Eur J Anat, 15 (1): 10-16 (2011)

Epicardial development

Ramón Muñoz-Chápuli

Department of Animal Biology, Faculty of Science, University of Málaga, 29071 Málaga (Spain)

SUMMARY

The physiological functions of the embryonic epicardium have been neglected for decades. However, we currently know that this tissue is an essential element in cardiac development. In this review we summarize the available knowledge about how the embryonic epicardium arises from an extracardiac progenitor tissue, the proepicardium, a cluster of coelomic cells that adhere to and spread over the myocardial surface. We describe the control, at molecular level, of the epicardial epithelial-mesenchymal transition leading to the formation of the epicardial-derived cells (EPDC), which are highly invasive mesenchymal cells that invade the subepicardial space and the myocardium. We briefly discuss the developmental fate of the EPDC and their contribution to the development of the coronary vessels. Furthermore, epicardial cells and EPDC interact with the developing ventricular myocardium, promoting its maturation and growth, through a poorly-known mechanism that seems to be dependent on retinoic acid signaling. We finish this review with a discussion of the evolutionary origin of the proepicardium and epicardium, and their relationship with the primitive excretory system of vertebrates. This relationship can account for some of the peculiar features of epicardial development.

Key words: Epicardium – Cardiac development – Coronary vessels – Snail – E-cadherin – Wilms' tumor suppressor gene

INTRODUCTION

Developmental origin of the epicardium

The epicardium is the outer cell layer of the heart. Despite earlier concepts about the derivation of the epicardium from an assumedly common epicardial-myocardial progenitor (the so-called "epimyocardium"), the epicardium actually develops from an extracardiac progenitor tissue: the proepicardium. This had been noted by Kurkiewicz (1909), but those observations were not taken in account in embryology textbooks for decades. We now know that shortly after the looping of the cardiac tube, the coelomic epithelium proliferates in an area immediately posterior to the inflow tract of the heart, either on the pericardial surface of the septum transversum (mammals) or at the limit between the sinus venosus and the liver primordium (avians). This cluster of coelomic cells, the proepicardi-

Submitted: November 26, 2010
Accepted: December 17, 2010

Corresponding author:
Dr. Ramón Muñoz-Chápuli. Department of Animal Biology, Faculty of Science, University of Málaga, 29071 Málaga (Spain). Phone: 34-952 131853; Fax: 34-952 131668. E-mail: chapuli@uma.es

um, attaches directly to the inner curvature of the heart or releases vesicles that float freely in the pericardial cavity and adhere to the myocardial surface. The first mechanism predominates in avian embryos, while the second one has been described in mammalian and fish embryos. In both cases, proepicardial cells flatten on the myocardial surface and spread throughout the cardiac wall to give rise to the epicardial lining. The epicardial spreading starts on the atrioventricular groove and then progresses over the ventricles, in a basoapical direction, finally lining the atria (Vrancken Peeters et al., 1995; Manner et al., 2001; Schlueter et al., 2006 ; Schulte et al., 2007; Rodgers et al., 2008; Schlueter and Brand, 2009, see Mikawa and Brandt, 2010, for a thorough review of the issue). The distalmost part of the outflow tract is lined by an epicardial epithelium of a different origin, since it grows on the cardiac surface from the conotruncal pericardial epithelium (Pérez-Pomares et al., 2003).

The adhesion of the proepicardial cells to the heart surface is mediated by extracellular matrix proteins, such as fibronectin, and also by the adhesion molecule VCAM-1, expressed by the myocardium, and the epicardial integrin $\alpha 4ß1$, which is a receptor for both, VCAM-1 and fibronectin (Kwee et al., 1995; Yang et al., 1995; Sengbusch et al, 2002). In fact, loss of function of the VCAM-1 or $\alpha 4$ integrin genes in transgenic mice causes epicardial detachment, leading to embryonic death by mid-gestation. This illustrates the essential role played by the epicardium in cardiac morphogenesis, an issue that we shall discuss below.

Epithelial-mesenchymal transition of the epicardium and origin of the EPDC

Soon after the epicardium has covered the outer cardiac surface, a space appears between the epicardial lining and the myocardium. This space, filled with an amorphous extracellular matrix, is rapidly populated by cells delaminating from the epicardium through a process known as the epithelial-mesenchymal transition (EMT) (Figure 1A). The cells delaminated from the epicardium, called epicardial-derived cells (EPDC), populate the whole of the subepicardial space, after which they invade the myocardium: first the ventricular wall and then the atrium, and even reaching the atrioventricular endocardial cushions (Pérez-Pomares et al., 1998; Gittenberger-de-Groot et al., 1998; Dettman et al., 1998). This invasion coincides in time with the thickening of the myocardial wall and the formation of the compact ventricular layer, and in fact invasion of the EPDC is essential for the myocardial compaction of the ventricle, as will be seen later.

The epicardial EMT is governed by a set of genes that are also involved in other EMT events during embryonic development, such as the origin of the mesoderm in the primitive streak, the formation of neural crest cells, the disintegration of the epithelial somite, or the origin of the valvuloseptal mesenchyme of the endocardial cushions that constitute the primordia of the cardiac valves. The Snail transcription factor is a main effector of the embryonic EMT. This protein represses a number of genes related to the epithelial phenotype (E-cadherin, VE-cadherin, claudins, occludins, desmoplakins, cytokeratins) and activates genes related to the mesenchymal phenotype (fibronectin, vimentin) and to migration and invasion (RhoB and matrix metalloproteinases) (Barrallo-Gimeno and Nieto, 2006). The transient colocalization of epithelial (cytokeratin) and mesenchymal (vimentin) markers in the embryonic epicardium and EPDC is shown in figure 1B.

It has recently been shown that in the epicardium Snail is regulated by another zinc-finger transcription factor called Wt1 (encoded by Wilms' tumor suppresor gene) (Martínez-Estrada et al., 2010). *Wt1* is regionally expressed in the coelomic epithelium and especially in the kidneys and gonads (Moore et al., 1999). Loss of function of the *Wt1* gene in transgenic mice leads to failure of kidney, gonad, adrenal galnd and spleen morphogenesis. However, although *Wt1* is only expressed in the epicardium and EPDC (Figure 1C), *Wt1*-deficient mouse embryos die at mid-gestation due to cardiac failure, showing an abnormal thinning of the myocardium (Kreidberg et al., 1993).

Returning to the origin and developmental fate of the EPDC, Wt1 is not only an activator of Snail, the main EMT effector, as stated above, but it also is a repressor of the epithelial gene E-cadherin, maintaining EPDC in a mesenchymal state (Martínez-Estrada et al., 2010). This phenotype is probably responsive to local signals for differentiation in endothe-

lial, smooth muscle, and fibroblastic lineages. This was suggested after an experiment in which embryoid bodies were derived from Wt1-deficient embryonic stem cells. These embryoid bodies, which represent the different cell lineages of the embryo, showed a normal expression of ectodermal, endodermal and early mesodermal markers, but they lacked the expression of all the cardiovascular lineage differentiation markers, including the endothelial, myocardial or hematopoietic markers. At the same time they showed an abnormal up-regulation of E-cadherin. Significantly, when Snail expression, which also had disappeared from the Wt1-deficient embryoid bodies, was forced in them, expression of the

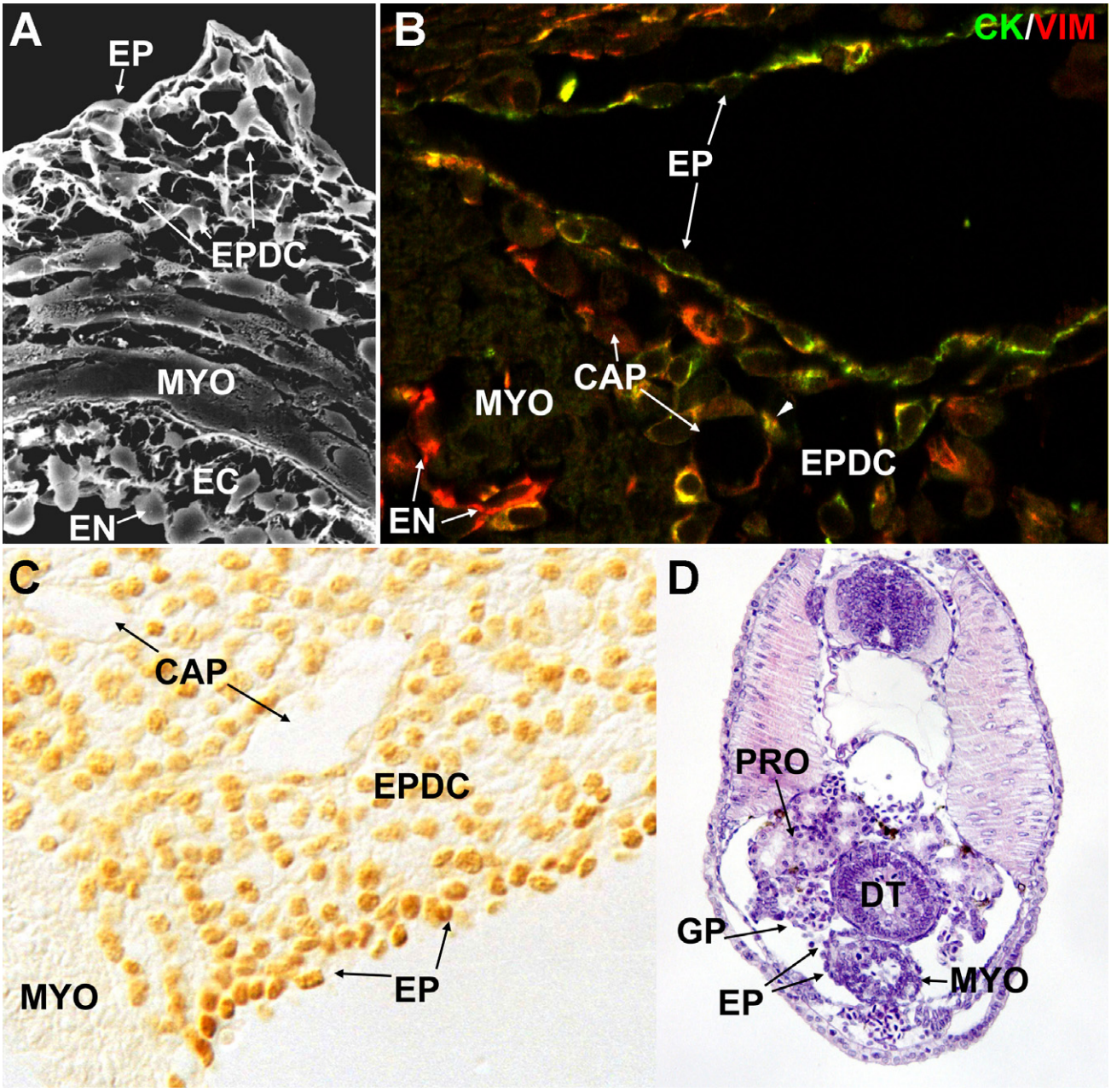

Figure 1. **A.** Transverse section of the cardiac wall of a chick embryo showing the three cell layers of the developing heart. Scanning electron microscopy. The epicardium (EP) covers a subepicardial space filled with epicardial-derived cells (EPDC). The endocardium (EN) gives rise to the mesenchymal cells of the endocardial cushions (EC). MYO: myocardium. **B.** Double immunostaining of the embryonic epicardium of a hamster embryo (E11.5), showing the localization of an epithelial marker (cytokeratin, in green) and a mesenchymal marker (vimentin, in red). Epicardial cells (EP) show double immunostaining (yellow signal). In EPDC, the cytokeratin staining drops declines in intensity, as they acquire a more defined mesenchymal phenotype or become differentiated. Endocardial cells (EN) are vimentin+ and cytokeratin-. Developing coronary capillaries (CAP) show vimentin+ endothelial cells, but double-stained cells (i.e. EPDC) are incorporated to the capillary wall (arrowhead). **C.** Expression of Wt1 in the atrioventricular groove of a chick embryo, HH28. Immunoperoxidase staining. Wt1 expression is strongest in the epicardial cells and seems to be dow-regulated in EPDC, although most cells still show immunoreactivity. Developing coronary capillaries (CAP) are shown. **D.** Origin of the epicardium in a lamprey prolarva. The epicardium (EP) originates from the right external glomerulus primordium (GP) of the pronephros (PRO). Note attachment of epicardial cells to the right and dorsal surface of the myocardium (MYO). DT: digestive tract.

cardiovascular markers was rescued (Martínez-Estrada et al., 2010). This experiment suggests that EMT could be a necessary process to originate cells able to differentiate into the distinct cardiovascular lineages.

Developmental fate of EPDC

The contribution of EPDC to the coronary vessels has been demonstrated in avian embryos in experiments in which a quail proepicardium was implanted into the pericardial cavity of a chick embryo (Vrancken-Peeters et al., 1999; Guadix et al., 2006). Quail cells can be readily identified by a specific antibody. Cells of quail origin (i.e., the EPDC) were observed within the coronary endothelium, vascular smooth muscle cells, and also in adventitial fibroblasts. In mammals, studies addressing the lineage of cells expressing Tbx18 and Wt1 have confirmed the significant epicardial contribution to the tunica media of the coronary vessels, although only a minor part of the coronary endothelium seems to derive from EPDC (Cai et al., 2008; Zhou et al., 2008). It is conceivable that the contribution of EPDC to the endothelium could be restricted to the early stages of coronary development, later being diluted by other sources of endothelial cells (eg. endocardium, circulating progenitors). In any case, in mouse embryos, the epicardial-specific ablation of ß-catenin or Wt1 leads to a complete failure of the coronary arteries to form (Zamora et al., 2007; Martínez-Estrada et al., 2010), showing that coronary development is dependent on the EPDC.

Epicardial-myocardial interactions

Besides the contribution of EPDC to the development of the coronary vessels, it seems clear that these cells, together with the epicardium, promote the growth, compaction and maturation of the ventricular myocardium. In fact, a number of mutations in genes expressed in the epicardium that cause epicardial maldevelopment lead to ventricular hypoplasia and cardiac failure at mid-gestation (Reviewed in Sucov et al., 2009). The signals involved in this epicardial-myocardial interaction are not known, but secreted proteins belonging to the FGF and Wnt families have been proposed as candidates (Pennisi et al., 2003; Merki et al., 2005; Lavine et al., 2005; Lu et al., 2008). The inductive function of the epicardium is dependent on autocrine retinoic acid (RA) signaling, since the epicardium and EPDC express RALDH2, the main enzyme responsible for RA synthesis in mesodermal tissues, and specific ablation of the RXRα retinoid receptor causes defective development of the epicardium and ventricular hypoplasia (Chen et al., 2002; Stuckmann et al., 2003; Merki et al., 2005). We have recently shown that RALDH2 is under the transcriptional control of Wt1 (Guadix et al., in press). Thus, Wt1 not only controls the epicardial EMT through Snail transactivation and E-cadherin repression, but also the synthesis of RA in the epicardium, which is, as stated above, essential for epicardial and EPDC development.

Evolutionary origin of the epicardium

The epicardium and the coronary vessels are an evolutionary innovation of vertebrates, since invertebrate hearts lack of these tissues. Some large hearts from cephalopods are endowed with vessels, but these always derive from an ingrowth of the external vasculature; i.e., they do not develop from intrinsic vascular progenitors. Thus, an intriguing question is how epicardium, EPDC and coronary progenitors arose along evolution. A second question is why the epicardium and the excretory system share the expression of so many genes that in some cases are critical for their development. We have already mentioned Wilms' tumor suppressor gene, whose loss of function causes anomalous development of the epicardium and the kidneys. Pod1/epicardin is a gene that was simultaneously discovered in podocytes and in the epicardium. Its deficiency causes maldevelopment of the renal glomeruli (Quaggin et al., 1999). Tbx18 shows a prominent expression in the inflow tract of the heart, including the proepicardium and epicardium, and it is critical for the development of the kidneys (Airik et al., 2006). Podoplanin is an extracellular matrix protein that is highly expressed by podocytes and the epicardium. Podoplanin-deficient mice die by mid-gestation or during the first weeks of life, showing severe cardiac defects (Mahtab et al., 2009).

A hypothesis about the relationship between epicardial and renal development has

been forwarded by our group (Pombal et al., 2008). This hypothesis is based on a description of epicardial development in a representative of the phylogenetically most primitive lineage of vertebrates, the lamprey *Petromyzon*. In lamprey embryos and prolarvae the epicardium develops from a cluster of coelomic cells situated on the pronephros, which in this species is located over the heart. Interestingly, after originating the epicardium, the cluster of cells gives rise to an external glomerulus, an excretory structure composed of vessels lined by podocytes, the cells responsible for blood filtration (Figure 1D). In this primitive organization of the vertebrate excretory system, which can also be observed in amphibian larvae, the filtrate is released to the coelomic cavity, and is then aspirated by ciliated nephrostomes and evacuated throughout a system of collecting ducts. Along the evolution of vertebrates, the external glomeruli became encased in compartments of the coelomic cavity (the capsulae of Bowman) connected directly to the collecting ducts. Thus, the proepicardium observed in most vertebrates is a derivative from an ancient glomerular primordium that became uncoupled from the pronephros (due to the migration of the excretory system in a caudal direction) and lost its excretory function, maintaining the role of providing the heart with vascular progenitors. This accounts for the expression of kidney-related genes in the epicardium, as well as the high vasculogenic ability of the proepicardium, whose original function was to give rise to the glomerular vessels. In any case, the supply of the heart with vascular progenitors probably allowed an increase in cardiac size and performance in vertebrates.

We believe that the primitive pronephric-cardiac connection that we have unveiled in agnathans could be related with a more primitive structure, the so-called "heart-kidney complex" of enteropneusts, a group of large marine worms belonging to the phylum Hemichordates. This complex, located on the proboscis of these animals, consists of a pulsating vessel lined by a layer of podocytes and resting (*and rests* si se trata del *pulsating vessel)* on a rigid tissue, the stomochord, derived from the mouth epithelium. The pressure increase within this pulsating vessel allows the filtration of hemal fluid throughout the podocytes, releasing the filtrate to the coelomic cavity of the proboscis. A relationship of the heart-kidney complex with the chordate heart had traditionally been discarded, since the former structure is dorsal in hemichordates while the vertebrate heart is always ventral. However, the dorsoventral axis of hemichordates is reversed with respect to that of chordates (Lowe et al., 2006). Thus, it is conceivable that the renal pump of hemichordates and the branchial heart of chordates could be phylogenetically related (Pombal et al., 2008). In this case, the proepicardium-myocardium connection would be reminiscent of the heart-kidney complex of Deuterostomes.

Conclusion

We have achieved surprising advances in our knowledge of the proepicardium and epicardium and their physiological functions during development. This new knowledge unveils the highly active role played by the epicardium and EPDC in cardiac morphogenesis, a role that is essential for the normal development of the heart. However, many points remain obscure and they will surely deserve the attention of developmental biologists in the near future. For example, where does the triggering of the epicardial EMT occur? It has traditionally been considered that the epicardium lining the atrioventricular groove is the earliest location of the EMT. However, the embryonic epicardium lacks E-cadherin expression (which is consistent with the strong expression of Wt1 and Snail, two E-cadherin repressors, as we have described above) and it also lacks basal lamina. Strictly speaking, therefore, despite its flattened, epithelial-like appearance the embryonic epicardium can be considered a population of mesenchymal-like cells rather than a true epithelium. It is conceivable that the epicardial EMT starts in the proepicardium, where activation of Wt1 and Snail would repress E-cadherin. At a later stage, epicardial cells would invade the subepicardial space and the myocardium. Thus, epicardial EMT would be different from other embryonic EMTs since the activation of mesenchymal genes/loss of epithelial markers and migration would be temporally and spatially uncoupled. A second issue is how the epicardial cell decision between migrating or remaining in the epicardial layer is made. This issue is closely related to two other questions: are embryonic epicardial cells genetically homogenous? Do EPDC

have the ability for multilineage differentiation or are they commited to a specific vascular lineage since their inception? Third, how is Wt1 expression regulated? Finally, and this is an issue with some potentially therapeutic interest, do adult epicardial cells and EPDC retain multipotentiality, as suggested by some experiments? Can adult EPDC be used for cell therapy of cardiac disease?

Acknowledgements

This work was supported by grants BFU2008-02384, BFU2009-07929 and SAF2008-1883, (Ministerio de Ciencia e Innovación), RD06/0010/0015 (TerCel network, ISCIII), RD96/0014/1009 (RECAVA network, ISCIII), P08-CTS-03618 (Junta de Andalucía) and LSHM-CT-2005-018630 (VI framework, UE).

References

Airik R, Bussen M, Singh MK, Petry M, Kispert A (2006) Tbx18 regulates the development of the ureteral mesenchyme. *J Clin Invest*, 116: 663-674.

Barrallo-Gimeno A, Nieto MA (2006) Evolution of the neural crest. *Adv Exp Med Biol,* 589: 235-244.

Cai CL, Liang X, Shi Y, Chu PH, Pfaff SL, Chen J, Evans S (2003) *Isl1* identifies a cardiac progenitor population that proliferates prior to differentiation and contributes a majority of cells to the heart. *Dev Cell,* 5: 877-889.

Chen TH, Chang TC, Kang JO, Choudhary B, Makita T, Tran CM, Burch JB, Eid H, Sucov HM (2002) Epicardial induction of fetal cardiomyocyte proliferation via a retinoic acid-inducible trophic factor. *Dev Biol,* 250: 198-207.

Dettman RW, Denetclaw W, Ordahl CP, Bristow J (1998) Common epicardial origin of coronary vascular smooth muscle, perivascular fibroblasts, and intramyocardial fibroblasts in the avian heart. *Dev Biol,* 193: 169-81.

Gittenberger-DE Groot AC, Vranchen Peeters MP, Mentink MM, Gourdie RG, Poelmann RE (1998) Epicardium-derived cells contribute a novel population to the myocardial wall and the atrioventricular cushions. *Circ Res,* 82: 1043-1052.

Guadix JA, Carmona R, Muñoz-Chápuli R, Pérez-Pomares JM (2006) *In vivo* and *in vitro* analysis of the vasculogenic potential of avian proepicardial and epicardial cells. *Dev Dyn,* 235: 1014-1026.

Guadix JA, Muñoz-Chápuli R, Hastie ND, Pérez-Pomares JM, Martínez-Estrada ME (in press) Wt1 controls retinoic acid signaling in embryonic epicardium through transcriptional activation of the Raldh2 enzyme. *Development*.

Kreidberg JA, Sariola H, Loring JM, Maeda M, Pelletier J, Housman D, Jaenisch R (1993) WT-1 is required for early kidney development. *Cell,* 74: 679-691.

Kurkiewicz T (1909) O histogenezie miesnia sercowego zwierzat kregowych. *Bull Acad Sci Cracovie,* 1909: 148-191.

Kwee L, Baldwin HS, Shen HM, Stewart CL, Buck C, Buck CA, Labow MA (1995) Defective development of the embryonic and extraembryonic circulatory systems in vascular cell adhesion molecule (VCAM-1) deficient mice. *Development,* 121: 489-503.

Lavine KJ, YU K, White AC, Zhang X, Smith C, Partanen J, Ornitz DM (2005) Endocardial and epicardial derived FGF signals regulate myocardial proliferation and differentiation in vivo. *Dev Cell,* 8: 85-95.

Lowe CJ, Terasaki M, Wu M, Freeman RM Jr, Runft L, Kwan K, Haigo S, Aronowicz J, Lander E, Gruber C, Smith M, Kirschner M, Gerhart J (2006) Dorsoventral patterning in hemichordates: insights into early chordate evolution. *PLoS Biol.* 4:e291.

Lu SY, Sheikh F, Sheppard PC, Fresnoza A, Duckworth ML, Detillieux KA, Cattini PA (2008) FGF-16 is required for embryonic heart development. *Biochem Biophys Res Commun,* 373: 270-274.

Mahtab EA, Vicente-Steijn R, Hahurij ND, Jongbloed MR, Wisse LJ, Deruiter MC, Uhrin P, Zaujec J, Binder BR, Schalij MJ, Poelmann RE, Gittenberger-De Groot AC (2009) Podoplanin deficient mice show a RhoA-related hypoplasia of the sinus venosus myocardium including the sinoatrial node. *Dev Dyn*, 238: 183-193.

Männer J, Pérez-Pomares JM, Macías D, Muñoz-Chápuli R (2001) The origin, formation, and developmental significance of the epicardium: a review. *Cell Tiss Org,* 169: 89-103.

Martínez-Estrada OM., Lettice LA, Essafi A, Guadix JA, Slight J, Velecela V, Hall E, Reichmann J, Devenney PS, Hohenstein P, Hosen N, Hill RE, Muñoz-Chápuli R, Hastie ND (2010) Wt1 is required for mesenchymal cardiovascular progenitor cell formation in epicardium and ES cells through direct transcriptional control of Snail and E-cadherin. *Nat Genet*, 42: 89-93.

Merki E, Zamora M, Raya A, Kawamaki Y, Wang J, Zhang X, Burch J, Kubalak SW, Kaliman P, Izpisúa-Belmonte JC, Chien K, Ruiz-Lozano P (2005) Epicardial retinoid X receptor is required for myocardial growth and coronary artery formation. *Proc Natl Acad Sci USA.* 102: 18455-18460.

Mikawa T, Brand T (2010) Epicardial lineages: Origins and fates. In: Rosenthal N and Harvey RP (eds.) *Heart development and regeneration*. Elsevier, Amsterdam, pp 325-344.

Moore AW, Mcinnes L, Kreidberg J, Hastie ND, Shedl A (1999) YAC complementation shows a requirement for Wt1 in the development of epicardium, adrenal gland and throughout nephrogenesis. *Development,* 126: 1845-1857.

Pennisi DJ, Ballard VL, Mikawa T (2003) Epicardium is required for the full rate of myocyte proliferation and levels of expression of myocyte mitogenic factors FGF2 and its receptor, FGFR-1, but not for transmural myocardial patterning in the embryonic chick heart. *Dev Dyn*, 228: 161-72.

Pérez-Pomares JM, Macías D, García-Garrido L, 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 JM, Phelps A, Sedmerova M, 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.

Pombal MA, Carmona R, Megías M, Ruiz A, Pérez-Pomares JM, 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.

Quaggin SE, Schwartz L, Cui S, Igarashi P, Deimling J, Post M, Rossant J (1999) The basic-helix-loop-helix protein pod1 is critically important for kidney and lung organogenesis. *Development,* 126: 5771-5783.

Rodgers LS, Lalani S, Runyan RB, Camenisch TD (2008) Differential growth and multicellular villi direct proepicardial translocation to the developing mouse heart. *Dev Dyn*, 237: 145-152.

Schlueter J, Brand T (2009) A right-sided pathway involving FGF8/Snai1 controls asymmetric development of the proepicardium in the chick embryo. *Proc Natl Acad Sci USA*, 106: 7485-7490.

Schlueter J, Manner J, 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, Manner 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 JK, He W, Pinco KA, Yang JT (2002) Dual functions of [alpha]4[beta]1 integrin in epicardial development: initial migration and long-term attachment. *J Cell Biol,* 157: 873-882.

Stuckmann I, Evans S, Lassar AB (2003) Erythropoietin and retinoic acid, secreted from the epicardium, are required for cardiac myocyte proliferation. *Dev Biol,* 255: 334-349.

Sucov HM, Gu Y, Thomas S, LI P, Pashmforoush M (2009) Epicardial control of myocardial proliferation and morphogenesis. *Pediatr Cardiol,* 30: 617-625.

Vrancken Peeters MP, Mentink MM, Poelmann RE, Gittenberger-De Groot AC (1995) Cytokeratins as a marker for epicardial formation in the quail embryo. *Anat Embryol*, 191: 503-508.

Vrancken Peeters MP, Gittenberger-De Groot AC, Mentink MM, Poelmann RE (1999) Smooth muscle cells and fibroblasts of the coronary arteries derive from epithelial-mesenchymal transformation of the epicardium. *Anat Embryol*, 19: 367-378.

Yang JT, Rayburn H, Hynes RO (1995) Cell adhesion events mediated by alpha 4 integrins are essential in placental and cardiac development. *Development,* 121: 549-560.

Zamora M, Männer J, Ruiz-Lozano P (2007) Epicardium-derived progenitor cells require beta-catenin for coronary artery formation. *Proc Natl Acad Sci USA,* 104: 18109-18114.

Zhou B, Ma Q, Rajagopal S, Wu SM, Domian I, Rivera-Feliciano J, Jiang D, Von Gise A, Ikeda S, Chien KR, Pu WT (2008) Epicardial progenitors contribute to the cardiomyocyte lineage in the developing heart. *Nature*, 454:109-113.