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Common Epicardial Origin of Coronary Vascular Smooth Muscle, Perivascular Fibroblasts, and

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Previous studies have shown that during avian heart development, epicardial and coronary vascular smooth muscle precursors are derived from the proepicardium, a derivative of the developing liver. This finding led to a model of coronary vascular development in which epicardial cells migrate over the postlooped heart, followed by migration of committed endothelial and smooth muscle precursors from the proepicardium through the subepicardial matrix where the coronary arteries develop. Here we show that epicardial cells undergo epithelial–mesenchymal transformation to become coronary vascular smooth muscle, perivascular fibroblasts, and intermyocardial fibroblasts. We began by establishing primary cultures of quail epicardial cells that retain morphologic and antigenic identity to epicardial cells *in vivo.* Quail epicardial monolayers stimulated with serum or vascular growth factors produced invasive mesenchyme in collagen gels. Chick epicardial cells labeled *in ovo* with DiI invaded the subepicardial extracellular matrix, demonstrating that mesenchymal transformation of epicardium occurs *in vivo.* To determine the fates of epicardially derived mesenchymal cells, quail epicardial cells labeled *in vitro* with *LacZ* were grafted into the pericardial space of E2 chicks. These cells attached to the heart, formed a chimeric epicardium, invaded the subepicardial matrix and myocardial wall, and became coronary vascular smooth muscle, perivascular fibroblasts, and intermyocardial fibroblasts, demonstrating the common epicardial origin of these cell types. A general model of coronary vascular development should now include epicardial–mesenchymal transformation and direct participation of mesenchyme derived from the epicardium in coronary morphogenesis.

INTRODUCTION

During heart development, coronary vessels arise by discontinuous vasculogenic events in which endothelial cells assemble into nascent capillaries within the extensive subepicardial extracellular matrix separating the myocardial wall from the epicardial epithelium (Mikawa and Fischman, 1992). Once formed, nascent coronary vessels invade the aorta to establish forward coronary perfusion (Bogers *et al.*, 1988, 1989; Waldo *et al.*, 1990; Bouchy *et al.*, 1996). Aortic and coronary vascular smooth muscle are both physiologically and ontologically distinct (Topouzis and Majesky, 1996; Glukhova *et al.*, 1990; Majesky *et al.*, 1992; Aikawa *et al.*, 1993; Frid, *et al.*, 1994). Whereas smooth muscle in the great vessels is derived from the cardiac neural crest, the origin of coronary vascular smooth muscle cells (CVSMCs)[2] and pericytes is unknown (LeLievre and LeDouarin, 1975; Hood and Rosenquist, 1992; Waldo *et al.*, 1994; Kirby and Waldo, 1995). In general models of vasculogenesis, smooth muscle cells and pericyte precursors are postulated to be recruited from the local mesenchyme to the vascular wall by chemotactic factors secreted by endothelial cells (Tomanek, 1996; Folkman and D'Amore, 1996; Lindahl *et al.*, 1997). We therefore reasoned that the key to determining the origin of CVSMC and perivascular fibroblasts was to define the developmental pathway taken by cells to become subepicardial mesenchyme.

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[2] Abbreviations used: CVSMC, coronary vascular smooth muscle cell; bFGF, basic fibroblast growth factor; EGF, epidermal growth factor; VEGF, vascular endothelial growth factor; TGFβ, transforming growth factor β; mAb, monoclonal antibody.

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Formation of the coronary vessels is intimately associated with epicardial development in vertebrates (Manasek, 1969; Ho and Shimada, 1978; Viragh and Challice, 1981; Komiyama, *et al.*, 1987; Kuhn and Liebherr, 1988; Fransen and Lemanski, 1990; Manner, 1992; Viragh *et al.*, 1993; Muñoz-Chapuli *et al.*, 1996). In the chick and quail, the epicardium is derived from the proepicardial outpouching of the primordial liver which protrudes from the ventral aspect of the vitelline vein and attaches to the dorsal wall of the sinus venosus by stage 16 (E2) (Viragh *et al.*, 1993). As the epicardium forms, epicardial cells proliferate and migrate cranially to envelop the heart from the sinus venosus to the outflow tract. The importance of the epicardium during coronary vascular development was demonstrated in the mouse by inactivation of the genes encoding integrin $\alpha 4$ (Yang *et al.*, 1995) and vascular cell adhesion molecule-1 (VCAM-1, Kwee *et al.*, 1995). In these mutant mice, formation of the epicardium is deficient and coronary vasculature fails to form, suggesting a direct relationship between epicardial and coronary vascular development.

Injection of *LacZ* expressing replication-defective retroviruses into the chick proepicardium demonstrated that CVSMC precursors are present in the proepicardium and staining of the proepicardium with smooth muscle markers demonstrated expression of smooth muscle genes in cells of the precursor organ (Mikawa and Gourdie, 1996). These findings suggested that committed smooth muscle founder cells migrate directly from the proepicardium into the subepicardium via the dorsal mesocardium. However, in our preliminary experiments, founder cells expressing early smooth muscle markers could not be identified in the subepicardial mesenchyme. Thus, we propose an alternate mechanism by which CVSMC and perivascular fibroblast precursors can reach the subepicardial matrix. We present direct evidence that the epicardium gives rise to the subepicardial mesenchyme through epithelial–mesenchymal transformation. Invasive epicardially derived mesenchyme is then capable of differentiation into CVSMCs, perivascular fibroblasts, and intermyocardial fibroblasts.

MATERIALS AND METHODS

Eggs and Cell Culture

Chick (*Gallus gallus domesticus*) and quail (*Cotournix cotournix japonica*) eggs were incubated in humidified egg incubators at 38°C. All cell culture was carried out at 37°C in humidified tissue culture incubators with 5% CO_2 in air. Staging of chick and quail embryos is described throughout the text using either the staging method of Hamburger and Hamilton (1951) or in terms of embryonic day (e.g., E2 is embryonic day 2). Hearts were removed from embryos for primary culture in Ca^{2+}, Mg^{2+}-free PBS and then placed in 30-mm tissue culture dishes containing M199 medium (Earle's salts). Epicardial monolayers grew circumferentially from beating hearts in 1–2 days. Some endothelial cells but no myocytes were found by immunostaining the monolayers. Endothelial cells were counted using the light microscope and the total number of epicardial cells was estimated by measuring the area of each monolayer and dividing by the average diameter of an epicardial cell. Percentage of endothelial cells was determined by dividing the number of QH-1-positive cells by the total estimated number of epicardial cells and multiplying by 100%. Monolayers were estimated to be greater than 97% epicardial cells by this method.

Collagen Gel Invasion Assays

Collagen gels (Vitrogen 100, Collagen Corp.) were prepared by the pH balancing method in 24-well plates. Invasion assays were carried out by explanting quail proepicardia or E3–E8 hearts onto drained collagen gels. Epicardial monolayers were allowed to form on the surface of the collagen for 1–5 days in serum-free M199 medium prior to stimulation with 10% (v/v) fetal bovine serum or growth factors for 24 h. Prior to fixation, medium was removed from each well and formaldehyde (4% v/v) in PBS with Triton (0.1% v/v) was added for 2 h. Wells were washed twice with PBS and either incubated with antibodies or dehydrated in ethanol for paraffin embedding. Embedded collagen gels were sectioned at 10-μm thickness and individual sections were mounted on Superfrost Plus slides (Fisher). After sections were dewaxed and rehydrated, cells were visualized with acridine orange (Molecular Probes) and imaged on a Zeiss inverted confocal microscope. Vascular growth factors that stimulated epithelial–mesenchymal transformation of cultured quail epicardial cells in our experiments were bFGF (R&D Systems), rhVEGF (R&D Systems), and rhEGF (Collaborative Research).

Fluorescent Microsphere Labeling of Epicardial Cells

E4 quail embryos were taken from eggs and placed in M199 medium. The pericardial sac was carefully removed and the medium was replaced with M199 medium supplemented with 2% fetal bovine serum and 10 μl of latex green fluorescent microspheres (0.2 μm, Polysciences). Embryos were placed in the incubator for 1 h. After incubation, embryos were washed several times with M199 medium, and hearts were removed and either explanted onto drained collagen gels or placed in OCT (Fisher) and frozen.

DiI Labeling of Chick Epicardium

Chick embryos were incubated at 38°C in humidified incubators until the fourth day of development (stage 25). The vitelline membrane was opened to expose the pericardial sac. The pericardial membrane was cut with 0.3-mm scissors (Fine Science Tools) in a caudal to cranial direction leaving a vertical incision exposing the beating heart. Embryos were placed at 4°C for 20–30 min to slow the heart beat. Borosilicate microinjection needles were pulled over a flame and the tip of the needle was melted into a glass ball with a flame. Needles were dipped in DiI/ethanol (Molecular Probes) several times to apply the dye to the glass tip. Dye-coated needles were placed in a micromanipulator and positioned over the exposed epicardium of chilled embryos. Labeling needles were lowered to touch the surface of the epicardial monolayer. Dye was allowed to transfer to the cells for 2 min. Hearts were usually labeled at several sites on the surface of the right ventricle and in the right atrioventricular groove. After the needle was touched to the surface of the heart it was raised and eggs were placed back into the incubator. After incubation, hearts were removed from the embryos, fixed for 2 h in 4% (v/v) formaldehyde in PBS, and washed twice in PBS prior to mounting for confocal microscopy.

Adenoviral Infection and LacZ Staining

The adenovirus (*AdCMVlacZ*) used in this study was a replication-defective type-5 adenovirus carrying the *lacZ* gene driven by the CMV promoter. β-galactosidase expressed from *AdCMVlacZ* included a nuclear localization signal, resulting in a stained nucleus in infected cells. The adenoviral genome is rarely integrated into the chromosome of infected cells and is not usually clonally passaged to daughter cells during mitosis (Brody and Crystal , 1994; Crystal *et al.*, 1994; Zabner *et al.*, 1994). Thus, labeled cells in grafted hearts are likely to be the cells originally infected. High titer virus was serially diluted in M199 medium. Plates containing quail epicardial cells in serum free medium were washed in Ca^{2+}, Mg^{2+}-free PBS and the medium containing virus was added. Cells were incubated in this medium for 1 h at room temperature before placing back into the incubator. After 24 h of incubation, plates were removed and washed in PBS and cells were fixed in formaldehyde (4% v/v) PBS. After fixation, plates were washed with X-gal (Gibco) staining buffer for 12–24 h (Vernet *et al.*, 1993). Titration experiments allowed us to determine the optimal dilution required for a given lot of virus to achieve greater than 95% infection without cytopathological effect.

Grafts of Cultured Cells

In grafting experiments, primary cultures of epicardial cells were made from explanted E3 quail hearts on 30-mm tissue culture plates (Falcon). Approximately 10–15 hearts were immersed in serum-free M199 medium and allowed to touch the plates for 12–18 h. Hearts were removed to new plates and the remaining epicardial cells were incubated in M199 medium until the grafting experiment was carried out; usually 5–7 days. Hearts were transferred to new plates for up to 3 days without loss of purity in the culture. Infection was carried out for 1 h prior to the time when cells were harvested for grafts. After infection, plates were washed six times with equal volumes of M199 medium and twice with Ca^{2+}, Mg^{2+}-free PBS. Cells were harvested from plates using trypsin (0.05%) under standard conditions except that the use of serum to inactivate the trypsin was omitted. Resuspended cells were placed into drawn glass microcapillary needles (FMC) according to the method developed by B. A. Williams (personal communication). E2 (stage 15) chick embryos were surgically prepared by making an incision to open the vitelline membrane, thereby exposing the pericardial cavity. The tip of the loaded needle was broken on the surface of the sterilized egg shell. A bolus of cells was placed inside the pericardial sac, between the chick proepicardium and the beating myocardial wall. The myocardial wall of the host was not injured or injected during this procedure. After the graft, eggs were sealed with parafilm and placed in the incubator for 1–17 days.

Antibodies, Immunofluorescence, and Immunohistochemistry

E3 quail hearts were explanted to glass slides (Superfrost Plus, Fisher) immersed in M199 medium. Cellular monolayers were fixed in 4% (v/v) formaldehyde, 0.1% (v/v) Triton X-100 in PBS and then stained with antibody (Ab) markers for (1) endothelial cells; mAb *QH-1;* (Coffin and Poole, 1988) (2) cardiac myocytes; myosin specific mAb MF20 (Developmental Studies Hybridoma Bank), and (3) epicardial cells; rabbit anti-bovine Cytokeratin (Dako). Immunofluorescence was carried out using Texas red and fluorescein-coupled secondary Abs or rhodamine phalloidin (Molecular Probes). Vector red (Vectastain) was used in immunohistochemical studies using visible light microscopy. Other primary Abs used in this study were (1) *smooth muscle actin* mAb (Sigma); (2) and *caldesmon* clone CD-5 (Sigma).

RESULTS

Epithelial–Mesenchymal Transformation of Epicardial Cells in Vitro

We set out to determine the developmental pathway taken by CVSMC precursors during migration to the subepicardium. In the avian heart, the developing epicardium is a polarized keratinized epithelium which is highly reactive with antibodies to cytokeratins (Vrancken Peeters *et al.*, 1995). The epicardial surface of a whole quail heart stained with pan anti-cytokeratins is shown in Fig. 1A. Cells are uniform in size and regularly spaced, with cell–cell attachments on all sides. When proepicardial organs from E2 quail embryos or hearts from E3–E8 embryos were explanted onto cell culture plates or collagen gels in serum-free medium, cells migrated from the surface to form uniform cellular monolayers (Figs. 1B and 1C) that were morphologically identical to the epicardial surface of the heart *in vivo* (Fig. 1A). When proepicardia were removed from E2 hearts prior to explanting them to collagen, monolayers did not form (data not shown), indicating that these monolayers did not result from endocardial or myocardial cell migration.

Cells within cultured epicardial monolayers expressed cytokeratins (Fig. 1C), and staining of cytoplasmic monofilaments in cultured epicardial cells was morphologically identical to epicardial cells covering the surface of fixed whole E3 hearts (Fig. 1A). Immunolocalization of markers for epicardial cells, endothelial cells, smooth muscle, and myocardial cells in monolayers revealed that these primary cultures were 97% epicardial cells. The remaining 3% of the cells were endothelial cells as determined by staining with the QH-1 antibody (Coffin and Poole, 1988). Cultured epicardial cells were not immunoreactive with MF-20 (muscle myosin) but were ubiquitously immunoreactive with anticytokeratin. These experiments demonstrate that cellular monolayers growing from explanted proepicardia or hearts were epicardial.

To define the invasive potential of epicardial cells in response to growth factors proposed to function during vasculogenesis, we explanted E3 or E8 quail hearts to the surface of collagen gels and allowed epicardial monolayers to form on the surface. Hearts and monolayers were then cultured in serum-free M199 medium (Fig. 2A), M199 medium supplemented with serum (10% v/v, Fig. 2B) or serum-free M199 medium supplemented with growth factors postulated to be important for vasculogenesis (Gendron *et al.*, 1996; Folkman and D'Amore, 1996). We found that the epicardial monolayer was minimally invasive when maintained in M199 medium. When epicardial monolayers were stimulated by serum, basic fibroblast growth factor (bFGF, Fig. 2C), recombinant human vascular endothelial growth factor (rhVEGF, data not shown), or recombinant human

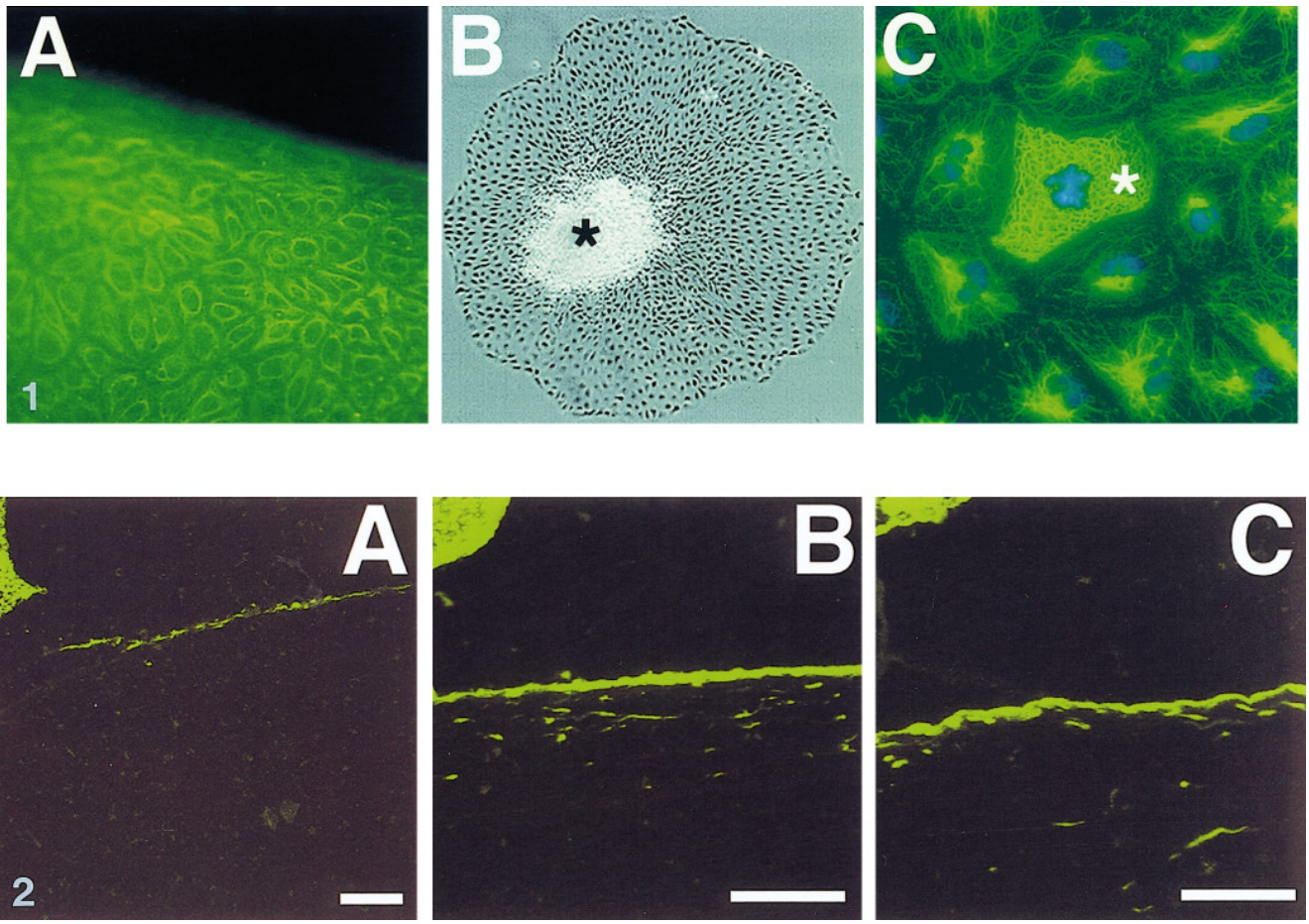

FIG. 1. Culture of epicardial cells and *in vitro* observations of epithelial–mesenchymal transformations. (A) Epifluorescent image of the surface of a quail heart stained in whole mount with anticytokeratin. (B) Monolayer of epicardial cells grown from a proepicardial organ (*). Epicardial monolayers can be grown in serum-free culture from proepicardial organs or explanted hearts of various ages. (C) Epifluorescent image of cultured epicardial cells, similar to (B), stained with anticytokeratin and DAPI showing that the shape of the cells and subcellular accumulation of keratin monofilaments in cultured cells is identical to that observed in cells on the surface of the developing heart (A). In **C,** the large cell in the center (*) is dividing.

FIG. 2. Serum and bFGF stimulate quail epicardial monolayers to invade collagen gels *in vitro.* Confocal fluorescent images of 10-μm cross sections of quail epicardial monolayers grown on collagen gels either maintained in serum-free M199 medium (A) or stimulated by either serum (10% v./v) (B) or bFGF (50 ng/ml) (C). A–C show collagen gel cross sections containing hearts (upper left hand corner) and cellular monolayers. The gel matrix is oriented to the bottom in each panel. Prior to imaging, sections were stained with acridine orange to stain both nuclei and cytoplasm. Bars represent 100 μm.

epidermal growth factor (rhEGF, data not shown), we observed a much greater number of invasive cells in the collagen matrix. The epicardium on the gel surface was also thicker than in serum-free controls, suggesting that epicardial cells had proliferated during serum and growth factor treatment. We concluded from these results that epicardial cells had the capacity to undergo epithelial–mesenchymal transformation *in vitro* in response to stimulation by fetal bovine serum and vascular growth factors.

Mesenchymal cells in collagen gels derived from epicardial monolayers *in vitro* were similar in shape to mesenchymal cells observed in the subepicardium *in vivo* (Figs. 3A and 3B). To demonstrate that epicardial cells on the surface of collagen gels underwent epithelial–mesenchymal transformation to give rise to mesenchymal cells, we labeled epicardial cells at the heart surface in E4 cultured quail embryos with 0.2-μm fluorescent microspheres. Fluorescent microspheres are actively taken up in cells by pinocytosis to label cytoplasmic vesicles and only cells directly exposed to the medium containing fluorescent microspheres are labeled. After labeling, hearts were either fixed immediately for sectioning or removed and explanted onto collagen gels. Sections of labeled hearts revealed that only cells at the surface of the heart had taken up fluorescent microspheres (Fig. 3C). Cells labeled with fluorescent microspheres were found within either the epicardium or the subepicardial matrix. In

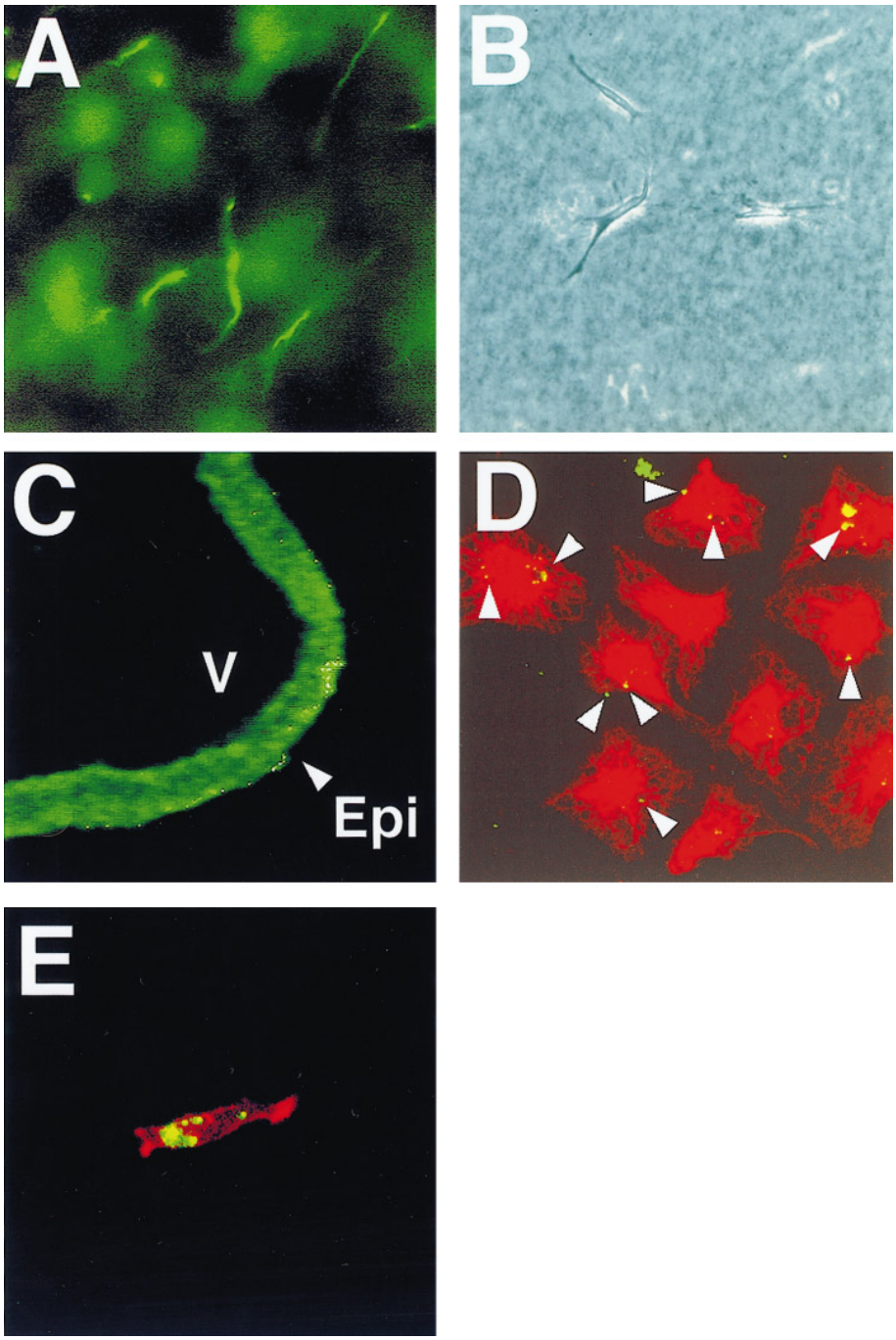

FIG. 3. *In vitro* observation of epicardial epithelial-mesenchymal transformations. (A) Epifluorescent image of subepicardial mesenchyme in a whole E4 quail heart stained with anticytokeratin. The cells in this field are approximately 15 μm below the epicardial surface. (B) Phase-contrast image of mesenchyme-like cells in a collagen gel found after epicardial monolayers were stimulated with serum. (C) Frozen section of an E4 quail heart labeled with green fluorescent microspheres and immediately fixed. Epicardial surface (Epi) is indicated by the arrowhead, the ventricular cavity is indicated by a V. Fluorescent microspheres can be observed at the epicardial surface and within the subepicardium. (D) Cells on the surface of a collagen gel stained with anticytokeratin (red) and labeled with green fluorescent microspheres (arrowheads). (E) Phase-contrast image of a cytokeratin-expressing (red) mesenchymal cell within the collagen gel matrix labeled with fluorescent microspheres.

subsequent cell invasion assays, labeled hearts were removed from collagen gels and monolayers were stimulated with serum. Immunofluorescent analysis of collagen gels revealed that cells within the monolayer expressed cytokeratins and that a subset of the cells within the monolayer was labeled with fluorescent microspheres (Fig. 3D). Following

serum stimulation, we observed cytokeratin-positive mesenchymal cells within the collagen matrix labeled with fluorescent microspheres, confirming that invasive cells originated from the epicardial layer of the heart (Fig. 3E). In no instance did we observe fluorescent latex microsphere labeling in non-cytokeratin-expressing cells either on the gel surface or within invasive cells.

Epicardial–Mesenchymal Transformation in Vivo

To determine whether epicardial cells invade the subepicardial matrix during normal development *in vivo*, we labeled patches of cells on the ventricular or atrioventricular epicardial surface of E5 chick embryos with the plasma membrane vital dye DiI (Honig and Hume, 1989). In control experiments designed to verify that DiI labeling was confined to cells within the epicardium, hearts were fixed either immediately or 2–4 h after labeling. We observed that labeling with DiI occurred in circular patches of about 400 μm on the heart surface (Fig. 4A). Transverse serial *z*-sections of DiI-labeled hearts fixed immediately after dye transfer demonstrated that DiI was present exclusively in the epicardium (Fig. 4B). We did not detect any DiI-labeled cells in the subepicardium in hearts fixed up to 4 h after labeling, indicating that DiI did not transfer to subepicardial mesenchymal cells during the labeling step and that epicardial cells did not undergo epithelial–mesenchymal transformation within the time tested. We labeled 24 E5 chick hearts at 68 individual epicardial sites and allowed them to develop for 48 h prior to fixation. When we analyzed these hearts with the laser confocal microscope, we observed labeled mesenchymal cells either at the epicardial surface or within the subepicardial matrix at depths of up to 30 μm (Figs. 4C and 4E). Figure 4D shows an epicardial surface in which no DiI-labeled cells were found and Fig. 4E shows a deeper focal plane in which two DiI-labeled cells were found 16 μm below the epicardial surface shown in Fig. 4D. The position of DiI-labeled mesenchymal cells relative to the epicardial surface was equivalent to that of cytokeratin-expressing mesenchymal cells observed in whole-mount immunohistochemical experiments (Fig. 3A). Surprisingly, after 48 h, DiI labeling was no longer found in large patches. While individual labeled cells were found within the epicardium (not shown), most were observed to be within the subepicardial matrix. Rapid dispersion of the DiI-labeled patches on the epicardial surface was unexpected and suggested that epicardial cells may either migrate within the epicardial epithelium or leave the epicardial surface via epithelial–mesenchymal transformation. Together, these observations provided direct evidence for epicardial–mesenchymal transformation within the subepicardial matrix *in vivo*.

Developmental Potential of Epicardially Derived Mesenchyme

Our cell culture and DiI-labeling experiments demonstrated that epicardial cells are capable of epithelial–mesenchymal transformation *in vitro* and that they invade the subepicardial matrix *in vivo*. However, these experiments did not reveal the fates of mesenchyme derived from epicardium. To determine the fate(s) of epicardial cells, we infected primary cultures of quail epicardial cells with an adenoviral strain (*AdCMVlacZ*) that drives expression of *LacZ* in the nucleus. In titering experiments we observed that *AdCMVlacZ* readily infected quail epicardial cells without any obvious deleterious effect. We were able to define conditions which resulted in nearly 100% infection of the epicardial cells grown in primary cultures (Fig. 5A). *AdCMVlacZ*-infected quail epicardial cells were then grafted to the myocardial wall of E2 (stage 15) chick hearts and embryos were allowed to develop for several days. Our method is shown in Figs. 5B and 5C. In preliminary experiments, we removed, fixed, and stained hearts for *LacZ* expression after 1–3 days of development. In each case we found patches of *LacZ*-expressing cells on the dorsal surface of the heart (Fig. 5D). Labeled cells within the epicardium were observed peripheral to the central patch as if cells were migrating away from the initial site of adhesion. When these hearts were sectioned, we observed labeled cells at the epicardial surface (Figs. 5F and 5G), within the subepicardium (Figs. 5E–5G) and, in some hearts, scattered through the myocardium (Fig. 5H). Since quail cells were infected 1–2 h prior to the grafting surgery, the presence of large patches of *LacZ*-expressing cells on the dorsal heart surface indicated that many of the cells had survived the grafting procedure. Because labeled cells were principally found within the epicardium or in the subepicardium we concluded that when cultured quail epicardial cells were reintroduced into a stage appropriate host, they participated in normal epicardial development.

To determine the fates of grafted cultured epicardial cells and to test our hypothesis that epicardial cells undergo epithelial–mesenchymal transformation to become CVSMC and perivascular fibroblasts, we allowed grafted embryos to develop an additional 10–16 days. Strikingly, in 9 of 12 successful grafts, we observed visible clusters of labeled cells in the posterior interventricular or atrioventricular groove in the vicinity of the developing coronary arteries (Fig. 6A). For the three remaining hearts, labeled patches of cells were found in ventral aspects of the heart in the epicardium over the ventricle, atrium, or outflow tract. The propensity for grafts to adhere to the dorsal aspect of the heart (Figs. 5D and 6A) most likely reflected the initial position of adhesion of grafted cells near the dorsal mesocardium. The occasional presence of cells on the anterior aspect of the heart may reflect cell migration away from the point of adhesion on the dorsal mesocardium or original sites of adhesion on anterior myocardial surfaces.

Serial sections of hearts with labeled cells over the dorsal aspect indicated that there were labeled cells in the smooth muscle component of coronary arteries and veins as well as in perivascular fibroblasts surrounding the smooth muscle of the posterior descending coronary artery and in interstitial fibroblasts in the myocardium (Figs. 6B–6F). To confirm that labeled cells in mature vessels were CVSMCs, we stained sections with the smooth muscle antibody markers:

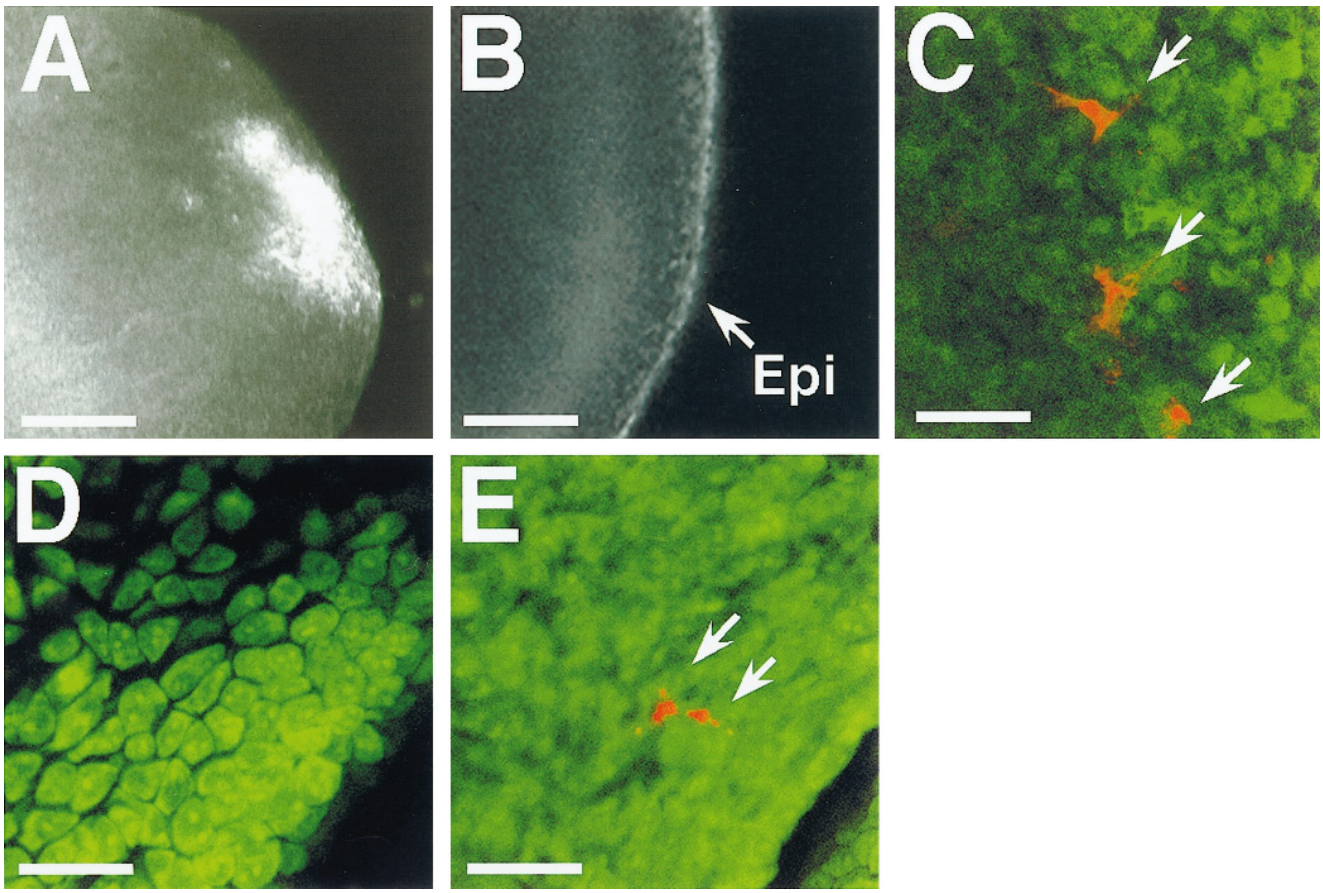

FIG. 4. *In vivo* observation of epicardial epithelial–mesenchymal transformations. (A) Surface confocal image of an E5 chick heart labeled with DiI and immediately fixed. This view of the heart surface demonstrates the size (400 μm diameter) and intensity of the DiI-labeled area produced with this technique. Bar is 200 μm. (B) Single image compiled from 25 transverse serial *z*-scans (2.5 μm) showing epicardial-specific staining (arrows) in a DiI-labeled heart fixed immediately after labeling. No DiI-stained cells were detected in the subepicardial matrix when hearts were fixed up to 4 h after labeling. Bar is 100 μm. (C–E) In cell-tracing experiments, hearts were fixed 48 h after DiI labeling and counterstained with fluorescein anti-mouse IgG. (C) Confocal image of DiI-labeled cells (arrows) extending 5–10 μm below the heart surface. (D) Confocal image of the epicardial surface of a second labeled heart in which no DiI-labeled cells can be seen. (E) Confocal image showing labeled cells (arrows) 16 μm below the epicardial surface shown in D. Bars in C–E are 20 μm.

anti-smooth muscle actin and anticaldesmon. We analyzed the *LacZ*-expressing cells in serial sections from eight grafted hearts and scored 2250 labeled cells in four distinct contexts (Table 1). Approximately 52% of the cells were identified as CVSMCs and 16% were identified as perivascular fibroblasts. CVSMCs expressed either smooth muscle actin or caldesmon when present in the vascular wall (Figs. 6B–6F). Neither *LacZ*-labeled perivascular fibroblasts nor neighboring chick mesenchyme were stained above background with either antibody, consistent with the hypothesis that smooth muscle is not specified until it reaches the vascular wall. We also found 28% of the total number of *LacZ*-labeled cells within the cardiac interstitium (Fig. 6E). Labeled cells in the interstitium expressed smooth muscle actin or caldesmon only when found in association with nascent vessels (Fig. 6C). Thus, the labeled intermyocardial cells could be cells migrating to neovascular locales, cardiac fibroblasts, or an unidentified cell-type within the myocardium. We also observed about 4% of the labeled cells in the endocardium (Fig. 6G) and atrioventricular cushions (data not shown), which most likely represent minor contamination of primary cultures with endothelial cells. Thus, while other fates of epicardially derived mesenchyme are possible, our data support a role for epicardium in providing mesenchymal cells to the subepicardium which ultimately become CVSMCs and perivascular and interstitial fibroblasts.

Labeled cells were found not only within epicardial coronary vessels but deeper within the developing vessels of the interventricular septum (Figs. 6C–6E). This observation suggests that the CVSMCs found in both types of coronary vessels are derived from the same pool of cells. The pathway by which these cells reach the deeper vessels has not been defined but may be correlated with our observations of labeled cells within the ventricular wall of earlier stage hearts

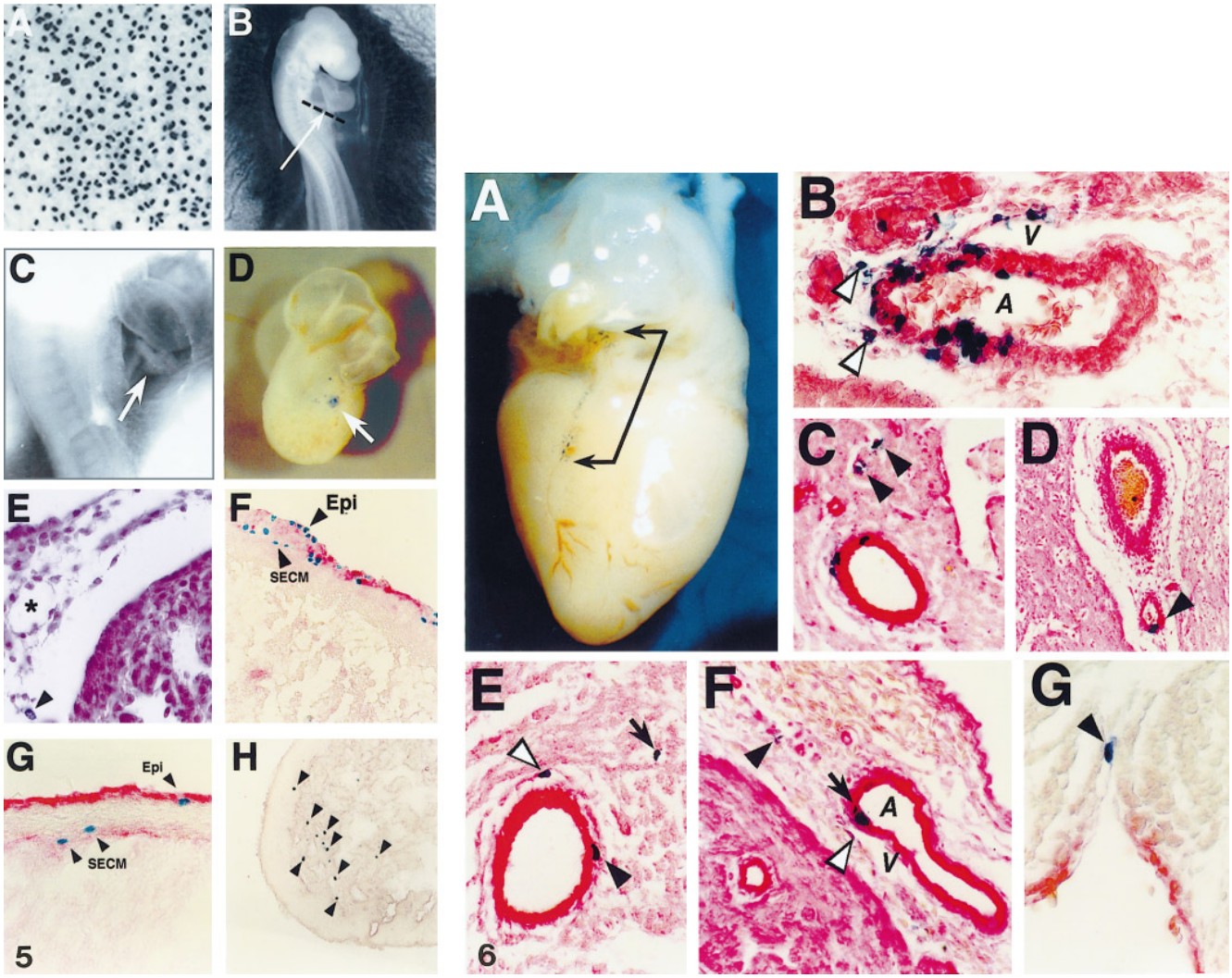

FIG. 5. Grafted epicardial cells participate in formation of the epicardium and invade the heart. Representative data from 12 grafted hearts. (A) High titer adenovirus (*AdCMVlacZ*) was tested in titration assays on parallel cultures to determine the optimal conditions (>95% infection) with which quail epicardial cells could be infected. Cells were fixed in the plate and stained with X-Gal buffer. Note the nuclear localization of *LacZ* staining (B) E2 (stage 15) chick embryo typical of host embryos for grafting studies. Dashed line shows the site of incision made during the surgery and the arrow points to the site of delivery of cells. (C) Grafted embryo in which the incision is clearly visible and the bolus of quail cells (arrow) can be seen along the sinus venosus region just posterior to the heart tube. (D) E4 (stage 23) chimeric heart stained with X-Gal buffer. Labeled cells can be seen in a central patch (arrow) and peripheral to this location. (E) Section of the heart shown in D showing a labeled nucleus (arrowhead) in the subepicardial matrix of the posterior atrioventricular groove in the vicinity of a nascent capillary (*). This section is counterstained with hematoxylin. (F–G) Cross sections of chimeric hearts postfixed and stained with X-Gal buffer. (F) Grafted heart with multiple *LacZ*-expressing cells in the epicardium (Epi) and within the subepicardial matrix (SECM). Section is stained with anticytokeratin (red). (G) Higher magnification of a second grafted heart showing one *LacZ* cell in the epicardium (Epi) and two *LacZ*-expressing cells invading the subepicardial matrix (SECM, arrowheads). Section is stained with anticytokeratin (red). (H) Section of a grafted heart in which *LacZ*-expressing cells were detected within the ventricular myocardium (arrowheads).

FIG. 6. Fate diversity of cultured epicardial cells. Representative data from 12 grafted hearts. Grafted hearts carrying quail epicardial cells infected with *AdCMVlacZ* were fixed in 4% formaldehyde (v/v) for 12–24 h and incubated in X-Gal buffer containing Bluogal (1 mg/ml, Gibco) for 12–24 h. After staining and imaging of labeled cells on the surface of whole hearts, hearts were embedded in paraffin for sectioning. All sections in this figure are at 10-μm thickness. Sections were stained with either anti-smooth muscle actin (B, D, F, G) or anticaldesmon (C, E). Background staining was observed in controls using only the horse anti-mouse IgG secondary Ab. (A) E14–E15 chimeric heart showing a line of *LacZ*-expressing cells on the posterior aspect of the heart found in the interventricular groove (between black arrows). Sectioning of similar grafted hearts revealed that these cells were primarily located in the smooth muscle lining of the posterior descending coronary artery and vein. (B) Cross section of the posterior aspect of a chimeric heart. Labeled cells can be seen in the smooth muscle lining of the posterior descending coronary artery (*A*) and in the associated vein (*V*). White arrowheads point to

TABLE 1
Outcome of Grafts of *AdCMVlacZ*-Labeled Quail Epicardial Cells

Graft	CVSMC	Endocardial cell	Perivascular cells	Intermyocardial cell	Total
1	163 (47%)	5 (1%)	131 (37%)	51 (16%)	350
2	218 (40%)	33 (6%)	102 (19%)	195 (36%)	548
3	449 (63%)	30 (4%)	43 (6%)	196 (27%)	718
4	94 (45%)	7 (3%)	28 (13%)	82 (39%)	211
5	61 (82%)	1 (2%)	1 (2%)	11 (15%)	74
6	52 (30%)	0 (0%)	56 (33%)	63 (37%)	171
7	97 (74%)	3 (2%)	3 (2%)	28 (21%)	131
8	32 (68%)	0 (0%)	6 (13%)	9 (9%)	47
Total	1166 (52%)	79 (4%)	370 (16%)	635 (28%)	2250

Note. Twelve grafted embryos were allowed to develop until E12–18 before they were fixed and stained in X-Gal buffer. Of these, three had isolated patches of quail cells on ventral aspects the heart and nine had visible clusters of labeled cells on dorsal aspects of the heart in the interventricular or atrioventricular groove near developing coronary arteries. Eight of nine hearts in the second group were sectioned, inspected for labeled quail cells, and stained with antibody markers to smooth muscle markers. Labeled cells were scored for their presence in the smooth muscle layer of the vascular wall or for expression of smooth muscle markers near neovascular structures (CVSMC, column 2), within the endocardium or atrioventricular cushions (endocardial cells, column 3), fibroblasts negative for smooth muscle markers and adjacent to vascular structures (perivascular cells, column 4), and fibroblasts negative for smooth muscle markers within the myocardium (intermyocardial cells, column 5). Total in column 6 indicates the total number of grafted cells scored for each graft and total in row 9 indicates the sum of scored cells for all sectioned grafts. Percentages in parentheses across rows indicate the percentage of each cell type for that graft. Percentages in parentheses across row 9 indicate the percent of each cell type for all sectioned grafts.

(Fig. 5H). We also observed labeled cells within the vessels of the atrial wall, demonstrating that these vessels also contained epicardially derived CVSMCs (Fig. 6F). In no case did we observe labeled cells in smooth muscle of the great vessels or the mesenchyme surrounding the great vessels. This observation supports the contention that the subepicardial mesenchyme does not contribute to the smooth muscle of the great vessels.

DISCUSSION

In this paper we present evidence that epithelial–mesenchymal transformation of epicardial cells occurs during avian cardiac development and that the mesenchymal cells produced by this epithelial–mesenchymal transformation can become CVSMC, perivascular fibroblasts, and intermyocardial fibroblasts. Our study, as well as evidence from several other studies, demonstrates the importance of the proepicardial organ and the epicardium in normal coronary vascular development (Manasek, 1969; Ho and Shimada, 1978; Viragh and Challice, 1981; Komiyama, *et al.,* 1987; Kuhn and Liebherr, 1988; Fransen and Lemanski, 1990; Manner, 1992; Mikawa and Fischman, 1992; Viragh *et al.*, 1993; Mikawa and Gourdie, 1996; Munoz-Chapuli *et al.*, 1996). Our findings refine the model of Mikawa and Gourdie (1996) which suggested that committed smooth muscle founders migrate from the proepicardium directly to the subepicardium. Although we have not ruled out the possibility that committed smooth muscle precursors exist in the proepicardium, we have now shown that epicardial cells undergo epithelial–mesenchymal transformation to give rise to the mesenchyme which participates directly in coronary vascular development.

Our grafting experiments were designed to minimize the heterogeneous nature of the cell population transferred to the host embryo. Utilizing retroviral injections to the proepicardium, grafting DiI-labeled quail proepicardia to chick hosts, and early smooth muscle markers, Mikawa and Gourdie (1996) convincingly demonstrated that the proepicardium is the source of epicardial precursors and smooth muscle precursors. The experiments presented here now allow us to refine the model of the cellular migrations that take place during epicardial and coronary vascular develop-

associated perivascular fibroblasts. (C) Labeled quail cells near neovascular structures in the interventricular septum. Arrowheads point to interstitial cells clustered around newly formed vessels that have accumulated caldesmon stain. Labeled quail CVSMCs can also be seen in the larger vessel below. (D) Two labeled quail CVSMCs in a mature interventricular septum artery. (E) *LacZ*-expressing perivascular fibroblast (white arrowhead), CVSMC (black arrowhead), and interstitial cell (arrow) in the interventricular septum of a grafted heart. (F) Labeled quail cells in the atrioventricular subepicardium. Arrow points to a cluster of labeled CVSMC, white arrowhead points to a labeled nucleus in the associated vein (V), and the black arrowhead points to labeled nuclei in neovascular structures. (G) Labeled quail endocardial cell (arrowhead). Staining of the endocardium with anti-smooth muscle actin results from nonspecific binding of the horse anti-mouse IgG secondary Ab.

ment. We demonstrated, using DiI labeling, that cells within the epicardium are stimulated, during normal development, to invade the subepicardial matrix and become mesenchyme. Grafts of substantially pure cultures of epicardial cells verified that epicardially derived mesenchymal cells assemble with endothelial cells in the coronary vascular plexus to form mature coronary vessels. These experiments thus provide direct evidence that epicardial—mesenchymal transformation is a fundamental developmental process through which the subepicardial mesenchyme is established, and that this fundamental cellular change gives rise not only to CVSMC but perivascular and intermyocardial fibroblasts as well.

One question that arises from our results is that of the frequency of epicardial invasion. Since the epicardium remains in adult hearts, any mechanism which stimulates epicardial–mesenchymal transformation must also maintain the integrity of the epithelium. Our observations suggest that epicardial invasion is frequent during the first week of development. In DiI-labeling experiments of the epicardium, most of the labeled cells were observed to have invaded the subepicardial matrix after 48 h. In grafting experiments, when hearts were fixed 1 to 2 days after cell grafting, many cells were observed in the epicardium (Fig. 5F). In contrast, when hearts were fixed 10 to 15 days after grafting, most of the labeled cells were observed to have invaded the heart. We do not believe that this invasive phenotype was promoted by culturing of epicardial cells, since similar results were obtained in our DiI-labeling experiments. It is likely that the developmental mechanism which maintains a high frequency of epicardial–mesenchymal transformation may be related to a highly proliferative state in the developing epicardium to meet the requirement for large numbers of mesenchymal cells in the subepicardial matrix.

Two possibilities exist for the role of the intermyocardial cells. First, these cells may be CVSMC precursors that have invaded the myocardium to participate in the formation of the deeper coronary vessels. Second, these cells may invade the myocardium to become intermyocardial fibroblasts (Zeydel *et al.,* 1991). While intermyocardial fibroblasts have been described to play important roles in patterning and remodeling of the myocardium, the origin of these cells is unknown. Since there is no unequivocal marker for intermyocardial fibroblasts and because CVSMCs do not express smooth muscle markers except within the vascular wall, we were unable to resolve whether interstitial myocardial cells were undifferentiated mesenchymal cells, intermyocardial fibroblasts, or migrating CVSMCs.

Our observations have led us to formulate the model for coronary vascular development shown in Fig. 7. First, cells within the primordial liver adjacent to the dorsal mesocardium form the proepicardial protrusion via an, as yet, undetermined mechanism. Second, the proepicardium establishes the dorsal mesocardium and keratinized epicardial cells migrate over the surface of the bare myocardium. During epicardial cell migration, the subepicardial matrix is produced, most likely by both epicardial cells and myocardial cells in contact with the emerging epicardium and it is likely that as epicardial cells begin to migrate cranially, epicardial cells are stimulated to invade the newly forming subepicardial matrix. It is during epicardial cell migration that endothelial cells first populate the subepicardial matrix to form the coronary vascular plexus (Poelmann *et al.,* 1993).

The third stage of epicardial development is epithelial–mesenchymal transformation of epicardial cells. Although the precise mechanism through which this epithelial–mesenchymal transformation is stimulated is unknown, numerous signaling molecules have been implicated in embryonic epithelial–mesenchymal transformation, including growth factors such as those in the TGFβ family (Potts and Runyan, 1989; Brown *et al.,* 1996; Ramsdell and Markwald, 1997), the FGF family (Savagner *et al.,* 1997), hepatocyte growth factor (Uehara and Kitamura, 1992), and matrix proteins such as ES-130 (Rezaee et al, 1993; Krug *et al.,* 1995). Of the vascular growth factors that could potentially induce epicardial–mesenchymal transformation, we have determined that bFGF, EGF, and VEGF, can stimulate epicardial cells to invade collagen gels. One hypothesis is that epicardial cells are stimulated to invade by factors secreted by the myocardium. Each of the growth factors that we have observed to stimulate epicardial–mesenchymal transformation *in vitro* is present in hearts during development (Parlow *et al.,* 1991; Gamiz *et al.,* 1993; Flamme *et al.,* 1995; Zhu *et al.,* 1996). Myocardial-secreted factors that stimulate epicardial–mesenchymal transformation may overlap with those myocardial factors which stimulate epithelial–mesenchymal transformation of endothelial cells of the atrioventricular canal. This idea could certainly be supported anatomically because the myocardium of the atrioventricular canal is flanked both subepicardially and subendocardially by an extensive matrix filled with mesenchyme. Moreover, the matrix glycoprotein ES130, which has been shown to be sufficient to induce endocardial cushion epithelial–mesenchymal transformation *in vitro,* is also expressed in the epicardium (Krug *et al.,* 1995). We have not yet tested the precise role for ES130 in epicardial–mesenchymal transformation.

Subepicardial mesenchyme then further differentiates into CVSMCs, perivascular fibroblasts and invasive intermyocardial fibroblasts. As the epicardium forms and epicardial cells undergo epithelial–mesenchymal transformation to invade the heart, endothelial cells establish the coronary vascular plexus within the subepicardial matrix. The final step in coronary vascular development is the association of nascent endothelial capillaries with subepicardial mesenchymal cells to form mature vessels. For this step, it is postulated that endothelial cells produce inductive agents such as EGF and platelet-derived growth factor-BB which function to recruit mesenchyme to the vascular wall (Folkman and D'Amore, 1996). Our ongoing studies will investigate the role of known growth factors and matrix elements in epicardial–mesenchymal transformation and cell fate specification of CVSMCs, perivascular fibroblasts, and intermyocardial fibroblasts.

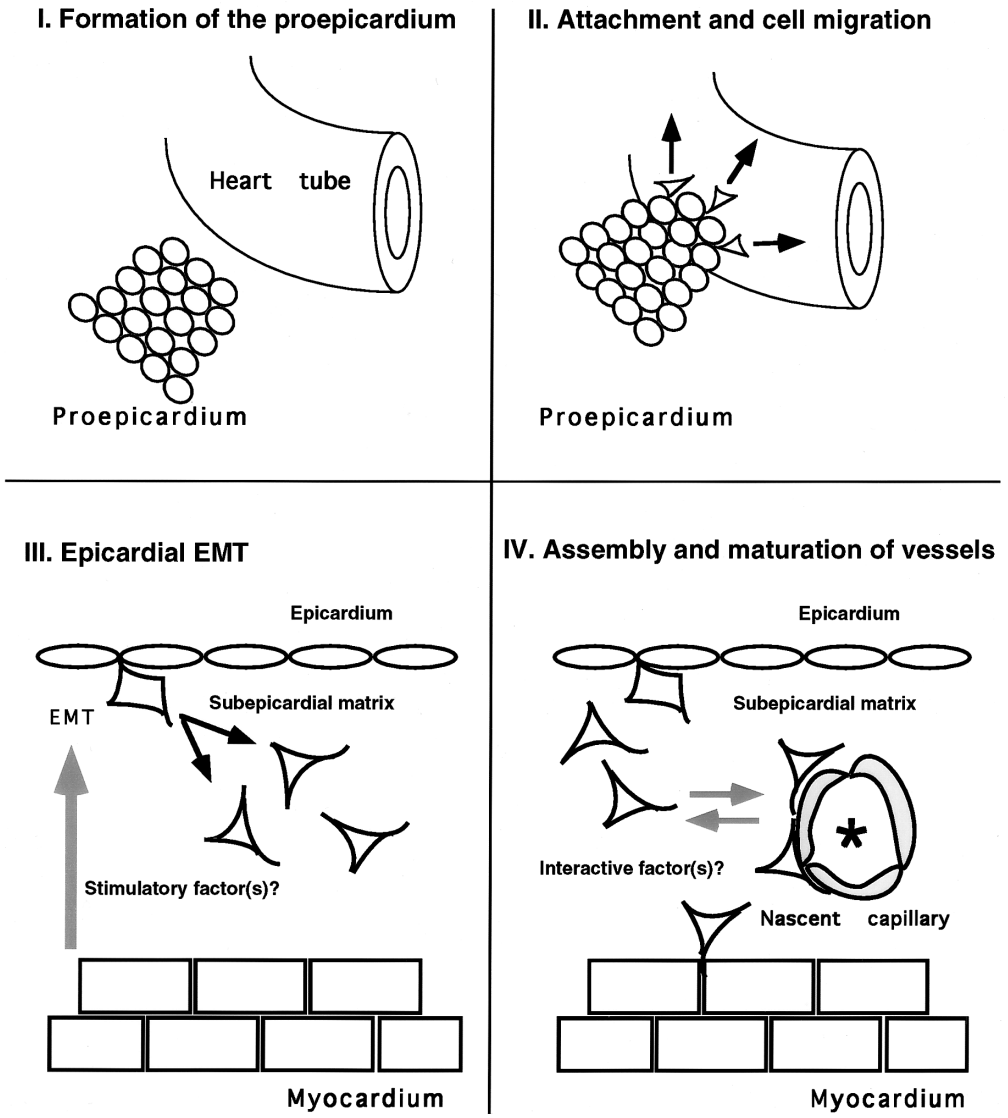

FIG. 7. Model for coronary vascular development. The early stages of epicardial development are described in Viragh *et al.* (1993). (I) Formation of the proepicardium occurs by embryonic stage 14 (E2). (II) Attachment of the proepicardium to the dorsal aspect of the sinus venosus occurs by stage 16 (E2) and migration of keratinized epithelial cells over the myocardial surface in a caudal to cranial direction is completed by stage 26 (E4). It is during this stage that endothelial precursors migrate to the subepicardial matrix from the liver via the secondary dorsal mesocardium (Poelmann *et al.*, 1993). (III) Epicardial–mesenchymal transformation gives rise to a population of mesenchymal cells in the subepicardial matrix. The timing of epicardial–mesenchymal transformation has not been precisely determined, although it may occur both during epicardial cell migration and after the epicardium is fully formed. The molecular stimulus for epicardial–mesenchymal transformation has not been defined, although it is likely that cardiac myocytes may stimulate this process (arrow). (IV) Assembly and maturation of coronary vessels occurs as primitive endothelial tubes (*) interact with surrounding mesenchymal cells to recruit epicardially derived CVSMC and perivascular fibroblast precursors to the vascular wall. Interactive signals produced by endothelial and mesenchymal cells regulate the assembly of mature vessels (arrows).

Our experiments, and those of others (Asahara *et al.*, 1997), demonstrate the validity of culturing vascular and angiogenic cell types or precursors before reintroduction into a vasculogenic or angiogenic pathway *in vivo.* Our results are consistent with proposed models for vasculogenesis and suggest that coronary vasculogenesis is similar to other vasculogenic processes in developing embryos (Folkman and D'Amore, 1996). Future studies of epicardial cells *in vitro,* coupled with grafting experiments using genetically manipulated epicardial cells, will allow us to analyze molecules proposed to function in epicardial–mesenchymal transformation and coronary vasculogenesis. This may ulti-

mately help to identify genes important for coronary vascular development and neovascularization in the adult heart.

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REFERENCES

Aikawa, M., Sivam, P., Kiro-o, M., Kimura, K., Nakahara, K., Takewaki, S., Ueda, M., Tamaguchi, H., Yazaki, Y., Periasamy M., and Nagai., R. (1993). Human smooth muscle myosin heavy chain isoforms as molecular markers for vascular development and atherosclerosis. *Circ. Res.* **73,** 1000–1012.

Asahara, T. Murohara, T., Sullivan, A., Silver, M., van der Zee, R., Li, T., Witzenbichler, B., Shatteman, G., and Isner, J. M. (1997). Isolation of putative progenitor endothelial cells for angiogenesis. *Science* **275,** 964–967.

Bogers, A. J., Gittenberger-de Groot, A. C., Poelmann, R. E., Peault, B. M., and Huysmans, H. A. (1989). Development of the origin of the coronary arteries, a matter of ingrowth or outgrowth? *Anat. Embryol.* **180,** 437–441.

Bogers, A. J. C., Gittenberger-de Groot, A. C., Dubbledaum, J. A., and Huysmans, H. A. (1988). The inadequacy of existing theories on development of the proximal coronary arteries and their connections with the arterial trunks. *Int. J. Cardiol.* **20,** 117–123.

Bouchey, D., Drake, C. J., Wunch, A. M., and Little, C. D. (1996). Distribution of connective tissue proteins during development and neovascularization of the epicardium. *Cardiovasc. Res.* **31,** E104–E115.

Brody, S. L., and Crystal, R. G. (1994), Adenovirus-mediated *in vivo* gene transfer. *Ann. NY Acad. Sci.* **716,** 90–101.

Brown, C. B., Boyer, A. S., Runyan, R. B., and Barnett, J. V. (1996). Antibodies to the type II TGFβ receptor block cell activation and migration during atrioventricular cushion transformation in the heart. *Dev. Biol.* **174,** 248–257.

Coffin, J. D., and Poole, T. J. (1988). Embryonic vascular development: immunohistochemical identification of the origin and subsequent morphogenesis of the major vessel primordia in quail embryos. *Development* **102,** 735–748.

Crystal, R. G., McElvaney, N. G., Rosenfeld, M. A., Chu, C. S., Mastrangeli, A., Hay, J. G., Brody, S. L., Jaffe, H. A., Eissa, N. T., and Danel, C. (1994). Administration of an adenovirus containing the human CFTR cDNA to the respiratory tract of individuals with cystic fibrosis *Nat. Genet.* **8,** 42–51.

Flamme, I., Breier, G., and Risau W. (1995). Vascular endothelial growth factor (VEGF) and VEGF receptor 2 (flk-1) are expressed during vasculogenesis and vascular differentiation in the quail embryo. *Dev. Biol.* **169,** 699–712.

Folkman, J., and D'Amore P. A. (1996). Blood vessel formation: What is its molecular basis? *Cell* **87,** 1153.

Fransen, M. E., and Lemanski, L. F. (1990). Epicardial development in the axolotl, *Ambystoma mexicanum*. *Anat. Rec.* **226,** 228–236.

Frid, M. G., Moiseeva, E. P., and Stenmark, K. R. (1994). Multiple phenotypically distinct smooth muscle cell populations exist in the adult and developing bovine pulmonary arterial media *in vivo*. *Circ. Res.* **75,** 669–681.

Gamiz, J. M., Melguizo, C., Prados, J., Fernandez, J. E., Alvarez, L., and Aranega, A. (1993). Expression of epidermal growth factor receptor in chick embryo myocardiocytes: relation with desmin expression during cardiac development. *Int. J. Cardiol.* **42,** 107–114.

Gendron, R. L., Tsai, F.-Y., Paradis, H., and Arceci, R. J. (1996). Induction of embryonic vasculogenesis by bFGF and LIF *in vitro* and *in vivo*. *Dev. Biol.* **177,** 332–346.

Glukhova, M. A., Frid, M. G., and Koteliansky, V. E. (1990). Developmental changes in expression of contractile and cytoskeletal proteins in human aortic smooth muscle. *J. Biol. Chem.* **265,** 13042–13046.

Guan, J.-L., and Hynes, R. O. (1990). Lymphoid cells can recognize an alternatively spliced segment of fibronectin via the integrin receptor $\alpha 4\beta 1$. *Cell* **60,** 51–63.

Hamburger, V., and Hamilton, H. L. (1951). A series of normal stages in the development of the chick embryo. *J. Morphol.* **88,** 49–92.

Hay, E. D. (1995). An overview of epitheliomesenchymal transformation. *Acta Anat.* **154,** 8–20.

Ho, E., and Shimada Y. (1978). Formation of the epicardium studied with the scanning electron microscope. *Dev. Biol.* **66,** 579–585.

Honig, M. G., and Hume, R. I. (1989). Dil and diO: Versatile fluorescent dyes for neuronal labeling and pathway tracing. *Trends Neurosci.* **12,** 333–335.

Hood, L. C., and Rosenquist T. H. (1992). Coronary artery development in the chick: origin and deployment of smooth muscle cells and the effects of neural crest ablation. *Anat. Rec.* **234,** 291–300.

Kirby, M. L., and Waldo, K. L. (1995). Neural crest and cardiovascular patterning. *Circ. Res.* **77,** 211–215.

Komiyama, M., Ito, K., and Shimada, T. (1987). Origin and development of the epicardium in the mouse embryo. *Anat. Embryol.* **176,** 183–189.

Kuhn, H. J., and Liebherr, G. (1988). The early development of the epicardium in *Tupaia belangeri*. *Anat. Embryol.* **177,** 225–234.

Krug, E. L., Rezaee, M., Isokawa, K., Turner, D. K. , Litke, L. L., Wunsch, A. M., Bain, J. L., Riley, D. A., Capehart, A. A., and Markwald, R. R. (1995). Transformation of cardiac endothelium into cushion mesenchyme is dependent on ES/130: temporal, spatial, and functional studies in the early chick embryo. *Cell. Mol. Biol. Res.* **41,** 263–277.

Kwee, W. L., Baldwin, H. S., Shen, H. M., Stewart, C. L., Buck, C., and Buck, C. A. (1995). Defective development of the embryonic and extraembryonic circulatory systems in vascular cell adhesion molecule (VCAM-1) deficient mice. *Development* **121,** 489–503.

Le Lievre, C. S., and Le Douarin, N. M. (1975). Mesenchymal derivatives of the neural crest: analysis of chimaeric quail and chick embryos. *J. Embryol. Exp. Morphol.* **134,** 125–154.

Lindahl, P., Johansson, B. R. Levéen, P., and Betsholtz, C. (1997). Pericyte loss and microaneurysm formation in PDGF-B-deficient *Science* **277,** 242.

Majesky, M. W., Giachelli, C. M., Reidy, M. A., and Schwartz, S. M. (1992). Rat carotid neointimal smooth muscle cells re-express a developmentally regulated mRNA phenotype during repair of arterial injury. *Circ. Res.* **71,** 759–768.

Manasek, F. J.(1969). Embryonic development of the heart. II. Formation of the epicardium. *J. Embryol. Exp. Morphol.* **22,** 333–348.

Manner, J. (1993) Experimental study on the formation of the epicardium in chick embryos. *Anat. Embryol.* **187,** 281–289.

Mikawa, T., and Fischman, D. A., (1992). Retroviral analysis of cardiac morphogenesis: discontinuous formation of coronary vessels. *Proc. Natl. Acad. Sci. USA* **89,** 9504–9508.

Mikawa, T., and Gourdie, R. G. (1996). Pericardial mesoderm generates a population of coronary smooth muscle cells migrating into the heart along with ingrowth of the epicardial organ. *Dev. Biol.* **174,** 221–232.

Muñoz-Chapuli, R., Macias, D., Ramos, C., Gallego, A., and De Andres, V. (1996). Development of the subepicardial mesenchyme and the early cardiac vessels in the dogfish (*Scyliorhinus canicula*) *J. Exp. Zool.* **275,** 95–111.

Parlow, M. H., Bolender, D. L., Kokan-Moore, N. P., and Lough, J. (1991). Localization of bFGF-like proteins as punctate inclusions in the preseptation myocardium of the chicken embryo. *Dev. Biol.* **146,** 139–147.

Poelmann, R. E., Gittenberger-de Groot, A. C., Mentink, M. M., Bokenkamp, R., and Hogers. B. (1993). Development of the cardiac coronary vascular endothelium, studied with antiendothelial antibodies, in chicken–quail chimeras. *Circ. Res.* **73,** 559–568.

Potts, J. D., and Runyan, R B. (1989). Epithelial–mesenchymal cell transformation in the embryonic heart can be mediated, in part, by transforming growth factor β. *Dev. Biol.* **134,** 392–401.

Ramsdell, A. F., and Markwald, R. R. (1997). Induction of endocardial cushion tissue in the avian heart is regulated, in part, by TGFβ-3-mediated autocrine signaling. *Dev. Biol.* **188,** 64–74.

Rezaee, M., Isokawa, K., Halligan, N., Markwald, R. R., and Krug, E. L. (1993). Identification of an extracellular 130-kDa protein involved in early cardiac morphogenesis. *J. Biol. Chem.* **268,** 14404–14411.

Tomanek, R. J. (1996). Formation of the coronary vasculature: A brief review *Cardiovasc. Res.* **31,** E46–E51.

Topouzis, S., and Majesky, M. W. (1996). Smooth muscle lineage diversity in the chick embryo. Two types of aortic smooth muscle cell differ in growth and receptor-mediated transcriptional responses to transforming growth factor-β. *Dev. Biol.* **178,** 430–445.

Uehara, Y., and Kitamura, N. (1992). Expression of a human hepatocyte growth factor/scatter factor cDNA in MDCK epithelial cells influences cell morphology, motility, and anchorage-independent growth. *J. Cell Biol.* **117,** 889–894.

Vernet, M., Bonnerot, C., Briand, P., and Nicolas, J. F. (1993). Application of LacZ gene fusions to preimplantation development. *Methods Enzymol.* **225,** 434–451.

Viragh, S., and Challice, C. E. (1981). The origin of the epicardium and the embryonic myocardial circulation in the mouse. *Anat. Rec.* **201,** 157–168.

Viragh, S., Gittenberger-de Groot, A. C., Poelmann, R. E., and Kalman, F. (1993). Early development of quail heart epicardium and associated vascular and glandular structures. *Anat. Embryol.* **188,** 381–393.

Vrancken Peeters, M. P., Mentink, M. M., Poelmann, R. E., and Gittenberger-de Groot, A. C. (1995). Cytokeratins as a marker for epicardial formation in the quail embryo. *Anat. Embryol.* **191,** 503–508.

Vrancken Peeters, M. P., Gittenberger-de Groot, A. C., Mentink, M. M., Hungerford, J. E., Little, C. D., and Poelmann, R. E. (1997). The development of the coronary vessels and their differentiation into arteries and veins in the embryonic quail heart. *Dev. Dyn.* **208,** 338–348.

Waldo, K. L., Willner, W., and Kirby, M. L. (1990). Origin of the proximal coronary artery stems and a review of ventricular vascularization in the chick embryo. *Am. J. Anat.* **188,** 109–120.

Waldo, K. L., Kumiski, D. H., and Kirby, M. L. (1994). Association of the cardiac neural crest with development of the coronary arteries in the chick embryo. *Anat. Rec.* **239,** 315–331.

Yang, J. T., Rayburn, H., and Hynes, R. O. (1995). Cell adhesion events mediated by alpha 4 integrins are essential in placental and cardiac development. *Development* **121,** 549–560.

Zabner, J., Petersen, D, M., Puga, A. P., Graham, S. M., Couture, L. A., Keyes, L. D., Lukason, M. J., St. George, J. A., Gregory, R. J., Smith, A. E., *et al.* (1994). Safety and efficacy of repetitive adenovirus-mediated transfer of CFTR cDNA to airway epithelia of primates and cotton rats. *Nat. Genet.* **6,** 75–83.

Zeydel, M., Puglia, K., Eghbali, M., Fant, J., Seifter, S., and Blumenfeld O. O. (1991). Properties of heart fibroblasts of adult rats in culture. *Cell Tissue Res.* **265,** 353–359.

Zhu, X., Sasse, J., McAllister, D., and Lough, J. (1996). Evidence that fibroblast growth factors 1 and 4 participate in regulation of cardiogenesis. *Dev. Dyn.* **207,** 429–438.

Zuk, A., and Hay, E. D. (1994). Expression of beta 1 integrins changes during transformation of avian lens epithelium to mesenchyme in collagen gels. *Dev. Dyn.* **201,** 378–393.

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