Epicardium-Derived Cells Contribute a Novel Population to the Myocardial Wall and the Atrioventricular Cushions

Adriana C. Gittenberger-de Groot, Mark-Paul F.M. Vrancken Peeters, Monica M.T. Mentink, Robert G. Gourdie, Robert E. Poelmann

Abstract—The epicardium and dorsal mesocardium are known to be the source of structures that form the wall of the coronary vessels. Because mouse knockout studies have shown that proper epicardial formation is also essential for myocardial development, we have studied in detail the migration and differentiation of epicardium-derived cells (EPDCs) within the developing heart. We constructed chicken-quail chimeras by grafting the quail epicardial organ, including a piece of primordial liver, at essentially stages 16 and 17. The embryos were studied at stages 25 to 43. To detect quail-derived EPDCs, an anti-quail nucleus antibody was used in combination with several differentiation markers, eg, for muscle actin, for vascular smooth muscle cells, for procollagen-I, for quail endothelium, and for Purkinje fibers. At stages 25 to 31, EPDCs are encountered in the myocardial wall and the subendocardial region. The latter deposition is spatially facilitated as the endocardium protrudes through transient discontinuities in the myocardium to contact the subepicardial layer. Later on, at stages 32 to 43, EPDCs invaded, by way of the atrioventricular sulcus, the atrioventricular cushion tissue. The localization is apparent at the interface with the myocardium, as well as subendocardially, but never within the endocardial lining. The origin of endothelium, smooth muscle cells, and fibroblasts of the coronary vessel wall from the epicardial graft were confirmed in accordance with already published data. The functional role of the novel EPDCs in the subendocardium, myocardium, and atrioventricular cushions remains to be investigated. A close positional relationship is found with the differentiating Purkinje fibers. Furthermore, a regulatory role is postulated in the process of endocardial-mesenchymal transformation. The ultimate fate of EPDCs seems to be a cardiac fibroblast cell line involved in the formation of the fibrous heart skeleton. (Circ Res. 1998;82:1043-1052.)

Key Words: atrioventricular cushion ■ cardiac development ■ epicardium ■ myocardial fibroblast ■ Purkinje fiber

Initially, the wall of the heart tube consists of myocardium and endocardium with cardiac jelly sandwiched in between. The myocardium and part of the endocardium have been previously described as originating from the bilateral cardiogenic plates.1,2 The endocardium is also postulated to originate from the visceral yolk sac mesoderm that gives rise to the vascular endothelium.3,4

To enable proper cardiac function, other cellular components and the formation of a myocardial interstitium are essential.5 For this, it is important to consider a third cell layer (which develops over the myocardium), the epicardium.6-9 When this epicardial layer does not develop, as in VCAM-1-deficient mice,10 the myocardium is thin and not well organized, which leads to embryonic death.

In addition to myocytes, cardiac fibroblasts are essential for formation of a normal myocardial wall. One of the functions of fibroblasts is to produce part of the collagen-rich matrix of the heart. The origin of these cardiac fibroblasts is still unclear. The cell lineage studies of Mikawa and coworkers11,12 show the epicardium to be the source of coronary endothelium, smooth muscle cells, and fibroblasts. Studies from our own group using chicken-quail epicardium chimeras13 correlate with those of the retroviral tracing studies,11,12 in that coronary endothelium reaches the heart via the subepicardial layer. The endothelial cells, however, are not epicardium-derived.8 Our present data from chicken-quail chimeras confirm that smooth muscle and adventitial fibroblasts are derived from the epicardium.

A number of proepicardial cells migrating to the myocardial wall, however, were unrelated to the coronary vessels. In the present study, we describe the origin of this novel EPDC population and its migration into the myocardial interstitium and AV endocardial cushion tissue and discuss the possible function in cardiogenesis and heart pathology.

Materials and Methods

For the present study, we used embryos of the White Leghorn chick (Gallus domesticus) and embryos of the Japanese quail (Coturnix coturnix japonica). We staged the embryos according to the criteria

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of Hamburger and Hamilton. The number of successfully assessed chimeras and their survival stages after the surgical implantation are indicated in the Table. A total of 16 chimeras from stage 25 to near hatching, at stage 43, were finally analyzed for this study.

### Chimerization Technique

The experiments were performed when a chick egg had been incubated at 37°C (80% humidity) for ~3 days and had developed to stage 16 or 17 (Table). Only a few experiments included stage 15 or 18 embryos. The chick egg was opened, and the vitelline membrane was locally removed. Subsequently, the pericardial cavity of the embryo was reached through the naturally existing body wall hiatus at stage 15 or through a slit made in the amniotic and pericardial membranes with watchmaker forceps at stages 16 to 18. A piece of proepicardial organ, including an adjacent piece of liver, was harvested from a quail embryo of similar incubation time and developmental stage (Table). The technique of chimerization has been described previously by our group. As in the latter study, a piece of liver tissue was included to ensure endothelial seeding. In the present set of experiments, care was taken to insert the piece of tissue under the heart in the region of the sinus venosus that was the origin of normal epicardial outgrowth. Because we did not remove the native chick proepicardium, both chick and quail epicardium spread together, resulting in a mixed covering of the myocardium. In two cases, the grafted tissue was inserted in the inner curvature of the looped heart.

### Immunohistochemistry

For detection of quail cells, an anti-quail nuclear antibody (QCPN, Hybridoma Bank) was used. The QCPN antibody, in combination with the quail-specific anti-endothelium antibody (QH1, Hybridoma Bank), allows us to differentiate between quail epicardial cells and quail endothelium.

To follow the differentiation pathway of the quail cells, we used the anti-endothelial antibody QH1, the smooth muscle cell marker 1E12, the procollagen type I marker M38, and the anti-actin muscle marker HHF35. The QH1, M38, and HHF35 antibodies were obtained from the Hybridoma Bank. To follow the possibility of an influence of EPDCs on conduction cell induction, the EAP-300 antibody (kindly provided by Dr G.J. Cole, Charleston, SC) was used to indicate the position of Purkinje fiber cells.

The fixation and staining procedures for the QCPN, QH1, 1E12, M38, and HHF35 antibodies were as follows: From the embryos that were harvested at consecutive stages, the thorax was dissected and fixed in a solution of 2% acetic acid in 100% alcohol for at least 12 hours. Subsequently, the pieces were embedded in paraffin and serially sectioned at 5 μm and transferred to albumin/glycerin-coated objective slides. The dehydrated sections were immersed in PBS to which was added 0.006% H2 O2 for the localization of peroxidase activity. After they were washed in PBS, the sections were stained with hematoxylin (10 seconds) and coverslipped in Entellan (Merck). The sections were investigated by light microscopy and photographed with a Leitz Dialux microscope.

For the fixation and staining procedure for the EAP-300 antibody, the dehydrated sections were rinsed twice in PBS and once in PBS-Tween for 10 minutes. Thereafter, they were incubated overnight with the EAP-300 antibody diluted 1:1000 (in 1% ovalbumin in PBS-Tween). After they were rinsed in PBS, the slides were incubated with a second antibody (1:50), fluorescein-conjugated rabbit anti-mouse immunoglobulin (Dakopatts F232), for 1.5 hours. After they were rinsed in PBS, the sections were dripped with diazabicyclo(2.2.2)octane (Sigma) and propidium iodide (Sigma) for fluorescence detection and coverslipped. The sections were investigated by the Leitz Dialux fluorescence microscope.

### Results

In all chicken-quail chimeras, we detected grafted quail cells that were present in the epicardial lining and the subepicardium. Furthermore, quail EPDCs had invaded the myocardium, the subendocardial space, and the AV cushion tissue and were seen in the lining of the developing coronary vasculature. In the present study, we concentrate on the EPDCs that are unrelated to the coronary vessels, and data on the latter will be presented only when they are essential for differentiation between coronary and noncoronary EPDCs. The deposition of EPDCs is described at subsequent developmental stages showing several successive migration waves.

### Myocardial and Subendocardial Invasion (Stages 25 to 31, Embryos 1 to 6)

The 6 embryos studied during this time period all showed quail-derived epicardium, as indicated by quail nuclear staining, covering the dorsal wall of the left ventricle, the left AV canal, and part of the left atrium. We could not establish a

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**Selected Abbreviations and Acronyms**

- AV = atrioventricular
- EPDC = epicardium-derived cell
- IV = interventricular
- VCAM = vascular cell adhesion molecule

**Successfully Assessed Chicken-Quail Chimeras**

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<tr>
<th>Embryo</th>
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sv indicates sinus venosus; ic, inner curvature.
pattern of outgrowth that related to the stage of harvesting of the graft or stage of implanting in the host, and we have therefore provided general data. In one embryo (Figure 1A and 1B and embryo No. 5 in the Table), the major part of the epicardium was quail-derived, except for the right atrium and the outflow tract. Using the quail anti-endothelial antibody, we confirmed in embryo 3 that the endothelium-lined tubes of the developing coronary vasculature were of mixed quail and chick origin (Figure 1C).

Apart from the quail cells that take part in formation of the vasculature, there is a distinct subpopulation of scattered cells that invades the myocardium. These EPDCs are usually isolated and do not stain for endothelium (compare panels A and B of Figure 2), M38, and HHF35 (not shown). There is a marked

**Figure 1.** A, Section of the AV sulcus interposed between atrium (A) and ventricle (V). The epicardium (E) covers the myocardium (M) that is still continuous between atrium and ventricle. The dark QCPN-stained quail cells form the epicardial lining and also form the lining of a coronary vessel (CV). B, Section subsequent to panel A showing quail-specific anti-endothelial antibody QH1 in the quail-derived endothelium. C, Distribution of EPDCs at stage 27 (embryo 3). EPDCs, with darkly stained nuclei, are present within the epicardium (E) and the myocardium (M) as isolated cells (arrowheads) and in the chicken (pale) and quail (dark) mixed lining of a coronary vessel (CV). Bar=50 μm.
preferential localization of these quail cells in the subendocardial region (Figure 2C). The way in which they reach the subendocardial position is remarkable in that there exist invaginations of endocardium through discontinuities in the thin-walled myocardium. In this way, the subepicardial and subendocardial space are in contact, allowing for a direct route of EPDCs to a subendocardial position (Figure 2D and 2E). Only occasionally does the endocardial lining of the ventricles and atria harbor quail endothelial cells. In one embryo (No. 6), there is colocalization of procollagen (M38)-positive cells and of quail-derived cells in the subendocardial region (not shown).

**Endocardial Cushion Invasion (Stages 32 to 43, Embryos 7 to 16)**

The distribution of the quail-derived epicardium varied from the right ventricular dorsal wall and part of the IV septum and right AV transition (embryos 7, 9, and 10) to a mainly left ventricular orientation (embryo 8). In one chimera, there was almost a complete quail-derived covering of the heart, including the outflow tract (embryo 11). In another heart, the covering epicardium was chick-derived, and the subepicardium was filled with quail cells (embryo 14). In the remaining chimeras, only parts of the heart were covered with quail cells, eg, the outflow tract (embryos 13 and 15), the IV septum (embryo 12), and the right ventricular apex (embryo 16). The distribution of the migrated EPDCs is tangential to the quail-derived areas of epicardial covering. In this way, we encountered two embryos (Nos. 11 and 14) with atrial myocardial invasion (Figure 3A).

The quail EPDCs were found inside the compact myocardium, the trabeculae, and the subendocardium. The myocardial discontinuities were not seen anymore. In three embryos (Nos. 9, 11, and 14), we found cells positive for

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**Figure 2.** A, Section of the ventricular wall of embryo 3. Dark EPDCs are seen scattered through the myocardium (M) and in a subendocardial position (arrowheads) along the trabeculae of the ventricle (V). E indicates epicardium. B, Section consecutive to panel A showing the EPDCs (indicated by arrows in panel A) that have not differentiated into endothelial cells. The quail-derived endothelial cells participating in coronary vessel formation have been stained with QH1. C, Detail of the subendocardial region (SE) overlying the myocardium in embryo 6. EPDCs (arrowheads) are markedly present in this area. D, Detail of embryo 3 of a trabecular space (T) lined by endocardium (arrows) that breaks through a myocardial discontinuity to contact the SE (arrowheads). E, Detail of embryo 1 showing the close relationship of subepicardium (E) and subendocardium (SE) through a myocardial discontinuity (arrowhead). Bar=50 μm.
the procollagen marker M38 in the same region as EPDCs (not shown).

A remarkable observation was the presence of a large number of quail EPDCs in the AV endocardial cushions. In embryos 7, 9, and 10, the cushions of the developing tricuspid valve had been invaded (Figures 3B, 4A to 4C, and 5B), and in embryos 11 and 14, the developing mitral valve had been invaded as well (Figure 5A). The location correlated with the overlying extent of quail-derived epicardium. In this region, there was a direct contact between the subepicardium and the endocardial cushion tissue as the atrial and ventricular myocardial connection was interrupted, enabling a relatively short migration route (Figures 3B, 4A, 4B, and 5A). The quail EPDCs in the endocardial cushions favored two positions. One was at the myocardial/endocardial cushion interface (Figures 3B, 4B, and 5B). The second site of accumulated quail EPDCs was found subendocardially at the luminal face of the AV cushion (Figures 3B, 4A to 4C, 5A, and 5B). In the AV cushions, we were never able to demonstrate incorporation of grafted cells into the endocardial lining that we had seen occasionally in endocardium lining the ventricular myocardium (Figure 4C). Once again, the scattered EPDCs that had gained an intramyocardial and subendocardial position could easily be distinguished from those quail cells that had differentiated into the endothelial lining of the coronary vessels (Figure 5B and 5C). A clear correlation was shown between procollagen formation and EPDCs in the developing annulus fibrosis (Figure 6A and 6B) as well as in the subendocardial region (Figure 6C and 6D).

In 4 embryos (Nos. 11, 13, 14, and 15), in which the epicardium also covered the outflow tract, we found scattered quail EPDCs in the muscular outflow tract septum. The mesenchyme of the endocardial outflow tract cushions had
disappeared at this time. The semilunar valves were negative for EPDCs.

Spatial Correlation of EPDC Distribution With Purkinje Fibers

When the EAP-300 antibody was used for detection of differentiating Purkinje fibers, a remarkable colocalization was observed. The Purkinje fibers were localized subendocardially (Figure 7A) and around coronary arteries in a later stage of development (Figure 7C). This staining correlated very closely with the subendocardially positioned quail EPDCs (Figure 7B) as well as with the coronary arterial adventitial quail-derived fibroblasts (Figure 7D). There was also a colocalization of clusters of myocardial cells that stained with the EAP-300 marker in the AV cushions (Figure 7E), and EPDCs were present in the adjacent section (Figure 7F).

Discussion

The present study shows for the first time that there is an extensive contribution of epicardial cells not only to the coronary vasculature but also to the myocardial wall, the subendocardial region, the AV cushions and valves, and the fibrous heart skeleton.

The importance of an epicardial covering for cardiac survival is substantiated by several experimental models in which epicardial formation is hampered. An example is given in the VCAM-1–deficient mice.10 These mice die at embryonic days 11.5 to 12.5. A somewhat comparable model, but with a less severely affected myocardium, is given in the α4 knockout mice that also die between embryonic days 12 and 12.5.24 In these embryos, the coronary vessels normally residing within the AV sulcus, together with the complete epicardial covering, are missing. Epicardial attachment is believed to be established by the α4 integrin/VCAM-1 interaction between epicardial cells and the outer myocardium.

It remains to be investigated what the actual triggering effect for the embryo lethality is. We postulate that in all models in which the epicardial outgrowth is disturbed not only is coronary vascular formation hampered but also an early important population of EPDCs does not enter the myocardium, the subendocardial layer, or, later on, the AV cushions.

There is evidence that we are dealing with precursors of the cardiac fibroblasts. In a number of cases, we could demonstrate costaining with the procollagen marker M38, especially in the older stages (stage 43), where formation of the fibrous heart skeleton between the atria and the ventricles takes place. Furthermore, the localization of the scattered EPDCs in the myocardium, in the subendocardial region, and, later on, in the AV cushion tissue complies with the presence of a fibroblastic cell lineage.

In the present study, we have identified a close positional relationship between subendocardial and periarterial EPDCs and the differentiation of Purkinje fibers. Our data implicate EPDCs in the development of conductive cells and resolve an interesting enigma that has emerged in recent years concerning the differentiation of this specialized myocardial lineage. It has been established that intramural Purkinje fibers ramify in close association with coronary arteries in the chick heart.25,26 In cell lineage studies, Gourdie et al25 have shown that perivascular Purkinje cells share common progenitors with working myocytes. They hypothesized that cells not further defined but associated with the arterial bed have induced the differentiation of myocytes into conductive cells. A caveat of this model has been that Purkinje fiber cells occur
at subendocardial locations far from arteries as well as at locations adjacent to arteries in birds. Indeed, in most mammalian species, including humans, the peripheral conductive network is confined exclusively to the subendocardium. The migration patterns of EPDCs described in the present study may provide the basis for an explanation of this interesting difference. We hypothesize that the specific distribution pattern of EPDCs implicates this extracardiac population in the induction and organization of Purkinje fibers at both subendocardial and periarterial sites in birds.

Figure 6. A, Section through an already well-differentiated mitral valve leaflet (MV) between the left atrium (LA) and left ventricle (LV) in embryo 14. At the site of the formation of the fibrous annulus (asterisk), there is marked procollagen-I staining, as indicated by the M38 antibody. B, Section subsequent to panel A showing the QCPN-stained EPDCs (asterisk) in annulus fibrosis formation. C and D, Two subsequent sections of the wall of the LA of embryo 14. The arrowheads in panel C show the procollagen-I–positive cells in the subendocardial region (SE). In panel D, the dark QCPN-stained EPDCs are in the same area. E indicates epicardium; M, myocardium. Bar=100 μm.
From stage 32 onward, we have detected EPDCs in the AV cushions and in later stages in the AV valves. This is the first time that such a population is described. The presence of the EPDCs is particularly prominent in the subendocardium, where endocardial cells have been reported to transform into AV cushion mesenchymes.2 This suggests competition for the same localization in the subendocardial position of the endocardium-derived cells and the EPDCs. It is, however, not possible to mix up populations, because the EPDCs are clearly quail-derived (QCPN-positive) and, in this location, nonendothelial (QH1-negative).

Another site of EPDCs is between cardiomyocytes that are found at the endocardial cushion/myocardium interface before valve formation. At this site, the cushion tissue has to delaminate from the underlying myocardium to form a free valve leaflet.4 Transformation of myocardial cells to fibro-

Figure 7. A, The Purkinje fiber (P) within a ventricular trabecula (T) of embryo 14 is positive for EAP-300. RV indicates right ventricle; M, myocardium. B, There is a colocalization with EPDCs (arrowheads), as shown in a consecutive section. C, Purkinje fibers (arrowheads) are localized within the myocardium surrounding the coronary arteries (CA) of embryo 14. E indicates epicardium. D, Colocalization with the adjacent coronary arterial adventitia containing quail fibroblasts (darkly stained) occurs. E, Myocardial cells present within the AV cushion tissue (AC) of embryo 14 are positive for EAP-300. LA indicates left atrium; LV, left ventricle. F, Again, many EPDCs (darkly stained) are lying in between the myocardial cell clusters. Bar=100 μm.
blasts has been postulated to be essential for this process.\textsuperscript{28} In the AV cushions, there were clusters of myocardial cells, intermingled with EPDCs, that showed Purkinje cell characteristics (EAP-300 staining). It remains to be investigated what the fate of the latter cells is and whether they might be the precursors of the described occasional nerve fibers in AV valves.\textsuperscript{29}

The EPDCs are also located at the site of the fibrous annulus between atrium and ventricle, being positive for procollagen-I. It is evident that migration of EPDCs is necessary for the formation of the fibrous heart skeleton and the AV valves. This is not achieved by infolding of the epicardium, as was postulated by Wenink et al.,\textsuperscript{30} but by migration of individual EPDCs. Our findings do not contradict the results of Wessels et al.,\textsuperscript{32} who showed different immunohistochemical characteristics of epicardium and AV cushions.

Besides the already mentioned potential roles of EPDCs in cardiac fibroblast formation and the induction of Purkinje fibers, we would like to postulate a third role during cardiac development. Remarkable is the subendocardial localization of the EPDCs and their close relationship with areas in which endocardial-mesenchymal transformation takes place. EPDCs may have a transient regulatory role in these transformation processes, with an inhibitory role being most likely. The line of arguing is that the quiescent mesothelial lining of epicardial cells becomes activated and is transformed into a subepicardial population of EPDCs. The presence of the molecular transformation markers JB3 and ES/130 at these sites\textsuperscript{32,34} supports this concept. Thereafter, the EPDCs migrate to subendocardial and intramyocardial positions. Migration is facilitated by convergence of the subepicardial and the subendocardial region during the early stages of development. This is seen first in the developing ventricles, where discontinuities in the myocardium are present.\textsuperscript{35,36} In the present study, we could not find these discontinuities in the AV canal, which explains the absence of EPDCs in the endocardial AV cushions in early stages. Later, during formation of the AV groove and separation of atrial and ventricular myocardium, the abundant subepicardial tissue in the AV groove comes into direct contact with the endocardial cushion tissue. We have summarized our observations on EPDC migration in schematic drawings of two different stages (Figure 8). EPDCs could prevent the transformation, in early stages, of the ventricular endocardial lining into mesenchymal endocardial cushion cells, whereas in the AV canal, the formation of endocardial cushions is not hampered because EPDCs are absent. The role of the endocardial transformation molecule ES/130\textsuperscript{33,34} is suggestive. Remarkably, the transformation marker JB3 is present in endocardium overlying the AV cushions, whereas it is absent in the ventricular endocardium.\textsuperscript{32}

Supporting the above-mentioned concept are preliminary data from experiments in which we block epicardial outgrowth and EPDCs in the quail embryo. These ultimately lethal experiments show a high cellularity of the AV cushions as well as thickened and altered endocardium over the ventricular trabeculae.

With regard to the ultimate fate of the EPDCs in the mature heart, there are a number of discussion points. Apoptosis is not involved, inasmuch as we could not find a coexpression of quail markers and in situ nick end labeling (authors’ unpublished data, 1996). The most attractive alternative is that these cells remain in position as an embryonic fibroblast population with a role in normal development and specifically in the formation of the fibrous heart skeleton. The scattered cells in the myocardium are probably the fibroblasts that have been observed in the mature myocardium\textsuperscript{1} with an active role in the formation of the myocardial connective tissue interstitium. A special role can be attributed to the subendocardial EPDCs in the ventricles and atria, with the latter containing more cells. Under pathological conditions, these cells could be involved in the development of endocardial fibroelastosis in which, preferentially, endocardium and subendocardium form abundant fibroelastic tissue that cannot be explained simply as ischemic fibrous scar tissue.\textsuperscript{37,38} Interestingly, the atria show more EPDCs and, even under normal circumstances, eventually develop a layer of endocardial fibroelastosis.

Experimental studies focusing on the role of the EPDCs will be continued not solely by blocking epicardial outgrowth but also by extending our experiments with chimeras in which epicardial tissue, carrying various growth regulating genes, has been implanted.
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References
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