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The Origin of the Subepicardial Mesenchyme in the Avian Embryo: An Immunohistochemical and Quail–Chick Chimera Study

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It has been proposed that the subepicardial mesenchymal cells (SEMC) originate from the primitive epicardium and also from migration of extracardiac mesenchyme from the liver area. We have studied the possibility of an origin of SEMC through transformation of the proepicardial mesothelium, as well as the potential of the early proepicardium to generate epicardium and SEMC in quail–chick chimeras. The study was carried out in quail and chick embryos between HH16 and HH29 stages. Most proepicardial cells, mesothelial as well as mesenchymal, were cytokeratin and vimentin immunoreactive, suggesting a cytoskeletal shift from the epithelial to the mesenchymal type. Furthermore, we immunolocated, in the proepicardial mesothelium, three proteins specifically expressed during the endothelial–mesenchymal transition of the endocardial cushions, namely the JB3/fibrillin-associated antigen, the ES/130 protein and the smooth muscle cell α-actin. Grafts of proepicardial tissue from HH16–17 quail embryos into chick embryos of the same age originated large areas of donor-derived epicardium, including mesothelial, mesenchymal, and vascular cells. The donor-derived primitive epicardium showed segment-specific features, being squamous and adhered to the myocardium on the atrial wall and showing morphological signs of ingression in the atrioventricular groove and outflow tract. These morphological traits together with the distribution of vimentin, the ES/130 protein, and the JB3/fibrillin-associated antigen suggested a localized transformation of some epicardial mesothelial cells into mesenchyme. Most of the donor-derived cells, mesothelial and mesenchymal, showed the vascular marker QH1, which frequently colocalized with cytokeratin. Heterotopic grafts of quail splanchnopleura into the pericardial cavity of chick embryos originated a squamous, epicardial-like, cytokeratin-immunoreactive cell layer on the heart surface, as well as a few QH1$^+$ subepicardial and intramyocardial cells. The results suggest that a substantial part of the subepicardial mesenchyme, including the progenitors of the cardiac vessels, originates from the transformation of proepicardial and epicardial mesothelial cells into mesenchyme, and that the epicardial transition could be driven by a segment-specific myocardial signal. © 1998 Academic Press

Key Words: **epicardium; subepicardial mesenchyme; epithelial–mesenchymal transition; coronary vessels; quail–chick chimeras.**

INTRODUCTION

The subepicardial mesenchymal cells (SEMC) appear between the epicardium and the myocardium closely following the epicardial investment of the heart. This mesenchyme is probably involved in the differentiation of the coronary vessels (including endothelial and smooth muscle cells) and also contributes to the fibroblasts which synthesize the epicardial connective tissue (Tidball, 1992; Tomanek, 1996; Mikawa and Gourdie, 1996). Two different sources have been proposed as the origin of the SEMC. Some of them migrate into the heart throughout the proepicardium, i.e. the mesothelial outgrowths located on the ventral part of the hepatic-cardiac limit, which constitute the primordium of the epicardium (Hiruma and Hirakow, 1989; Männer, 1992; Viragh *et al.*, 1993; Poelmann *et al.*, 1993; Van den Eijnde *et al.*, 1995). Other SEMC seem to originate from an epithelial–mesenchymal transition in-

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volving the epicardium (Markwald *et al.*, 1996; Pérez-Pomares *et al.*, 1997). At present, the relative weight of these two sources of SEMC, the migratory and the epicardial-derived, and whether this dual origin is related with the vasculogenic potential of a subset of the SEMC are not known. Viragh *et al.* (1993) and Poelmann *et al.* (1993) proposed that the earliest endothelial cells of the cardiac vessels differentiate from migrating angioblasts which arrive to the heart from the liver region. According to these authors, the epicardial-derived mesenchyme is not involved in the development of the cardiac vessels. Another point which remains unclear is the origin of the mesenchymal cells contained into the proepicardium. It is possible that they enter the proepicardium from the splanchnopleural mesoderm, but it cannot be discarded that at least a part of them originate *in situ* from the transformation of mesothelial cells (Markwald *et al.*, 1996).

We have designed a study about the relative importance of the mesothelial-derived contribution to the cardiac mesenchyme. The first part of our work consisted of a morphological and immunohistochemical study of the proepicardium. We immunolocated a number of antigens involved in processes of epithelial–mesenchymal transition. We first investigated the distribution of the cytokeratin (CK) and vimentin (VIM) immunoreactivity in the proepicardial cells. CK and VIM are the usual intermediate filaments of the epithelial and mesenchymal cells, respectively. During the epithelial–mesenchymal transition, the transient persistence of CK in the mesenchymal cells (Fitchett and Hay, 1989; Hay, 1990) as well as the expression of VIM in premigratory epithelial cells has been reported (Franke *et al.*, 1982; Hay, 1990). Other antigens whose presence was tested in the proepicardium were the fibrillin-associated antigen JB3, the protein ES/130, and the smooth muscle cell-specific α-actin (SMC α-actin). As will be explained under Discussion, these molecules are expressed in the endocardium during its transformation into valvuloseptal mesenchyme (Markwald *et al.*, 1996) and they seem to play key roles in the process of epithelial–mesenchymal transition (Rezaee *et al.*, 1993; Wunsch *et al.*, 1994; Krug *et al.*, 1995; Nakajima *et al.*, 1997).

The main component of our study was experimental. We aimed to know if the early proepicardium of the avian embryo contains competent elements to constitute a fully developed epicardium including mesothelium, SEMC, and subepicardial vessels, or if a further contribution of cells external to the proepicardium is needed. We were also interested in the possible development of segment-specific features in the donor-derived epicardium as well as the possibility of cell migration between the host and donor epicardial tissues.

The experiments consisted of the grafting of quail proepicardia into the pericardial cavity of chick embryos. We followed the development of the graft-derived epicardium and SEMC with antibodies that recognize quail cells (QCPN, Selleck and Bronner-Fraser, 1995) and quail endothelial and hematopoietic cells (QH1, Pardanaud *et al.*, 1987). In order to test if other nonproepicardial splanchnopleural mesoderm is also competent to originate epicardium and SEMC, we also grafted, in the pericardial cavity of chick embryos, slices of quail posterior digestive tube including endoderm, mesenchyme, and mesothelium.

MATERIALS AND METHODS

The investigation conformed with the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institute of Health (NIH Publication No. 8523, revised 1985). Quail and chick eggs were kept in a rocking incubator at 38°C. The embryos were staged according to the Hamburger and Hamilton (1951) stages of chick development.

The histomorphology of the early proepicardium was studied in four HH17 quail embryos which were fixed by immersion for 2 h in 1% glutaraldehyde and 1% paraformaldehyde in phosphate-buffered saline (PBS). The embryos were then washed in PBS for 30 min and postfixed in 1% OsO_4 in PBS for 90 min. After being washed in distilled water (30 min) the embryos were dehydrated in an ethanolic series finishing in acetone and embedded in Araldite 502. Semithin sections were obtained in a Reichert UMO-2 ultramicrotome and stained with toluidine blue.

The immunohistochemical study was performed in 19 embryos of quail (*Coturnix coturnix japonica*), and 14 embryos of chick (*Gallus gallus*), ranging from HH16 to HH29. These embryos were processed as described below and used for the immunohistochemical characterization of the studied tissues and also as controls to compare the development of the normal and donor-derived tissues in the quail–chick chimeras.

A first set of quail–chick chimeras were obtained through grafting of HH16–17 quail proepicardial tissue in the pericardial cavity of chick embryos of the same age (Fig. 1). Quail embryos

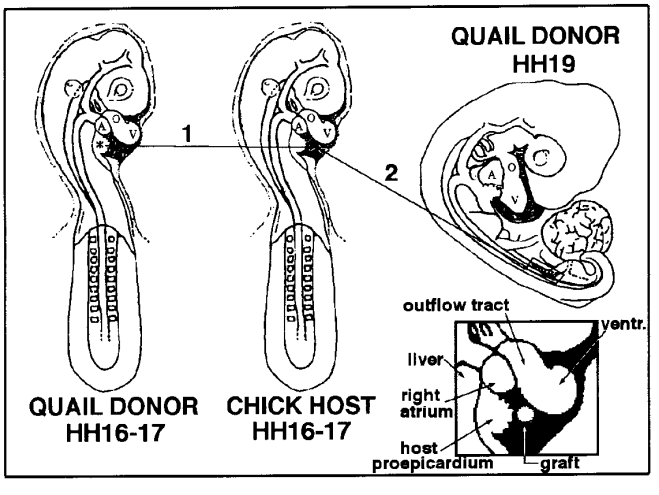

FIG. 1. Diagram of the graft experiences performed in the chick embryos. Proepicardia (star) or slices of posterior digestive tube from donor quail embryos were placed in the pericardial cavity of chick embryos (lines 1 and 2, respectively) as shown in the lower right corner. A, atrium; O, outflow tract; V, ventricle.

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TABLE 1
Summary of the Grafting Experiments of Quail Tissue into Chick Embryos

Number	Donor HH stage	Host HH stage	Fixation HH stage	Grafting	Labeling
1	17	16	19	PR	PDTV
2	16	17	20	PR	EP, SEMC
3	17	17	22	PR	EP, SEMC
4	17	17	22	PR	EP, SEMC, V
5	17	16	23	PR	EP, SEMC
6	17	16	25	PR	AH
7	16	17	25	PR	SEMC, V
8	17	17	25	PR	EP, SEMC, V
9	17	17	25	PR	EP, SEMC, V
10	17	16	25	PR	EP, SEMC, V
11	19	16	19	PDT	AH
12	19	16	19	PDT	AH
13	19	16	20	PDT	AH
14	19	16	26	PDT	SEMC, V
15	19	16	29	PDT	EP
16	19	16	29	PDT	EP, SEMC, V
17	19	17	29	PDT	SEMC, V
18	19	17	29	PDT	AH, EP

Note. The grafted tissue consisted of proepicardium (PR) or posterior digestive tube (PDT). Quail cells were found attached to the heart surface (AH), integrated into the epicardium (EP), forming part of the subepicardial mesenchyme (SEMC) or forming vascular structures (V). In one case, the graft attached to the posterior digestive tube where donor-derived vessels developed (PDTV).

were excised, placed in sterile Tyrode solution, and dissected with tungsten needles. The quail proepicardium was grafted between the host proepicardium and the heart. Twenty-six experiments of transplantation were made. A screening of the sections was performed with the QCPN and QH1 antibodies, and we obtained positive results (quail cells integrated in the chick tissues) in 10 embryos (Table 1).

A second set of experiments consisted in the heterotopic transplantation of the splanchnopleural tissue associated to the posterior digestive tube (Fig. 1). Slices of quail posterior intestine including the endodermal tissue were cut and grafted in the prospective pericardial cavity of chick embryos. We obtained 8 positive results from 11 experiments of transplantation (Table 1).

The operated eggs were sealed with adhesive tape and reincubated. At intervals, the embryos were excised and fixed in 40% methanol, 40% acetone, and 20% distilled water for 8–12 h. After fixation, the embryos were dehydrated in an ethanolic series finishing in butanol and paraffin-embedded. Five- and 10-μm serial sections were obtained with a Leitz microtome and collected on poly-*l*-lysine-coated slides.

The sections were dewaxed in xylene, hydrated in an ethanolic series, and washed in Tris–phosphate-buffered saline (TPBS, pH 7.8). For the immunoperoxidase technique, endogenous peroxidase activity was quenched by incubation for 30 min with 3% hydrogen peroxide in TPBS, and endogenous biotin was blocked with the avidin–biotin blocking kit (Vector, Burlingame, CA). After washing with TPBS, nonspecific binding sites were saturated for 30 min with 16% sheep serum, 1% bovine serum albumin, and 0.5% Triton X-100 in TPBS (SBT). The slides were then incubated overnight at 4°C in the primary antibody diluted in SBT. Control slides were incubated in SBT only.

For the immunoperoxidase technique, the slides were washed in TPBS, incubated for 1 h at room temperature in biotin-conjugated anti-mouse or anti-rabbit goat IgG (Sigma) diluted 1:100 in SBT, washed again and incubated for 1 h in avidin–peroxidase complex (Sigma) diluted 1:150 in TPBS. Peroxidase activity was developed with Sigma Fast 3,3′-diaminobenzidine (DAB) tablets according to the indications of the supplier. Some slides were counterstained with hematoxylin.

For immunofluorescent double labeling, the slides were incubated with a mix of monoclonal and polyclonal primary antibodies, washed, and incubated for 45 min in TRITC-conjugated goat anti-mouse IgG (Sigma) diluted 1:50. This solution had been preadsorbed for 1 h with 10% rabbit serum. After being washed, the slides were blocked again in SBT, incubated for 1 h in biotin-conjugated goat anti-rabbit IgG (Sigma) diluted 1:100 in SBT, washed, and incubated in extravidin–FITC conjugate (Sigma) diluted 1:100. Controls were incubated only with one primary antibody and then with both secondary antibodies, in order to detect any cross-reaction between the secondary and the primary antibodies. The sections were observed in a laser confocal microscope Leica TCS-NT, using filters specific for the FITC and TRITC fluorochromes. Selected images were captured and printed in a Sony UP-860CE video printer.

The QCPN and QH1 monoclonal antibodies, which stain quail cells and quail endothelial and hematopoietic cells, respectively, were supplied by the Developmental Studies Hybridoma Bank, University of Iowa. They were used at a 1:10 and 1:500 dilution of the supernatant, respectively. Polyclonal anti-bovine epidermal cytokeratin (Z622, Dakopatts, Denmark) is used for wide screening of keratins in several epithelial tissues. It has been used to describe the epicardial covering of the heart of quail embryos (Vrancken Peeters *et al.,* 1995). This antibody was diluted at 1:1000 for immunoperoxidase and 1:100 for immunofluorescence. The monoclonal anti-chick vimentin (clone AMF-17b) was also from the Hybridoma Bank, and it was used at a 1:200 and 1:100 dilution in immunoperoxidase and immunofluorescence techniques, respectively. The monoclonal anti-SMC α-actin (clone 1A4, Sigma) is specific for this actin isoform and shows a wide reactivity in vertebrates. It was used at a 1:2000 dilution of the supernatant. The polyclonal anti-ES/130 and the monoclonal JB3 were a generous gift from Dr. E. Krug (Medical University of South Carolina). The anti-ES/130 was obtained by immunizing a rabbit with a synthetic peptide of the chicken sequence DGPVKKKSASKKKAEPAPADS-DGP. This antiserum was diluted 1:500. The monoclonal anti-JB3 was obtained using membrane-enriched fractions of atrioventricular endocardial cushions as immunogen (Wunsch *et al.,* 1994). The supernatant was used pure in immunofluorescence and diluted 1:100 for immunoperoxidase.

RESULTS

Histomorphology and Immunohistochemistry of the Avian Proepicardium

The proepicardium of HH16–17 quail and chick embryos appeared as an outgrowth of the squamous mesothelium into the coelomic cavity (Figs. 2A–2F). It was placed on the ventral surface of the liver, close to the limit between this

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organ and the sinus venosus. The proepicardium enclosed a large amount of amorphous extracellular matrix and a number of mesenchymal cells. The mesothelial cells showed deep invaginations. Most mesothelial cells, in the surface and especially in the invaginations, were large and rounded, and many of them showed clear signs of migration into the proepicardial matrix, such as long basal cytoplasmic processes, cell overriding, and reduced adhesion to the neighbor cells (Fig. 2A).

In these embryonic stages, most proepicardial cells, either mesothelial or mesenchymal, were cytokeratin immunoreactive (Fig. 2B). Vimentin staining was also prominent in most mesenchymal proepicardial cells but also in many mesothelial cells, some of which were apparently migrating into the proepicardial matrix (Fig. 2C). The SMC α-actin antibody stained a number of mesothelial and mesenchymal proepicardial cells. The immunoreactivity was stronger in the mesothelial cells showing signs of migration toward the proepicardial matrix (Fig. 2D). The QH1 immunostaining was very weak and restricted to a few mesenchymal cells and, occasionally, to some mesothelial cells (Fig. 2E). QH1 immunoreactive vessels were not detected in the proepicardium of these embryos. Finally, ES/130 immunoreactivity was also conspicuous in some mesothelial and mesenchymal cells of the proepicardium (Fig. 2F).

By stage HH20 the proepicardium has moved to the inner curvature of the heart, being located between the sinus venosus and the AV groove (Figs. 2G–2L). As a consequence, there was a broad continuity between the proepicardial matrix and the subepicardial space of the AV groove. The amount of mesenchymal cells has increased. Most of the proepicardial and epicardial cells, mesenchymal as well as mesothelial, were CK^+ (Fig. 2G) and VIM^+. The QH1 antibody labeled a few mesothelial cells from the primitive epicardium and proepicardial villi (Fig. 2H). However, $QH1^+$ mesothelial cells were not observed in the primitive epicardium of embryos aged above HH21. The mesothelial cells of the proepicardium and epicardium were also stained with the ES/130 and JB3 antibodies (Fig. 2G). Although most of the JB3 immunoreactivity was extracellular, some mesothelial and SEMC showed an intracellular staining, as demonstrated by the successive confocal planes and also by the colocalization of JB3 with the CK immunoreactivity (Figs. 2I and 2J). CK and QH1 immunoreactivity colocalized in some mesenchymal cells from the proepicardium and subepicardium. These $CK^+/QH1^+$ cells were sometimes connected, forming short cords (Figs. 2K and 2L). The contact of these double-labeled cells with $CK^+/QH1^-$ SEMC was frequent (Fig. 2L).

FIG. 2. Histomorphological and immunohistochemical features of the proepicardium and subepicardium of the quail and chick embryos. (A) Semithin section of a proepicardium in a HH17 quail embryo. Sagital section. Many mesothelial cells (MT) invaginate within the proepicardial matrix (stars) and show extensive signs of delamination such as reduced intercellular adhesion (arrowheads), cell overriding (large arrow), or long basal cytoplasmic projections (small arrows). M, myocardium; PR, proepicardial matrix. Scale bar, 12 μm. (B–F) Proepicardium of HH16–17 quail embryos. Sagital sections. Immunostaining with antibodies against cytokeratin (CK, B), vimentin (VIM, C), smooth muscle cell α-actin (SMC α-actin, D), the vascular antigen QH1 (E), and the ES/130 protein (F). Most of the proepicardial cells, either mesothelial or mesenchymal, were CK and VIM immunoreactive. In C, the VIM immunoreactivity of a number of mesothelial cells (arrowheads) is evident, as well as the conspicuous labeling of the early mesenchymal cells in the developing endocardial cushions (EC). In D the marked SMC α-actin immunoreactivity of a number of proepicardial cells is shown. The labeling was more intense in the mesothelial cells bearing basal cytoplasmic projections (arrow). The myocardium (M) is also SMC α-actin immunoreactive in this developmental stage, although its immunoreactivity disappears later. The QH1 antibody (in E) faintly labeled a few mesenchymal (arrow) and mesothelial cells (arrowhead). The ES/130 protein (in F) was located in the endocardium (EN) and in some mesenchymal (arrow) and mesothelial proepicardial cells (arrowheads). M, myocardium; S, sinus venosus. Scale bars B, C, E, and F: 25 μm. Scale bar D: 12 μm. (G, H) Proepicardial villi (PR) and subepicardium (SE) of a HH21 quail embryo. Transverse section. Cytokeratin (G) and QH1 (H) immunoreactivity. Many mesenchymal cells of the proepicardial villi, subepicardium, and splanchnopleural mesoderm of the liver (LI) are CK immunoreactive. Note the presence of basal cytoplasmic processes in the mesothelial cells of the proepicardial villi (arrowhead in G). The QH1 antibody labeled some mesothelial cells of the proepicardial villi (arrowhead in H) as well as the endocardium (EN). M, myocardium. Scale bars, 12.5 and 13.5 μm, respectively. (I) Proepicardial villi (PR) and subepicardium (SE) of a HH23 chick embryo. Transverse section at the level of the atrioventricular junction with the JB3/fibrillin-associated antigen immunostaining. Laser confocal microscopy. Some mesothelial cells are labeled in the proepicardium (arrows) and the epicardium (arrowhead), apparently at the cytoplasmic level as suggested by the successive confocal planes. Note the basal cytoplasmic processes and the invagination of the labeled proepicardial cells. EP, epicardial mesothelium; M, ventricular myocardium. Scale bar, 9 μm. (J) Subepicardium of a HH24 quail embryo. Transverse section at the level of the atrioventricular junction. Double immunostaining with antibodies against cytokeratin (green) and the JB3 antigen (red). Laser confocal microscopy. The long basal processes of the epicardial mesothelial cells (arrowheads) are clearly visible, as well as the JB3 staining of the basal surface of the epicardial (EP) and endocardial (EN) cells. CK/JB3 colocalization was observed in some cells (arrow). M, myocardium. Scale bar, 8 μm. (K, L) Subepicardium of a HH20 quail embryo, at the level of the atrioventricular junction. Double CK (green) and QH1 (red) immunostaining. Laser confocal microscopy. K and L show two consecutive confocal planes. In K, two $QH1^+$ cells are connected forming a short cord. These cells show areas of CK immunoreactivity (arrows). Most cells of the epicardium (EP) and subepicardium (SE) are CK immunoreactive. The double-labeled cell of the right is shown in L at other confocal plane (white arrow). Note the connection of this cell with other CK-immunoreactive mesenchymal cell (arrowhead). Another QH1/CK colocalization can be seen in the upper right corner. The yellow arrow show a basal cytoplasmic projection of an epicardial cell. Scale bar, 8 μm.

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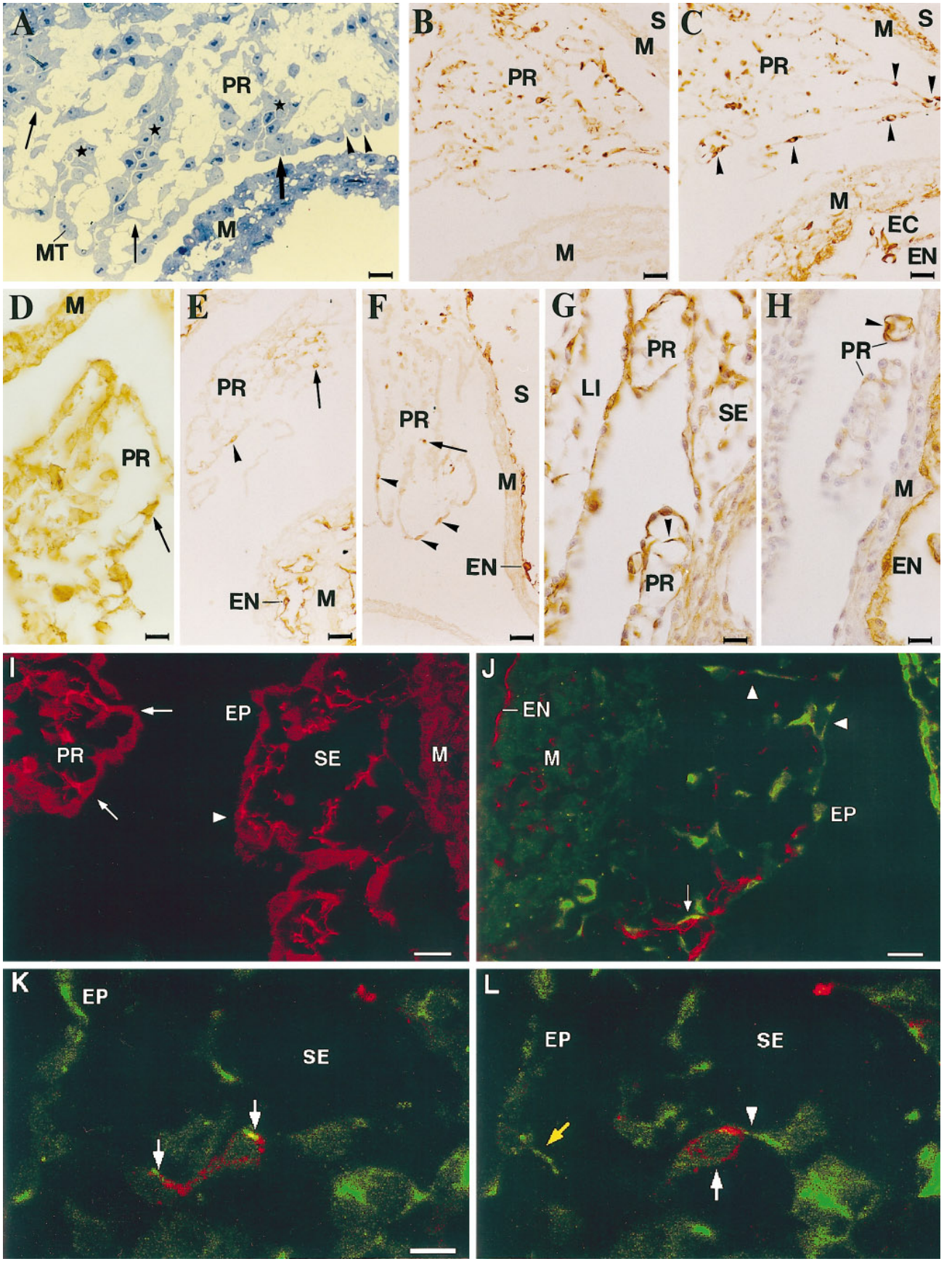
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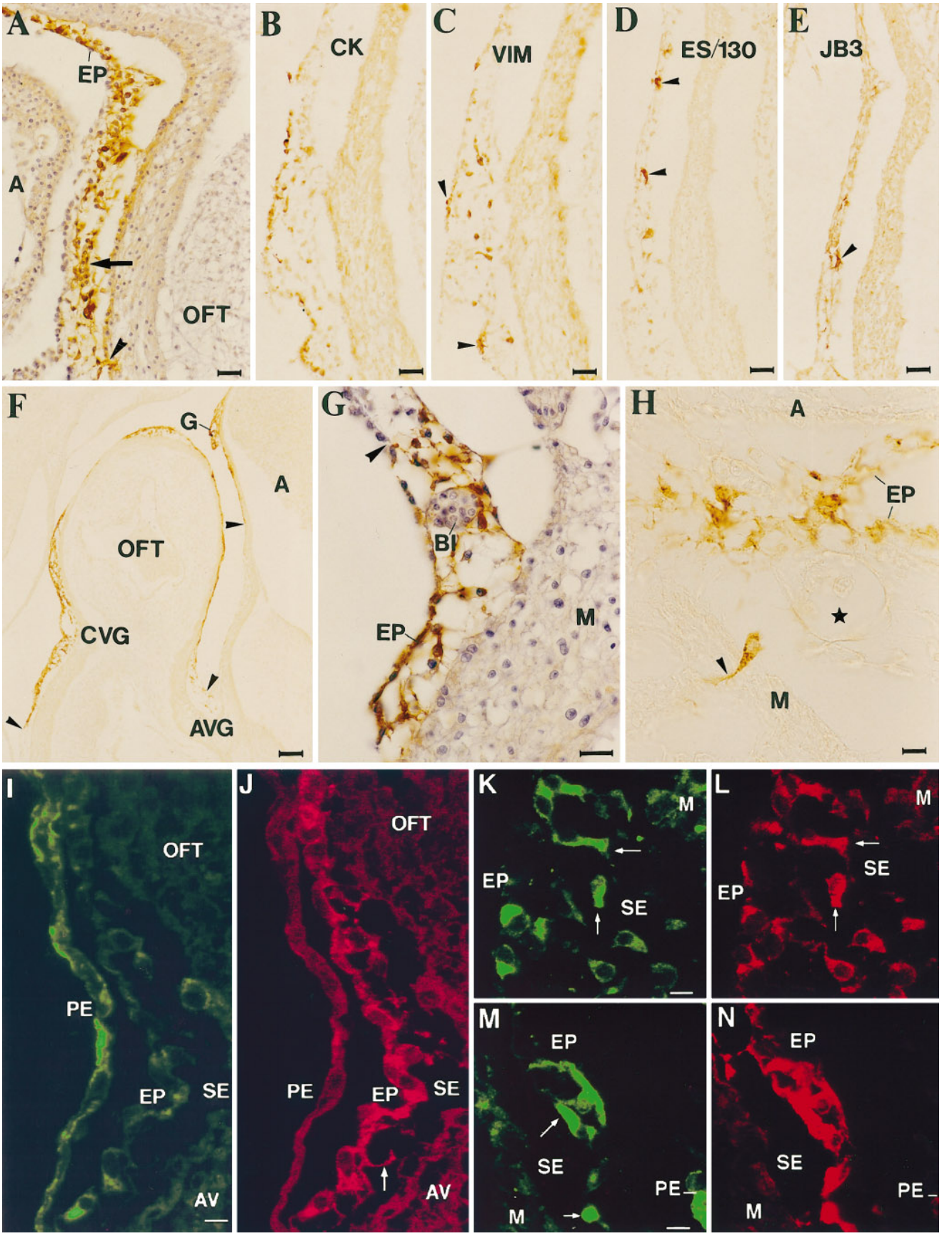
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Quail–Chick Chimera Study

Proepicardial grafts from HH16–17 quail embryos into chick embryos of the same age originated donor-derived patches of primitive epicardium and SEMC (Fig. 3 and Table 1). The donor-derived cells were detected through immunostaining with the QH1 and/or the QCPN antibodies (Figs. 3A, 3F, and 3G). This result was obtained even when the bulk of the grafted tissue did not attached to the heart. Quail cells were rare in other areas of the host embryos. In one case the grafted proepicardium adhered to the intestine and extensively contributed to its vascularization. In other cases a few isolated quail cells were detected into the parietal pericardium.

The donor-derived epicardium and SEMC revealed a close immunohistochemical similarity with the corresponding tissues of normal control embryos of the same age, although some differences can be remarked. Most donor-derived cells, either epithelial or mesenchymal, were CK^+ (Fig. 3B) although the CK immunoreactivity of the donor-derived epicardium showed, in some areas, a lesser intensity than the epicardium of the host or the control embryos (Fig. 3I). VIM immunoreactivity (and CK/VIM colocalization) was conspicuous in the donor-derived epicardium of the AV groove and OFT (Fig. 3C). The donor-derived epicardial cells and SEMC were more frequently and intensely labeled with the QH1 antibody than the corresponding cells of the same areas and developmental stages from the control quail embryos (Figs. 3A, 3G, 3J, 3L, and 3N). Consequently, colocalization of CK and QH1 was far more frequent and distinct in the donor-derived tissues than in the control quail embryos (Figs. 3I–3N). We could not find differences between donor-derived, host and control epicardial and subepicardial mesenchymal cells in the pattern of staining with the JB3 antibody, but the ES/130 immunoreactivity increased markedly in the donor-derived SEMC (Figs. 3D and 3E).

The histomorphological features of the donor-derived epicardium of the host embryos closely matched those of the normal epicardium of quail and chick embryos of the same age (Fig. 3F). This similarity included the specific characteristics that the epicardium shows in each cardiac segment. Thus, the donor-derived epicardium of the atria was always squamous, adhered to the myocardium, and without underlying SEMC, except for the areas close to the AV groove and for the base of the septum primum. However, when the donor-derived epicardium formed on the AV groove or OFT, it always covered a thick layer of subepicardium which enclosed abundant SEMC. In these cases, part of the epicardial cells were large and rounded and they showed signs of reduced cell adhesion as well as basal cytoplasmic projections (Fig. 3J).

FIG. 3. Immunohistochemical features of the epicardium and subepicardium derived from the grafting of proepicardial tissue from HH16–17 quail embryos in chick embryos of the same age. (A–E) Donor-derived epicardium and subepicardium on the outflow tract (OFT) of a HH25 chick embryo. The quail origin of the epicardium of this area was confirmed by QCPN and QH1 immunolabeling. QH1 (A), cytokeratin (CK, B), vimentin (VIM, C), ES/130 (D), and JB3 (E) immunostaining. Sagital consecutive sections. Most of the donor-derived cells, in the primitive epicardium (EP) as well as in the subepicardium (SE) are QH1 immunoreactive, as shown in A. Note the apparent penetration of $QH1^+$ cells into the myocardium (arrowhead) and the blood island-like structure (arrow) A, atrium. The CK and VIM staining shown in B and C is apparent in mesothelial and mesenchymal cells, and similar to the staining observed in the subepicardium of normal control embryos. However, the ES/130 immunoreactivity shown in D was stronger in the donor-derived mesenchymal cells (arrowheads) than in the controls. The fibrillin-associated JB3 antigen was immunolocated in a similar fashion in the donor-derived and normal subepicardium, forming a network of thin filaments as shown in E. Note the strong JB3 immunoreactivity of some mesenchymal cells (arrowhead). Scale bars, 25 μm. (F) Segment-specific features of the donor-derived epicardium and subepicardium shown by QH1 staining. HH25 chick embryo. Sagital section. Donor-derived mesenchymal cells can be observed in the outflow tract (OFT), atrioventricular groove (AVG), and conoventricular groove (CVG), where the primitive epicardium show signs of ingression. However, the donor-derived epicardium was squamous on most of the atrium (A) and remained adhered to the atrial myocardium, except for the base of the septum primum. These segment-specific features were not related with the place of attachment of the graft (G), which can be seen in this photograph adhered to the atrium. Note the neat limits (shown by the arrowheads) between the donor-derived and the host primitive epicardia. Scale bar, 66 μm. (G) Donor-derived tissue on the conoventricular groove of a HH25 chick embryo. QH1 staining. The donor-derived and host primitive epicardia are continuous, and this limit (shown by an arrowhead) seems to be also a boundary for the dispersal of the donor-derived subepicardial cells. A blood island-like structure (BI) is lined by $QH1^+$ cells. Note the conspicuous QH1 staining of the epicardial cells (EP). M, myocardium. Scale bar, 20 μm. (H) A donor-derived, $QH1^+$ isolated cell is migrating within the ventricular myocardium (M) of a HH25 chick embryo. Note the long filopodium (arrowhead). A, atrium; EP, primitive epicardium of the atrioventricular groove. Star, host blood vessel. Scale bar, 9 μm. (I, J) Donor-derived epicardium and subepicardium in a HH25 chick embryo at the level of the conoventricular groove. This section is consecutive to that shown in F. Double CK (I) and QH1 (J) immunostaining. Laser confocal microscopy. The CK immunoreactivity seems to be reduced in this area where the signs of epithelial–mesenchymal transition (loss of the squamous phenotype and the intercellular adherence, basal cytoplasmic processes as shown by the arrow) are evident. AV, atrioventricular junction; EP, donor-derived epicardium; OFT, outflow tract; PE, host pericardium; SE, subepicardium. Scale bar, 6.5 μm. (K–N) HH25 chick embryo. Laser confocal microscopy. Cytokeratin (immunostained in green) and QH1 (immunostained in red) colocalization was evident and frequent in the donor-derived cells of the epicardium (EP) and subepicardium (SE). Some cases are shown by the arrows. Myocardium (M) and host pericardium (PE) serve as controls. Scale bars, 7 μm.

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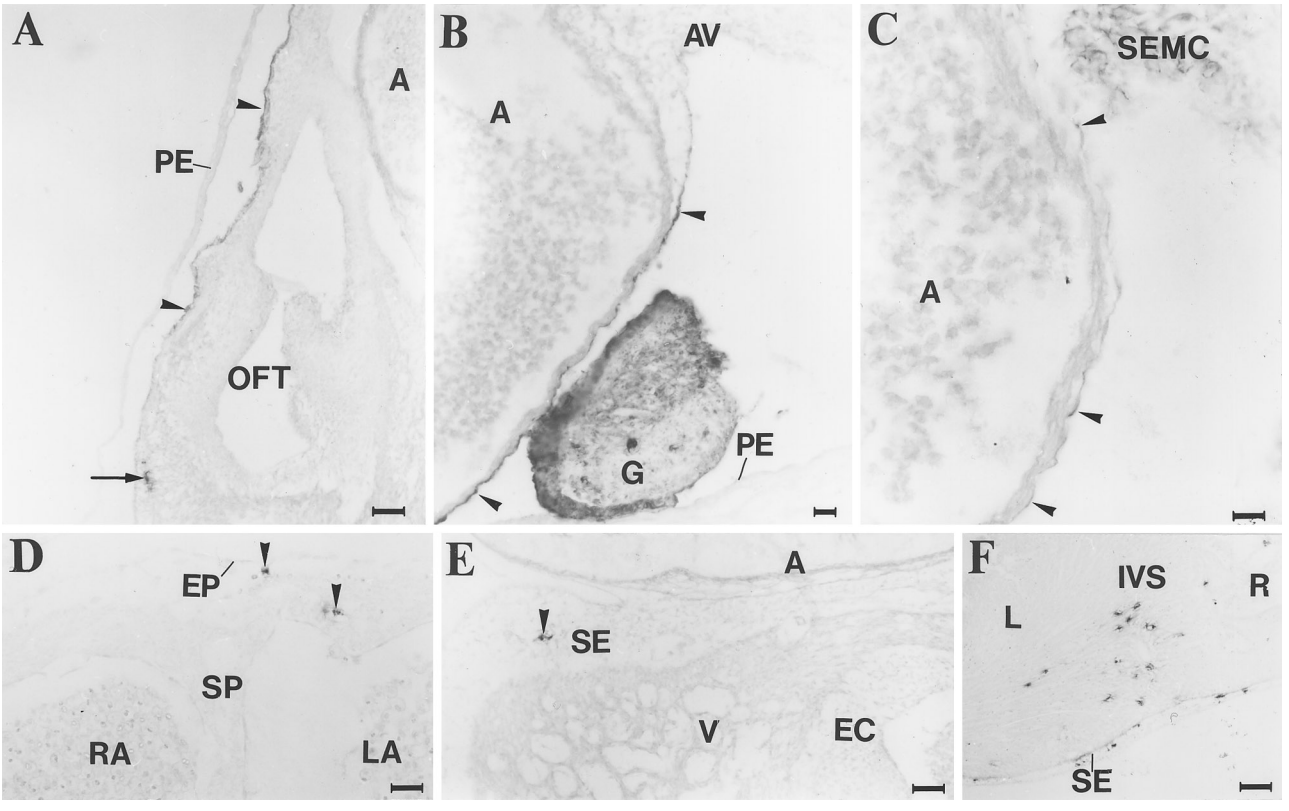

FIG. 4. Immunohistochemical features of the cells derived from the heterotopic grafting of slices of posterior digestive tube from HH19 quail embryos in the pericardial cavity of HH16–17 chick embryos. (A–C) An epicardial-like tissue (arrowheads) formed on the outflow tract (OFT) and atrium (A) of two different HH29 chick embryos after transplantation of a gut slice. QH1 (A, B) and cytokeratin (C) immunostainings. The graft (G) did not attach to the heart, as shown in B. The donor-derived tissue remained squamous, and subepicardial donor-derived cells (arrow in A) were scarce. Donor-derived cells were very rare in other areas of the embryo, even in the proximity of the grafts. Note the unlabeled pericardium (PE) in B. In C it is shown a section consecutive to that of B, stained with the anti-cytokeratin antibody. The epicardial-like squamous layer (arrowheads) is CK immunoreactive, likewise the normal primitive epicardium. AV, atrioventricular groove; SEMC, CK immunoreactive subepicardial mesenchymal cells. Scale bars, 50, 16, and 12.5 μm, respectively. (D–F) Subepicardial and intramyocardial $QH1^+$ cells obtained after heterotopic graft of posterior digestive tube slices. HH29 chick embryos. In D, labeled cells (arrowheads) were located in the roof of the atrium at the level of the septum primum (SP) which separates the left atrium (LA) and the right atrium (RA). In E, the donor cells (arrowheads) were located in the atrioventricular groove subepicardium (SE). The chick embryo shown in F was the only instance in which a number of intramyocardial $QH1^+$ cells, integrated within the host capillaries, were observed. These cells were at the level of the interventricular septum (IVS). Other abbreviations: A, atrium; EC, endocardial cushion; EP, epicardium; L, left ventricle; R, right ventricle; V, ventricle. Scale bars, 25, 50, and 110 μm, respectively.

The donor-derived and the host primitive epicardia were neatly connected, and this limit appeared also as a boundary for the dispersal of the donor-derived SEMC (Fig. 3G). This observation suggests the existence of constraints for the horizontal movement of the donor-derived SEMC. However, isolated $QH1^+$ donor-derived cells were observed migrating apparently into the host myocardium (Fig. 3H).

The last set of experiments consisted of heterotopic grafting, in the pericardial cavity of chick embryos, of quail slices of posterior digestive tube containing splanchnopleural mesothelium, mesenchyme, and endoderm. These experiences resulted in clusters of quail cells attached to the heart surface (in short-term reincubations, Table 1) or in the formation of a $CK^+/QH1^+$ epicardial-like layer in the areas of the heart closest to the graft (Fig. 4). The donor-derived epicardial-like layer was usually squamous irrespective of the cardiac segment in which it was located (Figs. 4A–4C). In two cases, a few $QH1^+$ cells were detected at the subepicardium and inside the myocardium, either isolated or integrated in the host capillaries, even in areas situated far away from the graft (Figs. 4D and 4E). In one case of long-term reincubation of a chick embryo bearing a graft of posterior digestive tube, a number of discontinuous stretches of cardiac vessels were labeled with the QH1 antibody (Fig. 4F). Most of these donor-derived vessels were

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located intramyocardially at the ventricle. This embryo apparently lacked of donor-derived epicardium.

DISCUSSION

Histomorphology and Immunohistochemistry of the Avian Proepicardium

Despite the importance of the proepicardial tissue, this structure has received little attention from the developmental biologists, probably because of the long-lasting belief about the differentiation of the epicardium *in situ* from a so-called ''myoepicardial mantle'' (Mollier, 1906). This tenet has been finally rejected, and it is now clear that the mesothelial protrusions of the liver–cardiac limit play a key role in the constitution of the epicardium in all the vertebrates. We think that our study can provide new insights about the features of the proepicardial tissue and the way in which the proepicardium contributes to the differentiation of the epicardium and the cardiac mesenchyme.

Our results suggest that at least a considerable part of the mesenchyme contained in the avian proepicardium originates from the delamination of the proepicardial mesothelium. Although this had been suggested by some authors (Markwald *et al.*, 1996), there was no indication of this process. The evidence provided by our study was morphological and immunohistochemical. CK and VIM immunoreactivity was detected in most proepicardial cells, either mesothelial or mesenchymal. It was known that the splanchnopleural mesothelium is characterized by a strong CK immunoreactivity (Vrancken Peeters *et al.*, 1995). We have also shown elsewhere that SEMC originate from the epicardial mesothelium in chick and hamster embryos, and that these mesothelial-derived mesenchymal cells are characterized by a transient CK/VIM immunoreactivity (Pérez-Pomares *et al.*, 1997). The persistence of the original epithelial-type intermediate filaments after the transdifferentiation of an epithelium has been reported both *in vivo* and *in vitro* systems (Fitchett and Hay, 1989; Hay, 1990). On the other hand, the expression of VIM prior to the transformation of an epithelial cell is probably involved in premigratory shape changes (Hay, 1990) and it has been demonstrated in the primitive streak and neural tube (Franke *et al.*, 1982). For these reasons, we suggest that the extensive colocalization of CK and VIM in the mesothelial proepicardium and in the underlying mesenchymal cells can be a first indication of a mesothelial origin of the proepicardial mesenchyme.

A second support of our hypothesis is the remarkable parallelism between the proepicardium and the endocardial cushions in the expression of three molecules, the JB3 fibrillin-associated antigen, the ES/130 protein, and the SMC α-actin. These proteins are specifically expressed by the transforming endocardium and the endocardial-derived mesenchymal cells. Only the subset of endocardial cells expressing the JB3 antigen is susceptible to be transformed in cushion mesenchyme cells (Wunsch *et al.*, 1994; Barton *et al.*, 1995; Markwald, 1995; Bouchey *et al.*, 1996). The ES/130 protein, secreted by the myocardial and endocardial cells, seems to be involved at a critical step in the initiation of the mesenchymal transformation of the cardiac endothelium (Rezaee *et al.*, 1993; Markwald, 1995; Markwald *et al.*, 1996) and it is located in several embryonic sites where inductive interactions occur (Krug *et al.*, 1995). Both antigens, JB3 and ES/130, seem to be also involved in the origin of SEMC from the primitive epicardium (Pérez-Pomares *et al.*, 1997), but their presence in the proepicardium had not been hitherto reported. Finally, the SMC α-actin is expressed by the premigratory transforming endocardial cells, probably as a consequence of the cytoskeletal reorganization required for the acquisition of a migratory phenotype (Nakajima *et al.*, 1997). A fourth molecule which is expressed in a similar fashion in the proepicardium and endocardium is the transcription factor ets-1 (Macías *et al.*, accepted for publication in *Anat. Embryol.*), which seems to play a main role in the epithelial–mesenchymal transition by transactivation of several proteinase genes (Kola *et al.*, 1993).

In summary, the current knowledge about the antigens included in this study allows one to presume that epithelial cells, such as those from the avian proepicardial mesothelium, which express vimentin, JB3 antigen, ES/130, and SMC α-actin, are undergoing a transition to a mesenchymal phenotype.

Quail–Chick Chimera Study

The grafts of HH16–17 quail proepicardia into the pericardial cavity of chick embryos resulted in large patches of donor-derived epicardium which was always continuous with the host epicardium. In fact, the limits between the donor and host tissues could only be determined by immunolabeling with specific quail markers, since there was no significant morphological differences. It is important to remark that the donor-derived epicardium adopted the morphological features corresponding to the cardiac segment in which it was located. Thus, the donor-derived epicardium formed on the atrium was squamous, adhered to the myocardium, and lacking of a subepicardium populated by mesenchymal cells (except at the level of the septum primum). In contrast, the donor-derived epicardium formed on the AV groove and OFT covered a wide subepicardium and it was composed of rounded cells showing traits of delamination such as reduced adhesion and basal cytoplasmic processes. The markers of a shift from epithelial to mesenchymal phenotypes (CK/VIM colocalization, JB3 and ES/130 immunoreactivity) were conspicuous in this donor-derived epicardium lining the AV groove and OFT. The distribution of these markers closely corresponded with that of the control embryos, although the expression of the ES/130 protein seemed to be increased in the donor-derived SEMC. During the epithelial–mesenchymal transi-

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tion which originates the endocardial cushion mesenchyme, this protein is secreted by the myocardial signaling cells and, upon induction, by the responsive $JB3^+$ endothelial cells and the derived cushion mesenchyme (Markwald, 1995; Krug *et al.*, 1995). Thus, it is tempting to interpret the apparent up-regulation of the ES/130 production in the donor-derived cells as a consequence of an inductive signal acting on responsive cells.

Our results suggest that the differential characteristics of the epicardial mesothelium derived from the proepicardial graft (squamous and quiescent or transforming into mesenchyme) are driven by a segment-specific signal arising from the underlying myocardium. This conception is very similar to the currently accepted model which accounts for the localized transformation of the AV and OFT endocardial endothelium into valvuloseptal mesenchyme (Markwald *et al.*, 1996), and it is conceivable that common molecular mechanisms can be regulating both processes.

The most striking difference observed in the donor-derived tissues with respect to the controls is the increased labeling of the epicardial mesothelium obtained with the monoclonal antibody QH1. Although we have found some $QH1^+$ mesothelial cells in the proepicardium and epicardium of normal quail embryos by HH17–20, their abundance was much higher in the donor-derived primitive epicardium. Consistently with this observation, the frequency of colocalization of CK and QH1 immunoreactivity was higher in the donor-derived epicardium and SEMC than in the normal quail embryos. According to the acknowledged value of the QH1 antigen as a hemangioblastic marker (Pardanaud *et al.*, 1987) and our proposal that CK immunoreactivity transiently remains in mesothelial-derived cells (Pérez-Pomares *et al.*, 1997), our observation suggests that cells derived from the proepicardial and epicardial mesothelium could have a vasculogenic potential. In fact, the proepicardial graft-derived cells frequently formed vessels and integrated within the host vessels in the hearts of the chick embryos. It is important to remark that $QH1^+$ cells were very scarce in the proepicardium at the stage in which transplantations were made (Fig. 3E).

As we have stated above, QH1 immunoreactivity was transiently detected in some early mesothelial cells of the control embryos. It is possible that these QH1-immunoreactive cells belong to a subpopulation of splanchnopleural cells, integrated within the mesothelial lining, which originates mesenchymal cells with vasculogenic potential. For some reason, perhaps related with the early stage in which the transplantations were made, the donor-derived primitive epicardium of our experiments seemed to be enriched in this $QH1^+$ subpopulation.

A number of donor $QH1^+$ cells and vessels were found inside the ventricular myocardium. Furthermore, the observation of $QH1^+$ isolated cells which seemed to be penetrating inside the myocardium from its outer surface was relatively frequent (Fig. 3H). It is tempting to relate this observation with the formation of the intramyocardial network of vessels. Thus, although it is generally considered that the subepicardial capillary network gives rise to the intramyocardial vessels through angiogenesis (i.e., sprouting from the already established vessels), the occurrence of intramyocardial vasculogenesis, i.e., *in situ* assembly of angioblasts, cannot be discarded. It would also be supported by our observation of donor-derived discontinuous segments of intramyocardial capillaries after the grafting of splanchnopleural mesoderm.

There are some differences between our findings and those reported by Poelmann *et al.* (1993), who also grafted quail proepicardial tissue in chick embryos and obtained chimeric cardiac vessels, but only when the grafted tissue contained fragments of liver tissue. Part of the disagreement could be accounted by the different experimental design. We have used earlier donor embryos than the quoted authors (HH16–17, while most donor embryos of the quoted authors were at the stages HH19–HH24) and we have followed the changes of the grafted tissue after shorter reincubation periods (HH19–25 versus HH30-hatching). On the other hand, Poelmann *et al.* (1993) only considered the $QH1/MB1^+$ donor-derived cells, which were integrated in the host vessels, but they did not check the presence of $QH1/MB1^-$ quail cells (e.g., in the epicardium, where the QH1 labeling is probably transient, as stated above). We think that longer reincubation periods of our embryos would have resulted in results more similar to those obtained by Poelmann *et al.* (1993).

When we grafted posterior splanchnopleural mesoderm in the pericardial cavity, we obtained a cytokeratin-immunoreactive epicardial-like layer covering some areas of the heart and continuous with the host epicardium. Several differences were found between this epicardial-like tissue and that formed after proepicardial grafting. In most cases, the epicardial-like tissue remained squamous and adhered to the myocardium. Donor-derived subepicardial and intramyocardial cells were scarce (except for one case). A possible interpretation of these results is that the myocardium is able to induce the proliferation of the splanchnopleural mesothelial cells and their adhesion to the myocardial wall. In the normal embryos, this induction would be effective only on the splanchnopleural mesothelium closest to the heart, i.e., that of the liver–cardiac limit which is virtually in contact with the heart, but it would become ineffective at larger distances. When we place pieces of tissue containing splanchnopleural mesothelium close to the heart, it responds to the myocardial induction and releases cells or cell clusters which attach to the heart surface and form the mesothelial patches observed. However, this epicardial-like tissue seems to be less responsive to the hypothetical induction which later causes the differentiation of the SEMC, and it remains largely squamous and quiescent.

Figure 5 shows a model about the hypothetical contribution of the proepicardium and the primitive epicardium to the SEMC. This model is based on the results herein presented as well as on findings published elsewhere (Bolender *et al.*, 1990; Pérez-Pomares *et al.*, 1997). In our

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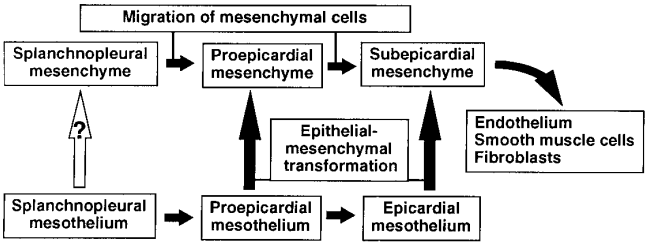

FIG. 5. Hypothetical model about the origin of the embryonic subepicardial mesenchyme. This model is based in the observations shown in this paper and in Bolender *et al.* (1990), Pérez-Pomares *et al.* (1997), and others. The mesenchymal compartment of proepicardium and subepicardium seems to receive a substantial contribution from the transformation of mesothelial cells. This process could be also operating in other areas of the embryo (arrow with question mark), as suggested by the distinct and transient cytokeratin/vimentin colocalization reported in the splanchnopleural mesothelium and its underlying mesenchyme (Viebahn *et al.*, 1988, and our own observations). Our finding of extensive QH1/CK colocalization in the mesothelium and subepicardial cells of the donor-derived epicardium suggests that the progenitor cells of the cardiac vessels might originate, at least in part, from the splanchnopleural mesothelium.

quail–chick chimera study, most SEMC of the areas covered by the donor-derived epicardium seemed to originate through the transformation of the surface mesothelium in precise areas of the heart. Donor mesenchymal cells were certainly carried within the proepicardial grafts, but we have obtained morphological and immunohistochemical evidence of a mesothelial origin of a major part of these cells. On the other hand, the proepicardial graft was sufficient to generate a normally constituted epicardium in which migration of host SEMC seemed to be not significant. Thus, although we cannot discard that some SEMC have a non-mesothelial origin, we conclude that a substantial part of the SEMC, in the chick embryo, probably arises from the transformation of the mesothelium, either proepicardial or epicardial.

Due to the existence of two potential sources of SEMC, from migration or from mesothelial transformation, there would be two possible origins for the progenitors of the cardiac vessels, i.e., migrating extracardiac angioblasts or mesothelial-derived cells. However, we think that our observation of $QH1^+/CK^+$ cells in the normal quail embryos and, particularly, in the proepicardial-derived tissue of the quail–chick chimeras, strongly supports the second alternative.

Our model and our suggestion of a mesothelial source of vascular progenitors is consistent with some descriptive and experimental studies. Bolender *et al.* (1990), working with explanted quail embryo hearts onto collagen, showed that during the first day in culture an outgrowth of epithelial cells extends from the cut ends of the explants onto the surface of the collagen lattice. These epithelial cells (originated from the primitive epicardium according to Markwald *et al.*, 1996) generate a population of mesenchymal cells that migrate into the collagen lattice and interconnect into primitive vascular networks which react positively with QH-1. Significantly, only limited mesenchyme formation was observed when hearts were explanted prior to the epicardial covering. On the other hand, Mikawa and Fischman (1992) and Mikawa and Gourdie (1996) demonstrated, through retroviral cell labeling, that the precursors of the chick coronary arteries are contained into the proepicardium and enter the heart during the epicardial morphogenesis. Finally, we have shown, in a fish model, that virtually all the SEMC develop through localized transformation of the primitive epicardium since there is no physical passage for the migration of cells from extracardiac areas until late embryonic stages. The SEMC coalesce in vascular structures before a connection between the subepicardium and the splanchnopleural mesoderm is established. (Muñoz-Chápuli *et al.*, 1996).

If the vasculogenic potential of proepicardial and epicardial-derived cells is confirmed by further studies, it might throw some light about a key question of the developmental biology of the circulatory system: When and how the angioblasts segregate from the mesoderm? (Dieterlen-Lievre and Pardanaud, 1992). Is this vasculogenic potential restricted to the proepicardial and epicardial mesothelium or is it a property of all the splanchnopleural mesothelium? Maybe an epithelial–mesenchymal transition, involving this mesothelium, a source of angioblasts for the developing vertebrate embryo?

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