Int. J. Dev. Biol. 46: 1005-1013 (2002)

Original Article

Origin of coronary endothelial cells from epicardial mesothelium in avian embryos

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ABSTRACT It has been established that coronary vessels develop through self-assembly of mesenchymal vascular progenitors in the subepicardium. Mesenchymal precursors of vascular smooth muscle cells and fibroblasts are known to originate from an epithelial-to-mesenchymal transformation of the epicardial mesothelium, but the origin of the coronary endothelium is still obscure. We herein report that at least part of the population of the precursors of the coronary endothelium are epicardially-derived cells (EPDCs). We have performed an EPDC lineage study through retroviral and fluorescent labelling of the proepicardial and epicardial mesothelium of avian embryos. In all the experiments only the surface mesothelium was labelled after 3 h of reincubation. However, endothelial cells from subepicardial vessels were labelled after 24-48 h and endothelial cells of intramyocardial vessels were also labelled after 48-96 h of reincubation. In addition, the development of the coronary vessels was studied in quail-chick chimeras, obtaining results which also support a mesothelial origin for endothelial and smooth muscle cells. Finally, quail proepicardial explants cultured on Matrigel showed colocalization of cytokeratin and QH1 (mesothelial and endothelial markers, respectively) after 24 h. These results, taken together, suggest that EPDC show similar competence to that displayed by bipotential vascular progenitor cells [Yamashita *et al.*, Nature 408: 92-96 (2000)] which are able to differentiate into endothelium or smooth muscle depending on their exposure to VEGF or PDGF-BB. It is conceivable that the earliest EPDC differentiate into endothelial cells in response to myocardially-secreted VEGF, while further EPDC would be recruited by the nascent capillaries via PDGFR-β signalling, giving rise to mural cells.

KEY WORDS: *epicardium, endothelium, vasculogenesis, heart development, avian embryo*

Introduction

The embryonic epicardial tissue, also referred to as "epicardial mesothelium" or "primitive epicardium", is constituted of a monolayered epithelium that originates from the proepicardium, a cluster of splanchnic mesothelial cells located in the caudal regions of the developing vertebrate heart. This epicardial mesothelium covers an epicardial-associated subepicardial mesenchyme that fills the so-called subepicardial space, i.e. the extracellular-matrix located between the epicardial mesothelium and the myocardium (reviewed in Männer *et al.,* 2001).

Most of the subepicardial mesenchymal cells derive from an epithelial-to-mesenchymal transformation of the epicardial mesothelium (Pérez-Pomares *et al.,* 1997, 1998a; Dettman *et al.,* 1998) and have therefore been named Epicardially-Derived Cells (EPDCs, Gittenberger-de Groot *et al.,* 1998). EPDCs remain in the subepicardium but also infiltrate the myocardium, eventually reaching the atrioventricular cushions (Gittenberger-de Groot *et al.,* 1998; Männer, 1999). The developmental fate of these EPDCs has hitherto been related with the subepicardial and myocardial fibroblasts as well as with the developing subepicardial coronary vessels (Dettman *et al.,* 1998; Gittenberger-de Groot *et al.,* 1998; Pérez-Pomares *et al.,* 1998a,b; Landerholm *et al.,* 1999; Reese *et al.,* 1999; Vrancken-Peeters *et al.,* 1999; Lu *et al.*, 2001; Wada *et al.,* 2001).

Abbreviations used in this paper: CCFSE, 5,6 carboxy 2',7' dichlorofluorescein diacetate succynimidil ester; CK, cytokeratin; EPDCs, epicardially-derived cells; FGF 1, 2, 7, fibroblast growth factor types 1, 2 & 7; H/H, Hamburger and Hamilton stages of chick development; PDGF-BB, platelet derived growth factor BB; PDGFR-β, platelet derived growth factor receptor β; VEGF, vascular endothelial growth factor; VEGFR-2/Flk-1, vascular endothelial growth factor receptor type 2/fetal liver kinase type 1.

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0214-6282/2002/$25.00

Printed in Spain
www.ijdb.ehu.es

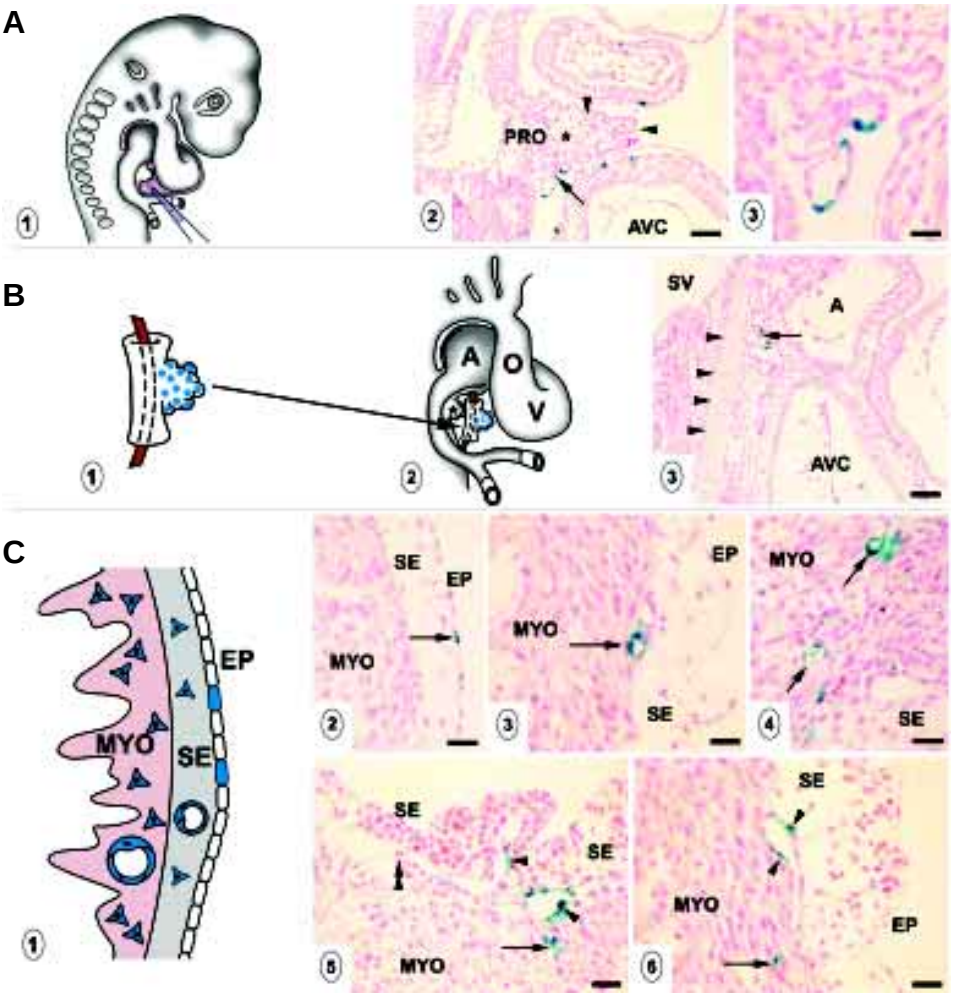

Fig. 1. Retroviral labelling and transplantation of the proepicardial mesothelium. *Cartoons in panel sections (A), (B) & (C) depict the basic steps of the chick (retrovirally labelled)-to-chick proepicardial transplantation as well as the results obtained.* **(A)** *Result of a retroviral injection into the pericardial cavity of H/H16-17 chick embryos* **(1)**. *The infection affects only the layer of cells on the surface of the tissues exposed to the virus as shown in* **(2)** *and* **(3)** *(H/H18, neutral fast red counterstain). Some mesothelial cells (blue cells, arrow) are β-Gal-positive while most of the mesothelium (arrowheads) and all the mesenchyme of the proepicardial matrix (asterisk) is uninfected. A detail of the infected mesothelium in the proepicardial villi is shown in (3). Scale bars: (2), 50 μm, (3), 33 μm.* **(B)** *The technique of proepicardial transplantation is shown. After a short reincubation period, the tagged proepicardium (shown as a blue dotted structure in* **(1)** *is transplanted as indicated in* **(2)**. *The result of the transplantation is shown in* **(3)** *(neutral fast red counterstained section from a stage H/H19 host embryo). Labelled mesothelial cells (blue cells, arrow) face the myocardium of the atrioventricular region and are hold by the eggshell membrane (arrowheads). Scale bar, 50 μm.* **(C)** *Results of the proepicardial labelling and transplantation. This section is illustrated with photographs of X-Gal stained embryos counterstained with fast red. β–Gal-positive cells (in blue) are found in all the heart layers* **(1)**. *In* **(2)** *an isolated epicardial epithelial cell is labelled in a H/H31 embryo (arrow).* **(3)** *and* **(4)** *show capillary-like structures formed over and inside the myocardial layers of a stage H/H35 embryo (arrows). In* **(5)** *and* **(6)** *the formation of vessels is shown. Arrowheads indicate the location of endothelial cells, the arrows the presence of isolated β-Gal-positive cells in the myocardium and the double arrowhead the presence of isolated labeled subepicardial cells. Scale bars, 50 μm. Abbreviations: A, atrium; AVC, atrioventricular cushions; EP, epicardium; MYO, myocardium; O, outflow tract; PRO, proepicardium; SE, subepicardium; SV, sinus venosus.*

Experimental evidence has strongly supported the epicardial-mesothelial origin of coronary smooth muscle cells (Mikawa and Gourdie, 1996; Vrancken-Peeters *et al.,* 1999) and transdifferentiation of epicardial cells into smooth muscle cells has been achieved *in vitro* (Dettman *et al.,* 1998; Landerholm *et al.,* 1999; Lu *et al*., 2001) but the origin of the endothelial cells of the coronary vessels is still under discussion. Some authors have proposed that the primitive subepicardial vascular plexus differentiates from assembly of angioblasts from the liver and the septum transversum area (Poelmann *et al.,* 1993). These angioblasts would invade the heart migrating throughout the proepicardium and the subepicardial space. Although this possibility cannot be ruled out, relevant descriptive and experimental studies from our group indicate that EPDCs are an alternative source for the precursors of the coronary endothelium (Pérez-Pomares *et al.,* 1998a,b).

The evidence supporting our proposal is diverse and it can be divided into two different kinds. The first one includes data about the vasculogenic formation of subepicardial vessels at developmental stages in which the subepicardial mesenchyme is isolated from non-cardiac sources of angioblasts. We have shown that in a primitive vertebrate, the dogfish (*Scyliorhinus canicula*), all the early subepicardial mesenchyme is composed exclusively of EPDCs, i.e. of cells derived from the epicardial mesothelium (Muñoz-Chápuli *et al.,* 1996, Macías *et al.,*1999). In this unique animal model subepicardial vessels appear much before a way for migration of extracardiac angioblasts is established. Thus, in the dogfish, EPDCs are the only possible source for the assembly of the subepicardial vascular plexus. These conclusions basically coincided with those obtained in an independent study where mouse avascular hearts were grafted *in oculo* into adult rats. In this experience it was shown that the coronaries of the transplanted hearts developed without any vascular contribution from the host (Rongish *et al.,* 1994). Both studies are in agreement with the idea that splanchnic angioblast differentiation occurs *in situ* without significant migration of vascular cell precursors (Drake *et al.,* 1997).

The second kind of evidence includes the colocalization of mesothelial and angioblastic markers. In the developing subepicardium cytokeratin (CK, a mesothelial marker, Vrancken Peeters *et al.,* 1995) has been shown to colocalize with the vascular endothelial growth factor receptor-2 (VEGFR2/Flk-1, an early vascular marker)

in the Syrian hamster (Pérez-Pomares *et al.,* 1998b) and with QH1 (an endothelial and hemopoietic marker) in quail embryos (Pérez-Pomares *et al.,* 1998a).

The aim of this paper is to present new *in vivo* and *in vitro* data supporting a differentiation of coronary endothelial cells from EPDCs. We have carried out a study on the fate of the EPDC population using three different but complementary lineage tracing methods. We have used a fluorescent tracer (CCFSE) and β-Gal transfecting retroviruses to specifically label the mesothelial proepicardial and epicardial cells as well as their derivatives. Results arising from these experiments are conclusive (although quantitatively limited) clonal evidences. For that reason, we transplanted quail proepicardia into host chick embryos to construct quail-to-chick proepicardial chimeras. This interspecific grafting procedure supplies the host heart with quail EPDCs, although it does not really exclude the possibility of differentiation of cardiac structures from non-proepicardial/epicardial mesothelial progenitors. Nevertheless, the construction of chimeras provides quantitative information on the fate of proepicardial/epicardially-derived cells and can be compared with the findings obtained in other studies about epicardial and coronary development (Dettman *et al.,* 1998; Gittenberger-de Groot *et al.,* 1998; Pérez-Pomares *et al.,* 1998a, 2002; Männer, 1999; Vrancken Peeters *et al.,* 1999).

In the *in vitro* study we have cultured proepicardial cells on Matrigel. It is well-known that this substance promotes the differentiation of endothelial cells, probably due to its basement membrane-like composition and its content of growth factors. Endothelial cells in culture form tubes on Matrigel in only 18 h, while it takes several days to form a tube on collagen gels (Grant *et al.,* 1989; Passaniti *et al.,* 1992). We hypothesized that a rapid angioblastic differentiation of the EPDC on Matrigel would allow us to colocalize vascular and mesothelial markers more easily than in other culture systems such as the collagen gels.

The results obtained support our claim that endothelial cells can differentiate from EPDCs. As it will be discussed below, this finding might be regarded in the context of the recent discovery of a bipotential vascular progenitor isolated from embryonic stem cells (Yamashita *et al.,* 2001).

Results

Combined Retroviral Labelling and Transplantation

Retroviral labelling allowed us to trace epicardially-derived cells (EPDCs) in chick/chick transplantations of tagged proepicardia (Fig. 1). Only discrete cells in the mesothelial surface of the transplanted proepicardia were labelled in the control embryos, reincubated for 8-12 h after retroviral injection (Fig. 1 A,B). After reincubation until stages HH29-35, only a few epicardial epithelial cells were β-Gal-positive (Fig. 1C). Instead, a number of subepicardial and intramyocardial cells were clearly labelled, specially around the inner curvature of the heart and around the conoventricular (CV) and atrioventricular (AV) grooves. Some cells

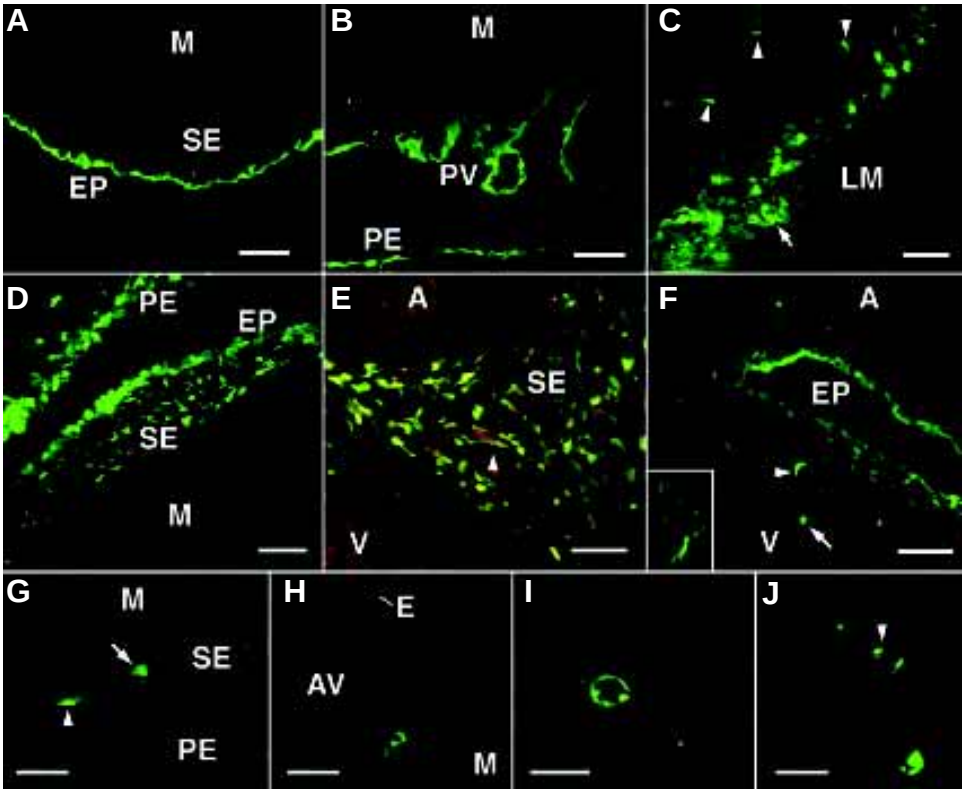

Fig. 2. Fluorescent labelling of the epicardium and the epicardial-derived cells. (A,B) *Embryos reincubated for 3 h after CCFSE injection. Only the mesothelial cells of the epicardium (EP in A) and the proepicardial villi (PV in B) appear labelled. Note the abundance of long basal cytoplasmic projections in these cells. Scale bars, 25 μm.* **(C)** *Liver mesothelium (LM) labelled with CCFSE and reincubated for 24 h. Note the abundance of mesenchymal cells in the submesothelial tissue as well as the presence of a number of discrete labelled endothelial cells (arrowheads). Some mesothelial cells remain fluorescent (arrow). Scale bars, 25 μm.* **(D)** *Abundant labelled cells in the subepicardium (SE) of an embryo reincubated for 24 h. Most of the cells seem to be isolated and fibroblast-like. The epicardium (EP) is thoroughly fluorescent, as well as the pericardium (PE). Scale bar, 25 μm. (E,F) Embryos incubated for 48 h. Some fluorescent cells are forming vascular structures (arrowheads) in the subepicardium (SE) of the atrioventricular junction. Background red fluorescence has been kept in* **(E)** *to highlight the presence of blood cells inside the vessel. The intramyocardial labelled cell shown in* **(F)** *(arrow) seems to be invading the myocardium from the vessel wall, as demonstrated in other confocal plane (insert). In (F), the lesser amount of labelled EPDCs in the subepicardium of the atrium (A) compared to the ventricle (V), correlates with a stronger fluorescence in the epicardial mesothelium. Scale bar, 25 μm. (G-J) Embryos reincubated for 96 h after CCFSE injection. Fluorescent cells are scarcely found in the epicardial mesothelium (arrowhead in G), and most of them are fibroblast-like cells in the subepicardium (arrow in G), the myocardium, the atrioventricular (AV) valves (fluorescent cell in* **H***) or incorporated into vascular structures* **(I,J)**. *Compare these labelled cells to those shown in Fig. 1C. Scale bars, G, 20 μm; H,I,J, 15 μm. Abbreviations: E, endocardium lining the AV valves; M, myocardium; PE, pericardium; SE, subepicardium.*

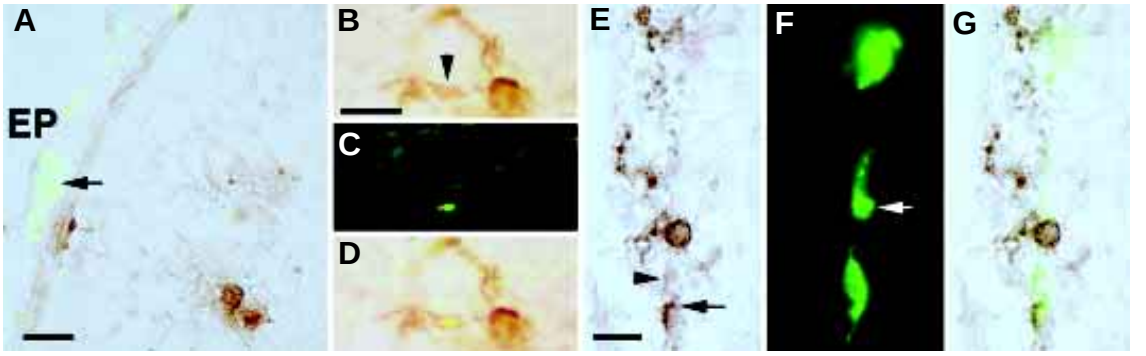

Fig. 3. Quail hearts labelled with CCFSE *in toto*, excised and cultured *in vitro*. **(A)** *Control heart cultured for 3 h after CCFSE labelling. Fluorescent cells are located in the surface and they are QH1-negative (arrow). Scale bar, 15 μm. (B,C,D) QH1-positive, vascular structure located in the subepicardium of a heart cultured for 48 h.* **(B)** *and* **(C)** *show the light transmission and the epifluorescent images, respectively, while the blended image in* **(D)** *shows colocalization in a single cell of the vascular structure (arrowhead in B). Scale bars, 10 μm.* **(E,F,G)** *A small capillary-like (arrowhead) and other cell (arrow) are also QH1/CCFSE-positive in other heart cultured for 48 h. Compare to other fluorescent, QH1-negative cells (arrow in F). Scale bar, 15 μm.*

in the myocardial and the subepicardial areas were arranged in small capillary like structures, formed by one or two cells (1C), that were often associated to large developing coronary vessels (Fig. 1C). β-Gal-positive cells were also found in the endothelial lining or in the perivascular cell population of well developed coronary vessels (Fig. 1C), though the mixture of the two cell types was not frequent. Furthermore, β-Gal-positive EPDCs were found into the AV cushions where they were interspersed between the valvuloseptal mesenchyme (data not shown).

Fluorescent Labelling of the EPDC In Ovo *and* In Vitro

Embryos labelled *in ovo* with CCFSE and reincubated for 3 h showed fluorescence only in the epicardial mesothelium, an observation which was consistent with the reported inability of the dye to cross the epithelial barrier (Fig. 2 A,B). However, a number of subepicardial and intramyocardial cells became fluorescent after 24-96 h of reincubation (Fig. 2 D-J). The number of labelled epicardial cells decreased with the time of reincubation. They were abundant in the embryos reincubated for 3 and 24 hours but only very few

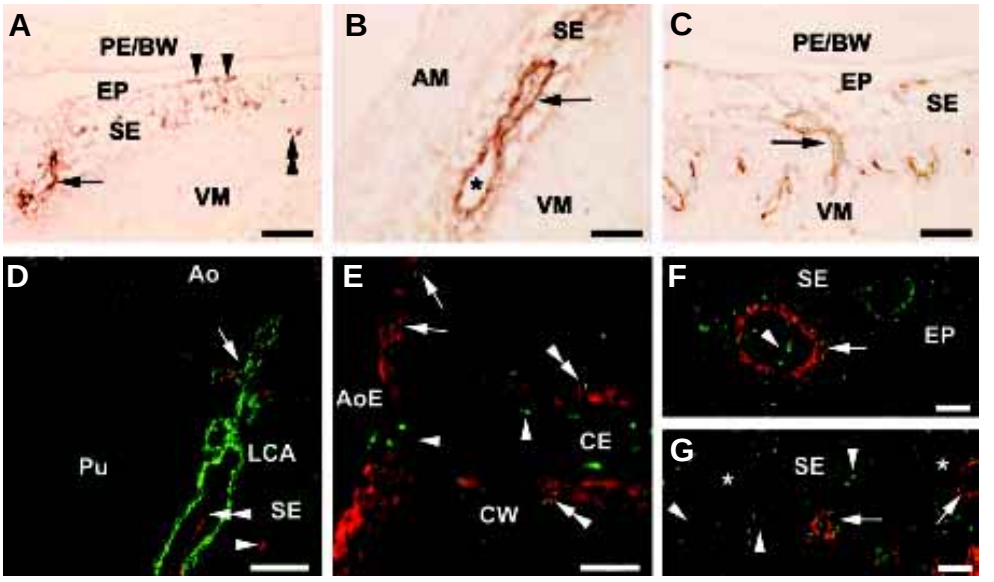

Fig. 4. Origin of endothelial and smooth muscle cells from quail-derived epicardium developed on chick host embryos. *(A,B,C) Different features of coronary vasculogenesis are shown in a proepicardial chimera (H/H29) after QH1 staining. In* **(A)** *QH1-positive cells are indicated in the epicardium (arrowheads), myocardium (double arrowhead) and subepicardium of the ventricle where they form part of the developing coronary vessels (arrow). Scale bar, 50 μm. In* **(B)**, *a long section of a subepicardial AV vessel lined with QH1-positive endotelium is shown (arrow). Note the lumen of the vessel (asterisk). Scale bar, 37.5 μm. Ventricular subepicardial vessels are shown in* **(C)** *invading the compact myocardium (arrow). Scale bar, 50 μm.* **(D)** *The main left coronary subepicardial branch of a stage H/H36 chimera is shown making contact with the root of the aorta (arrow). Quail endothelial cells are labeled with the QH1 antibody (red) and the vessel wall is caldesmon-positive (green). Note the presence of small QH1-positive vessels that have not developed a muscular wall (QH1 red labelling, arrowhead) and the presence of some QH1-negative endothelium within the coronary (double arrowhead). Scale bar, 150 μm.* **(E)** *Higher magnification of the contact between the coronary endothelium and the aortic wall of the specimen shown in (D). Note that caldesmon expression is now shown in red. QCPN-positive donor-derived cells (green nuclei) are found in the inner endothelium (arrowheads) and the outer media (double arrowheads). The sharp boundary of QCPN-positive cells in the border between coronary and aortic endothelium is indicated by double arrowheads. QCPN/caldesmon-positive cells are occasionally found dispersed in the aortic wall of this area (arrows). Scale bar, 25 μm. (F,G) Details of the vascular network in the same H/H36 chimera. Quail derived cells are QCPN-positive (green dots) while smooth muscle cells of the arterial media appear in red (caldesmon expression, arrow). In* **(F)**, *endothelial cells are basically QCPN-positive in arteries (arrowhead) which are surrounded by a multilayered wall formed by QCPN/caldesmon-positive cells (arrow). In* **(G)**, *some vascular structures show a scarce perivascular tissue that presents only traces of caldesmon immunoreactivity coinciding with QCPN-negative endothelium (asterisks). Small arterial sections appear formed by QCPN-positive endothelium and QCPN/caldesmon-positive media (arrows). Note quail-derived mesenchymal cells surrounding the vessels (arrowheads). Scale bars, 30 μm. Abbreviations: AM, atrial myocardium; Ao, Aorta; AoE, aortic endothelium; CE, coronary endothelium; CW, coronary wall; EP, epicardium; LCA, left coronary artery; MYO, myocardium; PE/BW, pericardium/body wall; Pu, pulmonary artery; SE, subepicardium; VM, ventricular myocardium.*

epicardial cells remained labelled in the 96 h embryos (Fig. 2G). Many isolated subepicardial and intramyocardial labelled cells were fibroblast-like mesenchymal cells (Fig. 2 E,H), and some of them were located in the walls of the coronary vessels. However, labelled cells were also found integrated into the endothelial lining of vessels located in the subepicardium after 24-48 h of reincubation (Fig. 2 E,F). Endothelial cells of intramyocardial capillaries were also labelled in embryos reincubated for 96 h (Fig. 2 I,J). Incidentally, when the liver mesothelium was also stained with CCFSE, discrete endothelial cells from the sinusoids were labelled after 24 h (Fig. 2C).

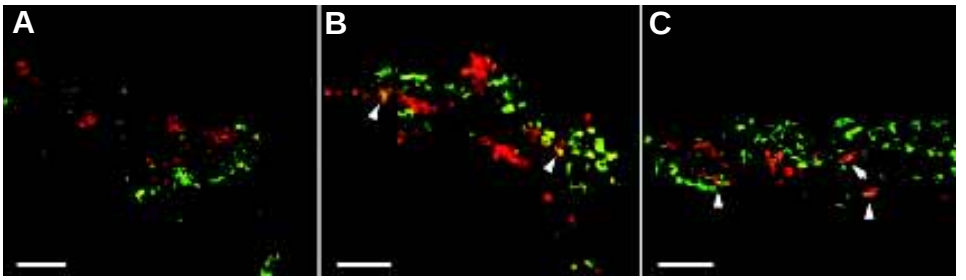

Fig. 5. Quail proepicardial explants cultured on Matrigel. *Explants in* **(A)** *where cultured for 3 h whereas cultures in* **(B)** *and* **(C)** *were cultured for 24 h. In these confocal images mesothelial cells are stained in green and endothelial cells are stained in red (cytokeratin and QH1 immunostaining, respectively). Note the abundant colocalization of both markers in the proepicardial tissue cultured for 24 h (yellow color, arrowheads). Some double-labelled cells are forming capillary-like structures (arrow in C). Scale bars, 25 μm.*

Similar results were obtained when the whole embryos were labelled in a CCFSE solution and the excised hearts were cultured. After 3 h of reincubation only the surface, QH1-negative, epicardial cells were fluorescent (Fig. 3A). However, subepicardial cells were labelled in hearts kept in culture for 24 and 48 h. Some labelled cells, especially in the cardiac cultures performed for 48 h, were QH1-immunoreactive and they were integrated into the endothelium of vessel-like structures (Fig. 3 B,C).

Quail-Chick Chimeras

In the chimeras studied, virtually all the epicardium generated over the chick myocardium was QCPN-positive indicating a donor (quail) origin. Some epicardial mesothelial cells as well as subepicardial mesenchymal cells were strongly QH1-positive (Fig. 4A). In stages H/H29-35 most of the QH1-positive cells formed part of the endothelial lining of the developing coronary vessels although some isolated cells appeared scattered in the subepicardial space or inside of the myocardial layers (Fig. 4 A-C). Long QH1-positive vessels were located in the AV and CV grooves, being the vessels of wider diameter located in the dorsal wall of the AV groove and neighboring ventricular areas (Fig. 4B). Some of these vascular structures contained blood cells even before the developing subepicardial plexus connected to the aortic root by the stage H/H31 and became perfused by the systemic blood flow. Coronary vessels developing in the subepicardium of the free walls of the ventricles frequently invaded the outer compact myocardium (Fig. 4C).

The caldesmon antibody strongly stained a subset of epicardial and subepicardial EPDCs in the chimeras at the stage H/H36, specially some groups of cells in the AV and CV grooves close to the developing coronaries (data not shown). At this stage the arterial subset of the coronary system has started to form a complex multilayered smooth-muscle media from the perivascular precursors. This wall is strongly QCPN/caldesmon-positive indicating its quail origin and the differentiation of the perivascular cells into the smooth muscle lineage (Fig. 4 E-G). The endothelium of coronary arteries was also of donor origin as indicated by the QH1 stainings (Fig. 4D). QH1-positive endothelium was very frequently found in large vessels walled by a multilayered caldesmon-positive media (very likely developing arteries) but it was also present in vessels of small caliber that lacked a well-developed media (Fig. 4F). Donor-derived endothelium was, however, less frequent in large, vein-like vessels. In these vascular structures only a few subendothelial QCPN/caldesmon-positive cells were found whereas numerous, randomly distributed, QCPN-positive/caldesmon-negative as well as QCPN/caldesmon-negative perivascular cells distributed around the endothelium (Fig. 4G). After stage H/H31 the subepicardial web of developing vessels was connected to the right and left coronary sinuses (Fig. 4D). In the contacting points, i.e in those places were the coronaries penetrated the aortic walls to form the coronary ostia, the coronary endothelium was constituted by a perfectly chimeric mosaic in which some cells were of donor origin (quail) and others were of host origin (chick). Some scattered quail-derived smooth muscle cells (QCPN/caldesmon-positive) were found in the wall of the aortic sinuses but quail endothelial cells were never seen in the endothelium of the aortic lumen (Fig. 4E).

Mesothelial-Derived Cells express Vascular Markers when cultured on Matrigel

After 3 h of culture on Matrigel, mesothelial and mesothelial-derived cells could be easily identified on histological sections by their cytokeratin immunostaining, but colocalization of this antigen with the vascular marker QH1 was very scarce. QH1 positive/cytokeratin negative cells were present inside the proepicardial matrix of the control explants (Fig. 5A). However, after 24 h of culture on Matrigel, colocalization of cytokeratin and QH1 became relatively frequent in the mesothelial-derived cells (Fig. 5 B,C).

Discussion

We have shown experimental evidence that EPDCs can differentiate into endothelial cells of the primitive coronary vessels. Three different tracing strategies were used to track the EPDC lineage and to obtain information about the origin of coronary vascular progenitors and the morphogenesis of the primitive coronary plexus. DiI tracing experiments by Dettman *et al.* (1998) showed similar results. Direct retroviral and fluorescent labelling of epicardial mesothelial cells coincided in the recovery of the label in endothelial cells of the coronary vascular plexus. On the other hand, a short-term culture of proepicardial cells on Matrigel demonstrated a dramatic increase in the colocalization of the vascular marker QH1 with cytokeratin, a mesothelial marker, in mesenchymal EPDCs. Furthermore, quail proepicardia transplantation into chick hosts generated a network of coronary vessels from isolated mesenchymal precursors. Both the endothelial and media layers of the chimeric vessels were built from quail cells. The development of the coronary endothelium could thus be claimed as a classic example of vasculogenesis type I (differentiation of angioblasts *in situ*) instead of a type II vasculogenic process (migration of angioblasts into an organ) (Coffin and Poole, 1991).

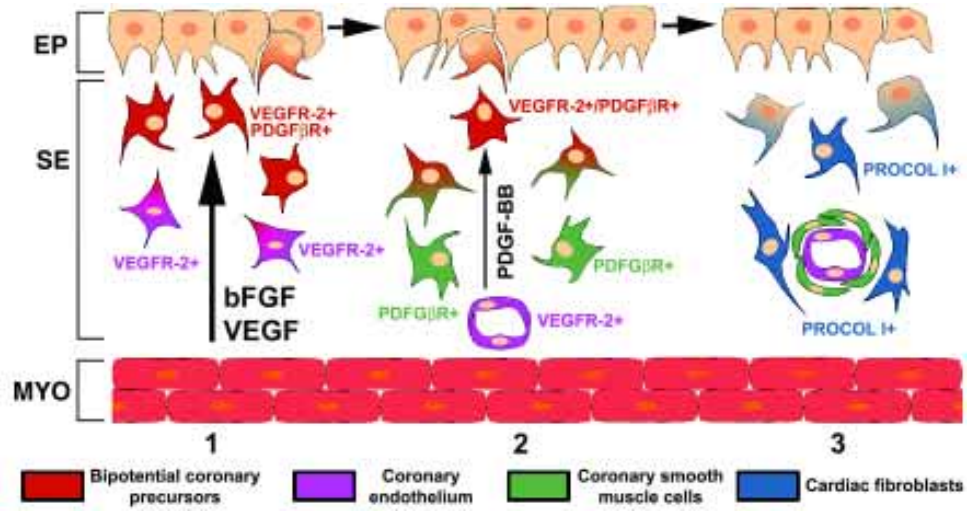

Fig. 6. A hypothetical model of coronary vasculogenesis from epicardially-derived cells (EPDCs). *The model is based on data from this work and other published studies (Mikawa and Fischman, 1992; Mikawa and Gourdie, 1996; Perez-Pomares* et al., *1998a,b; Tomanek* et al., *1998, 1999; Vrancken Peeters* et al., *1999; Tevosian* et al., *2000; Yamashita* et al., *2000). The first step of the epicardial-mesenchymal transition is shown in* **(1)**. *Early EPDCs, expressing specific receptors for VEGF and PDGF, would basically differentiate into angioblasts (mesenchymal cells in purple) induced by myocardial-secreted VEGF. Angioblasts coalesce to form primary blood vessels, indicated as flattened purple cells in* **(2)**. *Subsequent populations of EPDCs provide smooth muscle cell precursors (green cells in 2) to form the vascular wall. Coronary endothelium would drive the differentiation and recruitment of these smooth muscle precursors by secretion of PDGF-BB. As shown in* **(3)**, *late perivascular EPDCs do not contact the endothelium. Their lack of induction by endothelial-derived signals might induce their differentiation into a fibroblastic tissue (blue cells expressing procollagen-I). Abbreviations: EP, epicardium; MYO, myocardium; SE, subepicardium.*

This consideration does not exclude a non-epicardial extracardiac angioblastic contribution to coronary development.

Quail-to-chick chimera experiments have suggested a differential contribution of EPDCs to arterial and venous-like developing coronary vessels. First of all, venous-like endothelium seems to be more heterogeneus in its origin (with mixed contribution of quail and chick cells), whereas the endothelium of well-developed arterial vessels was mainly of donor (quail) origin. The only exception to this rule was found around the point where the main left and right coronary branches join the aortic root. In these areas, the predominantly quail-derived endothelium showed a significant number of chick endothelial cells. On the other hand, our findings about an epicardial origin for coronary smooth muscle and perivascular fibroblastic cell populations confirm already published results (Mikawa and Gourdie, 1996; Dettman *et al.*, 1998; Gittenberger-de Groot *et al.*, 1998; Vrancken-Peeters *et al.*, 1999; Pérez-Pomares *et al.,* 2002). The coronary arterial walls in the chimeras were basically composed of donor-derived, caldesmon-expressing cells, indicating their differentiation into the smooth muscle cell lineage. Interestingly, venous-like structures present a thinner media that included a low number of donor-derived caldesmon-positive cells and abundant fibroblast-like quail and chick-derived perivascular cells. It is possible that this phenomenon is related to the molecular nature of the venous endothelium as compared to the arterial one (Moyon *et al.*, 2001).

The origin of endothelium from mesothelial-derived mesenchymal cells is not a novel observation (reviewed in Muñoz-Chápuli *et al.*, 1999). It had already been demonstrated in the case of the hepatic sinusoids (Le Douarin, 1975; Moore *et al.,* 1998; Muñoz-Chápuli *et al.,* 2001). In earlier embryos, the endothelial cells of the endocardium arise from the precardiac mesoderm when it is constituted as a coelomic epithelium (Sugi and Markwald, 1996; Mjaatvedt *et al.* 1999; Lough and Sugi, 2000). This is not an isolated observation, since angioblasts segregate from the splanchnic mesoderm by an epithelial-mesenchymal transition by the 5 somites stage in avian embryos (Cox and Poole, 2000; Poole *et al.*, 2001). Thus, the endothelial differentiation from mesothelial-derived progenitors in other developing embryonic viscera could just be a lengthening of this phenomenon until later developmental stages (Muñoz-Chápuli *et al.*, 1999). Anyhow, given that mesothelial-derived cells differentiate into endothelial cells in the liver, even if a migration of angioblasts from the liver to the subepicardium occurs, it does not contradict the proposal of a mesothelial origin of the primary coronary endothelium.

It is tempting to speculate that the generation of mesothelial-derived cells and their endothelial differentiation specifically occur in the coelomic investment coinciding with the emergence of embryonic viscera such as the liver, lungs or heart. The primordia of these organs could be a source of growth factors involved in both, the mesothelial-mesenchymal transition and also in the differentiation of the mesothelial-derived mesenchyme. In fact, in the case of the heart, the myocardium secretes FGF-1, FGF-2, FGF-7 and VEGF during the period of coronary vasculogenesis and angiogenesis (Tomanek *et al.,* 1999; Morabito *et al.,* 2001). These growth factors have been claimed to be involved in processes of epithelial-mesenchymal transition as well as in endothelial differentiation (Dettman *et al.,* 1998; Cox and Poole, 2000; Morabito *et al.,* 2001).

According to this hypothetical scenario, most of the embryonic splanchnic angioblasts would be derived not only of an early population of mesodermal cells, as it has previously been proposed (Risau and Flamme, 1995), but rather locally differentiated through the suggested mechanism. This would allow for a rapid organization of a vascular plexus in fast-growing embryonic viscera. The vascularization of these viscera has frequently been attributed to invasive migrating angioblasts, but we think that local differentiation of mesothelial-derived cells can be an alternative source.

A key question for the future is: are coronary smooth muscle and endothelial cells derived from different stocks of EPDCs or do they differentiate from a common bipotential vascular precursor? This question is specially relevant after the recent discovery of a bipotential vascular progenitor cell population derived from embryonic stem cells (Yamashita *et al.,* 2000). Two tyrosine kinase receptor-binding proteins, VEGF and PDGF-BB would be main regulators of the differentiation of these cells. Culture of the bipotential progenitors in the presence of VEGF give rise to

endothelial cells but when they are exposed to PDGF-BB or to serum, they differentate into smooth muscle cells. The exposure to both growth factors gives rise to mixed cultures composed of endothelial cells and smooth muscle.

Thus, the balance between VEGF and PDGF-BB signalling might be a main regulator for the organization of the primary vascular plexus of the heart. It may be significant that the overexpression of VEGF causes not only oversized epicardial vessels but also an underdevelopment of the myocardium (Miquerol *et al.,* 2000). This observation suggests that the fine balance which might exist between three EPDCs-derived lineages (endothelium, smooth muscle and undifferentiated cells invading the myocardium) is broken due to an excessive differentiation of endothelial cells. This would reduce the number of undifferentiated EPDC invading the myocardium, thus precluding the formation of the compact ventricular layer, a process which is probably dependent on the invasion of EPDCs (Carmona *et al.*, 2001; Pérez-Pomares *et al.,* 2002).

Also according to the properties of the bipotential vascular progenitors of Yamashita *et al.* (2000), when these cells are cultured with serum, without significant amounts of VEGF, they differentiate into smooth muscle cells. It can explain why most of the experiments with culture of EPDC on collagen gels have resulted in a smooth muscle differentiation (Dettman *et al.,* 1998; Landerholm *et al.,* 1999).

The model depicted in Fig. 6 summarizes the hypothetical steps of coronary vasculogenesis in relation to epicardial development. Data considered arise from this work and other studies (Mikawa and Fischman, 1992; Mikawa and Gourdie, 1996; Pérez-Pomares *et al.*, 1998a,b; Tomanek *et al.,* 1998, 1999; Vrancken Peeters *et al.,* 1999; Tevosian *et al.,* 2000). We propose that myocardial-derived factors, likely FGFs (Tomanek *et al.,* 1999; Morabito *et al.,* 2001) with the possible involvement of other cytokines (Dettman *et al.,* 1998; Morabito *et al.,* 2001) can induce the epicardial EMT, generating a pluripotential mesenchyme. Myocardial-derived VEGF/bFGF (Tomanek *et al.,* 1999) would induce the differentiation of the earliest EPDCs into endothelial cells (Fig. 6-1) and these, through PDGF-BB secretion, would stimulate the differentiation of further EPDCs which are recruited to the vascular walls as pericytes and smooth muscle (Fig. 6-2) (Folkman and D'Amore, 1996). The outward location of the remaining EPDC impedes them to react to endothelial signals and they finally differentiate into fibroblasts (Fig. 6-3).

The suggested ability of the coelomic epithelium to give rise to bipotential vascular progenitor cells, if confirmed, would open new perspectives in the field of the biotechnology and tissue engineering. It will be most important in the future to check if this ability is definitively lost in the adult mesothelium.

Materials and Methods

The animals used in our research program were handled in compliance with the international guidelines for animal care and welfare. Chick and quail 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.

Combined Retroviral Labelling and Proepicardial Transplantation

Preparation of Retroviruses For these experiments a modified replicative defective Spleen Necrosis Virus (SNV) encoding the β-Gal gene was used (Mikawa and Fischman, 1992; Hyer and Mikawa, 1997). Retroviral solutions greater than 10^7 virions/ml were obtained by ultracentrifugation of culture supernatant (2 h, 15.000 rpm, 37°C) from CXL packaging cell cultures (DMEM + 7% Fetal Bovine Serum).

Labelling of the Donor Proepicardium Chick embryos were incubated until stages H/H16-17 and the eggs carefully windowed using small forceps and tungsten needles. Small volumes of the β-Galactosidase transfecting retroviruses were injected with a microinjector (PicospritzerII) into the pericardial cavity of the embryos (H/H16-17) as shown in Fig. 1A.

Transplantation of the Proepicardia into H/H16-17 Host Chick Embryos After a reincubation of 2 h, the tagged embryos were excised, extensively washed in EBSS and their proepicardia excised and transplanted into normal H/H16-17 chick embryos following Männer's (1999) transplantation procedure (depicted in Fig. 1B, see below for a short description). Host embryos were reincubated until stages H/H29-H/H35, fixed in 4% paraformaldehyde and stained overnight for β-Gal activity. All the specimens included in this study (10) were then embedded in Paraplast Plus®, sectioned and counterstained with nuclear fast red. Control embryos (4) were reincubated only for 8-12 h.

Fluorescent Labelling of the Epicardium

The epicardium was labelled with the dye CCFSE (5,6 carboxy 2',7' dichlorofluorescein diacetate succynimidil ester, Molecular Probes, Eugene, Oregon) which becomes fluorescent when it is incorporated into the cells and stands up to formaldehyde fixation and wax embedding (Sun *et al.*, 2000). This marker cannot permeate through epithelial barriers, including the embryonic epicardium (Sun *et al.*, 2000; Morabito *et al.*, 2001). In a number of experiments, the dye was directly injected into the pericardial cavity of 26 chick embryos from stages H/H21-25. The injection was done through a small incision performed in the pericardial sac. Approximately 2 μL of a 1/50 dilution of the stock solution of CCFSE (6,26 mg/mL in DMSO) in Pannet-Compton saline was injected per embryo. The embryos were reincubated for 3, 24, 48, 72 and 96 h (6, 11, 3, 3 and 3 embryos, respectively). Then, the embryos were fixed for 2 h in 4% paraformaldehyde, dehydrated and paraffin-embedded.

In other set of experiments we isolated 15 whole quail embryos at the stage H/H23-24 in sterile M199 medium. The pericardial sac was opened to expose the heart. Then, the embryos were placed in a 1/100 dilution of the CCFSE stock solution in M199 medium, and incubated for 1 h at 37°C. After washing the embryos with fresh medium (2x15 min), the heart was carefully excised and cultured in M199 for 3, 24 and 48 h (7, 4 and 4 embryos, respectively). Then, the hearts were fixed for 2 h in modified Amsterdam's fixative (methanol:acetone:water=2:2:1), dehydrated and paraffin-embedded. 10 μm sections were immunostained as described below. This experimental system has already been used to specifically label EPDCs (Morabito *et al.*, 2001).

In cultured quail hearts, colocalization of CCFSE-labelled cells and the QH1 antigen was performed by immunostaining first the sections with the QH1 antibody (immunoperoxidase technique) and then superposing the digital images of the fluorescent cells obtained in a fluorescence microscope to the images obtained by conventional light transmission microscopy. This superposition was performed using the Adobe Photoshop 6.0 software.

Proepicardial Quail/Chick Chimeras

To optimize the donor (quail) epicardial covering of the host chick heart, quail-to-chick proepicardial chimeras were prepared as described by Männer (1999) with the following modifications. Host chick embryos were incubated until stages H/H16-17. Using tungsten needles small openings were made through the vitelline and chorionic membranes to expose the pericardial cavity. For each embryo, a small piece of the eggshell membrane was cut with iridectomy scissors and made to fit between the sinoatrial sulcus and the caudal vitelline veins. Then, quail embryo donors (at the stages H/H16-17) were excised and perfused with EBSS (GIBCO). The heart was removed by cutting it through the outflow tract and the sinoatrial sulcus. The previously prepared eggshell membrane was introduced throughout the omphalomesenteric vein of the quail embryo and pushed until it reached the cardiac

lumen, so that the sinus venosus formed a cuff around the membrane holding the donor proepicardium on its surface. The membrane carrying the quail (donor) proepicardium was inserted facing the ventricular heart surface. After the surgery the eggs were sealed with Scotch tape and reincubated to obtain H/H25-26 (two chimeras), H/H28-29 (five chimeras), H/H32 (three chimeras) or H/H36 (two chimeras). Embryos were fixed in modified Amsterdam's fixative, dehydrated in a graded series of ethanol, cleared in toluene, carefully oriented, and embedded in paraffin (Paraplast Plus, OXFORD Labware). Finally, 5 μm serial sections were mounted on microscope slides (Superfrost/Plus, Fisher Scientific).

Immunohistochemical characterization of the chimeric tissues was performed using the QCPN antibody (quail pan-nuclear marker) or the QH1 antibody (quail endothelial and hemopoietic cell marker) as described below.

Culture of Proepicardial Explants

Quail proepicardia, at the stage H/H17-18, were excised and cultured on Matrigel. Briefly, the embryos were isolated in sterile Tyrode medium and the proepicardium was carefully dissected with sharpened tungsten needles, placed on a layer of Matrigel previously kept at 37°C for 30 min, and allowed to attach for 3 h. Then, we cultured the explants for 24 h with M199 medium supplemented with ITS and 1% chick serum. The cultures were fixed in modified Amsterdam's fixative, dehydrated and paraffin-embedded. Dewaxed sections were double immunostained for CK and QH1 as described below. Control assays consisted of proepicardia cultured only for 3 h.

Immunohistochemistry

For the immunoperoxidase technique, endogenous peroxidase activity was quenched by incubating the sections for 30 min with 3% hydrogen peroxide in Tris-PBS. After washing, non-specific binding sites were saturated for 30 minutes with 16% sheep serum, 1% bovine serum albumin and 0.5% Triton X-100 in Tris-PBS (SBT). Endogenous biotin was blocked with the avidin-biotin blocking kit (Vector, Burlingame, CA). The slides were then incubated overnight at 4°C in the primary antibody diluted in SBT. Then, the slides were washed in TPBS (3x5 minutes), incubated for 1 h at room temperature in biotin-conjugated anti-mouse goat IgG (Sigma) diluted 1:100 in SBT, washed again and incubated for 1 hour in avidin-peroxidase complex (Sigma) diluted 1:150 in TPBS. After washing, peroxidase activity was developed with Sigma Fast® 3,3'-diaminobenzidine (DAB) tablets according to the indications of the supplier.

For the fluorescent CK/QH1 double-labelling we used a mixture of primary anti-cytokeratin (polyclonal) and anti-QH1 (monoclonal) antibodies followed by incubation with TRITC-conjugated goat anti-mouse IgG and biotin-conjugated goat anti-rabbit IgG (1:100). Finally, sections were incubated with FITC-conjugated avidin (1:150). All these secondary antibodies were from Sigma. Fluorescent double-labellings in the quail-to-chick chimeras were performed as follows. Sections were first incubated with the monoclonal primary antibodies overnight at room temperature. After three washes in PBS the slides were incubated in anti-mouse IgG biotinilated antibody (Amersham; 1:100 in PBS, 2 hours, room temperature), washed again (3x10 minutes, PBS), and incubated in Extravidin-FITC or Extravidin-TRITC (Amersham). After washing in PBS (3x10 minutes) the sections were incubated overnight at room temperature in the polyclonal antibody. Then, 3x10 minutes washes were followed by an incubation (2 hours, room temperature) with AlexaFluor-568 or Alexa Fluor-488-conjugated goat anti-rabbit IgG (Molecular Probes). After extensive washes, the sections were mounted in an 1:1 PBS/glycerol solution and analyzed using a BioRad MRC 1024 laser scanning confocal microscope.

The monoclonal antibodies anti-QH1 and anti-QCPN were obtained from the Developmental Studies Hybridoma Bank. They were used at a 1:200 dilution and undiluted, respectively. The polyclonal anti-bovine epidermal cytokeratin (Z622, Dakopatts, Denmark) was used at a 1:200 dilution in immunofluorescence. The anti-caldesmon polyclonal antibody (kindly provided by Dr. R.G. Gourdie) was used at a 1:100 dilution.

Negative controls were always performed incubating the sections with SBT instead of the primary antibody.

Acknowledgements

The authors sincerely thank Dr. Manjón-Cabeza and Miss Tanya Rittman for their kind help designing the illustrative cartoons. We also want to thank Aimée Phelps and Martina Sedmerova for their valuable technical help and Dr. R.G. Gourdie for kindly providing the anti-caldesmon antibody. The monoclonal antibodies QH1 and QCPN were obtained from the Developmental Studies Hybridoma Bank maintained by the Department of Pharmacology and Molecular Sciences, John Hopkins University School of Medicine, Baltimore, MD 21205, and the Department of Biological Sciences, University of Iowa, Iowa City, IA 52242, under contract NO1-HD-2-3144 from the NICHD. This work was supported by grants PM98-0219 and 1FD97-0693 (Ministerio de Ciencia y Tecnología, Spain) to Dr. Muñoz-Chápuli and by funds from the NIH (grant NHLBI 1PO1 HL52813-06) and the AHA (grant AHA-GIA 005099U) to Dr. Wessels and Dr. J.M. Pérez-Pomares. Mauricio González-Iriarte is the recipient of a fellowship from Ministerio de Ciencia y Tecnología (Spain).

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Received: May 2002
Reviewed by Referees: July 2002
Modified by Authors and Accepted for Publication: October 2002