single human cell. *Science* **338**, 1622-1626.