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RESEARCH ARTICLE

Contribution of Mesothelium-Derived Cells to Liver Sinusoids in Avian Embryos

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The developing liver is vascularized through a complex process of vasculogenesis that leads to the differentiation of the sinusoids. The main structural elements of the sinusoidal wall are endothelial and stellate (Ito) cells. We have studied the differentiation of the hepatic sinusoids in avian embryos through confocal colocalization of differentiation markers, in ovo direct labeling of the liver mesothelium, induced invasion of the developing chick liver by quail proepicardial cells, and in vitro culture of chimeric aggregates. Our results show that liver mesothelial cells give rise to mesenchymal cells which intermingle between the growing hepatoblast cords and become incorporated to the sinusoidal wall, contributing to both endothelial and stellate cell populations. We have also shown that the proepicardium, a mesothelial tissue anatomically continuous with liver mesothelium, is able to form sinusoid-like vessels into the hepatic primordium as well as in cultured aggregates of hepatoblasts. Thus, both intrinsic or extrinsic mesothelium-derived cells have the developmental potential to contribute to the establishment of liver sinusoids. ***Developmental Dynamics 229:465–474, 2004.*** **© 2004 Wiley-Liss, Inc.**

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INTRODUCTION

The first evidence of avian liver organogenesis appears around Hamburger and Hamilton stage (H/H) 14–15 (50–53 hr of incubation) when endodermal diverticula arise from the floor of the foregut (Le Douarin, 1975). These diverticula extend forward into the transverse septum area. Distal portions of the diverticula form the liver, which is already a macroscopic structure in the H/H18 embryo (3 days of incubation), whereas the more proximal segments give rise to the biliary ducts and gall bladder (Romanoff, 1960). The developing liver is vascularized through a complex vasculogenic process that leads to the differentiation of the portal vein, the hepatic artery, and the sinusoids (DeRuiter et al., 1993; Gouysse et al., 2002). The main structural elements of the sinusoidal wall are endothelial cells and stellate (Ito) cells. The adult sinusoidal endothelial cells show a highly specific phenotype, characterized by the presence of fenestrations, the lack of a basement membrane and markers of continuous endothelium (e.g., PECAM-1), and the expression of markers such as the CD4, CD14, and CD32 antigens (Couvelard et al., 1996). The stellate cells and perivascular elements are closely apposed to the endothelial cells, with supporting functions similar to those performed by pericytes or smooth muscle cells in other vessels (Enzan et al., 1997). In addition to their pericyte-like role, stellate cells have specific functions such as storage of retinoids (Blomhoff et al., 1990). Other specialized cell types from the sinusoids are the Kupffer cells, dendritic cells, and pit cells (Wake, 1999).

Vasculogenesis is the process of formation of the earliest embryonic vessels by assembly of endothelial progenitors (angioblasts) and recruitment of perivascular cells from the surrounding mesenchyme. According to this view, the origin of sinusoid components should be found in the mesenchyme in which the liver anlage grows. In mammals, endo-

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thelial and stellate cells differentiate from the mesenchyme of the transverse septum in a typical process of vasculogenesis (Enzan et al., 1983, 1997; Couvelard et al., 1996), although the origin of these vascular progenitors, as well as the precise mechanisms leading to the commitment and differentiation of the endothelial and stellate cells, are poorly known. In the avian embryo, however, the transverse septum develops relatively late, and the liver does not grow in a preexisting mesenchyme-rich environment. For that reason the origin of the hepatic vascular progenitors in avians is even less known than in mammals, despite careful histomorphologic studies (Wong and Cavey, 1992). Some authors have suggested that the hepatic mesenchyme arises, at least in part, from delamination of the liver mesothelium (Le Douarin, 1975; Moore et al., 1998; Muñoz-Chápuli et al., 2001). An origin from the septum transversum mesenchyme has been proposed for the stellate cells (Enzan et al., 1997), although no experimental evidence has yet been provided for all these proposals.

The question of the origin of the endothelial progenitors of the liver has acquired new interest because a study by Matsumoto et al. (2001) has shown that the angioblasts might play an active role in the morphogenesis and differentiation of the organs they vascularize. This role has been demonstrated in both liver and pancreas development. The molecular mechanisms of this interplay between the developing organs and their differentiating vascular network are unknown.

The aim of this study is to present new data about the origin and differentiation of liver sinusoids in avian embryos. We have carried out a study using four different techniques: colocalization of mesothelial, endothelial, and stellate cell markers; in ovo direct labeling of the liver mesothelium; induced invasion of the developing chick liver by quail proepicardial cells; and in vitro culture of aggregates composed of liver primordia and proepicardial cells. Our findings have shown an active contribution of mesothelium-derived mesenchymal cells to the developing liver and also suggest a common origin of endothelial and stellate cells from these mesothelium-derived mesenchyme. This proposal might even help to establish a link between the suggested signaling properties of the hepatic angioblasts with the inductive role played by the epicardium and epicardially derived cells (EPDCs) in the development of the myocardium.

RESULTS

Histology

At stage H/H17, mesothelial cells of the growing hepatic primordium showed morphologic traits that have been described in embryonic systems of epithelial–mesenchymal transition. These traits included a much reduced lateral adhesion, leaving even intercellular spaces, cell hypertrophy, and long basal cytoplasmic processes (Fig. 1). The signs of epithelial–mesenchymal transition disappear once the fast-growing phase of the liver is finished. Thus, this organ, by stage H/H30, shows a typical flat, squamous mesothelium.

Immunohistochemistry

Cytokeratins are good markers of the chick embryonic mesothelia. Accordingly, the hepatic mesothelium was found to be cytokeratin immunoreactive in all the stages studied. Most mesenchymal cells in the submesothelial space and several mesenchymal cells located between the hepatic cords were also cytokeratin-positive during the phase of fast growth of the liver (H/H18–H/H26). Mesenchymal cells usually have cytoskeletal intermediate filaments composed of vimentin, and for that reason, the transient presence of cytokeratin immunoreactivity in mesenchymal cells suggests a mesothelial origin, as discussed below. The sinusoid wall showed flat, cytokeratin-positive cells, some of which were apparently adlumenal (Fig. 2A,B). All these nonmesothelial, cytokeratin-positive cells have disappeared by stage H/H30, suggesting that the mesothelial contribution to the liver mesenchyme is a transient process (Fig. 2C).

To test the endothelial differentiation of the cytokeratin-positive sinusoidal cells, we performed double immunolabelings with the quail endothelial marker QH1. Cytokeratin immunoreactivity colocalized with the QH1 marker in some sinusoidal cells of quail embryos (Fig. 2A,B). Colocalization of QH1 with the transcription factor WT1, a marker of mesothelial-derived cells in the liver/septum transversum area, was also occasionally recorded (Fig. 2D–G), but these colocalizations were fainter and less frequent than in the heart, an organ in which endothelial differentiation of mesothelial-derived cells has been described (Pérez-Pomares et al., 2002b) and where WT1/QH1-positive cells were frequently detected in the atrioventricular subepicardium and invading the ventricular myocardium (Fig. 2H,I). Colocalization of WT1 with smooth muscle cell α-actin (SMC α-actin), a marker of smooth muscle and liver stellate cells, was also observed in the submesothelial areas of the liver (Fig. 2J). Thus, mesothelial markers colocalize with both endothelial and stellate cell markers in liver mesenchymal cells.

Direct Mesothelial Cell Labeling

When the chick embryos were injected in the coelom with the fluorescent dye CCFSE and reincubated for 3 hr, only the surface mesothelium was labeled (Fig. 3A). However, after 24 hr of reincubation, many submesothelial cells and a few sinusoidal cells of the outer area of the liver were fluorescent (Fig. 3B). Labeled cells were observed in the inner hepatic tissue after 48 hr of reincubation (Fig. 3C). We colocalized also the CCFSE fluorescence with the QH1 endothelial marker in quail embryos. After 3 hr of reincubation, only mesothelial cells were labeled by CCFSE (Fig. 3D). Occasional CCFSE/QH1 colocalizations were observed after 10 hr of reincubation (Fig. 3E), but only after 24 hr, double-labeled cells were seen intermingling between the sinusoids (Fig. 3F). Further CCFSE/QH1-positive cells were observed in the sinusoid walls between 48 and 96 hr of reincubation (Fig. 3G–I). We also found

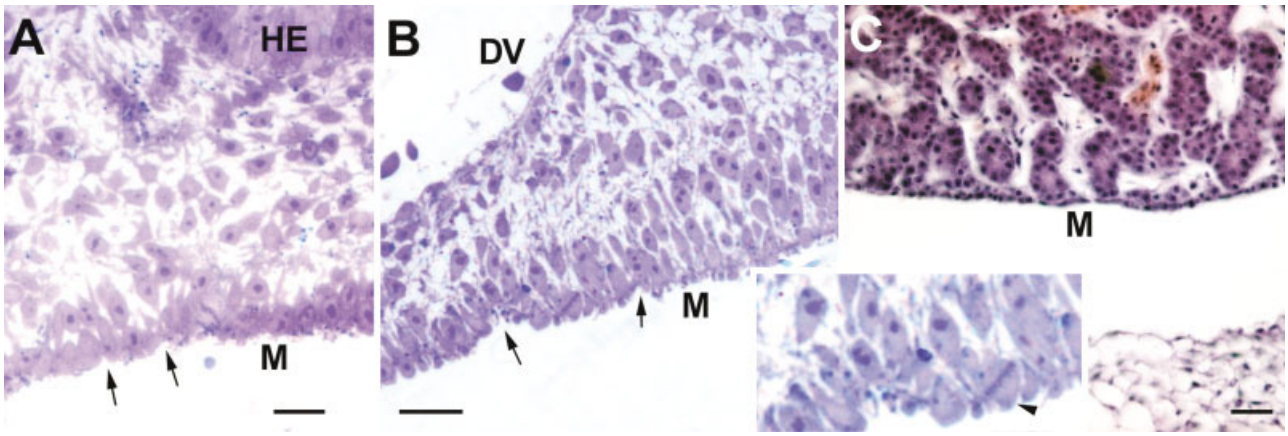

Fig. 1. **A,B:** Semithin sections (0.5 μm) of the liver surface in quail embryos, Hamburger and Hamilton stage 17, close to hepatoblast cords (A) and venous structures (B). Mesothelial cells (M) show traits of epithelial–mesenchymal transition, such as reduced intercellular adhesion, long basal cytoplasmic processes, and signs of migration into the submesothelial space (arrows). The insert is a higher magnification of the mesothelial cells shown in B; a mitotic cell is shown by the arrowhead. **C:** Paraffin section of the liver surface in a quail embryo, Hamburger and Hamilton stage 30. Hematoxylin and eosin staining. By this stage, the liver mesothelium is constituted of flat cells, without signs of epithelial–mesenchymal transition. DV, ductus venosus; HE, hepatoblasts. Scale bars = 10 μm in A–C.

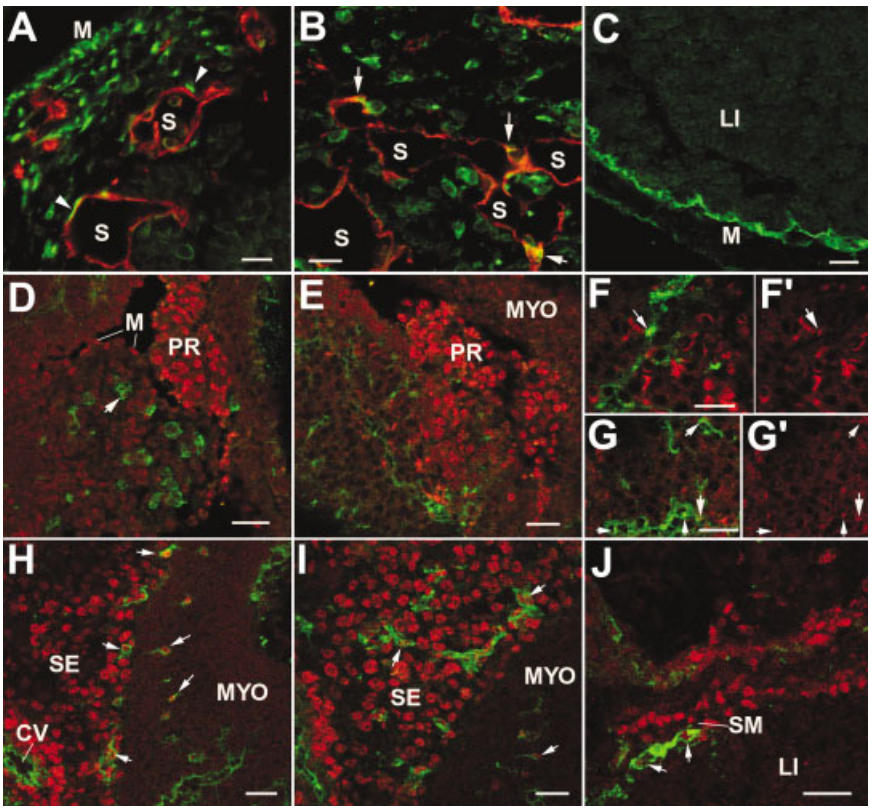

Fig. 2. Colocalization of differentiation markers in the liver of avian embryos. **A,B:** Colocalization of cytokeratin and QH1 (mesothelial and endothelial markers, green and red, respectively) in a quail embryo at Hamburger and Hamilton stage (H/H) 20. The mesothelium (M) and mesothelium-derived cells are cytokeratin immunoreactive. Mesothelium-derived cells can be seen incorporated to the wall of the sinusoids (S, arrowheads in A) and some of them also express QH1 (arrows in B). **C:** Cytokeratin staining of the liver mesenchyme is transient, as shown in this H/H30 chick embryo, where only the mesothelium is immunoreactive. **D–G′:** Colocalization of the transcription factor WT1 (red) with the endothelial marker QH1 (green) in the liver of a quail embryo, H/H25. Only a few QH1-immunoreactive cells show a faint WT1 immunoreactivity in their nuclei (arrows). F′,G′: The red channel is shown separately. Note the strong WT1 expression in the proepicardium (PR). **H,I:** Colocalization of the transcription factor WT1 (red) with the endothelial marker QH1 (green) in the heart of a quail embryo, H/H25. Cells expressing both markers are present in the subepicardium (SE) and also within the myocardium (MYO; arrows). A coronary vessel (CV) is shown. Note the abundant WT1-immunoreactive cells around this vessel and the lack of WT1 expression in the differentiated endothelium. **J:** Colocalization of the transcription factor WT1 (red) with smooth muscle cell α-actin–specific isoform (green) in the liver of a quail embryo, H/H25. Cells expressing both markers (arrow) are present in the submesothelium (SM). LI, liver; MYO, myocardium. Scale bars = 25 μm in A–J.

rounded, isolated, CCFSE/QH1-positive cells either in the sinusoid lumen or below the sinusoidal wall (Fig. 3H,I). On the other hand, colocalization of CCFSE and the stellate/smooth muscle cell marker SMC α-actin was relatively frequent after 48 hr of reincubation, either in cells from the sinusoidal walls or around the main hepatic veins (Fig. 3J–L).

Induced Proepicardial Invasion of the Liver

Before grafting them into chick embryo hosts, quail proepicardia were labeled in their surface with CCFSE, as described in the Experimental Procedures section. This labeling allows for a multiple tracking of the cells, as mesothelial-specific CCFSE labeling can be combined with the quail markers QH1 and QCPN. When the donor-derived proepicardium attached to the developing host liver, proepicardial cells migrated and contributed to the hepatic mesenchyme after 24 hr of reincubation (Fig. 4B,C). Some of these cells were probably mesothelial-derived, as suggested by the CCFSE labeling. Donor-derived cells become integrated in the sinusoidal walls and differentiated into endothelial and stellate-like cells, as shown by the QH1 marker (Fig. 4D,E), and by the CCFSE/SMC α-actin colocalization (Fig. 4F), respectively. These donor-derived sinusoidal cells were morphologically indistinguishable from the host endothelial and stellate cells.

In Vitro Culture of Chimeric Cell Aggregates

Chimeric cell aggregates of quail proepicardia and chick liver primordium did not show significant differences from control aggregates (liver primordium only) in size (around 300 μm of diameter for the spheric aggregates and 500 μm for the long axis of ovoidal ones) or morphology, although the compaction of the cell spheroids seemed faster in the chimeric cases. Histology of the chimeric aggregates showed that their morphology was very reminiscent to that of the developing liver primordium, including the formation of si-

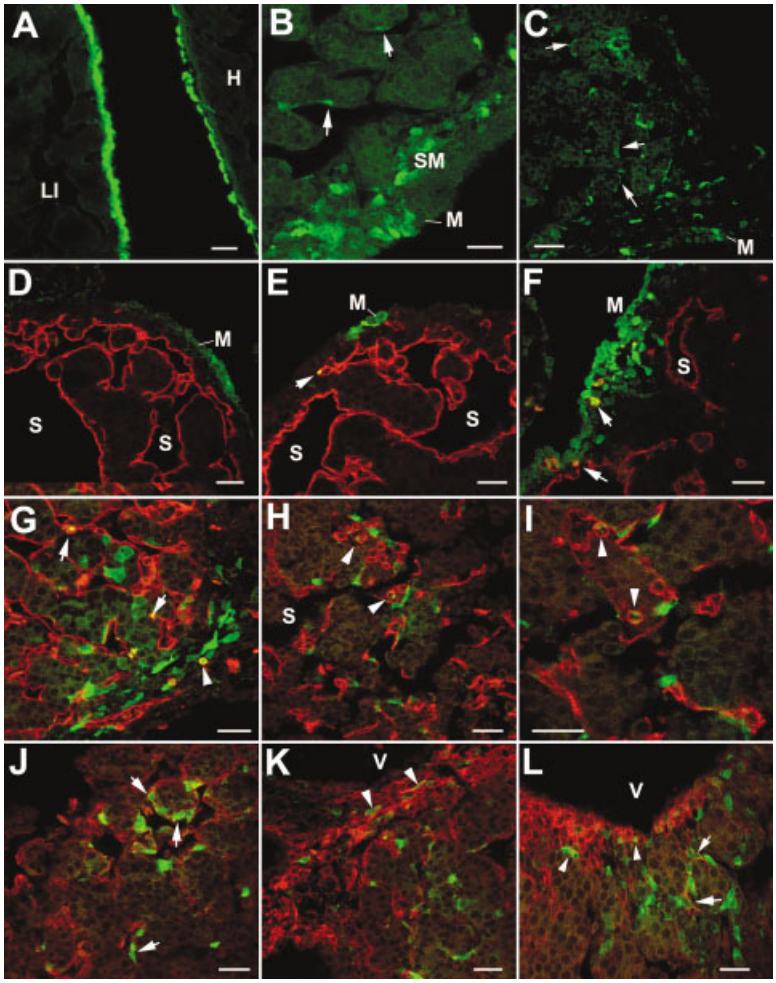

Fig. 3.

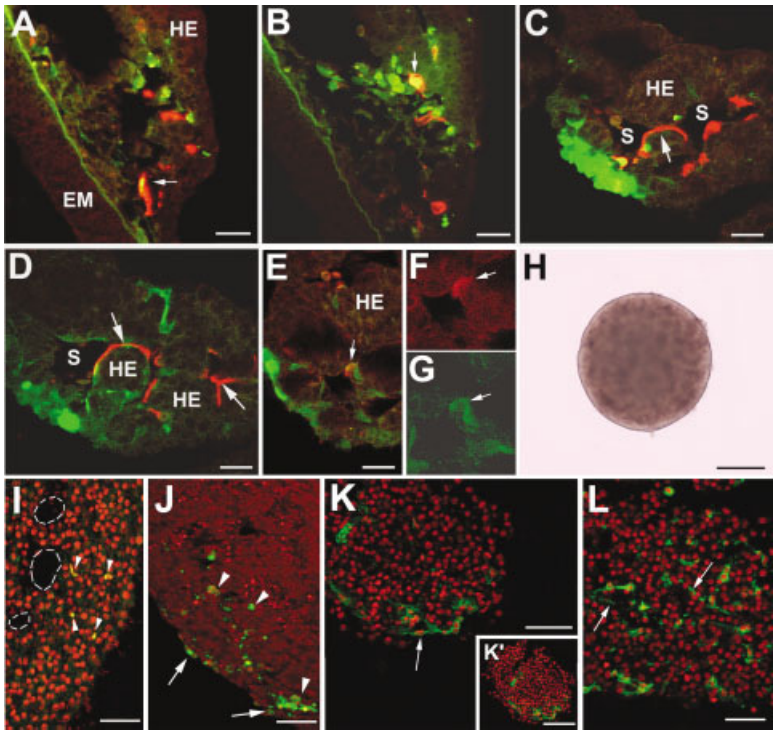

Fig. 4.

nusoid-like vascular beds that were lined by endothelium (Fig. 4I–L). Quail cells were found in the surface of the aggregates as shown by CCFSE or QCPN staining of the donor cells, but also invading the core of the spheroids and often close to hollow vascular vesicles (sinusoid-like structures; Fig. 4I,J). Some quail-derived cells that were found inside the aggregates had differentiated into vascular cells as shown by QH1 immunostaining (Fig. 4K,L).

DISCUSSION

The mammalian liver grows into the mesenchyme-rich environment of the transverse septum (Theiler, 1989). It has been shown how this mesenchyme intermingles with the growing hepatic cords and differentiates into sinusoidal cells, mainly endothelium and stellate cells. However, the liver of the avian embryo does not grow into a preexisting well-developed, highly cellularized transverse septum. For that reason, the origin of the hepatic mesenchyme and the precursors of the sinusoidal cells was poorly known. We have shown descriptive and experimental evidence of a significant contribution of mesothelial cells to the hepatic mesenchyme through a process of delamination or epithelial–mesenchymal transition. The descriptive evidence includes morphologic traits and a transient localization of mesothelial markers (cytokeratin and WT1) in the hepatic mesenchymal cells, with a clear gradient of labeling between the submesothelial and the inner liver areas. These features have disappeared by stage H/H30. On the other hand, the results of the direct cell labeling of the hepatic mesothelium with the fluorescent dye CCFSE closely agree with the descriptive findings. Labeled mesothelial cells, which stay in the mesothelial lining after 3 hr of reincubation, have invaded the submesothelium after 24 hr and become progressively incorporated to the sinusoidal walls between 24 and 72 hr after their labeling.

We have also collected information about the developmental fate of the mesothelium-derived cells. In our experimental setting, colocalization of CCFSE with SMC α-actin suggests that stellate perisinusoidal cells differentiate from mesothelium-derived cells in an interesting parallelism with the origin of the precursors of the coronary smooth muscle from epicardially derived cells (Dettman et al., 1998; Vrancken-Peeters et al., 1999; Wada et al., 2003). Of interest, we have also observed CCFSE/SMC α-actin–positive cells in the wall of large veins of the hepatic region, suggesting that mesothelium-derived mesenchymal cells can also be recruited by these vessels differentiating into smooth muscle cells and contributing in this way to their tunica media.

The hypothesis of the differentiation of mesothelium-derived cells into sinusoidal endothelial cells of the chick embryo was first proposed by Le Douarin many years ago, but it has received little attention from developmental biologists. We have shown herein some evidence supporting this possibility, namely the colocalization of endothelial and mesothelial markers as well as the expression of the

Fig. 3. Direct labeling of the liver mesothelium with the fluorescent dye CCFSE. All the embryos were injected by Hamburger and Hamilton stage (H/H) 17 and reincubated. **A:** Chick embryo reincubated for 3 hr. Only the mesothelial components of the heart (H) and liver (LI) appear labeled. **B:** Chick embryo reincubated for 24 hr. Most of the labeled cells are in the submesothelium (SM), but some of them remain in the mesothelium (M) and a few can be seen in the sinusoidal walls (arrows). **C:** Chick embryo reincubated for 48 hr. Most of the labeled cells are in the inner part of the liver. Note the labeled cells in the sinusoids (arrows). **D,E:** Quail embryos reincubated for 3 and 10 hr, respectively, and immunostained with the endothelial marker QH1 (red). After 3 hr of reincubation only the mesothelium (M) is labeled, but a few CCFSE-positive cells can already be seen inside the liver and expressing the QH1 antigen (arrow in E). **F:** Quail embryo reincubated for 24 hr and immunostained with the endothelial marker QH1 (red). Mesothelial-derived cells are migrating into the submesothelium, and some of them are expressing the QH1 antigen (arrows). **G–I:** Quail embryos reincubated for 48 hr and immunostained with the endothelial marker QH1. CCFSE-labeled cells can be seen between the cords of hepatoblasts, incorporated to the sinusoid walls, and expressing sometimes the QH1 antigen (arrows in G). Note the rounded cells that are double-labeled (arrowheads). Some of them are apparently within the sinusoidal lumen (S). **J–L:** Quail embryos reincubated for 48 hr (J,K) and 72 hr (L) and immunostained with an antibody against the smooth muscle cell α-actin–specific isoform (red). CCFSE-labeled cells expressing this protein, probably stellate (=Ito) cells, can be seen in the sinusoidal wall (arrows in J). Double-labeled cells can also be seen in the wall of the large hepatic veins (V) and are pointed to by arrowheads in K and L. Some double-labeled cells seem to be migrating toward the veins (arrow in L). S, sinusoids. Scale bars = 25 μm A–L.

Fig. 4. Experimental vascularization of chick liver primordia in vivo and in vitro. A–F: CCFSE-labeled quail proepicardia. QH1 immunostaining is shown in red. **A,B:** Proepicardial cells are migrating into the liver and expressing the QH1 antigen. Double-labeled cells (arrows) are probably mesothelium-derived angioblasts, because the proepicardia were labeled with CCFSE only in their surface. EM, egg membrane; HE, cords of hepatoblasts. **C,D:** Chimeric sinusoids (S) differentiated from quail proepicardial-derived cells, as shown by the QH1 (donor-specific) antigen, which stains the donor-derived endothelium (arrow). These sinusoids are morphologically similar to those from the host. **E:** Colocalization of smooth muscle cell α-actin–specific isoform (red) with the CCFSE label. **F,G:** Higher magnification of the cells indicated by an arrow in E. The double-labeled cells (shown by the arrows in separate channels) suggest that proepicardial mesothelium-derived cells can differentiate into perisinusoidal cells. H–K illustrate the in vitro culture of quail proepicardial and chick embryonic liver cells (chimeric aggregates). **H:** A general view of a chimeric cell aggregate in culture. **I:** Proepicardial cells can be tracked easily by using the QCPN antibody, which stains all quail nuclei. QCPN-positive nuclei appear yellow after a general propidium iodide counterstain (arrowheads). Sinusoidal-like spaces are indicated by broken lines. **J:** CCFSE-stained quail mesothelial/mesothelial-derived cells (green) are found in the surface (arrows) and inside (arrowheads) the cell aggregates. Unspecific red background has been kept to show the morphology of the tissue. **K:** The chimeric aggregates are partially vascularized by quail proepicardial endothelial cells as indicated by an arrow (QH1 immunoreactivity, green). Cell nuclei are counterstained with propidium iodide. **K′:** The aspect of the complete spheroid. **L:** A detail of the incorporation of proepicardial QH1-positive cells (green, arrows) into sinusoid-like vascular beds. Cell nuclei are counterstained with propidium iodide. Scale bars = 25 μm in A–G, 100 μm in H, 30 μm in I,J,L, 50 μm in K, 100 μm in K′.

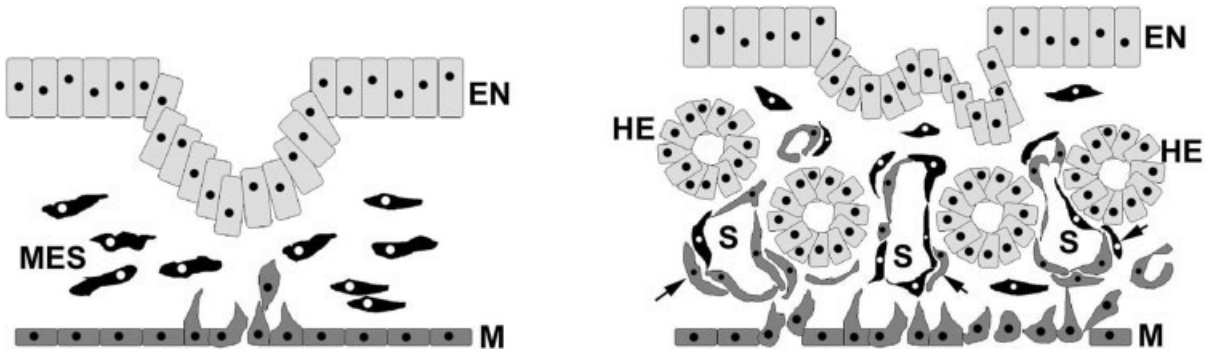

Fig. 5. Model of a contribution of mesothelium-derived cells to the developing liver sinusoids. Pluripotential cells (dark grey) arising from the mesothelium (M) through an epithelial-to-mesenchymal transition probably induced by the endodermal tissue (light grey, EN) would mix with the preexisting mesenchyme of the septum transversum (black cells, MES). Both endothelial cells from the sinusoids (S) and stellate cells (arrows) would differentiate from mesenchymal cells of both origins. HE, hepatocytes.

endothelial marker QH1 in CCFSE-positive cells which are presumably mesothelial-derived. However, cytokeratin/QH1 colocalizations were relatively scarce, perhaps due to a rapid degradation of the cytokeratin that remains in the hypothetical endothelial progenitors. These proteins constitute the intermediate filaments of many epithelial cells, including the mesothelium, but they are degraded when the mesothelial cells transform into mesenchyme (Pérez-Pomares et al., 1997). WT1/QH1 colocalization was also rare and much more scarce than in the developing heart, possible due to a faster down-regulation of the WT1 expression in the mesothelium-derived cells in the liver. In fact, while WT1+ cells are abundant in the subepicardium as well as intramyocardially, they can only be localized in a narrow submesothelial domain of the developing avian liver, as we had already shown (Carmona et al., 2001).

Although we think that the available data support Le Douarin's hypothesis of a mesothelial origin of at least several sinusoidal endothelial cells, alternative explanations should be considered. We have observed rounded, macrophage-like cells into the sinusoidal lumen, which showed a CCFSE/QH1 double labeling. Intraluminal clumps of macrophages had been described in the liver of human and mice embryos (Enzan et al., 1983, 1997; Sasaki, 1990) and an origin from yolk sac has been proposed for these clumps (Sasaki, 1990). These intrasinusoidal macrophages disappear in later developmental stages (Kelemen and Janossa, 1980), perhaps by transendothelial migration and differentiation into Kupffer cells (Enzan et al., 1983). It is conceivable that the CCFSE-positive macrophage-like cells that we have observed become labeled after phagocytosis of apoptotic mesothelium-derived cells. Because some authors have described that macrophages can adopt the flat appearance of the endothelial cells forming vessel-like structures (Moldovan et al., 2000), we should also consider this possibility as an alternative explanation for the CCFSE/QH1 colocalizations in the sinusoidal walls. An even more conjectural possibility is that CCFSE-labeled macrophages can differentiate from mesothelium-derived cells. Although this event has never been described in vertebrates, it is interesting to point out that this is a normal mechanism of origin of macrophages in invertebrates, even in adult ones. The coelomic lining of starfishes, for example, gives rise to phagocytic cells when injected intraperitoneally with carbon particles (Vanden Bossche and Jangoux, 1976). An origin of macrophage-like cells from the coelomic epithelium has also been described in Cephalochordates (*Branchiostoma*), the invertebrate group most closely related to vertebrates (Ruppert, 1997).

The possibility of a common origin of endothelial and stellate cells from mesothelium-derived mesenchymal cells might be related to the recent description of a bipotential vascular progenitor from embryonic stem cells (Yamashita et al., 2000). These bipotential progenitors differentiate into endothelial cells when they are cultured with vascular endothelial growth factor (VEGF), but they originate smooth muscle cells in cultures supplied with serum or platelet-derived growth factor (PDGF) -BB. If mesothelium-derived cells would possess this differentiation potential, a hypothetical scenario for the origin of sinusoids in the liver would be as indicated in Figure 5. The earliest mesothelium-derived mesenchymal cells intermingle between the growing cords of hepatocytes, which are secreting large amounts of VEGF, and differentiate into endothelial cells. Then, these cells secrete PDGF-BB and recruit further mesothelium-derived mesenchymal cells, inducing their differentiation into stellate cells. Thus, a balance is established between the populations of endothelial and stellate cells, depending on the predominance of endotheliogenic or musculogenic signals, derived from the hepatocytes or the endothelium, respectively. A similar model has been proposed to explain the contribution of EPDCs to coronary development (Pérez-Pomares et al., 2002b).

In a set of experiments, we have shown that the proepicardium, which is ontogenetically homologous and morphologically continuous with the liver mesothelium, is able to contribute endothelial and stellate cell progenitors to the hepatic sinusoids both in vivo and in vitro. Although the proepicardium has just been used as a clean source of mesothelial and mesothelium-derived cells, we have described the in

ovo quail-to-chick chimerism experiments as an "experimentally induced invasion," without excluding a real possible contribution, not experimentally forced, of proepicardial cells to the developing liver. We think that this finding is significant for several reasons. First, the proepicardium is a mesothelial proliferation that is normally transferred to the heart where it spreads and forms the epicardium, the subepicardium, and the cardiac vessels (Manner et al., 2001). We have published elsewhere experimental evidence that mesothelium-derived cells can contribute to the coronary endothelium and smooth muscle cells (Pérez-Pomares et al., 2002b). Because proepicardial cells can form sinusoid-like vessels in the hepatic primordium, it seems that intrinsic, hepatic-specific progenitors of the sinusoids are not the only required cells for the establishment of these vessels. On the other hand, some of the chimeric sinusoidal endothelial cells were CCFSE-positive, reinforcing the hypothesis of a mesothelial origin at least for part of the sinusoidal endothelium. The dynamics of mesothelial and mesothelium-derived cells as well as their differentiation abilities have also been tested in vitro by creation of chimeric (quail proepicardium/chick liver) cell aggregates. These series of experiments proved that proepicardial mesothelial cells can immigrate into the core of the aggregate from the outer surface and differentiate into vascular endothelial cells that contribute to the formation of the small sinusoidal spaces, which spontaneously form inside these aggregates.

We insist that, in our interpretation, these results do not directly suggest that proepicardium is a normal source of mesenchymal cells for the developing liver. Instead, we think that the embryonic coelomic mesothelium constitutes a source of pluripotential mesenchymal cells for the developing visceral organs, as we have previously hypothesized (Muñoz-Chápuli et al., 1999). These cells would contribute to the vascular and connective tissue of these organs. We should consider now, in relation with this proposal, an interesting question that has been raised by some studies which have shown that the endothelial progenitors of some endodermal organs, such as liver or pancreas, play an inductive role before they constitute the vascular plexus (Matsumoto et al., 2001). The inductive role of the transverse septum mesenchyme (and other mesenchymes derived from the lateral plate) for the growth and differentiation of the hepatic endodermal cells was known since the classic works of Le Douarin (1975). It has been shown that this inductive activity correlates with the expression of IGF-I in the septum transversum mesenchyme (Streck and Pintar, 1992). Since we are suggesting a common origin of endothelial cells of heart and liver from mesothelium-derived mesenchymal cells, it must be emphasized that the epicardium and the EPDCs (which include at least part of the coronary endothelial progenitors) also play an essential role in the cardiac development. Experimentally induced delay in the epicardial covering results in a hypoplastic compact myocardium (Pérez-Pomares et al., 2002a). The same result is obtained after knockout of genes whose expression is restricted to the epicardium, such as the transcription factor WT1 (Kreidberg et al., 1993; Moore et al., 1999). It has been suggested that an unknown signal is delivered from the epicardium and EPDCs to the myocardium, a signal whose production depends on the retinoic acid signaling system (Pérez-Pomares et al., 2002a). Liver mesothelium-derived cells express WT1 (Fig. 2) and RALDH2 (not shown), a key enzyme in the synthesis of retinoic acid, and probably contribute to the liver endothelium according to our findings. Therefore, it is possible that a common signaling system located in the embryonic mesothelium and mesothelium-derived cells can account for the angioblast/endodermal cell interaction, which has been revealed to be essential for the development of liver, pancreas, and other organs (Matsumoto et al., 2001).

EXPERIMENTAL PROCEDURES

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.

Histology

To obtain semithin sections, quail embryos from stages H/H17 and H/H18 were fixed in 1% paraformaldehyde, 1% glutaraldehyde in phosphate buffered saline (PBS), washed and post-fixed in 1% OsO_4 for 90 min. After washing, the embryos were dehydrated in an ethanolic series finishing in acetone and embedded in Araldite 502. Semithin (0.5–1 μm) sections were obtained in a Reichert UMO-2 ultramicrotome and stained with toluidine blue.

For WT1 immunohistochemistry, the embryos were excised and cryoprotected in 10%, 20%, and 30% sucrose solutions where they were kept at 4°C until they sank. Then the embryos were embedded in OCT and snap frozen in liquid nitrogen-cooled isopentane. The frozen embryos were sectioned in a cryostate, and 14-μm sections were collected on poly-L-lysine–coated slides and fixed for 10 min in 1:1 methanol-acetone at −20°C. Then the sections were rehydrated in PBS and processed as described below.

Fluorescent Labeling of the Liver Mesothelium

The liver mesothelium was labeled with CCFSE (5,6-carboxy-2′,7′-dichlorofluorescein diacetate succinimidyl ester, Molecular Probes, Eugene, OR), a dye that 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 (Sun et al., 2000; Morabito et al., 2001). In several experiments, the dye was directly injected into the coelomic cavity of 39 chick and quail embryos at stages H/H15–21. The injection was done through a small incision performed in the vitelline and chorionic membranes. Approximately 2 μl of a 1/50 dilution of the stock solution of CCFSE (6.26

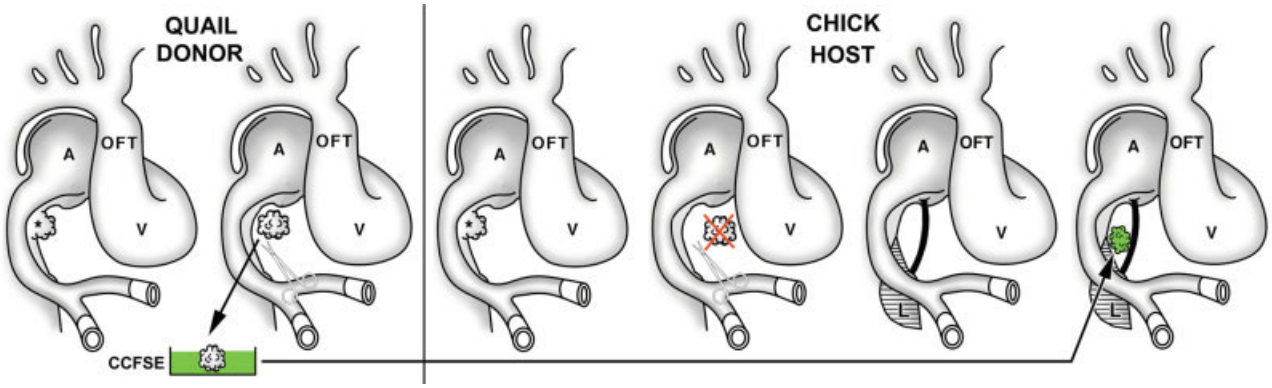

Fig. 6. Construction of quail-to-chick chimaeras. Quail proepicardia (asterisks) were isolated, labeled in a CCFSE solution and grafted into the coelomic cavity of chick embryos. In these chick host embryos, which had previously undergone a proepicardial ablation, a piece of the eggshell membrane (in black) was used to hold the donor proepicardium over the liver primordium. Modified from Pérez-Pomares et al. (2003) with permission of the editors. A, atrium; L, liver; V, ventricle; OFT, outflow tract. (Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.)

mg/ml in dimethyl sulfoxide) in Pannett-Compton saline was injected per embryo. The embryos were reincubated for 1, 3, 10, 24, 48, and 72 hr (3, 6, 5, 14, 9, and 2 embryos, respectively). Then the embryos were fixed for 2 hr in 4% paraformaldehyde, dehydrated, and paraffin-embedded.

Induced Proepicardial Invasion of the Liver

H/H16–17 quail embryos were injected in ovo with CCFSE as indicated above and reincubated for 30 min at 37°C. After the reincubation period, the embryos were excised, extensively washed in sterile EBSS, and the proepicardium isolated by using tungsten needles.

H/H16–17 host chick embryos were prepared as follows (see Fig. 6): small openings were made with tungsten needles through the vitelline and chorionic membranes to expose the coelomic cavity. The host (chick) proepicardium was manually ablated by using tungsten needles and iridectomy forceps (Pérez-Pomares et al., 2003) and discarded. Then, a small piece of the eggshell membrane was placed between the sinoatrial sulcus and the caudal vitellin veins so that the membrane faced the surface of the liver primordium. Finally, the CCFSE-labeled donor (quail) proepicardium was transferred into the donor and held against the surface of the liver primordium by means of the eggshell membrane insert.

After the operation, the eggs were sealed with Scotch tape and reincubated for 24 hr to obtain H/H20–21 embryos. Seven chimeras were obtained in this way, which were fixed in 1% paraformaldehyde, dehydrated in a graded series of ethanol, cleared in butanol, and embedded in Histosec (Merck). Finally, 10-μm serial sections were mounted on poly-L-lysinated (0.01%, Sigma) microscope slides (Menzel-Gläser). Immunohistochemical characterization of the chimeric tissues was performed as described below.

Immunohistochemistry

Briefly, deparaffinized slides were washed with Tris-PBS, and nonspecific binding sites were saturated for 30 min with 16% sheep serum, 1% bovine serum albumin, and 0.5% Triton X-100 in Tris-PBS (SBT).

For single-fluorescent labeling, the slides were incubated overnight at 4°C in the primary antibodies diluted in SBT (polyclonal anti-cytokeratin, and QH1, QCPN, and anti-α-actin monoclonals). Incubation of the primary antibody was followed by incubation in a secondary biotinylated goat anti-rabbit IgG (for cytokeratin), a biotinylated goat anti-mouse IgG (for SMC α-actin), or a fluorescein isothiocyanate (FITC) -conjugated goat anti-mouse IgG (Sigma). When required, a final incubation in FITC-conjugated ExtrAvidin (Sigma) at a 1:150 dilution in TPBS was performed.

Fluorescent double-labeling was always performed by using a combination of polyclonal and monoclonal primary antibodies. Briefly, deparaffinized slides were washed with Tris-PBS, and nonspecific binding sites were saturated for 30 min with 16% sheep serum, 1% bovine serum albumin, and 0.5% SBT. The slides were then incubated overnight at 4°C in the primary polyclonal anti-WT1. Then, the slides were washed in TPBS (3 × 5 min), and incubated for 2 hr at room temperature with Cy5-conjugated donkey anti-rabbit IgG (Jackson Laboratories). Incubation of monoclonal primary antibodies (anti-SMC α-actin or QH1) was performed overnight at 4°C. After washing, the sections were incubated in FITC-conjugated goat anti-mouse IgG (2 hr, room temperature).

Slides from tissue labeled with CCFSE were incubated with anti-SMC α-actin or QH1 primary antibodies (4°C, overnight), washed and incubated in a Cy5-conjugated donkey anti-mouse secondary antibody, and washed and incubated in FITC-conjugated ExtrAvidin (Sigma).

All the sections were mounted in an 1:1 PBS/glycerol solution and analyzed by using a Leica TCS/NT laser scanning confocal microscope. In some cases, propidium iodide was used as a nuclear counterstaining agent.

The monoclonal antibody anti-QH1 labels all quail endothelial, hemopoietic, and macrophage cells, whereas the QCPN antibody stains all quail nuclei. Both were obtained from the Developmental Studies Hybridoma Bank. The monoclonal anti-smooth muscle cell SMC α-actin (clone 1A4, Sigma) was used at a 1:100 dilution. The polyclonal anti-bovine epidermal cytokeratin (Z622, Dakopatts, Denmark) was used at a 1:200 dilution. The affinity-purified anti-WT1 polyclonal antibody (sc-192, Santa Cruz Biotechnologies) was developed by immunizing rabbits against the 19 carboxy-terminus amino acids of the human WT1 protein, but it specifically recognizes the avian WT1 protein (Carmona et al., 2001). It was used at a 1:50 dilution. FITC, Cy5-conjugated, and biotinylated secondary antibodies were diluted 1:100 in TPBS. FITC ExtrAvidin was diluted 1:150 in TPBS. Negative controls were always performed incubating the sections with SBT instead of the primary antibody.

Coculture of Embryonic Liver and Proepicardial Cells

H/H21 chick liver primordia were dissected and trypsinized (2.5% trypsin–ethylenediaminetetraacetic acid, Gibco) for 15 min at 37°C. Digestion was stopped with 10% fetal bovine serum (Gibco) in M199 medium (Gibco). After centrifugation (200 rpm, 5 min), cells were resuspended in fresh M199 supplemented with 100 ng/ml basic fibroblast growth factor (bFGF; Peprotech), 1% chick serum (Sigma), and 100 IU penicillin/streptomycin (Gibco). H/H16–17 quail proepicardial cells were trypsinized as described and pooled in fresh M199 supplemented with 100 ng/ml bFGF, 1% serum, and 100 IU penicillin/streptomycin. In some cases, quail proepicardial cells were CCFSE labeled (15 min incubation in medium, 37°C, 5%CO_2).

Hanging drop cultures (Rudnicki and McBurney, 1987) were set in 35-mm Petri dishes. In some cases, chick and quail cells were mixed before seeding. In this latter experiment, around 60,000 chick liver cells/2,000 quail proepicardial cells per drop (20 μl) were incubated at 37°C, 5% CO_2. In a different group of experiments, 60,000 chick liver cells per drop were allowed for a preaggregation period (2–3 hr, 37°C, 5% CO_2) and then quail proepicardial cells (2,000 per drop) were added. Control aggregates were made only of chick liver cells. The incubation period was 24 hr in all cases.

Cell aggregates were fixed in 1% paraformaldehyde, dehydrated in ethanol, cleared in butanol, embedded in Histosec, and sectioned at 5 μm. Quail proepicardial cells were tracked by CCFSE labeling or by QCPN immunohistochemistry as described above.

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