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# Pericardial Mesoderm Generates a Population



# into the Heart along with Ingrowth of the Epicardial Organ

Takashi Mikawa*,[1] and Robert G. Gourdie†

*Department of Cell Biology and Anatomy, Cornell University Medical College, 1300 York Avenue, New York, New York 10021; and †Department of Cell Biology and Anatomy, Cardiovascular Developmental Biology Center, Medical University of South Carolina, 171 Ashley Avenue, Charleston, South Carolina 29425-2204

The vascular smooth muscle cells of coronary arteries are distinguished from those of the proximal aorta by a number of structural and functional criteria which may include an increased propensity for atherosclerotic transformation. At present, the source of this variation between smooth muscle subpopulations is uncertain. Whilst smooth muscle of the proximal aorta is thought to be derived from neural crest, the origin of coronary vascular smooth muscle remains uncharacterized. We have previously shown that precursors of the coronary vasculature enter the tubular heart on the same day as the epicardial mantle starts to envelop the myocardium and that coronary vessels form by ingrowth of these migratory precursors and not by outgrowth from the aorta (Mikawa and Fischman, 1992). To study the origin of coronary smooth muscle cells, the proepicardial organ, from which epicardial cells arise, was tagged with either a vital dye (DiI) or replication-defective retroviruses encoding ***β***-galactosidase. Cellular lineage marking was achieved by either direct targeting of putative vasculogenic cells in the proepicardium *in ovo* or tagging dissected proepicardial cells *in vitro* followed by transplantation to stage-matched host embryos. Monitoring of tagged cells during coronary vasculogenesis indicate incorporation of proepicardial-derived cells into three vessel-associated populations; coronary mooth muscle, perivascular connective tissue, and endothelial cells. Immunoconfocal microscopy identified both endothelial and smooth muscle cell populations within the proepicardial organ. The results demonstrate that: (1) the proepicardium contains a progenitor population of coronary smooth muscle cells that migrates into the heart along with ingrowth of the epicardium and (2) prior to the migration, the coronary smooth muscle lineage is established. 

## INTRODUCTION

The function of the heart is dependent on an efficient coronary vascular system. At present, the mechanisms governing the development of this essential system are not well understood. This is due mainly to uncertainties concerning the ontogeny or lineage of cells comprising coronary vessels, including vascular smooth muscle cells. In earlier studies of noncoronary vessels, two embryonic origins, cervical neural crest and local mesoderm, were proposed as progenitor tissues for vascular smooth muscle. Studies using chick–quail chimeras demonstrated that smooth muscle cells in the proximal great vessels, including the thoracic aorta and aortic arch arteries, are derived at least in part from neural crest (LeLièvre and LeDouarin, 1975), with no discernible contribution by neural crest to coronary arteries (Waldo *et al.*, 1994). Distal vessels are derived from mesenchyme, and the interfaces of vessels contain smooth muscle cells of mixed origin (LeLièvre and LeDouarin, 1975). It is now recognized that there is a high degree of phenotypic heterogeneity within vascular smooth muscle. The basis of this variation is not fully understood; however, it has been suggested that cell lineage (e.g., neural crest vs mesenchyme) may be one of its determinants (Topouzis *et al.*, 1992; Rosenquist *et al.*, 1990; Schwartz and Liaw, 1993). Indeed, there is evidence that blood vessel ontogeny is a factor in the varying susceptibility of different components of the vascular sys-

[1] To whom correspondence should be addressed. Fax: (212) 746-8175.

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tem to atherosclerosis (Topouzis *et al.*, 1992; Hood and Rosenquist, 1992). It would seem pertinent therefore to more precisely characterize the origin of smooth muscle cell populations in the coronary arterial system.

The embryonic tubular heart is avascular on initiation of rhythmic contractions on Day 2 (E2, HH stage 10; Hamburger and Hamilton, 1951). At this stage, the heart is composed of myocytes and endocardial endothelial cells with no histologically distinguishable coronary vessels, neural elements, or conduction cells (Manasek, 1968). Studies using chick–quail chimeras have provided evidence that the endocardial endothelium is derived from angioblasts of the lateral plate mesoderm (Coffin and Poole, 1991). In previous retroviral cell lineage studies on avian heart, we have shown the lineage of coronary endothelia to be distinct from endocardial endothelia and that coronary vascular progenitors begin migration into the tubular heart on E3 (HH stage 17) (Mikawa and Fischman, 1992). By E6 (HH stage 29), these migratory cells have organized into discontinuous channels consisting of endothelial cells which subsequently form early arterial vessels that anastomose with the sinusoid (Bogers *et al.*, 1989; Waldo *et al.*, 1990). These results are consistent with the coronary vascular system being established by ingrowth of migratory vascular precursor cells, rather than by sprouting from the aortic root as has been suggested by some authors (Aikawa and Kawano, 1982; Hutchins, 1988; Hirakow, 1983; Conte and Pellegrini, 1984). The connection of the coronary orifices to the aorta and closure of the coronary vascular bed is completed after E14 (Rychter and Ostadal, 1971). Since the epicardial mantle begins to envelop the myocardium on E3 (Hiruma and Hirakow, 1989; Ho and Shimada, 1978), an event that coincides with the appearance of cardiac vascular progenitors, it has been postulated that coronary vascular cells migrate into the tubular heart along with epicardial connective tissue precursors (Mikawa and Fischman, 1992; Poelmann *et al.*, 1993).

The epicardial mantle originates from the proepicardial organ; protrusions of mesothelial cell clusters on the right side of the external surface of the sinus venosus (Hiruma and Hirakow, 1989). The protrusions of the proepicardium contact the dorsal wall of the tubular heart in the region of the atrioventricular junction at E3 (HH stage 17) and subsequently form a cellular monolayer which gradually covers the heart in a well-characterized progression (Hiruma and Hirakow, 1989). While our earlier studies have shown that cells committed to the coronary smooth muscle cell lineage are present in the E3 heart, they did not identify the origin of these cells (Mikawa and Fischman, 1992). Here, we use two cell tagging procedures, retroviral-mediated (Sanes *et al.*, 1986; Turner and Cepko, 1987) and vital fluorescent dye (Selleck and Stern, 1991), to track cells derived from the proepicardium during coronary morphogenesis *in vivo*. We demonstrate that the proepicardium contains progenitor populations that give rise to coronary smooth muscle cells (in addition to perivascular fibroblasts and coronary endothelial cells) and conclude that the coronary smooth muscle cell lineage is distinct from that of aortic smooth muscle cell populations.

## MATERIALS AND METHODS

*Retroviral vector.* The retrovirus used for genetic cell tagging is a spleen necrosis virus (SNV)-based replication-defective virus (Dougherty and Temin, 1986), encoding $\beta$-galactosidase ($\beta$-gal). Preparation of the $\beta$-gal viral vector, its propagation *in vitro* and proof of helper virus-free stocks have been presented elsewhere (Mikawa *et al.*, 1991; Mikawa and Fischman, 1992). Viral concentrations of greater than $10^7$ active virions/ml were obtained by ultracentrifugation of culture supernatant from packaging cells as has been described previously (Mikawa *et al.*, 1992a,b).

*Microsurgical procedures for tagging the proepicardium in ovo.* After opening a small window in the shell and shell membrane of fertilized chicken eggs (*Gallus domesticus*), a small cut was made with a tungsten needle in the chorion and amniotic membranes to expose the proepicardial organ. DiI (1,1′-dioctadecyl3,3,3′,3′-tetramethyl indocarbocyanine perchlorate: Molecular Probes, Inc.), at 0.25 % in ethanol containing dimethyl sulfoxide (DMSO) or viral suspensions of ≤5 nl, containing 100 $\mu$g/ml polybrene, were pressure injected *in ovo* (Mikawa *et al.*, 1992a,b; Mikawa and Fischman, 1992). The cells intended for targeting were easily recognized in HH stage 17–18 embryos as ''grape-like'' clusters of cells (Fig. 1) protruding from the external surface of the sinus venosus. A parallel series of embryos received virus injection into the myocardial wall and served as a control for specificity of targeting. The eggs were resealed with parafilm and placed in a humidified incubator at 37.5°C to allow injected embryos to develop.

*Isolation, in vitro infection, and transplantation of the epicardial primordium.* On E3, embryos were removed from the eggs and staged using a dissecting microscope. Embryos at HH stages 17–18 were placed ventral-side up and the tubular heart exposed by opening the amnion. After removal of the tubular heart, the ''grape-like'' clusters of cells described previously were isolated from the coelomic wall. The cell clusters were inoculated with virus solution containing 10 $\mu$g/ml polybrene at 37°C for 30–60 min (Fig. 1). Excess virus solution was removed from the cell clusters by several washes in buffer solution containing 7% fetal bovine serum (FBS) in Dulbecco's modified Eagle's medium (DMEM). The rinsed fragments were then grafted into the epicardial cavity, dorsal to the tubular heart of isochronic recipients *in ovo* as described by Poelmann *et al.* (1993). Embryos receiving transplants were then returned to the incubator and development was allowed to proceed. Other dissected fragments infected *in vitro* were cultured on a collagen lattice in DMEM containing 10% FBS and 2% chick embryo extract to test the efficiency of viral tagging and cell survival after surgical procedures.

*Histochemistry.* DiI-injected embryos were removed from eggs at E4–E9, fixed with 2% paraformaldehyde in PBS for 1–2 hr at 4°C, and examined under an inverted fluorescent microscope. The virus-infected embryos at E14–E18 were killed by cervical transection. Hearts were removed and fixed by immersion overnight in 2% paraformaldehyde (4°C, PBS) and processed for X-gal histochemistry in whole mounts as described previously (Mikawa and Fischman, 1992). Hearts exhibiting $\beta$-gal-positive cells within the epicardium and coronary vessels were paraffin embedded, serially sectioned at 10 $\mu$m thickness, and examined by bright-field or phase-contrast optics. For immunohistochemistry and confocal microscopy, hearts were dissected from HH stage 15–18, 22–25, and

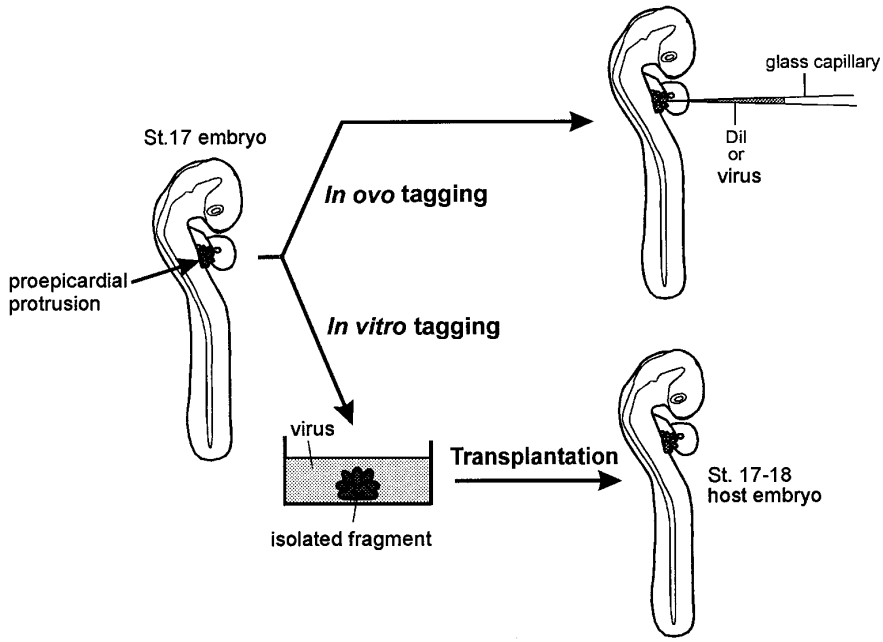


FIG. 1. Schematic illustration for tagging of cells at the proepicardium with DiI or a replication-defective retrovirus to trace their fate during coronary vessel development. A ''grape-like'' cluster of protruding cells (the proepicardium) (shaded) in HH stage 17 chicken embryos was labeled either by direct injection of $\beta$-gal virus *in ovo* or by *in vitro* inoculation of virus after isolation from donor embryos. Fragments marked in culture were transplanted into the cavity between the tubular heart and body wall of isochronic host embryos.

36 chick embryos and comparably staged quail embryos. The hearts were fixed by immersion in either 4% paraformaldehyde or 70% ethanol and then stored in 70% ethanol at −20°C for whole-mount immunolabeling (Germroth *et al.,* 1995) or directly processed for paraffin embedding. Paraffin-embedded hearts were serially sectioned at 10 $\mu$m and mounted on glass slides as previously described (Gourdie *et al.,* 1995). Immunolabeling using anti-caldesmon antibodies (CD5: Sigma, chick and quail smooth muscle marker) and QH1 (quail endothelial cell marker; Pardanard *et al.,* 1987) was carried out using overnight incubation with primary antibodies at 1:100 dilution. Indirect fluorescent labeling of localized primary antibodies was done as described previously (Gourdie *et al.,* 1995). All imaging was done on a Leica TCS 4D confocal microscope according to manufacturer's specifications.

## RESULTS

*DiI labeling of proepicardium.* The extracardiac source of epicardial precursors can be recognized as a protruding cluster of cells (i.e., proepicardium) on the external surface of the sinus venosus of HH stage 17–18 chicken embryos (Figs. 2A–2C). The clusters were marked with DiI and subsequent migration of labeled cells was traced during the early stages of epicardial formation. Injection of 0.5–1 nl of DiI-containing solution produced fluorescent signal localized to the targeted cell cluster (Fig. 2C). No significant diffusion was observed, even 1 hr after injection. Leakage of DiI into the epicardial cavity sometimes occurred if volumes of greater than 5 nl were used. As a precaution, migration of proepicardium-derived cells was traced only on those embryos that had received DiI injections of less than 5 nl.

After several hours, scattered DiI signal was detected on the surface of the tubular heart (Figs. 2D–2E). Strong signal also remained evident within the proepicardium. These results are consistent with the proepicardial organ being a significant pathway for cellular migration during the formation of the epicardial mantle. At E8, DiI signal was still detectable in the epicardium and subepicardial space (Figs. 2F–2G). However, due to dilution of dye by successive cell division, the fluorescent signal progressively weakened as the embryo developed.

*Viral-mediated genetic marking of proepicardial cells.* DiI proved an ideal marker for tagging cells in the living embryo during the initial 3–6 hr of epicardial migration. However, progressive dilution of DiI over time meant that this method could not be used for examining the long-term differentiation of coronary vessels (including accumulation of smooth muscle) over the subsequent 2–3 weeks *in ovo.* In order to follow events subsequent to epicardial mantle formation, stable genetic markers were introduced into the putative vasculogenic cells within the proepicardium using a retroviral-mediated gene-transfer method (Sanes *et al.,* 1986; Turner and Cepko, 1987; Mikawa and Fischman, 1992). A replication-defective variant of a retrovirus encoding the reporter gene, $\beta$-gal, was microinjected directly into the proepicardial protrusion of cells using the same micro-

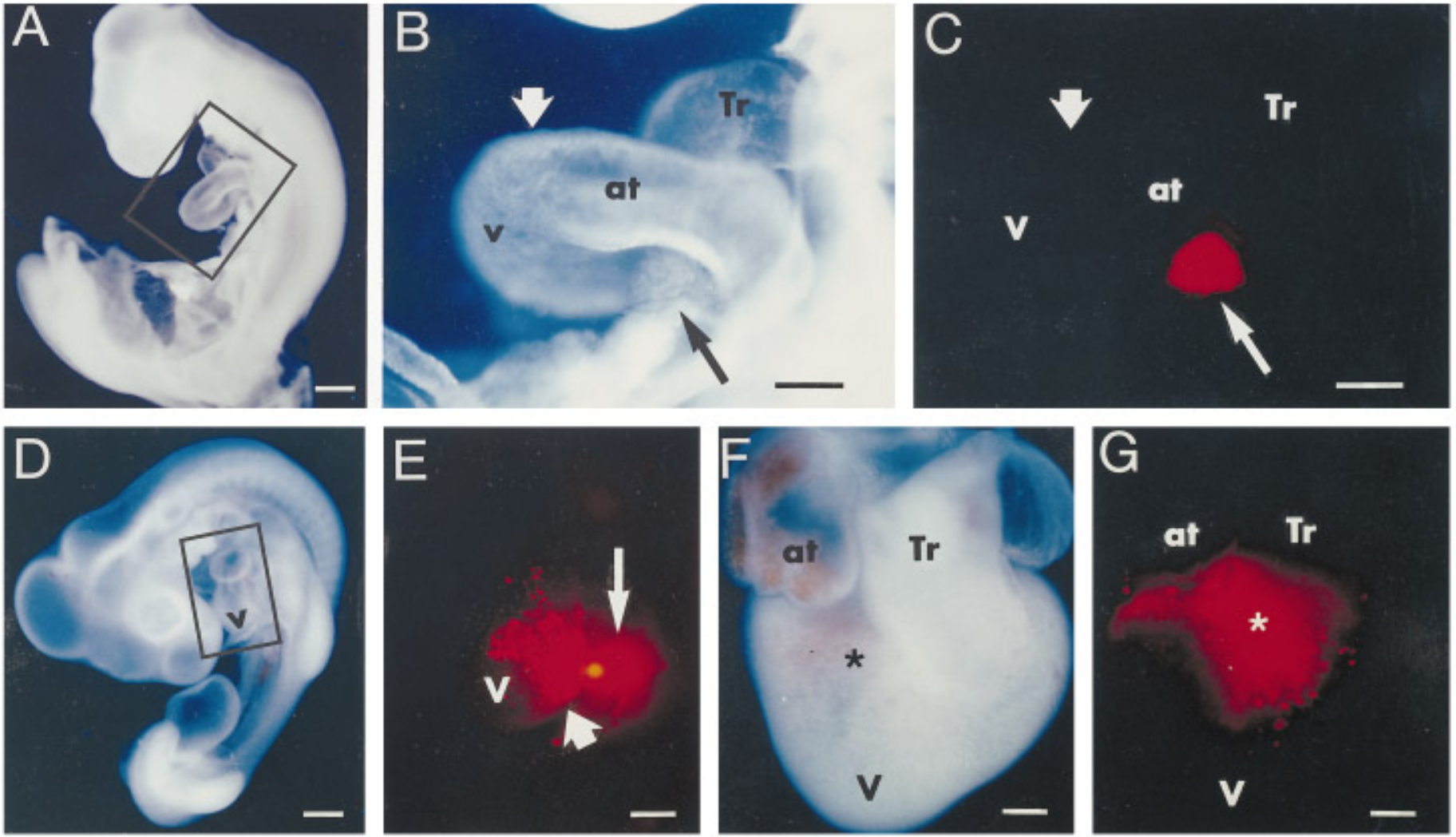


FIG. 2. Fate of DiI-marked proepicardium. DiI-containing solution was pressure-injected into the proepicardial protrusion *in ovo* at E3. The tagged embryos were examined just after injection (A–C), at E3.5 (D, E), or at E8 (F, G), under a dissecting microscope (A, B, D, F) and by fluorescent microscopy (C, E, G). The red signal in C, E, and G identifies DiI-tagged cells. B and C represent (higher magnification) details of the boxed area marked in A and show DiI signal within the injection site (long arrow), but not in the adjacent tubular heart (short arrow). E is a detail of the boxed area marked in D and demonstrates the distribution of DiI-labeled cells in the tubular heart (short arrow) and proepicardium (long arrow) of an E3.5 embryo. Asterisks in F and G mark the region containing detectable DiI signal in the E8 heart. v, ventricle; at, atrium; Tr, truncus arteriosus. Scale bars: 400 $\mu$m, A, D, E; 200 $\mu$m, B, C, F, G.

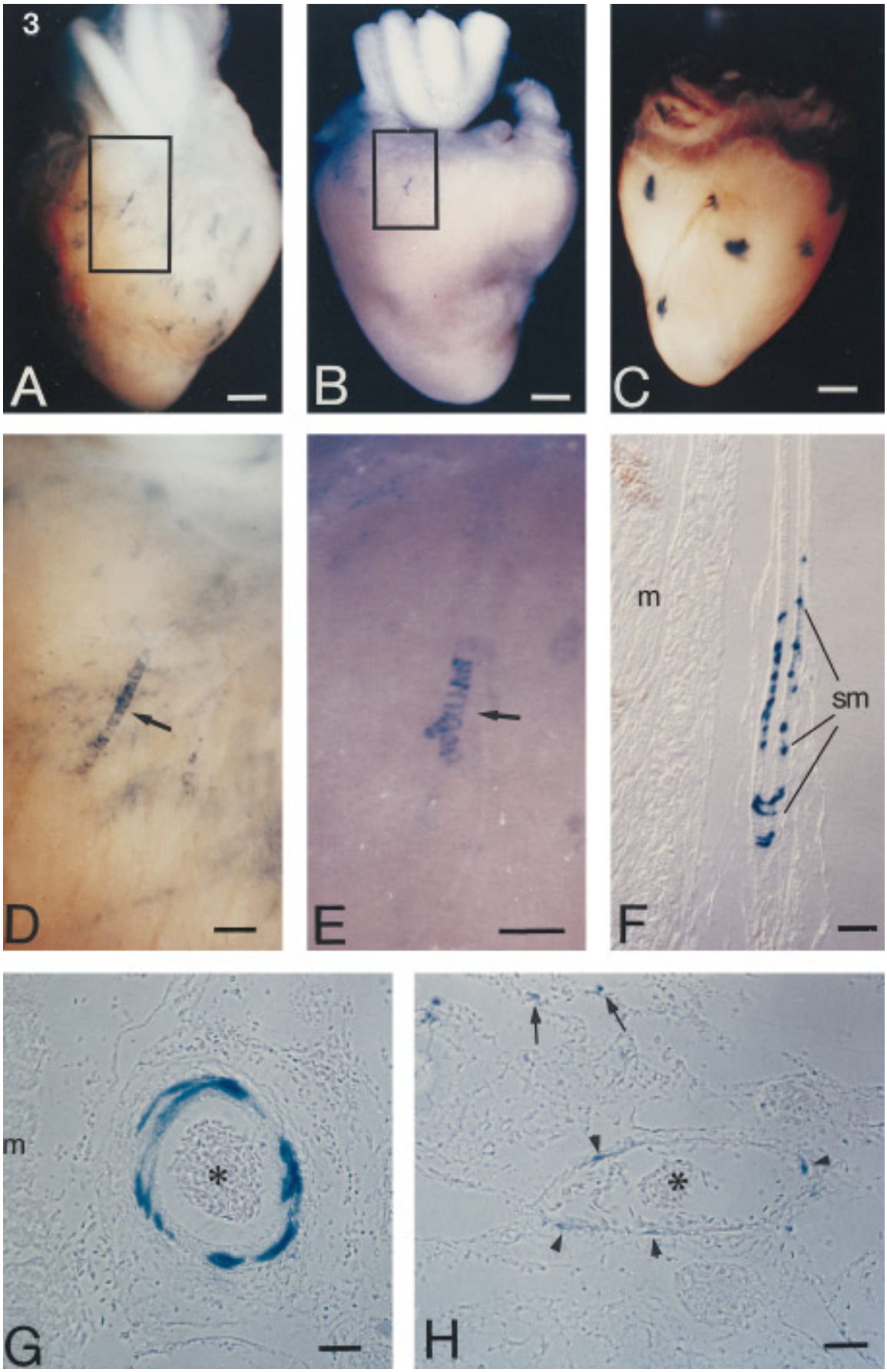
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TABLE 1
Distribution of $\beta$-Gal$^+$ Smooth Muscle Cells ($\beta$-Gal$^+$ SMC) in Coronary Vessels of E18 Hearts after *in Vivo* Infection or Transplantation of the Proepicardium

| Type of experiment containing | Viral titer (virions/ml) | Normal development/ No. infected embryos | Coronary vessels | |
|---|---|---|---|---|
| | | | $\beta$-gal$^+$ cells | $\beta$-gal$^+$ SMC |
| *In vivo* infection | $\sim 2 \times 10^8$ | 39/48 | 39 | 39 |
| *In vivo* infection | $\sim 5 \times 10^6$ | 47/60 | 21 | 12 |
| Transplantation | $\sim 5 \times 10^6$ | 49/140 | 38 | 30 |

surgical and microinjection procedures established for DiI injection. The fate of infected cells (and their daughter cells) was then followed using whole-mount X-gal staining of the heart for $\beta$-gal expression at various stages after infection. In the present study, 108 embryos were infected *in ovo*, and of these, 86 survived through to E18 when analysis of $\beta$-gal colonies was done (Table 1).

In the first set of experiments, $2–5 \times 10^2$ viral particles were injected to infect multiple cells in the proepicardium. X-gal staining of hearts from infected embryos revealed that $\beta$-gal-positive ($\beta$-gal+) cells had a similar distribution to DiI-labeled cells, i.e., predominantly in the epicardial layer (Figs. 3A and 3B). As we have demonstrated in a previous study (Mikawa *et al.*, 1992a,b), injection of the virus into the E3 myocardial wall gave rise to $\beta$-gal+ patches consisting of myocytes (Fig. 3C). These results suggest that targeting of the proepicardium results in highly specific and efficient infection of extracardiac populations of cells and that these cells maintain the viral marker upon migration into the heart. Detailed inspection of hearts from embryos targeted for proepicardial infection with higher viral titers often revealed multiple colonies displaying different types of organization (Fig. 3D). In addition to the groups of $\beta$-gal+ cells found intermingled with the epicardium, discrete groups of spirally arranged $\beta$-gal+ cells were also often identified along sharply defined segments of blood vessels (Fig. 3D). Histology of such sectors indicated group-to-group differences in $\beta$-gal+ cell phenotype, including clusters of smooth muscle, endothelial, and connective tissue cells (Fig. 3H). In order to investigate the nature of these colonies in isolation, viral solutions were titrated such that less than 10 virions were introduced upon infection (Figs. 3B and 3E, Table 1). This modification enabled hearts bearing only single colonies to be routinely obtained. Of those hearts displaying solitary vessel-associated clusters of $\beta$-gal+ cells, it was found that the spirally arranged cells were always restricted to a discrete segment of the coronary artery. In no case were vessels labeled along their entire length. The segmental distribution and spiral arrangement of $\beta$-gal+ cells were identical to smooth muscle cells characterized in earlier clonal analyses of developing coronary arteries (Mikawa and Fischman, 1992). Histological sections of these cellular clusters confirmed their identity as smooth muscle, since $\beta$-gal labeling delineated long, spindle-shaped cells organized in spirals comprising the tunica media of blood vessels (Figs. 3F and 3G). These distinctive clusters were never identified at luminal or perivascular locations within blood vessels, thereby excluding the possibility that they were phenotypically endothelial or connective tissue cells, respectively. These results indicate that the coronary smooth muscle lineage is already separated from both endothelial and connective tissue lineages, at the time of viral tagging in the proepicardium and prior to migration into the heart tube.

We examined the pericardial and cardiac spatiotemporal distribution of caldesmon, a marker for smooth muscle (Reckless *et al.*, 1993), from the appearance of the proepicardium until the first occurrence of coronary arterial structures. Occasional caldesmon-positive (CD+) cells were detected in the ''grape-like'' cluster of HH stages 17–18 embryos on whole mounts and histological sections (Fig. 4A). In addition, CD+ cells were also found at the epicardial layer of HH stage 17–18 embryonic tube hearts (Fig. 4A). In the ventricular wall of HH stage 22–25 hearts, CD+ cells were distributed throughout both myocardium and epicardium. In comparably staged quail embryos, discontinuous channels, or sinusoids, consisting of endothelial cells were apparent by immunostaining with QH1, an endothelial

FIG. 3. Retroviral tagging of the proepicardium *in ovo.* The proepicardial protrusion (A, B) or the myocardial wall of the tubular heart (C) of E3 chicken embryos were infected *in ovo* with 5 nl (A) or 0.5 nl (B, C) of $\beta$-gal virus suspensions containing $\sim 3 \times 10^7$ active virions/ml. On E18, hearts were fixed and stained with X-gal in whole mount. The colonies of $\beta$-gal+ smooth muscle cells in the boxed areas of A and B were examined at higher magnification (arrows in D, E). Semisagittal (F) and transverse (G) sections of coronary arteries were obtained from hearts bearing single $\beta$-gal+ smooth muscle cell colonies (B), while a section of epicardium (H) containing $\beta$-gal+ endothelial cells (arrowheads) and connective tissues cells (arrows) was prepared from hearts infected with higher viral titers (A). Asterisks, lumen of vessels; sm, smooth muscle cells; m, myocardium. Scale bars: 1 mm, A–C; 250 $\mu$m, D, E; 75 $\mu$m, F; 30 $\mu$m, G, H.

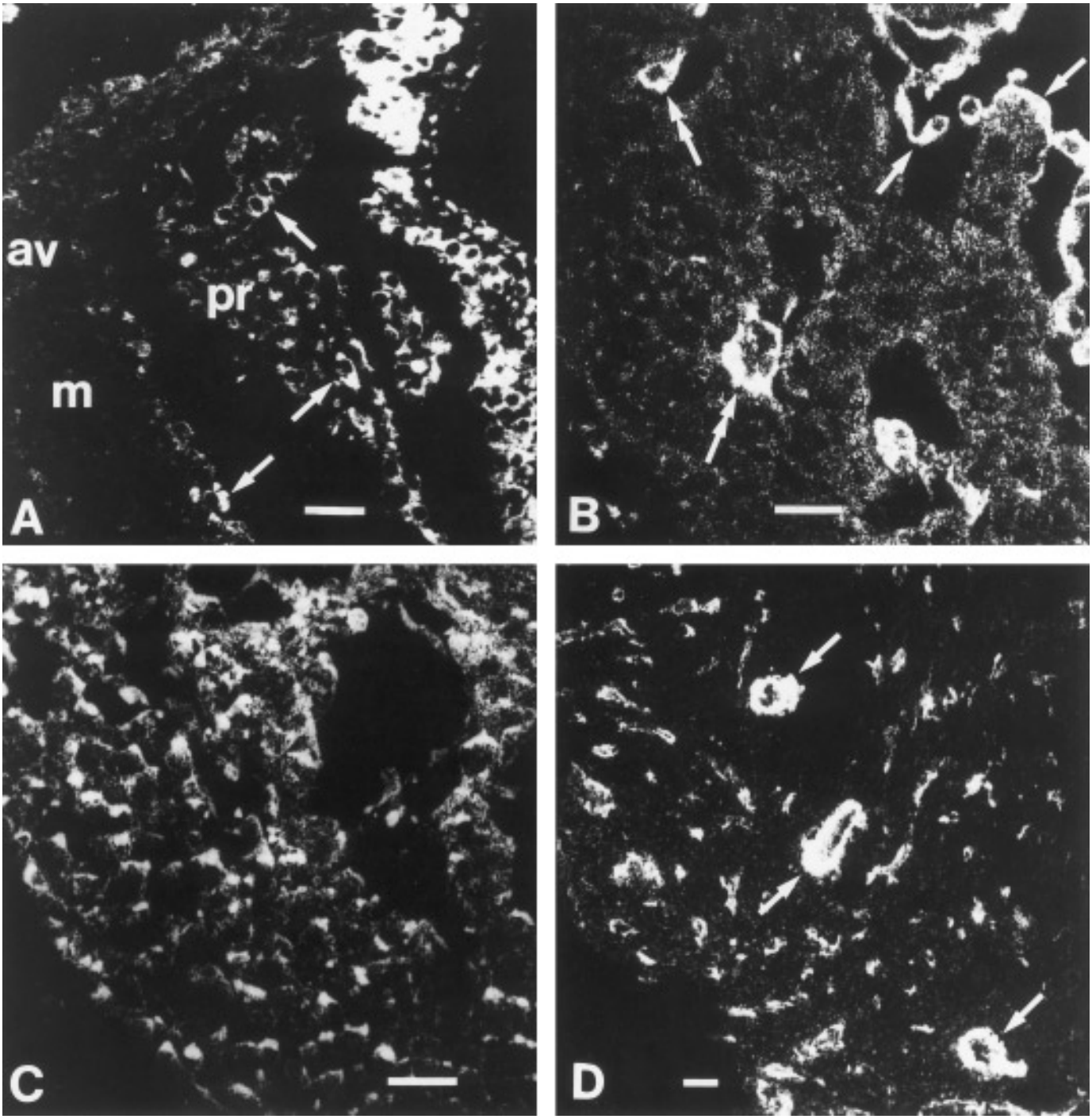



FIG. 4. Confocal analysis of coronary smooth muscle cells in embryonic hearts: (A) Caldesmon-positive (CD+) cells (arrows) in the proepicardial primordium and tubular heart of a HH stage 18 chicken embryo; pr, proepicardial primordium; av, atrioventricular junction; m, myocardium. (B) QH-1+ immunolabeled endothelial cells in the endocardium (arrows) and sinusoidal channels (double arrows) of a quail heart staged to match a HH stage 24 chicken embryo; (C) CD+ cells are scattered through the myocardium and epicardium of a CD5 immunolabeled section imaged at a corresponding location 30 $\mu$m serial to its near-sister section shown in 4B. (D) Accumulation of CD+ cells at developing coronary arteries of a stage 36 chicken heart. Scattered, non-vessel-associated CD+ cells are also observed at this stage. Scale bar: 20 $\mu$m.

marker (Fig. 4B). However, the scattered distribution of smooth muscle cells in the myocardium was distinct from these endothelial channels (Fig. 4C). Accumulation of smooth muscle cells to form coronary arteries was evident by E10 (HH stage 36) (Fig. 4D).

*Transplantation of β-gal-tagged proepicardial organ.* The results of the present study show that retroviral labeling of the proepicardium of E3 embryos gives rise to tagged cells which later become localized within subpopulations of cells in the heart. However, we were not able to rule out that tagging of smooth muscle progenitors resulted from potential leakage of virus from the intended injection site. Therefore, proepicardial clusters were microsurgically isolated from donor embryos at HH stages 17–18 (Figs. 5A and 5B). These clusters of cells were then tagged with β-gal-virus in culture and transplanted into the epicardial cavity of isochronic host embryos (Fig. 1). Some of the isolated fragments infected *in vitro* were cultured on a collagen lattice to test efficiency of viral tagging and survival following microsurgery (Figs. 5C–5E). After a few days in culture, outgrowths of mesothelial cells extended from the explant onto the collagen lattice (Fig. 5D). By 14 days in culture, epithelial cells from the explant had generated populations of mesenchymal cells that either migrated into the collagen lattice and dispersed or radiated from the explant in narrow tracts of interconnected cells (Fig. 5E). The dispersal of explanted cells and their formation into structures resembling vascular capillaries is consistent with the behavior of these cells *in vivo;* in the migratory events associated with epicardialization from HH stage 17 to 18 and the subsequent formation of endothelial-lined sinusoids in the developing heart. These experiments also demonstrate the stable maintenance of β-gal-expressing cells in culture for up to 14 days. On average, 5–10% of the cells in cultured explants were β-gal+ (Fig. 5E).

The fate of the transplanted cells in the host hearts was examined with the X-gal reaction in whole mount (Fig. 5F). In the present study, 49 embryos of 140 recipients survived through to E18. Of these 49, 38 hearts contained β-gal+ vascular cells (Table 1). In some cases, we found the spirally arranged β-gal+ clusters which are characteristic of smooth muscle cell colonies (Fig. 5F), in addition to other epicardially located cell populations. These results show that the graft-derived cells give rise to smooth muscle cell populations which are capable of being incorporated into the host coronary vascular systems. This data, together with that obtained by direct infection *in vivo* demonstrates that coronary smooth muscle cells, at least in part, are derived from the mesenchymal cells of the proepicardium.

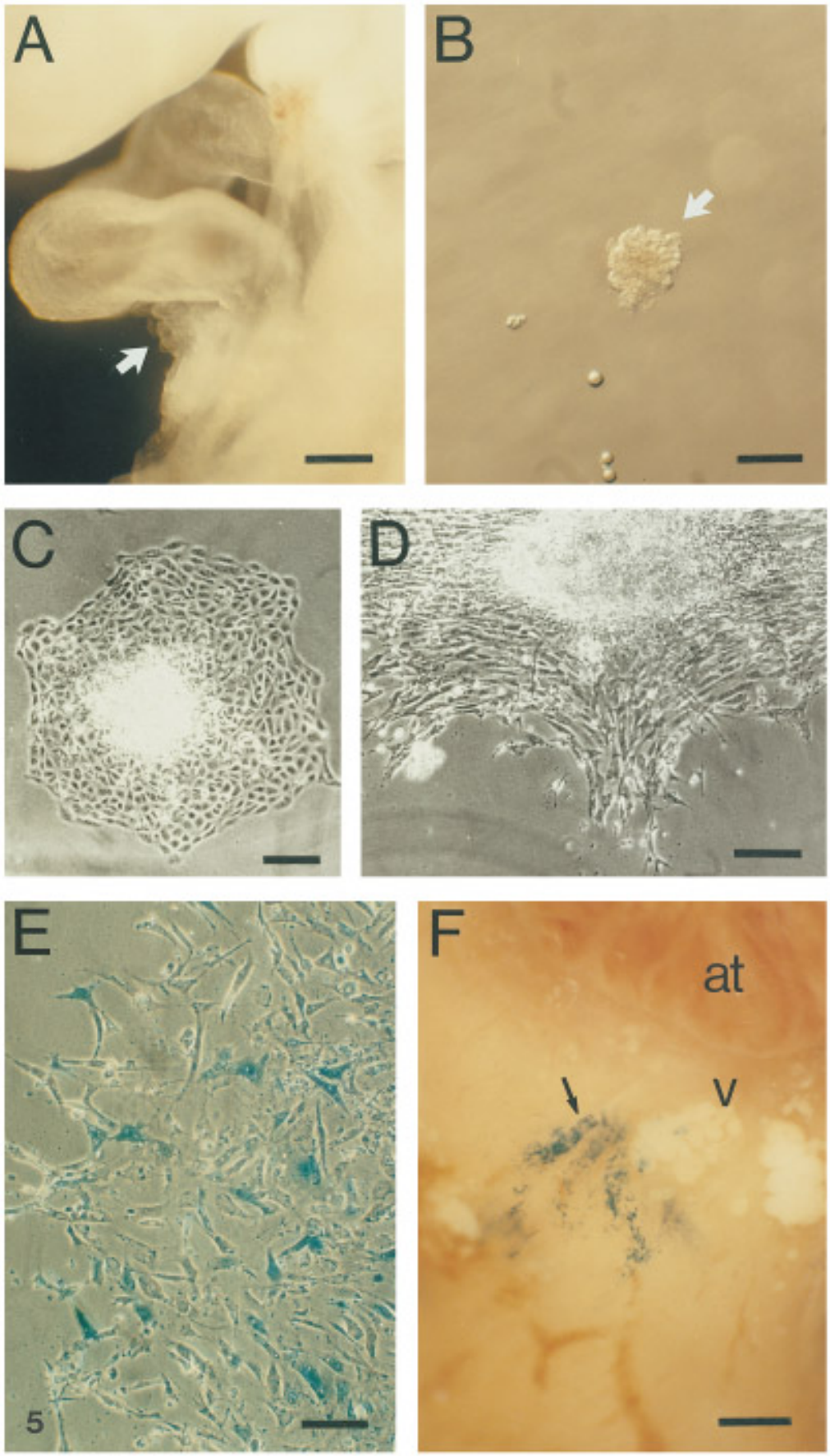
## DISCUSSION

The developmental mechanisms and origin of component cells of the coronary vascular system are controversial. The long-standing view that coronary vessels form by angiogenic outgrowth from the aortic root has been challenged by cell lineage (Mikawa and Fischman, 1992) and immunohistochemical studies (Bogers *et al.*, 1988; 1989; Waldo *et al.*, 1990). The present work shows that the proepicardium contains a progenitor population which generates the vascular smooth muscle cell lineage of coronary arteries and that cells of this lineage subsequently migrate, along with other coronary progenitors (e.g., vascular endothelium and connective tissue), into the tubular heart during formation of the epicardial mantle. This extracardiac origin and route of migration via the proepicardium is distinct from that of cardiac neural crest (d'Amico-Martel and Noden, 1983; Kirby *et al.*, 1983, 1993). Therefore, it is unlikely that coronary vascular precursors share common parentage with crest-derived populations thought by some workers to contribute to the smooth muscle of the proximal aorta and aortic arches (LeLièvre and LeDouarin, 1975; Hood and Rosenquist, 1992). Our conclusion is supported by embryonic chick/quail chimera (Kirby and Stewart, 1983) and DiI-labeling studies (Kirby *et al.*, 1993) in which contributions of ectomesenchyal derivatives to the heart were reported to be confined to the cardiac outflow tract and there were no discernible contribution to coronary arteries (Waldo *et al.*, 1994).

Since our methods labeled only a subset of cells within the proepicardial primordium, the genesis of coronary smooth muscle cells from sources other than that characterized by the present study cannot be ruled out. The present study demonstrates that a subset of cells from the proepicardium is one major source for coronary smooth muscle cells.

Our prior clonal analyses of coronary vascular cells in the embryonic chick heart (Mikawa and Fischman, 1992) showed that the smooth muscle lineage is established prior to stage 17 when these cells first enter the heart. Here, by clonal analysis of β-gal+ smooth muscle cells, we show that this specification occurs external to the tubular heart in the proepicardial organ. Chick/quail chimeric studies by Poelmann *et al.* (1993) have recently shown that the pericar-

FIG. 5. Retroviral tagging of the proepicardium *in vitro:* (A) The proepicardium is seen as a grape-like cluster of cells subjacent to the tubular heart (white arrow) of a HH stage 17 embryo; (B) an isolated proepicardial cluster (white arrow); (C) phase-contrast image of the isolated proepicardial anlagen cultured on a collagen lattice for 24 hr. Note the epithelial-like cell sheet migrating onto the collagen matrix; (D) mesenchymal transition of the epithelial-like cell sheet after 2 weeks in culture; (E) X-gal staining of an infected and cultured proepicardial anlagen; (F) β-gal+ cells in a host heart transplanted as in Fig. 1 examined at E18. Arrow, β-gal+ smooth muscle cells; v, ventriclar wall; at, atrium. Scale bars: 200 μm, A, B; 150 μm, C, D; 75 μm E; 400 μm, F.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dial villi contain endothelial cells which migrate into the myocardium to form the coronary endothelia. Our confocal analysis of immunolabeled cells showed the presence of smooth muscle markers in this structure. Other workers have also demonstrated the presence of cells expressing markers of smooth muscle phenotype within the pericardial villi of HH stage 17 embryo (Valder and Olson, 1994). These results suggest that at least a portion of vascular progenitors are committed to either the smooth muscle or endothelial cell lineage within the villi prior to their migration into the tubular heart. It remains to be seen whether the proepicardial cells expressing smooth muscle markers are progenitors of coronary smooth muscle cells. Although the intracardiac migration of vascular progenitors starts at E3, we did not detect significant accumulation of caldesmon-positive cells (i.e., vascular smooth muscle cells) in presumptive early coronary arteries until E10. Furthermore, Hood and Rosenquist (1992) have shown that the accumulation of smooth muscle cells first becomes prominent at the most proximal coronary arteries, and is later deployed downstream by E14. This proximal distal wave of smooth muscle cell accumulation to coronary arteries (Hood and Rosenquist, 1992) occurs coincidentally with the formation of the capillary network (Bogers *et al.*, 1988 and 1989; Waldo *et al.*, 1990) and is followed by establishment of closed coronary circulation (Rychter and Ostadal, 1971). The proliferation and migration of vascular smooth muscle cells are regulated by several peptide growth factors, including those expressed by endothelial cells responsive to changes in local blood flow (reviewed in Schwartz and Liaw, 1993). We therefore speculate that endothelial cells may act locally on smooth muscle precursors signaling them to enter active mitosis and/or migration, as well as inducing terminal differentiation of smooth muscle progenitors in the myocardial wall.

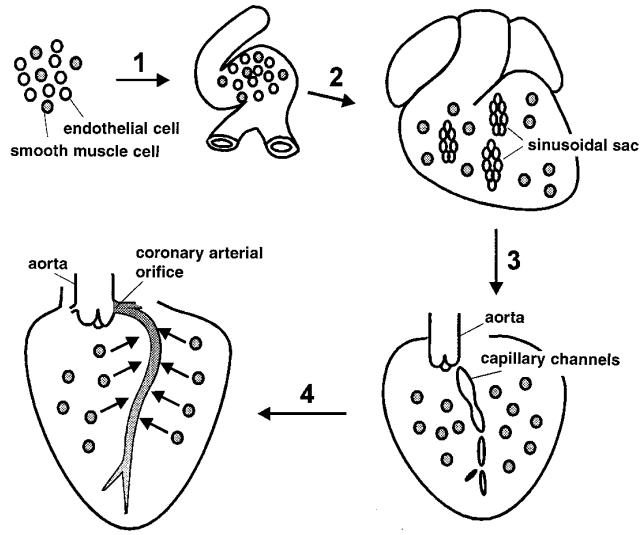


FIG. 6. Hypothetical model representing the differentiation of coronary arteries from migratory endothelial and smooth muscle progenitors. Independent endothelial (clear circles) and smooth muscle (shaded) precursors migrate from the proepicardium to the tubular heart during formation of the epicardial mantle (step 1). The endothelial cells differentiate to form sinusoidal sacs or channels (step 2). These sinusoids fuse along certain preferred axes, eventually forming capillary channels (step 3). Intracardiac smooth muscle cell progenitors migrate to defined segments of the endothelial channels (step 4) and differentiate to form the spiral segments observed following retrovirus tagging. Omitted from this diagram are the other progenitors which contribute to the epicardium and the tunica adventitia and surrounding interstitial connective tissue.

Our results and interpretations are summarized in a model presented in Fig. 6. This model represents four distinct steps in the development of avian coronary arteries and is based on the present data, our earlier cell tagging studies (Mikawa and Fischman, 1992) and other histological studies (Bogers *et al.*, 1989; Waldo *et al.*, 1990; Hood and Rosenquist, 1992). First, a progenitor population of coronary smooth muscle, as well as those of perivascular connective tissue and coronary endothelial cells, starts to migrate from the proepicardium to the tubular heart along with precursors of the epicardial mantle. Following this initial migration, endothelial cells form sinusoids or discontinuous channels which subsequently fuse to form a network of capillaries. Closure of the coronary vascular bed is then established by connection of capillaries to the root of aorta (formation of coronary orifices), coincident with initiation of proximal–distal accumulation of smooth muscle cells to early coronary arteries.

The model indicates that the ontogeny of coronary smooth muscle cells is separate from that of cardiac neural crest. However, as described earlier, there is growing evidence of roles for neural crest in spatial deployment and maintenance of the coronary vascular system (Hood and Rosenquist, 1992; Gittenberger-de Groot *et al.*, 1993; Waldo *et al.*, 1994). The coronary orifice is bounded by the aorta superiorly, the myocardial sheath of the truncus arteriosus inferiorly, and by components of the cardiac plexus laterally. When the cardiac neural crest is ablated, normal development of each of these components is disrupted (reviewed in Kirby, 1988), and consequently the coronary arteries show variance in their site of orifice formation at the aortic root (Hood and Rosenquist, 1992; Gittenberger-de Groot *et al.*, 1993). Neurons comprising the cardiac plexus originate from the cardiac neural crest (d'Amico-Martel and Noden, 1983; Kirby and Stewart, 1983), and it has been suggested that localization of these neural crest derivatives adjacent to blood vessels is important in the survival of definitive branches of coronary arteries (Waldo *et al.*, 1994). Hence, although making no direct contribution, cardiac neural crest-derived cells may be an important factor in defining spatial order in developing coronary arteries, as suggested by Hood and Rosenquist (1992). It remains to be shown why coronary vessels form only in certain areas of the subepicardial compartment (e.g., along the anterior interventricular sulcus) and whether coronary-directed mitogenic and migratory signals are restricted to those regions.

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