REVIEW ARTICLE

The Epicardium and Epicardial-Derived Cells: Multiple Functions in Cardiac Development

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The epicardium develops from an extracardiac primordium, the proepicardium, which is constituted by a cluster of mesothelial cells located on the cephalic and ventral surface of the liver-sinus venosus limit (avian embryos) or on the pericardial side of the septum transversum (mammalian embryos). The proepicardium contacts the myocardial surface and gives rise to a mesothelium, which grows and progressively lines the myocardium. The epicardium generates, through a process of epithelial-mesenchymal transition, a population of epicardial-derived cells (EPDC). EPDC contribute to the development of cardiac connective tissue, fibroblasts, and the smooth muscle of cardiac vessels. Recent data suggest that EPDC can also differentiate into endothelial cells of the primary subepicardial vascular plexus. If this is confirmed, EPDC would show the same developmental properties that characterize the stem-cell-derived bipotential vascular progenitors recently described, whose differentiation into endothelium and smooth muscle is regulated by exposure to VEGF and PDGF-BB, respectively. Aside from their function in the development of cardiac connective and vascular tissue, EPDC also play an essential modulating role in the differentiation of the compact ventricular layer of the myocardium, a role which might be regulated by the transcription factor WT1 and the production of retinoic acid.

Key words: *Epicardium. Epithelial-mesenchymal transition. Vasculogenesis. Differentiation.*

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El epicardio y las células derivadas del epicardio: múltiples funciones en el desarrollo cardíaco

Durante el desarrollo cardíaco, el epicardio deriva de un primordio externo al corazón, denominado proepicardio, que está formado por un acúmulo de células mesoteliales situado en la superficie ventral y cefálica del límite hígado-seno venoso (aves) o en la cara pericárdica del septo transverso (mamíferos). El proepicardio entra en contacto con la superficie miocárdica y da lugar a un mesotelio que crece y recubre progresivamente al miocardio. El epicardio genera, por un proceso localizado de transición epitelio-mesénquima, una población de células mesenquimáticas, las células derivadas de epicardio (CDEP). Las CDEP contribuyen al desarrollo del tejido conectivo del corazón y también dan lugar a los fibroblastos y las células musculares lisas de los vasos coronarios. Existen evidencias que sugieren la diferenciación de las CDEP en células endoteliales del plexo subepicárdico primitivo. De confirmarse esto, las CDEP mostrarían propiedades similares a los precursores vasculares bipotenciales derivados de células madre recientemente descritos, cuya diferenciación en endotelio y músculo liso se regula por exposición a VEGF y PDGF-BB, respectivamente. Además de las funciones señaladas en la formación de los tejidos vascular y conectivo del corazón, las CDEP podrían desempeñar un papel modulador esencial para la formación de la capa compacta ventricular del miocardio, un papel que podría estar regulado por el factor de transcripción WT1 y la producción de ácido retinoico.

Palabras clave: *Epicardio. Transición epitelio-mesénquima. Vasculogénesis. Diferenciación.*

INTRODUCTION

The epicardium is the outermost layer of the heart in vertebrates. In the adult organism, it is constituted by a cubical mesothelium that covers a space occupied by connective and vascular tissue. In the early embryo, the epicardium adopts the form of a squamous cell epithelium that either rests directly on the surface of the myocardium, or covers a subepicardial space that is more or less densely populated by mesenchymal

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ABBREVIATIONS

b-HLH: basic Helix-Loop-Helix
BMP: bone morphogenetic proteins
BVES: blood vessel/epicardial substance
CDEPs: cells derived from epicardium
cdks: cyclin-dependent kinases
FGFs: fibroblast growth factors
FOG-2: friend of GATA
NCAM: neural cell adhesion molecule
PDGF-BB: platelet-derived growth factor-BB
Rb: retinoblastoma
RALDH2: retinaldehyde dehydrogenase-2
SRF: serum response factor
TGF-β: transforming growth factor-β
VCAM: vascular cell adhesion molecule
VEGF: vascular endothelial growth factor
VEGFR-2: vascular endothelial growth factor receptor-2
WT: Wilms´ tumor

cells. In this article we will review the processes related with the development of the epicardium, appearance of the subepicardial mesenchyme, and its differentiation. We will also examine evidence about the essential role of the subepicardial mesenchyme in myocardial differentiation.

The epicardium is, without doubt, the cardiac component that traditionally has received the least amount of attention from embryologists and developmental biologists. The epicardium was long considered a simple derivative of the outermost layer of the heart tube. The term «epimyocardium» or «myoepicardium,» which refers to this primordium common to both tissues,[1] appeared in text books up until lately.[2] Nevertheless, in recent years a series of studies have appeared that have demonstrated, in first place, that the epicardium has a development independent of the myocardium and endocardium, a proposal that was made almost a century ago.[3] In second place, most of the connective and vascular tissues of the heart, including the coronary vessels, are epicardial derivatives. The most recent contribution to the subject has come from studies that suggest that cells derived from the epicardium provide essential signals for the formation of the compact layer of the ventricular myocardium. The epicardium, therefore, has evolved from an apparently passive role to prominence as a key protagonist of episodes of cardiac morphogenesis. Our aim in writing this article was to review current knowledge about these new functions of the epicardium and epicardium-derived cells.

The epicardium derives from an extracardiac mesothelial primordium called the proepicardium

The epicardium is the last layer of the heart to appear and is the only layer that originates outside the primitive heart tube. General aspects of its ontogenetic and phylogenetic development have been recently reviewed.[4] The epicardium forms from a clump of mesothelial tissue that appears ventrally, on the limit between the liver and sinus venosus. This rudiment, which is called the proepicardium,[5] can be simple and located to the right of the sagittal plane, as in the case of the chick embryo, or bilateral, as in the mouse embryo.

The structure of the proepicardium varies according to the species in which it is described. There are descriptions of the proepicardium in representatives of almost all the major vertebrate groups. The most primitive representative, phylogenetically speaking, is a small shark, the dogfish *(Scyliorhinus canicula)*.[6-8] In the embryos of these sharks, two large clumps of mesothelial cells develop on the ventral and anterior portion of the epithelium covering the liver, where they join the sinus venosus. Later, when the septum transversum develops in this limit, the proepicardial mesothelial cushions are displaced towards the pericardial surface of the septum. The proepicardium of the dogfish is formed by round mesothelial cells, with little extracellular matrix between them. In teleosts, the proepicardium has not been described, although has been reported that the epicardium of the plaice *(Pleuronectes platessa)* had an extracardiac origin, although its characteristics were not specified.[9] The presence of the proepicardium in amphibians is deduced from studies of the cardiac development of the axolotl *(Ambystoma mexicanum)*.[10] These authors affirm that the epicardium develops from the cells of the septum transversum. Information on this point is also scarce in reptiles, although has been reported that the epicardial development of the turtle is similar to that described in chick embryos.[11]

The organization of the proepicardium has been extensively studied in chick and quail embryos.[5,12-15] Its development begins around stages HH13-14, with rapid proliferation of the mesothelium that covers the horns of the sinus venosus ventrally on its limits with the hepatic rudiment (Figures 1 and 2). Unlike what occurs in other animal models, the proliferation of the left side ceases while that of the right side continues until it reaches a considerable size in stage HH17. The proepicardium in this stage is formed by multiple digitations or protrusions that give it a «cauliflower-like» appearance. The digitations are covered by mesothelium and contain numerous mesenchymal cells in an abundant extracellular matrix.

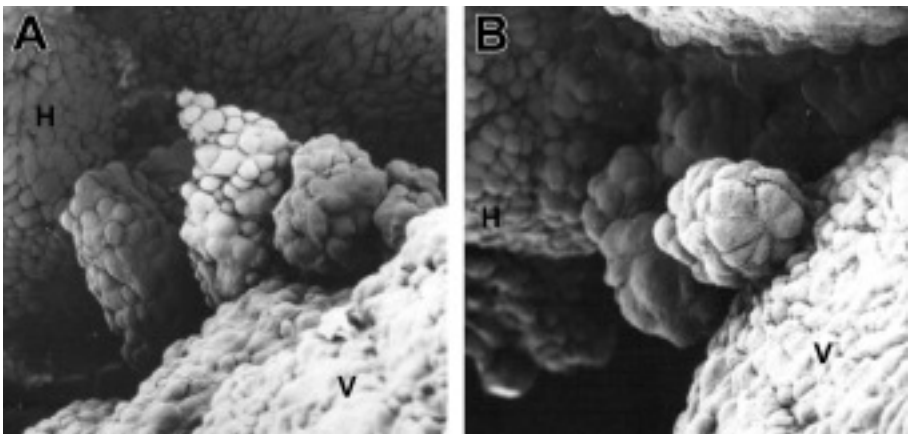

Fig. 1. The proepicardium of a quail embryo (stage HH21) by scanning electron microscopy. *A* and *B*. Two different aspects of the proepicardial protrusions in the phase of adhesion to the ventricle (V). H indicates hepatic surface.

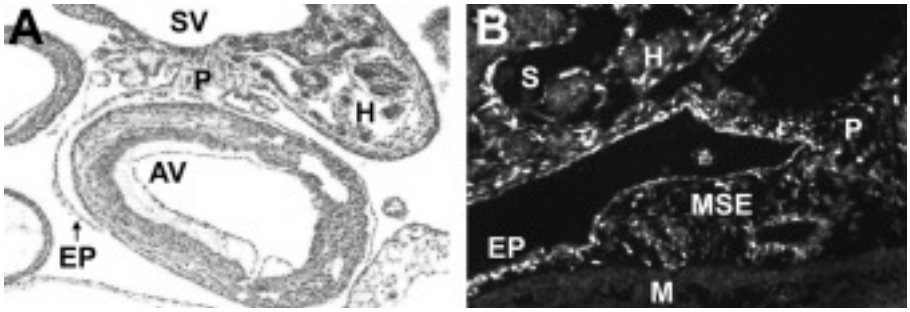

Fig. 2. Histological sections of the quail proepicardium in cross-section. *A*. Stage HH21. The proepicardium (p) is located on the limit between the sinus venosus (SV)/Liver (L). Observe how the epicardium (EP) extends over the myocardium of the atrioventricular canal (AV). *B*. Stage HH22, immunolocalization of the cytokeratin. The immunoreactivity of the proepicardial and epicardial mesothelium, as well as most of the subepicardial mesenchyme (SEM) is evident. Note that some cells of the hepatic sinusoids (s) are also immunoreactive. M indicates myocardium.

The proepicardium is bilateral in mammals, as has been noted, and it develops on the pericardial surface of the septum transversum, near the sinus venosus.[16-20] The mesothelial protrusions at first consist in cushions of round cells and later acquire an appearance similar to that described in the chick embryo, digitations covered by mesothelium and containing mesenchymal cells and extracellular matrix. In mouse embryos, the proepicardium is present from 9.5 to 11 days *post coitus*.

The factors that control the development of the proepicardium are not known. A mechanical effect has been proposed, an effect of «aspiration» produced by the cardiac contractions on the mesothelium of the septum transversum.[18] However, it is much more likely that the mesothelium of the zones in which the proepicardium develops receives some type of proliferative signal of unknown origin and nature. In fact, the proepicardium shows strong mitotic activity.[5,18]

The proepicardium is transferred to the heart and originates the epicardial mesothelium

The proepicardial cells, which originate and proliferate outside the limits of the heart, must migrate to the cardiac surface in order to constitute the primitive epicardium. Two main mechanisms of transfer of the proepicardium have been described, which sometimes coexist in the same species.

In certain cases, proepicardial cells are shed and float free in the pericardial cavity. These cells adhere to specific areas of the myocardium, mainly in the atrioventricular and conoventricular furrows. From the moment in which they adhere, the cells flatten and fuse to form an epithelium that progressively develops over the cardiac surface. This mechanism of proepicardial transfer is the only one present in the dogfish[8] and it has been described in amphibians and mammals.[10,17,18]

The other mechanism of transfer of the proepicardium involves the direct adhesion of proepicardial villi to the cardiac surface. Given the position of the proepicardium with respect to the heart, this adhesion usually occurs in the posterior (dorsal) part of the ventricles and atrioventricular furrow (Figure 2). Direct adhesion of the proepicardium seems to be the fundamental mechanism of transfer in the case of chick embryos, and coexists in amphibians and mammals with the adhesion of free clumps of

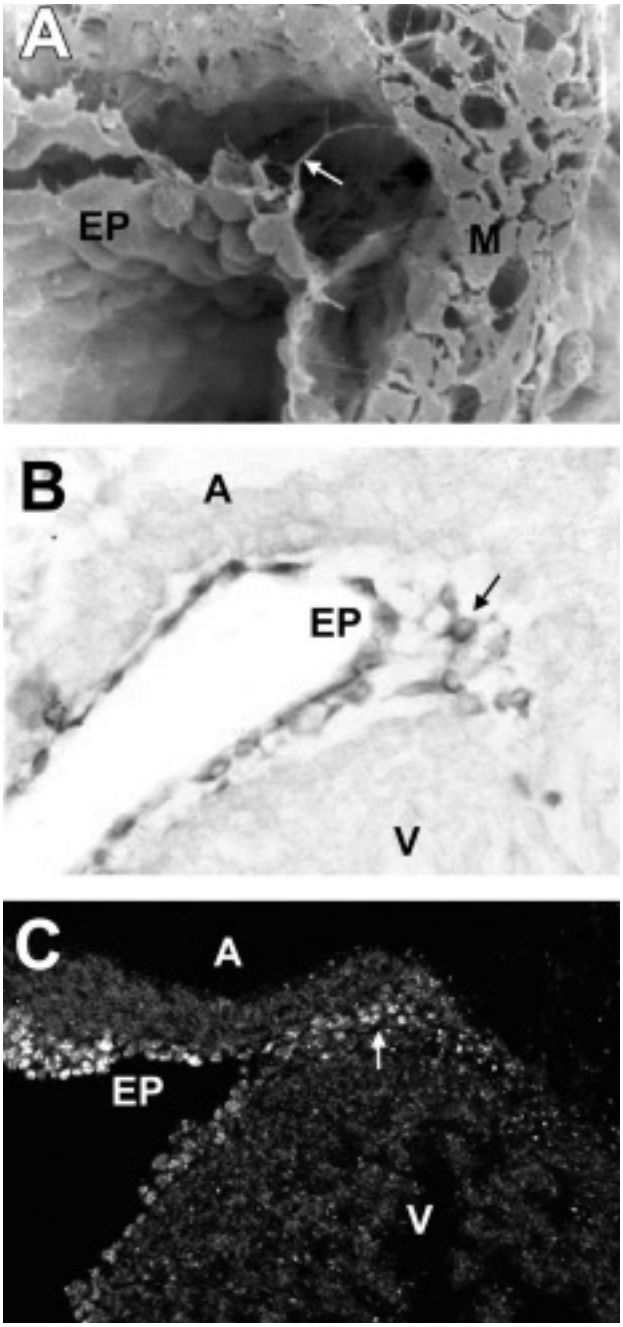

Fig. 3. Evidences of epicardium-to-mesenchyme transition in mammalian embryos. *A.* Mouse embryo, 11.5 days post coitus, scanning electron microscopy. Cells of the epicardium (EP) of the atrioventricular furrow emit long basal prolongations toward the underlying extracellular matrix. M: myocardium. *B.* Hamster embryo, 11 days post-coitus, immunolocalization of cytokeratin. The mesenchymal cells that are generated in the atrioventricular furrow are positive (arrow), demonstrating a mesothelial origin. A indicates atrium; V, ventricle. *C.* mouse embryo, 11.5 days post-coitus, immunolocalization of factor WT1. Observe the reactivity of the epicardium and cells of the atrioventricular furrow.

proepicardial cells described before. It is possible that the large communication between the pericardial cavity and general coeloma of the body in the bird embryo (due to the delayed development of the septum transversum) is related to this particularity.[10]

In all the cases, the epicardium progresses around the atrioventricular furrow, then extends to the left ventricle, ventral surface of the atrium, right ventricle and, finally, the roof of the atrium and outflow tract.[21] It is interesting to note that the growth of the epicardium stops at the limit between the myocardial and mesenchymal regions of the outflow tract, which is covered by the growth of pericardial mesothelium.[22]

The adhesion of proepicardial cells to the surface of the myocardium indicates the existence of a specific mechanism of recognition. The details of this mechanism are not known because, although candidate molecules have been proposed, no conclusive evidence exists in their favor. For example, NCAM (neural cell adhesion molecule) is expressed in both the epicardium and the developing myocardium.[23,24] Nevertheless, mutant mice deficient for NCAM do not present epicardial defects.[25] It has also been reported that the naked myocardium presents discrete areas covered by fibronectin,[20] and that the adhesion of free cells involves a strong increase in fibronectin expression in the epicardium-myocardium interphase.[26] Nevertheless, it is again observed that mice deficient for fibronectin do not have anomalies in epicardial development.[27]

In spite of the absence of significant information about the mechanism of adhesion of proepicardial cells to the myocardium, there is evidence that two molecules are essential for maintaining epicardial integrity in the initial moments of development. Mice deficient in VCAM-1 (vascular cell adhesion molecule)[28] or in the α_4 subunit of the integrins[29] show a similar defect in epicardial development. This coincidence is consistent with the role of the α_4 integrins ($\alpha_4\beta_1$ and $\alpha_4\beta_7$) as receptors for VCAM. The mice deficient for these two molecules show a normal phase of adhesion of proepicardial cells between days 9.5 and 11 of development, but the epicardium is shed immediately after and disappears. These mutations are lethal due, probably, to massive pericardial hemorrhage, the causes of which will be discussed below. VCAM-1 is expressed in the embryonal myocardium, whereas integrin α_4 is expressed in the epicardium and proepicardium.[30] Therefore, their interaction seems to be essential for maintaining epicardial integrity.

The epicardium generates a population of mesenchymal cells by an epithelium-to-mesenchyme transition process

As the epicardium covers the embryonal myocardium, a space develops between these two tissues. This space, which we will call the subepicardium, appears first around the atrio-ventricular and conoventricular furrows then later

extends over the surface of the ventricles and, in the case of mammals, the interventricular furrow. The subepicardium is little developed in the atrium, particularly on the roof, where the epicardium adheres directly to the myocardium. The formation of the subepicardial space can be determined by a change in the expression of adhesion molecules and/or by an increase in the production of extracellular matrix in the epicardium/myocardium interphase. This extracellular matrix is an extraordinarily complex medium that is rich in fibronectin and collagens I, IV, V and VI,[20,31-34] proteoglycans and laminin,[20] GP68,[35] vitronectin, fibrillin-2 and elastin,[33] tenascin-X,[36] and flectin.[37]

The subepicardial space is quickly populated by mesenchymal cells of fibroblastoid appearance. For a long time it was thought that these cells reached the subepicardium by migration from the region of the septum transversum.[15] Another possibility that has been described in mammalian embryos is the transfer of cells inside proepicardial vesicles that are released into the pericardial cavity and adhere to the surface of the heart.[38] In birds it is evident that the transfer of the proepicardium by direct adhesion drags along mesenchymal cells that are then incorporated by the subepicardium. Nevertheless, it is currently accepted that much of the subepicardial mesenchyme is constituted by cells derived from epicardium (CDEP).[39]

The CDEPs are generated by an epicardium-to-mesenchyme transition phenomenon, which is a particular case of a family of cellular processes that are very important for development, the epithelium-to-mesenchyme transitions.[40] These processes involve the acquisition by epithelial cells of mesenchymal characteristics, which allow them to separate from their neighboring cells, reorganize their cytoskeleton, break down the basement membrane and underlying extracellular matrix, and acquire the capacity to migrate through this membrane. Examples of epithelium-to-mesenchyme transitions are the formation of the mesoderm on the primitive line,[41] neural crest differentiation,[42] dermomyotome disintegration, or the formation of valvuloseptal mesenchyme in the endocardial cushions.[43]

In the case in question, the epicardium-to-mesenchyme transition begins in the atrioventricular and conoventricular furrows and later extends to other areas of the ventricular epicardium. However, most of CDEPs, at least in mammalian embryos, seem to be generated in the atrioventricular furrow (Figure 3A).

What proportion of subepicardial mesenchyme is constituted by CDEPs? Before answering this question, it is necessary to note that a large part of the proepicardial mesenchyme also originates by epithelium-to-mesenchyme transition from the mesothelium covering proepicardial vellosities.[44] This is suggested by morphological data, as well as by the presence of markers of the epithelium-to-mesenchyme transition in the proepicardial mesothelium and mesenchyme, as we will see below. This means that cells derived from the proepicardial mesothelium are incorporated into the population of CDEPs generated *in situ*, so that most of the subepicardial mesenchyme derives from the coelomic mesothelium, whether proepicardial or epicardial.

The epithelium-to-mesenchyme transition involves cytoskeletal reorganization, as mentioned before. The cells of the epicardium (and of the proepicardial mesothelium) have intermediate filaments constituted by cytokeratins.[21] During the transformation into mesenchyme, these filaments are replaced by filaments of vimentin (which are intrinsic to mesenchymal cells).[45] Of course, this substitution is not instantaneous. Vimentin expression begins even in premigratory phases, when the cell in transition still conserves its epithelial phenotype.[40] On the other hand, cytokeratin is degraded progressively throughout the process, but persists for a time during in mesenchyme derived from epithelium. This implies that the location of cytokeratin in mesenchymal cells is a marker of recent epithelial origin.[45] In fact, the great majority of the proepicardial and subepicardial mesenchymal cells are immunoreactive for anticytokeratin antibodies[39] (Figures 2B and 3B). On the other hand, numerous proepicardial and epicardial mesothelial cells are vimentin-positive, possibly indicating that they are in premigratory states.[39]

Likewise, the transcription factor associated with Wilms´ tumor (WT1) is expressed in the coelomic mesothelium, epicardium, and CDEPs, among other locations[46,47] (Figures 3C and 5B). The massive presence of WT1 protein in the proepicardial and subepicardial mesenchyme of the chick has been characterized as evidence of its mesothelial origin.[48] On the other hand, mice that are carriers of a reporter WT1 gene, with expression of β-galactosidase controlled by the WT1 promoter, show expression of this gene in practically all the subepicardial cells of the mouse.[46]

The mechanism that regulates the epicardium-to-mesenchyme transformation is little known, although there have recently been important advances in this sense. Transformation begins, and is more intense, at the level of the atrioventricular cushions and the outflow tract. Since another epithelium-to-mesenchyme transition is taking place simultaneously in these cushions, which generate the valvuloseptal mesenchyme,[43] from the first it was suspected that the same signal from the myocardium might initiate both processes. This signal was associated with the presence of «adherons,» which are complex particles composed by several proteins that have been found in the extracellular matrix of the cushions and

subepicardium.[49] At present it is thought that the growth factors of the BMP (bone morphogenetic proteins) and TGF-β (transforming growth factors-β) families are essential for endothelium-mesenchyme transformation.[50,51] However, the situation is much less clear in the case of the epicardium. The probable role of the FGFs (fibroblast growth factors), specifically FGF-1, 2 and 7, has been reported.[52] These factors stimulate epicardium-to-mesenchyme transformation *in vitro*, while TGF-β1, 2 and 3 inhibit it. Other authors insist, however, that stimulation by FGFs is possible only when the epicardial cells have been previously activated by BMP type signals (Markwald, personal communication). It is important to note that BMP-2 and BMP-4 are specifically expressed in the myocardium of the atrioventricular canal and outflow tract.[53]

In any case, after the signal or signals that induce the onset of the epithelium-to-mesenchyme transition, the implication of zinc-finger type transcription factors pertaining to the *Snail* family seems clear. Apparently, the functions of *Snail* in mammals are carried out in the avian embryo by the product of another gene, *Slug*.[54] These factors are essential to the formation of mesoderm and participate in different embryonal processes of epithelium-to-mesenchyme transformation, including the formation of the neural crest.[55] The expression of *Slug* has been shown to be essential for the transformation of the endocardium in endocardial cushions,[51,56] whereas the presence of *Slug* has been found in the epicardium and CDEPs of the chick embryo.[57]

The function of the *Slug/Snail* factors seems to be to repress the expression of cellular adhesion molecules. *Slug*, for example, represses the expression of desmoplakins and desmogleins.[58] On the other hand, it has been demonstrated that *Snail* represses the expression of E-cadherin in what could be a key event in epithelium-to-mesenchyme transformation.[59,60]

Other transcription factors of the zinc-finger type that are probably implicated in the epicardium-to-mesenchyme transition are Ets-1 and WT1, which has already been mentioned. Ets-1 activates the expression of proteolytic enzymes and seems to be a key factor in the degradation of the extracellular matrix that is associated with the migrator phenotype.[61] The presence of Ets-1 has been correlated with the areas of epicardium-to-mesenchyme transition in chick embryo.[62] WT1 seems to have a function of its own, if not in the epicardium-to-mesenchyme transition *per se*, then in the differentiation of the CDEPs. WT1 could repress the differentiation of these cells, keeping them in a mesenchymal and proliferative state.[48] This would explain the smaller number of CDEPs observed in the heart of mouse embryos deficient in WT1, as we will see below.

FOG-2 (friend of GATA) is a transcription factor expressed in the myocardium that also seems to have an essential function in epicardium-to-mesenchyme transition. FOG-2 is a cofactor of the transcriptional factors of the GATA family, three of whose members (GATA4, 5 and 6) are expressed in the embryonal heart.[63] FOG-2 deficiency produces a cardiac phenotype characterized by a low number of CDEPs, absence of coronary vessels, and hypoplasia of the ventricular myocardium.[64] FOG-2 could be implicated in the generation of the myocardial signal for beginning epicardium-to-mesenchyme transformation.

Next, other transcription factors expressed in the epicardium are cited that could participate in the generation of CDEPs. Epicardin[65] (also described as capsulin[66,67] and POD-1[68]) pertains to the bHLH family (basic Helix-Loop-Helix). It is expressed in the embryonal epicardium and in the mesothelium and submesothelial mesenchyme of the lungs, digestive tract, kidneys, and spleen. Its absence produces pulmonary and renal hypoplasia,[69] as well as agenesis of the spleen,[70] but cardiac disturbances have not been described in this model. On the other hand, two genes of epicardial expression, *Tbx5*[71] and *Tbx18*,[72] pertain to the family of the T-Box factors (related with *Brachyury*). Mutations in *Tbx5* are associated with the Holt-Oram syndrome.[73] Finally, the embryonal epicardial expression of the Rb (retinoblastoma) tumor suppressor, a protein involved in the control of the cell cycle that acts as a substrate of cdks (cyclin-dependent kinases), has also been detected. The pattern of expression of Rb suggests its involvement in endocardium-to-mesenchyme transformation,[74] which is why a parallel function in epicardial transformation cannot be excluded.

The epicardium-derived cells differentiate into connective and vascular tissue

The fibroblastoid appearance of CDEPs was the reason why they were first thought to be components of the subepicardial connective tissue. Nevertheless, evidence suggests a more active role in the development of the coronary vascular system.

For a long time coronary vessels have been considered to derive from buds of the aortic root that grow and invade the entire heart. At the end of the 1980s, a hypothesis (denominated the ingrowth hypothesis) was formulated that these vessels organize in the subepicardium to form a vascular plexus that connects with the right and left Valsalva sinuses in a given moment.[75] The sudden increase in intravascular pressure that originates this connection induces the arterialization of specific segments of the plexus that constitute the coronary arteries.

According to this hypothesis, the primary subepicardial vascular plexus organizes by the connection of vascular precursors, that is to say, by

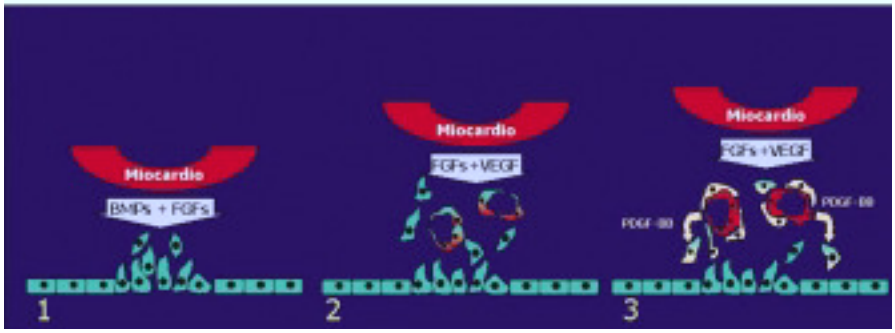

Fig. 4. Hypothetical model of the origin of the coronary vessels from cells derived from the epicardium (CDEP). *1.* In the first phase, growth factors of the BMP and FGF families induce the epicardium-to-mesenchyme transition. *2.* The presence of VEGF secreted by the myocardium and epicardium induces the endothelial differentiation of the CEDPs. *3.* In the second phase, the production of PDGF-BB by the endothelium formed induces the recruitment of new CDEPs and their differentiation into pericytes and smooth muscle.

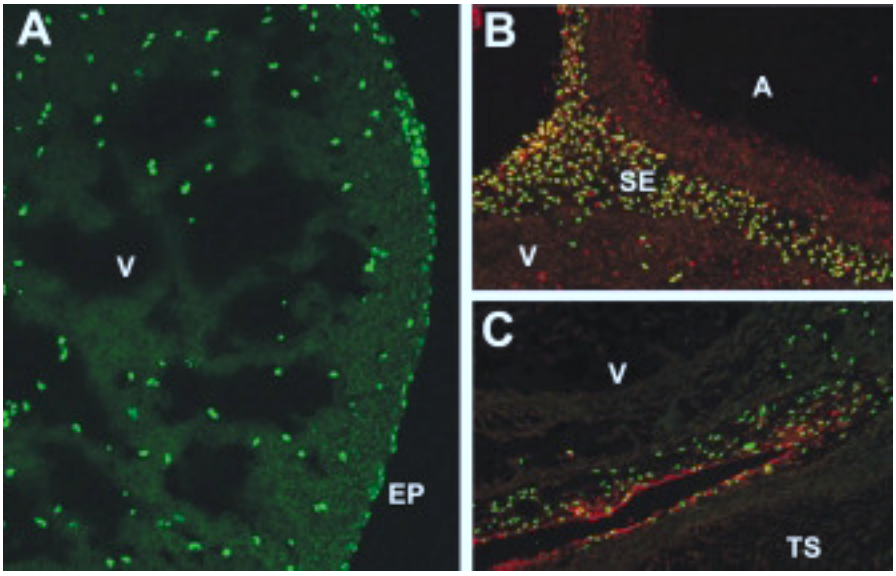

Fig. 5. Signaling function of cells derived from epicardium (CDEP), manifested in chick/quail chimera (quail epicardium on chick myocardium). The cells of the quail are recognized in all the cases by the immunolocalization of QCPN antigen (in green). *A.* Stage HH29. The ventricle (v) has been completely invaded by CDEPs. EP indicates epicardium. *B.* Stage HH32, atrioventricular furrow. The WT1 transcription factor has been immunolocalized in red. Observe the co-localization of QCPN and WT1 in most of the subepicardial mesenchyme (SE). Co-localization results in a yellow color. The presence of WT1-positive cells is also observed in the ventricle (V), but not in the atrium (A). *C.* Stage HH29, conoventricular furrow. The retinaldehyde dehydrogenase enzyme has been immunolocalized in red. Observe the reactivity of the epicardium and CDEPs, as well as the presence of CDEPs in the ventricle (V), but not in the outflow tract (OT).

vasculogenesis.[76] Once established, it grows by angiogenesis or proliferation of established vessels. The vasculogenic origin of the coronary vessels raised the question of the origin and differentiation of their cellular precursors, which necessarily had to be found in the subepicardial mesenchyme.

Experimental evidence has demonstrated that the smooth muscle precursors of the media of coronary vessels, as well as the fibroblasts of the adventitia, are differentiated from CDEPs. A fundamental technique for reaching this conclusion were the «epicardial chimera,» which are developed by grafting quail proepicardium in the pericardial cavity of chick embryos in the HH17 stage of development.[22] This generates chick embryos whose hearts are covered by quail epicardium. The destiny of CDEPs derived from this epicardium can be followed using antibodies that recognize quail cells, but not chicken cells (Figure 5).

Using epicardial chimera, it was demonstrated that CDEPs from the donor contributed to the formation of the coronary musculature, vascular adventitia, and fibrous skeleton of the heart.[77,78] In this system, the coronary endothelium also derived from the quail, although the explanation given by the authors of these studies did not contemplate a CDEP origin of the coronary endothelium, as we will see below. The epicardial chimera used in these experiments showed that CDEPs invade the myocardium and even colonize the atrioventricular cushions, reaching subendocardial levels. However, the cushions of the outflow tract do not show the presence of CDEP.

Other experiments have confirmed these findings. The culture of proepicardial cells on collagen gels generates a monolayer of epithelial cells identical to those of the epicardium. These cells, once isolated, marked, and injected into the pericardial cavity of chick embryos, give rise to the epicardium, coronary smooth muscle, and fibroblasts.[79] In another series of experiments it has been shown that the epicardial cells generated on collagen gels by the culture of proepicardial explants expressed markers of smooth muscle *in vitro*, such as caldesmon and specific

isoforms of actin and myosin.[80] This process is stimulated by the presence of PDGF-BB (platelet-derived growth factor-BB) and is dependent on the expression of SRF (serum response factor), a transcription factor of the MADS box superfamily, which is expressed by the epicardium in culture.[81] In fact, dominant-negative genetic constructions for SRF inhibited the differentiation of smooth muscle from CDEPs.

The BVES (blood vessel/epicardial substance) protein, which probably represents a new family of adhesion molecules, has been found throughout the entire proepicardium-epicardium-CDEP-vascular smooth muscle cell line, and constitutes further evidence of the epicardial origin of the coronary medial layer.[82,83]

The differentiation of smooth muscle cells and fibroblasts from CDEPs is a well established phenomenon. Nevertheless, the origin of the coronary endothelium is still debated. The first phase of the processes of vasculogenesis is the assembly of angioblasts and mesodermal endothelial precursors. In a second phase, the endothelial tubes recruit mesenchymal cells and induce their differentiation into perivascular cells (pericytes and smooth muscle cells). The origin of the angioblasts that give rise to the primitive subepicardial plexus is uncertain, although there are two possibilities that are not mutually exclusive. The first possibility is that the angioblasts migrate to the subepicardium from the region of the liver and septum transversum, either through the proepicardium, or directly, when the subepicardium has connected with the hepatic splachnopleura.[15] The second possibility is by the differentiation of CDEPs.[44] In this case, the epicardium would be the origin of both the endothelium and the smooth muscle of coronary vessels.

The first possibility is supported by the findings of certain experiments, in which the epicardial chimera only developed endothelium derived from the donor if the proepicardial graft was accompanied by a fragment of liver tissue.[84] This is why other chimera, which developed coronary endothelium from the donor, were not considered to be proof of the epicardial origin of this endothelium.[77] Nevertheless, the hypothesis of a hepatic origin of the coronary precursors encounters two difficulties. In the first place, epicardial chimera in which the donor epicardium forms a mosaic with the host epicardium showed how the limits between the respective mesenchymes were perfectly clear and coincided with the limits between epicardia.[44] Stated in other terms, beneath the quail epicardium only quail mesenchyme is found, and beneath the chicken epicardium, only chicken mesenchyme. Logically, the coronary vessels that developed in this system were a mosaic of endothelial cells from the donor and receptor. The absence of horizontal migration of the subepicardial mesenchyme does not fit the hypothesis of an invasion of the cardiac surface by extracardiac angioblasts.

The second argument to consider regarding a hepatic origin of the coronary angioblasts is based on evidence of hepatic vasculogenesis. It is likely that the endothelium of hepatic sinusoids is a coelomic derivative, an idea that was proposed a long time ago[85] and has recently received experimental support. For example, cytokeratin remains can be found in the early sinusoidal cells of the chick embryo[39] (Figure 2B), whereas in transgenic mice with the WT1 reporter gene, the hepatic endothelium expresses this mesothelial marker.[46] Direct labeling of the hepatic mesothelium of chick embryos with a fluorescent marker shows that endothelial cells in the sinusoids are marked after only 24 h of reincubation.[86]

The possibility that CDEPs differentiate into angioblasts is supported by experimental evidence. This evidence includes the combined presence in subepicardial cells of cytokeratin remains with VEGFR-2 (vascular endothelial growth factor receptor-2), also known as Flk-1,[87] the earliest known vascular marker, as well as a series of experiments with epicardial chimera,[44] and the findings of direct proepicardial mesothelial and epicardial labeling with fluorescent tracers and retrovirus.[88] If these findings are confirmed, both the embryonal heart and liver receive angioblastic cells from the coelomic mesothelium. This is a process that may be generalized in other organs in which vasculogenesis occurs (vitelline sac, lungs, digestive tube, allantois), and has been situated in a conceptual framework explaining the origin of the vertebrate circulatory system.[89]

Epicardium-derived cells could be pluripotential vascular precursors

The possibility that CDEPs give rise to at least three cell types (fibroblasts, smooth muscle cells and endothelium) raises interesting questions about the mechanisms that regulate this pluripotentiality. In this context, it is important to underline the recent discovery of bipotential vascular precursors derived from embryonal stem cells and selected by VEGFR-2 expression.[90] These cells, when cultured in the presence of serum or PDGF-BB, differentiate into smooth muscle cells. However, in the presence of VEGF (vascular endothelial growth factor) they give rise to endothelial cells. The exposure of these bipotential precursors to both growth factors induces the formation of mixed cultures in which smooth muscle cells surround endothelial cells.

CDEPs could constitute bipotential vascular precursors from the moment in which they both

express high-affinity receptors for the two growth factors previously mentioned, VEGFR-2 and PDGFRβ[87-91] (unpublished data). If this hypothesis is confirmed, an attractive scenario for the development of cardiac vascularization would be the following (Figure 4): myocardial signals (BMPs, FGFs) would induce the transformation of epicardium into pluripotential mesenchyme. The first populations of CDEPs would be induced to differentiate into endothelial cells by the high level of epicardial and myocardial production of VEGF.[92] These cells would organize into a primary vascular plexus that would recruit successive CDEPs through the production of PDGF-BB, a growth factor produced by the endothelium.[93]

Various observations seem to support this hypothetical model. The vascularization of the heart, like that of other embryonal organs, depends on a precise dose of VEGF. The absence of VEGF is lethal, even in heterozygosis,[94,95] and myocardial overexpression of VEGF leads to defective vascularization[96] and vascular dilation, very similar to what takes place in models in which signaling by PDGF-BB/PDGFRβ is disturbed.[97]

The phenomenon of the pluripotentiality of CDEPs could extend further than suggested here, since there are two points that have not been examined, but will have to be considered in the future. In the first place, in spite of numerous morphologic descriptions to this effect, the existence of hematopoiesis in the embryonal heart is still uncertain. The development of the subepicardial vascular plexus has been described in mammalian embryos (including humans) as a process of the fusion of «blood islets.»[18,98] Evidence in favor of the existence of a common precursor of blood and endothelial cells, known as the hemangioblast,[99] suggests that the process of differentiation of the coronary precursors could be more complex than described and leaves open the possibility of an even greater degree of CDEP potentiality. The role of the surprising epicardial expression of erythropoietin and its EPOr receptor is uncertain in this context.[100] On the other hand, on several occasions it has been suggested that the subepicardial mesenchyme could be different in myocardiocytes,[101] although no evidence of this possibility has been found in epicardial chimera.[22]

Epicardium-derived cells modulate myocardial development

A series of recent experiments have suggested that, in addition to contributing to the development of the vascular and connective tissue of the heart, as has been described, CDEPs could have an essential modulating role in the development of the ventricular myocardium and, in particular, of the compact layer. Evidence to this effect is described below.

Adult myocardial cells in primary culture suffer a process of dedifferentiation by which they change their structure, function and gene expression profile. Co-culture of epicardial and myocardial cells has been shown to delay this process and maintain the contractile phenotype of the myocardium for a longer time.[102] This phenomenon, which requires contact between the epicardium and myocardium, has been attributed to some type of interaction of a physiological nature.

In both mammalian and avian embryos, the first phases of ventricular compaction coincide with an invasion of the myocardium by CDEPs.[48,77] It is interesting to observe that this invasion takes place in the ventricle, but not in the atrium (Figure 5). The cells that penetrate the ventricular myocardium maintain the expression of the WT1 transcription factor, in contrast with the CDEPs that differentiate into vascular tissue.[103] One of the first indications of the modulating role of the CDEPs on myocardial differentiation comes from the study of the cardiac phenotype in mice deficient in WT1.[104] The loss of WT1 function produces severe anomalies in the development of gonads, kidneys, the spleen, and adrenal glands. Nevertheless, the lethal nature of this mutation is due to heart failure induced by ventricular hypoplasia, characterized by a thin ventricular wall and non-formation of the compact layer. In $WT1^{-/-}$ mice, the formation of the epicardium takes place normally and CDEPs are generated, but in smaller amounts than in normal mice. Since WT1 is expressed exclusively in the epicardium and CDEPs, and these cells specifically invade the ventricular myocardium, it seems that myocardial compaction is dependent on the CDEP invasion.

It is necessary to comment that this so-called thin myocardium syndrome that characterizes WT1 mutation also takes place in association with the impaired function of various genes involved in epicardial development, as has already been mentioned in this review, such as FOG-2, VCAM-1, α_4 integrin, erythropoietin, and its EPOr receptor.

The precise mechanism of epicardium/myocardium interaction is not known, although signaling by retinoic acid seems to be involved. In fact, the epicardium and CDEPs express retinaldehyde-dehydrogenase-2 (RALDH2), a key enzyme in the synthesis of retinoic acid[103,105] (Figure 5C). It has been confirmed that the cells that invade the myocardium also express this enzyme, although the expression tends to decrease with time and disappears with the differentiation of the CDEPs.[103] This observation is related with the fact that mice deficient in the RXRα receptor of retinoic acid present a ventricular hypoplasia identical to that of $WT1^{-/-}$ mice.[106,107] In the first case, it could be thought that the retinoic acid

produced by the epicardium and CDEPs is the signal that induces the formation of the ventricular compact layer. However, the process seems to be more complex, since conditioned mutations that specifically annul the expression of RXRα in the myocardium are innocuous.[108,109] Since CDEPs also express this receptor, it is more likely that retinoic acid is essential to the formation of an autocrine loop that maintains the CDEPs in a undifferentiated state and generating signals to the myocardium. These signals are of unknown nature, but they could maintain the ventricular myocardium in a proliferative state. The absence of WT1 may induce the premature differentiation of the CDEPs, which cease to produce both retinoic acid and signals to the ventricular myocardium.

The functions of the CDEPs in cardiac development seem to depend, therefore, on a very fine balance between signals that originate in both the myocardium and the CDEPs, a balance that allows specific subpopulations of CDEPs to enter differentiation pathways and contribute to the connective and vascular tissue, or to remain undifferentiated, migratory, and producing autocrine and paracrine signals. Evidence that this balance is exquisitely regulated comes from genetic manipulation that affects certain growth factors. For example, mice in which VEGF-A overexpression occurs in the myocardium develop large epicardial vessels, as could be expected, but they also show ventricular hypoplasia.[96] A possible interpretation of this phenomenon is that the rupture of the balance between CDEP subpopulations reduces the number of cells modulating the development of the ventricular compact layer.

A final topic to address in this chapter are the possible relations between the CDEPs and the differentiation of conduction tissue. Some suggestions to this effect have been made, indicating the spatial coincidence between the distribution of the CDEPs, the main coronary vessels that derive from them. and the Purkinje fibers.[77] It has been suggested that endothelin produced by the coronary endothelium could mediate the differentiation of conduction tissue.[110]

CONCLUSIONS AND PERSPECTIVES

Throughout this article we have shown how the importance of role of the epicardium in cardiac development has become progressively more apparent in recent years. This is due to the discovery that the epicardium provides an important population of mesenchymal cells and to the pluripotential nature of these cells, a characteristic whose significance will have to evaluated in the coming years.

The pluripotentiality of the CDEPs brings us to a concept that is beginning to circulate among researchers in cardiac development, the idea that the epicardium constitutes a population of potential cardiac stem cells. It should not be overlooked that the myocardium and endocardium derive from the precardiac mesoderm when this mesoderm is a true coelomic epithelium, which means that the same cell type continues ontogenetically in the proepicardium and epicardium. On the other hand, the potentiality of the coelomic epithelia or mesothelia is dramatically illustrated by the tissue heterogeneity of a group of malignant mesotheliomas that give rise to osseous, cartilaginous, muscular, or hemangioblastic elements. The term «mesodermoma» has been proposed for these tumors, since it is considered that the coelomic mesothelium recovers truly ancestral mesodermal properties in the process of tumor formation.[111,112]

It is possible that the adult epicardium retains a capacity for responding to signals that can induce their transdifferentiation into cell types of interest for the treatment of various pathologies. Many clinical possibilities could derive from such speculations.

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