

Assembly of Trunk and Limb Blood Vessels Involves Extensive Migration and Vasculogenesis of Somite-Derived Angioblasts

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Vascular development requires the assembly of precursor cells into blood vessels, but how embryonic vessels are assembled is not well understood. To determine how vascular cells migrate and assemble into vessels of the trunk and limb, marked somite-derived angioblasts were followed in developing embryos. Injection of avian somites with the cell-tracker DiI showed that somite-derived angioblasts in unperturbed embryos migrated extensively and contributed to trunk and limb vessels. Mouse–avian chimeras with mouse presomitic mesoderm grafts had graft-derived endothelial cells in blood vessels at significant distances from the graft, indicating that mouse angioblasts migrated extensively in avian hosts. Mouse graft-derived endothelial cells were consistently found in trunk vessels, such as the perineural vascular plexus, the cardinal vein, and presumptive intersomitic vessels, as well as in vessels of the limb and kidney rudiment. This reproducible pattern of graft colonization suggests that avian vascular patterning cues for trunk and limb vessels are recognized by mammalian somitic angioblasts. Mouse–quail chimeras stained with both the quail vascular marker QH1 and the mouse vascular marker PECAM-1 had finely chimeric vessels, with graft-derived mouse cells interdigitated with quail vascular cells in most vascular beds colonized by graft cells. Thus, diverse trunk and limb blood vessels have endothelial cells that developed from migratory somitic angioblasts, and assembly of these vessels is likely to have a large vasculogenic component.

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INTRODUCTION

Several distinct cellular processes contribute to embryonic blood vessel formation (reviews in Risau and Flamme, 1995; Beck and D'Amore, 1997; Cleaver and Krieg, 1999). In vasculogenesis, mesoderm-derived vascular precursor cells called angioblasts coalesce and differentiate to form blood vessels. Vasculogenesis is further divided into vasculogenesis type I, the *in situ* differentiation of angioblast-containing cords, and vasculogenesis type II, the aggregation and differentiation of migratory angioblasts into blood vessels. In contrast, angiogenesis is the proliferation and migration of endothelial cells as sprouts from preformed vessels to form new vessels. Embryonic organs are often

vascularized by both vasculogenesis and angiogenesis (Noden, 1989; Wilms *et al.*, 1991; Robert *et al.*, 1998), but the relative contributions of each process in most vascular beds are unknown.

Some angioblasts migrate extensively. In avian embryos, numerous individual cells express the quail vascular marker QH1 and are presumptive migratory precursor cells (Noden, 1989; Poole and Coffin, 1989). Moreover, a physical barrier placed between a potential source of angioblasts and the target resulted in malformation of the target vessel, suggesting that vascular cell migration was blocked (Coffin and Poole, 1991). Another embryonic cell type that migrates extensively, the neural crest, has specific controls directing migration, so neural crest migratory pathways are well established in time and space (Bronner-Fraser, 1994). In contrast, little is known of the pathways and timing of angioblast migration during development. Moreover, the

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signals that influence the migration of angioblasts are poorly understood. In *Xenopus*, a VEGF isoform that acts as a long-range chemoattractant diffuses from the hypochord, and it potentially controls the angioblast migration that precedes formation of the dorsal aorta (Cleaver and Krieg, 1998). However, the hypochord does not exist in higher vertebrates, where the dorsal aorta is thought to develop by vasculogenesis type I (Pardanaud *et al.*, 1987; Coffin and Poole, 1988).

The migratory potential of vascular cells was also revealed by avian chimera analysis. Vascular cells derived from quail somite grafts colonized several chick host vascular beds (Wilting *et al.*, 1995; Pardanaud *et al.*, 1996; Pardanaud and Dieterlen-Lievre, 1999). Many of the graft cell destinations, such as the limb, were some distance from the graft site, suggesting migration of graft cells. However, in most avian chimera studies, the distinction between the migration of angioblasts (vasculogenesis type II) and the migration of endothelial cells (angiogenesis) was not made. This was in part due to the lack of a marker to visualize host vascular cells, which prevented clear determination of the source of the cells that made up individual vessels.

It seemed feasible to address questions of angioblast migration and assembly using mammalian tissue as a source of precursor cells, since mouse cells can be genetically marked and mouse vascular cells express the marker PECAM-1 (Baldwin *et al.*, 1994). Mouse somite transplantation experiments suggested that mammalian somitic mesoderm also contains migratory vascular precursor cells (Beddington and Martin, 1989; and V.L.B., unpublished results), but limitations of mouse embryo culture have prevented extensive analysis in the mouse host. An alternative was to place mouse grafts in avian hosts and analyze the resulting chimeras. Somite development has been studied by using mouse-chick chimeras, since mouse somites develop normally in the avian host (for review, see Fontaine-Perus, 2000). Proper migratory patterns were maintained to form muscle and skin, as visualized with grafts genetically altered to express a *lacZ* reporter gene in a subset of muscle progenitor cells and the trunk dermis (Houzelstein *et al.*, 1999, 2000).

To study the migration and assembly of the embryonic trunk and limb vasculature, we injected avian somites with a lineage tracer, and we generated mouse-avian chimeras. Somite-derived angioblasts migrated and colonized vascular beds of the trunk and limbs in otherwise unperturbed embryos. Mesoderm graft-derived mouse angioblasts migrated extensively and contributed to the host vasculature in reproducible patterns, demonstrating that mammalian angioblasts can respond to vascular patterning signals in the avian host. Finely chimeric vessels at numerous sites indicated that many trunk and limb blood vessels in the embryo were formed by vasculogenesis type II.

MATERIALS AND METHODS

Mice

B6129S-Gtosa26 (Rosa26) mice (Friedrich and Soriano, 1991; The Jackson Laboratory) were outcrossed once to CD1 mice (Charles River), then intercrossed to obtain homozygous Rosa26 mice. Genotype was determined by using a PCR protocol developed by The Jackson Laboratory. C57BL/6-J-Kdr^{tm1Jrt} males hemizygous for the *flk-1* gene (Shalaby *et al.*, 1995; The Jackson Laboratory) were maintained by crossing C57BL/6-J-Kdr^{tm1Jrt} males to C57BL/6-J females. Homozygous Rosa26 and hemizygous C57BL/6-J-Kdr^{tm1Jrt} male mice were bred to wild-type CD1 females to obtain embryos. Embryos from the *flk-1* cross were genotyped by β -galactosidase detection after the presomitic mesoderm was removed. The day of the vaginal plug was considered 0.5 days post coitum (dpc).

DiI Labeling

DiI labeling was performed by using CellTracker CM-DiI (C-7000; Molecular Probes, Eugene, OR) that was resuspended in DMSO at 2 mg/ml, and then diluted in either PBS or Ringer's solution at a concentration of 200 μ g/ml. For labeling *in ovo*, the DiI solution was injected into the center of the last formed somite of an 11- to 20-somite quail embryo after removal of the vitelline membrane. Embryos were either fixed immediately or incubated for 72 h in a humidified 37°C incubator, then fixed in 4% paraformaldehyde for 16 h at 4°C. DiI labeling of mouse presomitic mesoderm from 8.5 dpc *flk-1*^{+/−} embryos was done by soaking the grafts in DiI solution for 3 min. The grafts were then either fixed immediately in 4% paraformaldehyde for 5 min or transferred to avian hosts as described and incubated for 48 h. Embryos were processed for β -galactosidase detection.

The cell tracker CM-DiI is stable during the paraffin embedding process, so labeled grafts were dehydrated through an EtOH series and incubated in 100% EtOH for 10 min before being transferred to 100% Hemo-De for 10 min. Embryos were processed as described, and grafts or embryos were embedded in paraffin and sectioned. DiI was visualized by epifluorescence in whole embryos and in sections by using an Olympus IX-50 inverted microscope.

Mouse-Avian Chimeras

Fertilized chick and Japanese quail eggs (Truslow Farms, Chestertown, MD) were incubated at 37°C for 2 days prior to surgery. Pregnant mice were sacrificed by cervical dislocation when embryos were 8.5 dpc, and embryos were dissected from the decidua in M2 medium (Quinn *et al.*, 1982).

Donor presomitic mesoderm was excised from mouse embryos with 6–12 somites that were hemizygous for Rosa26 (Rosa^{+/−}), *flk-1* (*flk-1*^{+/−}), or wild-type (^{+/+}). Embryos were pinned dorsal side up in a Sylgard (Corning) dish in PBS containing 5 μ g/ml trypsin (Sigma) and 25 μ g/ml pancreatin (Sigma). By using sharpened tungsten needles, the ectoderm was carefully removed, and three to five somites worth of presomitic mesoderm was removed and labeled with Nile Blue on the anterior end. Grafts were rinsed in Ringer's solution/5% FCS.

The HH 10–13 stage (Hamburger and Hamilton, 1951) avian hosts were prepared *in ovo*. The vitelline membrane was peeled back, and a small amount of 0.15% trypsin in Ca²⁺-Mg²⁺-free Tyrode solution was applied to the graft site. Ectoderm and segmental plate were removed by using tungsten needles, and the

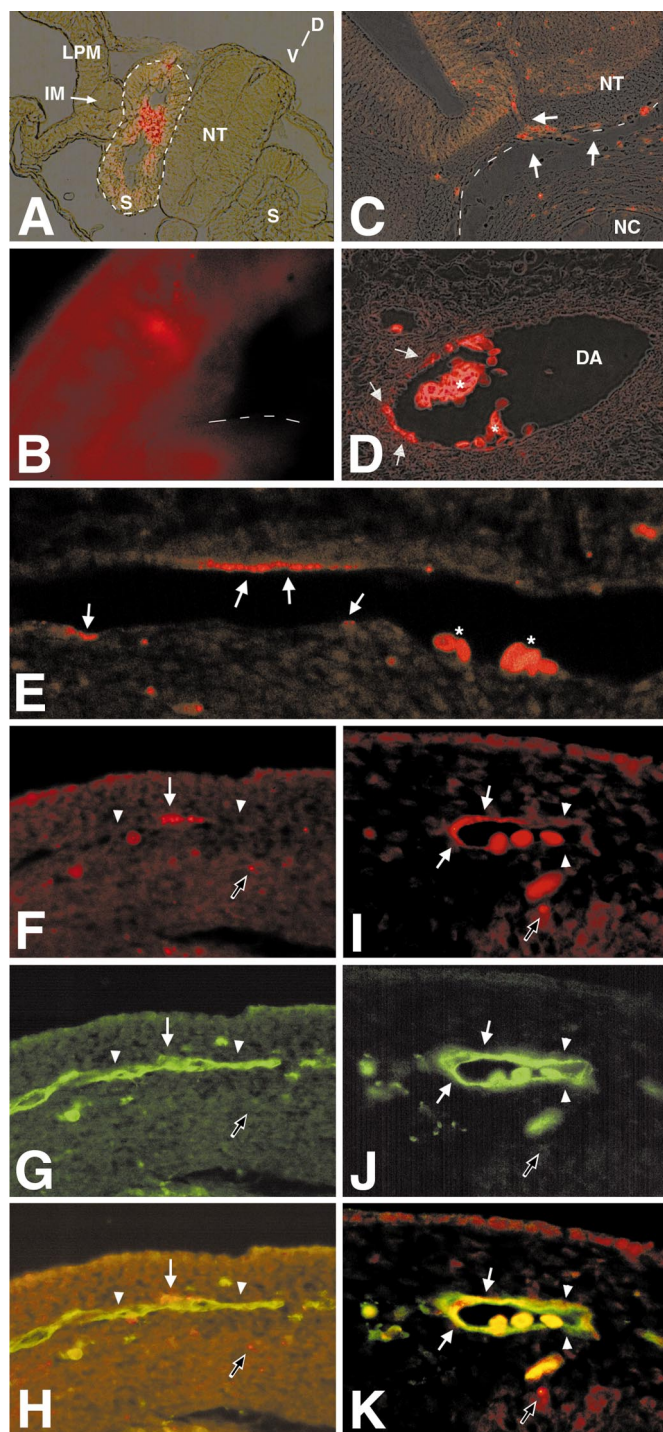


FIG. 1. DiI labeling of quail somites. (A) The last-formed somite of a quail embryo was injected with DiI solution. A transverse section of an embryo fixed just after injection shows DiI-labeled cells (red) within the somite (S). The adjacent neural tube (NT), intermediate mesoderm (IM), and lateral plate mesoderm (LPM) were unlabeled. The injection occurred through the ectoderm on the dorsal side of the embryo and resulted in the labeling of a few ectoderm cells. (B) Whole-mount visualization of an embryo 72 h

mouse graft was oriented as closely as possible to maintain the correct axes. The chimeric embryo was then sealed with tape or parafilm and allowed to incubate for 48 or 72 h at 37°C in a humidified incubator.

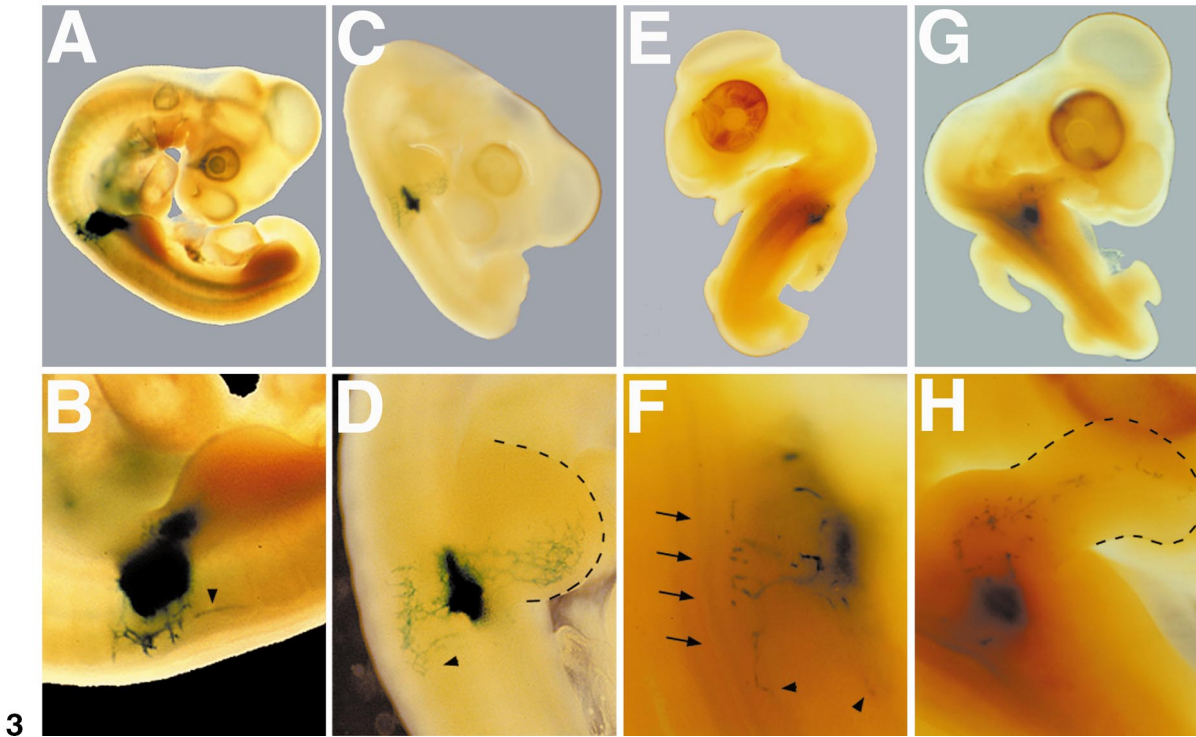
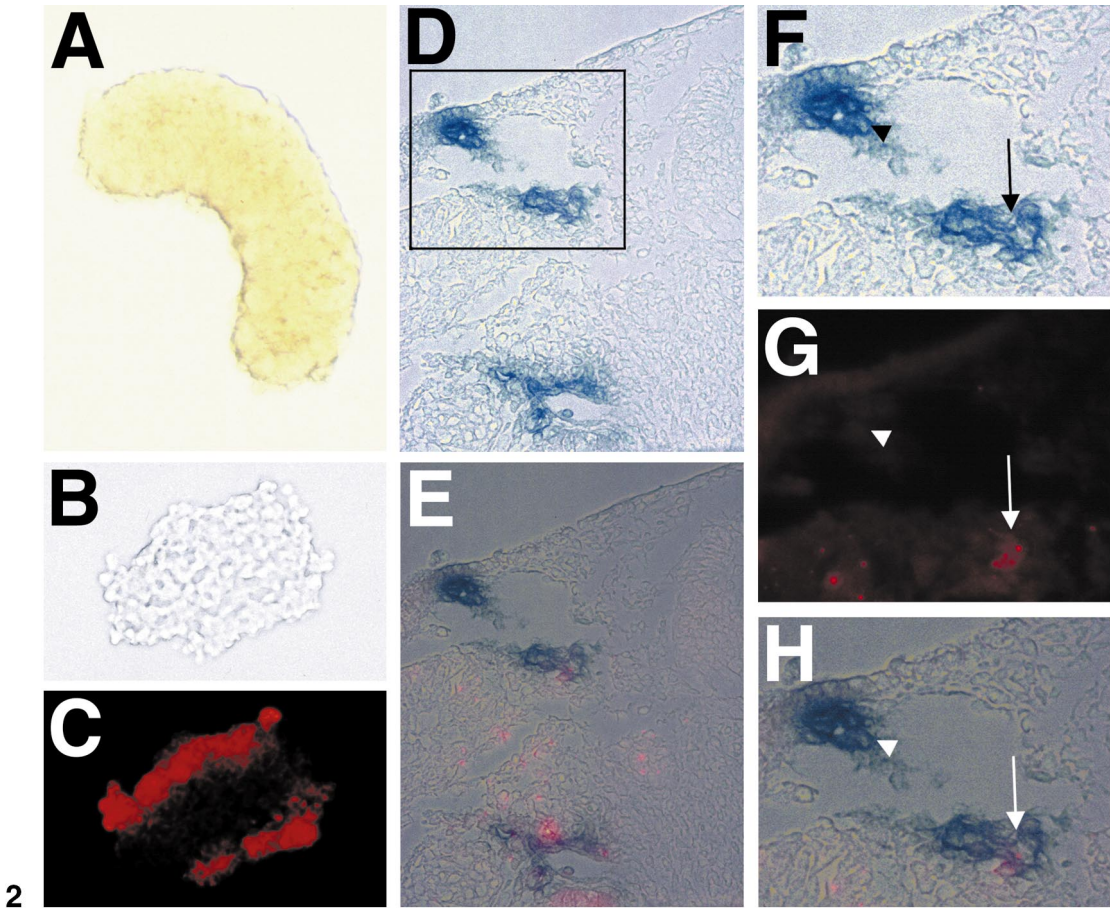
Whole-Mount β -Galactosidase Detection and Immunohistochemistry

For β -galactosidase detection (adapted from Beddington *et al.*, 1989), chimeric embryos were rinsed in PBS and fixed in 4% paraformaldehyde for 20 min at room temperature. Chimeric embryos were washed three times for 10 min each in wash buffer [0.1 M phosphate buffer (pH 7.3), 0.1% sodium desoxycholate, 0.02% NP40, 0.05% BSA (Sigma)], then transferred to wash buffer containing 1 mg/ml X-gal (Sigma), 0.24 mg/ml spermidine, 5 mM ferricyanide, and 5 mM ferrocyanide. After incubation for 3–18 h at 37°C in the dark, embryos were postfixed in 4% paraformaldehyde and stored at 4°C until photographed.

For whole-mount immunohistochemistry (adapted from Dent *et al.*, 1989), chimeric embryos were fixed in 4% paraformaldehyde overnight at 4°C, dehydrated through a MeOH series, and stored in 100% MeOH at –20°C. PECAM-1 was used to visualize the mouse graft vascular tissue because it does not cross-react with avian embryonic cells (data not shown). For immunohistochemistry, embryos were exposed to 3% H_2O_2 /MeOH overnight at 4°C, followed by rehydration to PBS. Embryos were incubated for 10 min in a solution of 1 $\mu\text{g}/\text{ml}$ proteinase K in 50 mM Tris (pH 8.0)/5 mM EDTA, and washed for 5 min in 2 mg/ml glycine in PBS with 0.1% Tween 20 (PBT). After blocking overnight at 4°C in Tris-buffered saline (pH 7.5)/0.1% Tween 20 (TBST)/20% FCS, the chimeric embryos were incubated overnight at 4°C with rat anti-mouse PECAM-1 (MEC 13.3; Pharmingen) at 1:200 in TBST/20% FCS. Five 1-h washes in TBST were followed by addition of an HRP-conjugated goat anti-rat secondary antibody (cat. no. JGRO35102; Accurate) at 1:200 in TBST/20% FCS. After incubation overnight, embryos were washed five times for 1 h in TBST. The antibody was detected by incubation in PBS/0.5 mg/ml DAB (Sigma)/0.03% H_2O_2 /3 mg/ml NiSO_4 for 10 min, resulting in a purple reaction product. Sometimes the NiSO_4 was eliminated to yield a brown reaction product.

To visualize the quail vasculature in chimeric embryos,

after injection. Most DiI-labeled cells were seen in the somitic region anterior to the limb (dashed line), but labeled cells were observed both anterior and posterior to the injection site by epifluorescence. (C–E) Transverse sections of a DiI-injected embryo show DiI-labeled cells (arrows) in vessels of the perineural vascular plexus (C, dashed line), the dorsal aorta (D), and the limb (E), visualized by epifluorescence (NC), notochord. Hematopoietic cells (*) exhibit autofluorescence that is distinguishable from DiI-labeling. (F–K) To confirm the presence of DiI-labeled cells within the embryonic vasculature, sections were additionally stained with the quail vascular marker, QH1 (green). (F–H) Limb blood vessel. (I–K) Dorsal perineural vessel. (F, I) DiI label (red); (G, J) QH1 antibody stain (green). (H, K) Overlay of (F) and (G) (H) or (I) and (J) (K). White arrows point to cells labeled with DiI that also react with QH1, white arrowheads point to cells not labeled with DiI that also react with QH1, and the black arrows point to DiI-labeled cells that do not react with QH1.



PECAM-1 staining was followed by QH1 staining, which is specific for quail hematoendothelial cells (Pardanaud *et al.*, 1987). Embryos were incubated overnight at 4°C in a 1:500 dilution of partially purified QH1 (Developmental Studies Hybridoma Bank, Iowa City, IA) in PBT/20% FCS. Five 1-h washes in TBST were followed by addition of an HRP-conjugated goat anti-mouse secondary antibody (cat. no. 115-035-003; Jackson ImmunoResearch) at a 1:200 dilution in PBT/20% FCS. Embryos were incubated overnight at 4°C, then washed five times for 1 h in TBST before antibody detection using a DAB (Sigma)/H₂O₂ reaction without NiSO₄ to produce a brown reaction product. Embryos were visualized and photographed with an Olympus SZH10 dissecting microscope.

Embryo Sectioning and Staining

Embryos were dehydrated through a MeOH series and then transferred to 100% EtOH for 20 min. The embryos were incubated in 50% EtOH/50% xylenes, 100% xylenes, and 50% xylenes/50% paraffin for 30 min each, then incubated with melted paraffin at 56°C for 3–48 h. Then, 10- μ m sections were cut on a microtome and dried before clearing in Hemo-De (Sigma). Sections were rehydrated in an ethanol series and mounted with Glycergel (Dako).

Detection of QH1 was performed on sections of chimeric embryos processed for whole-mount β -galactosidase detection. Coverslips were removed and slides were soaked in distilled H₂O at 60°C for 5 min. Slides were rinsed in PBS, then with 0.23% H₂O₂/PBS for 5 min. Slides were incubated overnight at 4°C with a 1:200 dilution of QH1 in PBS/20% goat serum. Slides were rinsed for 15 min in PBS followed by a 2-h incubation at 4°C with a 1:200 dilution of HRP-conjugated goat anti-mouse IgG in PBS/20% goat serum and a DAB (Sigma)/H₂O₂ reaction as described above. Slides were remounted with Glycergel (Dako), and visualized with a Nikon Optiphot 2 or a Nikon Eclipse TE300 microscope.

RESULTS

Labeled Somite-Derived Angioblasts Migrate Extensively

Although numerous avian studies have demonstrated that somite graft-derived angioblasts contribute reproduc-

ibly to host vasculature, the caveat that grafting may perturb normal developmental processes exists. To determine whether somite-derived cells migrated and contributed to the vasculature in a nongraft situation, the cell tracker DiI was injected into the center of quail embryo somites, and the labeled vasculature was analyzed after incubation (Fig. 1). Immediately after injection, the DiI label was found in the somite and not in surrounding structures such as the neural tube or intermediate mesoderm (Fig. 1A). Extensive migration of labeled cells from the injection site was seen after 3 days (Fig. 1B). The DiI label was found in presumptive endothelial cells of the perineural vascular plexus, the dorsal aorta, the limb, and the cardinal vein on the injected side of the embryo (Figs. 1C–1E; and data not shown). Double label with the quail vascular marker QH1 showed distinct labeling of blood vessels. The appearance of labeled cells in vessels of the limb (Figs. 1F–1H) and the dorsal perineural area (Figs. 1I–1K), areas distant from the injection site, showed that labeled angioblasts migrated significant distances prior to participating in vessel formation. Because of the limitations of DiI labeling, we chose to further analyze vascular migration and assembly with mouse–avian chimeras.

Mouse Presomitic Mesoderm Contains Vascular Precursor Cells

To determine whether mammalian presomitic mesoderm contains angioblasts that express the vascular marker *flk-1*, the presomitic mesoderm or the last-formed somites from 8.5 dpc *flk-1*^{+/-} embryos was removed and processed for β -galactosidase detection. β -galactosidase-positive cells expressed *lacZ* under control of the *flk-1* promoter, and they were considered to be *flk-1*-positive mouse vascular cells (Shalaby *et al.*, 1995). β -Galactosidase-positive cells were seen only at the edges of the excised somites, suggesting that they were endothelial cells from incompletely removed vessels such as the dorsal aorta (data not shown). In contrast, no positive cells were seen in or surrounding the

FIG. 2. The vascular potential of mouse presomitic mesoderm. (A) Presomitic mesoderm from a *flk-1*^{+/-} 8.5 dpc embryo processed for β -galactosidase detection. Note the absence of blue cells. (B, C) Presomitic mesoderm graft soaked in CellTracker DiI and sectioned sagittally through the center of the graft. Photographs of one section were taken in brightfield (B) and epifluorescence (C). Note that the DiI-labeled cells are restricted to the exterior region of the graft. (D–H) Day 8.5 presomitic mesoderm was soaked in CellTracker DiI, then transplanted into a chick embryo and incubated for 48 h. β -Galactosidase staining (blue) was performed to visualize *flk-1*^{+/-} mouse vascular cells, and DiI-labeled cells were detected by epifluorescence (red). Photographs of sections were taken in brightfield (D, F) and epifluorescence (G) and then overlaid (E, H) to visualize both β -galactosidase-positive cells and DiI-labeled cells. (E) Most DiI labeling was observed in β -galactosidase-negative cells. (F–H) Graft cells that labeled with DiI and were β -galactosidase-positive (arrows), as well as β -galactosidase-positive graft cells that did not label with DiI (arrowheads).

FIG. 3. Whole-mount analysis of mouse–avian chimeras. Mouse–avian chimeras were either *Rosa*^{+/-} presomitic mesoderm grafts (A–F) in chick hosts or *flk-1*^{+/-} presomitic mesoderm grafts (G, H) in a quail host. After incubation for 48 (A–D) or 72 h (E–H), chimeric embryos were fixed and stained for β -galactosidase. In the *Rosa*^{+/-} grafts, all mouse cells are identified by blue staining. (B, D, F) Higher magnification pictures of (A), (C), and (E), respectively, showing presumptive vascular outgrowth from the *Rosa*^{+/-} grafts. Graft cells were observed dorsally toward the neural tube (arrows, F) and laterally in the limb (limb margin denoted by dashed line, D and H). Vascular-like graft structures were also seen posterior to the incorporated graft (arrowheads, B, D, F). (G, H) Chimeric embryo with a *flk-1*^{+/-} presomitic mesoderm graft. (G) β -Galactosidase staining of graft-derived mouse presumptive vascular cells. At higher magnification (H), β -galactosidase-positive cells were seen in the limb.

presomitic mesoderm tissue (Fig. 2A). This result showed that excised presomitic mesoderm tissue was free of endothelial cells from formed vessels, and it suggested that vascular cells either are not determined to the vascular lineage in the presomitic mesoderm or do not express *flk-1*.

To determine whether mammalian cells fated to form vascular cells were present in either the inner or outer cell layers of the presomitic mesoderm, *flk-1*+/- presomitic mesoderm grafts were briefly labeled with DiI prior to transplantation. The short DiI exposure labeled the exterior cell layers of the graft through approximately two to three cell diameters, but the innermost cells, comprising about six to eight cell diameters, were not labeled (Figs. 2B and 2C). Grafts were then placed in the avian host and incubated for 48 h before β -galactosidase detection. A few β -galactosidase-positive graft cells labeled with DiI, but the majority of the β -galactosidase-positive graft cells were not labeled (Figs. 2D–2H). This result shows that mouse presomitic mesoderm contains vascular precursor cells, and it indicates that most graft precursor cells that will express *flk-1* initially reside in the interior of the presomitic mesoderm. This result also confirms that vascular cells derived from presomitic mesoderm grafts are unlikely to be derivatives of preformed mouse vessels, such as the dorsal aorta.

Mammalian Presomitic Mesoderm Contributes Migratory Vascular Cells to the Avian Host

A total of 42 of 52 (81%) surviving mouse–avian chimeric embryos had mouse cells as detected by β -galactosidase detection or antibody staining. To assess graft placement and migration of graft cells, presomitic mesoderm grafts from *Rosa*+/- mice were first analyzed. Whole-mount visualization showed that most chimeras had grafts that were placed in the trunk, with an extensive network of presumptive vasculature (Figs. 3A–3F). The chimeric embryos consistently had graft cells in structures resembling blood vessels that were positioned at significant distances from the graft site, such as the limb and the perineural area. Even chimeric embryos with partially incorporated grafts had graft-derived cells in these areas (Figs. 3C and 3D). Mouse cells were primarily restricted to the graft side of the embryos, but, on rare occasions, graft cells were found on the contralateral side, near the dorsal edge of the neural

tube (data not shown). Graft-derived mouse cells were found at large distances from the grafts in both the lateral plane of the graft and posterior to the graft site (Figs. 3B, 3D, and 3F, arrowheads). Occasionally, graft cells were also found anterior to the graft site (Fig. 3B). These results indicated that mouse graft cells migrated extensively in the avian hosts.

Chimeric embryos were sectioned (17 of 42 positive grafts) to further analyze graft cell identity and migration. Graft incorporation varied. Some grafts filled most of the somitic space and appeared to differentiate into dermomyotome (Fig. 4A, arrow) and sclerotome. The dorsal root ganglion formed from host chick cells (Figs. 4A and 4B, arrowheads), as predicted by its neural crest derivation. Other grafts only partially filled the somitic space, and graft tissue was often found ventral and lateral to the graft site (data not shown). Regardless of how the mouse graft incorporated in the avian host, morphologically distinct structures resembling blood vessels containing graft cells were always seen at significant distances from the graft proper.

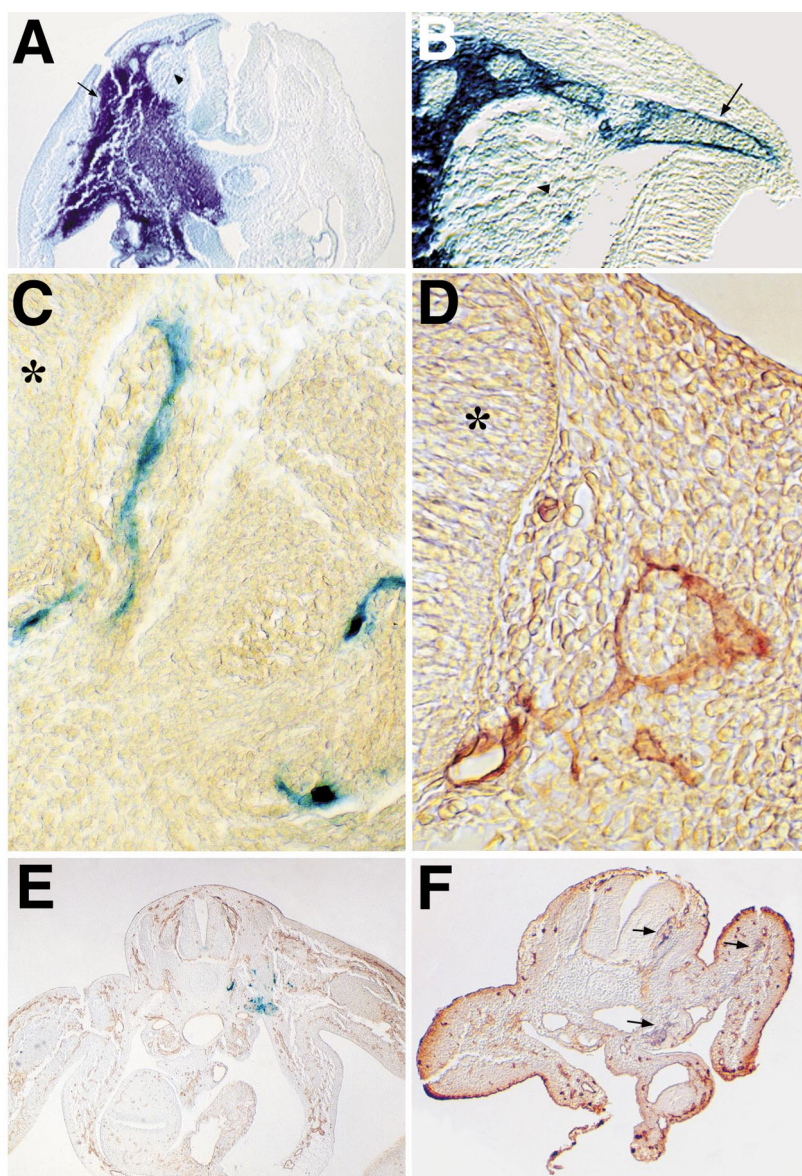
To confirm that mouse graft-derived vascular cells migrated from the grafts and differentiated in the avian host, chimeric embryos with *flk-1*+/- or wild-type grafts were analyzed. Mouse–avian chimeras with *flk-1*+/- grafts had β -galactosidase-positive graft cells (Figs. 3G, 3H, 4C, and 4E). β -Galactosidase-positive blood vessels were consistently seen in and around the graft site, as well as at some distance from the graft site. For example, the blue vessel next to the neural tube in Fig. 4C was in the perineural area, which was frequently some distance from the graft site. Antibody staining for PECAM-1, a mouse vascular marker, identified graft-derived vascular cells in chimeras with wild-type grafts (Figs. 4D and 4F). PECAM-1-positive mouse vasculature was seen in all areas identified as sites of graft migration, including the perineural area. These results show that the presumptive graft-derived vascular cells seen in the *Rosa*+/- grafts were indeed mouse vascular cells.

Mouse presomitic mesoderm was transplanted into quail embryos to visualize the relationship between host and graft blood vessels. Graft vasculature was visually distinct from host vasculature, and graft vessels were found in areas that also contained host vessels, such as the perineural area and the limb bud (Fig. 4F, arrows). Some graft vessels, such as those lateral to the cardinal vein (Fig. 4E) or the dorsal

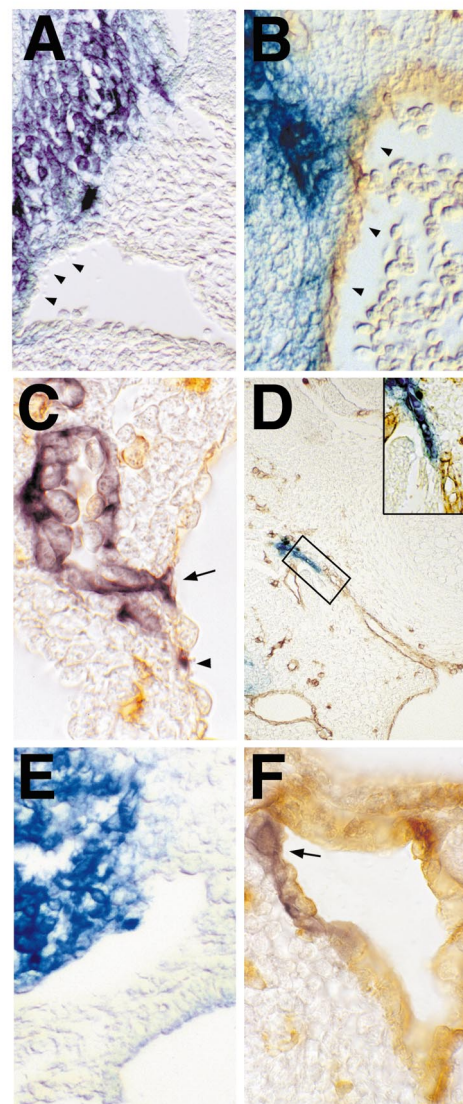
TABLE 1
Location of Mouse Graft Vascular Cells in Avian Hosts at 48–72 h

Dorsal aorta	Cardinal vein	Perineural vascular plexus	Vessels connected to the dorsal aorta	Embryonic kidney region	Limb
18% (3 ^a /17 ^b)	59% (10/17)	100% (17/17)	76% (13/17)	100% (17/17)	83% (10/12) ^c

^a All grafts contributing to dorsal aorta were incubated for 72 h.
^b Of 42 positive grafts, 17 were sectioned and analyzed microscopically.
^c Of the 17 grafts that were sectioned, 12 contained grafts at the limb level.



4



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FIG. 4. Microscopic analysis of mouse-avian grafts. (A, B) Transverse section of a mouse-chick chimera with a *Rosa*^{+/−} presomitic mesoderm graft. (A) The arrow denotes presumptive dermomyotome, and the arrowhead denotes presumptive dorsal root ganglion. (B) Higher magnification of (A) shows morphologically distinct β -galactosidase-positive vessel-like structures. (C, E) *Flk-1*^{+/−} presomitic mesoderm grafts or (D, F) wild-type presomitic mesoderm grafts in either a chick host (C, D) or a quail host (E, F). The grafts were processed for either β -galactosidase (blue, C and E) or PECAM-1 staining (brown, D, and purple, F), and mouse-quail chimeras (E, F) were also stained for QH1 (brown). (C, D) β -Galactosidase-positive graft cells or PECAM-1-positive graft cells were seen in the perineural area adjacent to the neural tube (*), and within the somitic region. (E, F) Lower magnification of transverse sections showing graft-derived β -galactosidase-positive or PECAM-1-positive cells relative to QH1-stained host vasculature. Arrows in (F) indicate the placement of PECAM-1-positive mouse cells (purple) relative to quail vessels (brown) in the perineural area, the limb, and the embryonic kidney region.

FIG. 5. Graft association with specific host vessels. (A, B) Transverse sections of chimeric embryos with graft cells adjacent to the dorsal aorta. (A, B) Chimeras with *Rosa*^{+/−} grafts were β -galactosidase-stained (blue), and (B) in the quail host, were labeled with QH1 (brown). Graft cells were usually absent from the endothelial lining (arrowheads, A, B). (C, D) Transverse sections of chimeras with graft cells in vessels connected to the dorsal aorta. (C) Chimera with a wild-type graft in a quail host was double-labeled for PECAM-1 (purple) and QH1 (brown). The arrow points to graft cells from a vessel that contacted the endothelium of the dorsal aorta. The arrowhead points to a rare instance of a graft cell in the endothelium of the dorsal aorta. (D) Chimera with a *flk-1*^{+/−} graft in a quail host was double-labeled for β -galactosidase (blue) and with QH1 (brown). A region of β -galactosidase-positive cells is seen adjacent to QH1-positive quail cells in a presumptive intersomitic vessel. (D, inset) A higher magnification of the boxed area. (E, F) Transverse sections of chimeric embryos with graft cells in the cardinal vein. (E) Chimera with a *Rosa*^{+/−} graft. Note the β -galactosidase-positive cells in the endothelial lining of the cardinal vein. (F) Chimera with a wild-type graft in a quail host was double-labeled for PECAM-1 (purple) and QH1 (brown). PECAM-1-positive graft cells were present in a portion of the cardinal vein along with QH1-positive quail vascular cells.

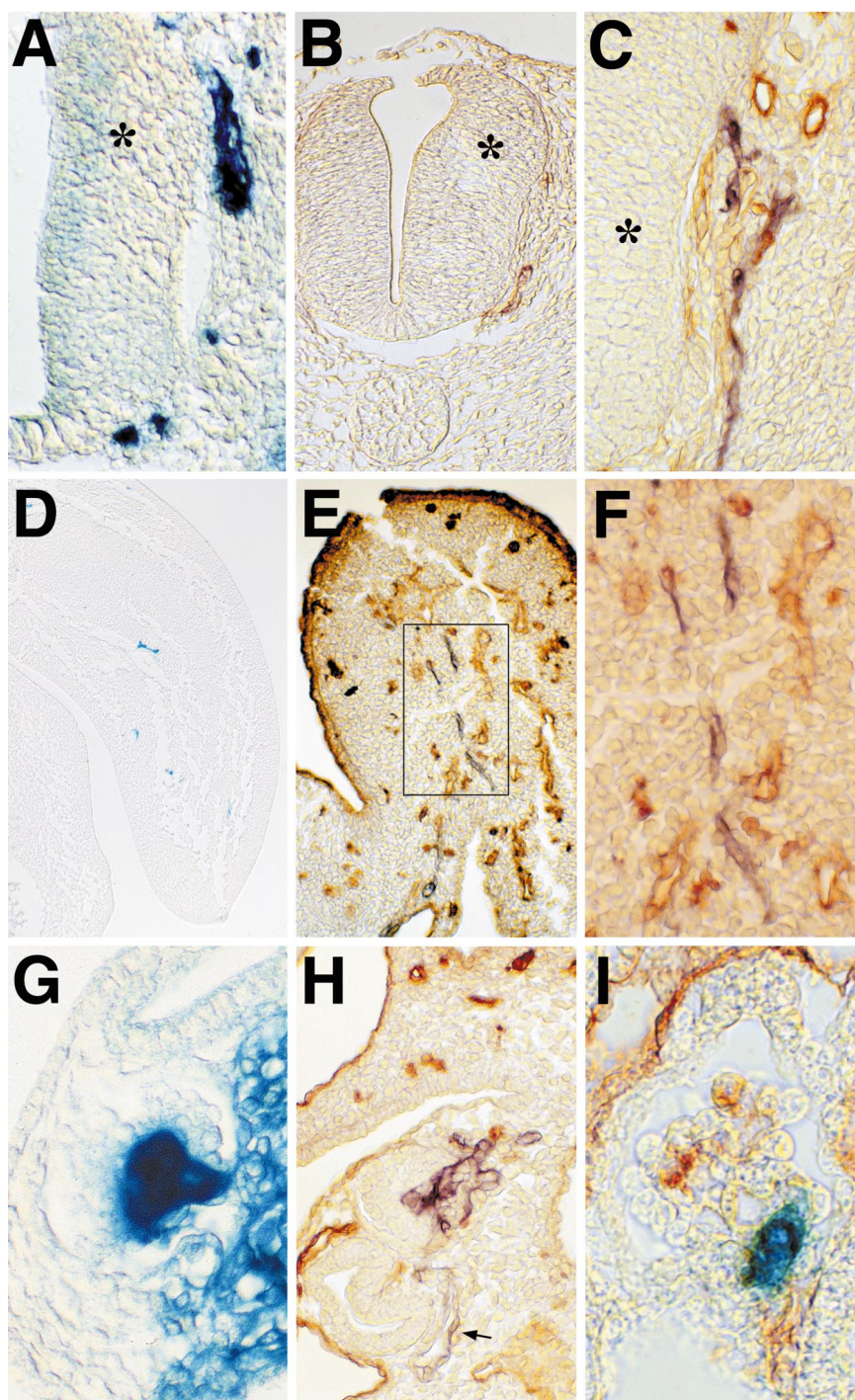


FIG. 6. Graft association with specific host vascular beds. (A–C) Transverse sections of chimeras with graft cells in the perineural area. (A) Chimera with a *Rosa*^{+/-} graft was β -galactosidase-stained (blue). (B) Chimera with a wild-type graft was stained for PECAM-1 (brown). (C) Chimera with a wild-type graft in a quail host was double-labeled for PECAM-1 (purple) and QH1 (brown). Note the mouse cells in all regions of the perineural vascular plexus surrounding the neural tube (*) on the graft side of the embryo. (D–F) Transverse sections of chimeras with graft cells in the limb. (D) Chimera with a *Rosa*^{+/-} graft. (E, F) Chimera with a wild-type graft in a quail host was double-labeled for PECAM-1 (purple) and QH1 (brown). Note the mouse graft cells at a distance from the graft site. (F) A higher magnification of the boxed region of (E). Note the PECAM-positive graft structures in the limb mesenchyme adjacent to and interdigitated with the QH1-positive quail vasculature. (G–I) Transverse sections of chimeras with graft cells in and around the embryonic kidney. (G) Chimera with a *Rosa*^{+/-} graft was β -galactosidase-stained (blue). Note the mouse cells within the avian kidney. (H, I) Mouse–quail chimeras with a wild-type graft stained for PECAM-1 (purple, H) or *flk-1*^{+/-} graft stained for β -galactosidase (blue, I) to show graft vascular cells, and stained for QH1 (brown) to show host vasculature. The arrow (H) points to graft vessels adjacent to a kidney tubule.

aorta (Fig. 4F), were in areas that did not have significant vasculature on the contralateral side. These could result from either ectopic vessel formation or sections that were not cut exactly perpendicular to the main body axis. Taken together, these results suggest that mammalian graft vascular cells migrated extensively in several directions in the avian host, often to areas rich in host vasculature.

Mouse Cells in the Vasculature of Chimeric Embryos

To determine whether the migration of mouse vascular cells was reproducible, the number of times that graft vascular cells were found in specific vessels and vascular beds of the host was determined (see Figs. 5-7 and Table 1). Of the 17 grafts analyzed microscopically, 8 were incubated for 48 h and 9 were incubated for 72 h postsurgery. Only in the dorsal aorta did graft incubation time affect the frequency of vascular contribution. Graft-derived cells were found in the endothelial layer of the dorsal aorta rarely (3/17, 18%), and, in all cases, these were 72-h grafts (Fig. 5C, arrowhead). However, graft tissue was often seen immediately adjacent to the dorsal aorta in chimeras with *Rosa*^{+/−} grafts (Figs. 5A and 5B, arrowheads).

Vessels connected to the dorsal aorta contained graft cells in 13 of 17 (76%) of the embryos analyzed (Figs. 5C and 5D, and Table 1). Vessels connected to the dorsal aorta often had graft-derived mouse cells incorporated into their endothelium (Fig. 5C, arrow), showing that this connection was not made via angiogenic sprouting from the dorsal aorta, but by fusion of independently derived vessels with the dorsal aorta. A *flk-1*^{+/−} graft in a quail host had a section of graft-derived vascular cells immediately adjacent to a presumptive intersomitic vessel that was host-derived (Fig. 5D and inset). This placement suggests that the vessel formed by a combination of vasculogenic and angiogenic processes.

Unlike the dorsal aorta, mouse graft-derived cells were frequently (10/17, 59%) seen in the endothelial lining of the cardinal vein (Figs. 5E and 5F, and Table 1). In embryos with *Rosa*^{+/−} grafts, graft cells were seen in the cardinal vein where it abutted the graft (Fig. 5E). In double-labeling experiments, graft vascular cells were found in the endothelial lining adjacent to host vascular cells (Fig. 5F, arrow).

Graft-derived mouse cells were observed in the perineural vascular plexus in all grafts analyzed (17/17, 100%, Figs. 6A–6C, and Table 1). Graft cells were present in this region even when the graft was shifted ventrally in the graft site (Fig. 6A). Graft cells were found in all regions along the perineural vascular plexus from the base of the neural tube to the dorsal end, but they were more frequently found next to the dorsal neural tube (Figs. 6A and 6C). A double-label experiment showed that, as at other sites, graft vascular cells were found closely juxtaposed to host vascular cells (Fig. 6C), suggesting that the perineural vasculature forms in part by vasculogenesis type II.

Graft-derived cells were seen in the limb in 10 of 12 (83%) chimeric embryos with graft placement in the limb field

(Table 1). An extensive network of limb vasculature developed from some grafts, as seen in the whole-mount embryos (Figs. 3D and 3H) and in sections (Figs. 6E and 6F). Graft cells were seen at long distances from the graft sites in the limb (Fig. 6D). Double labeling of a chimera showed that mouse vasculature developed in the limb mesenchyme, adjacent to and intermingled with QH1-positive quail vessels (Figs. 6E and 6F).

Graft-derived mouse cells were present in the embryonic kidney region in all grafts analyzed (17/17, 100%; Figs. 6G–6I, and Table 1). Graft-derived cells were observed inside the embryonic kidney and appeared to connect to the surrounding vasculature. Mouse vascular cells were also found surrounding the embryonic kidney tubules (Fig. 6H, arrow).

Assembly of Blood Vessels

To analyze how blood vessels of the trunk and limb are assembled, we took advantage of the ability to label both graft and host vascular cells in the same chimeric embryo. Double-label experiments with either *flk-1*^{+/−} grafts or wild-type mouse grafts in quail hosts were analyzed microscopically (Fig. 7). In some cases, mouse vascular cells appeared to replace host vascular cells. For example, a host vessel in the embryonic kidney had a graft-derived counterpart in a chimeric embryo with a *flk-1*^{+/−} graft (Figs. 7A and 7B). In other cases, graft-derived vessels were seen in the absence of vasculature on the contralateral side. For example, one chimeric embryo with a *flk-1*^{+/−} graft had a region of β -galactosidase-positive vascular cells unique to the graft side (Fig. 7C). However, in most cases, the “ectopic” vasculature was seen at or near the graft site, suggesting that graft-derived signals may have induced the vasculature. In some chimeric embryos, graft-derived vascular cells were clearly in the host vascular field (Fig. 7D). In these cases, it was not possible to determine whether the graft vascular structures were replacement structures or ectopic vasculature.

PECAM-1-positive mouse vascular cells were often seen immediately adjacent to quail vascular cells, consistent with the formation of mouse–quail chimeric vessels. This type of chimerism was seen in both major vessels, such as the cardinal vein (Fig. 5G, arrow), and minor vessels in the limb (Figs. 7E–7G) and in the perineural vasculature (Fig. 6C). The fine chimerism seen in many vessels strongly suggests that they formed by vasculogenesis. In one minor chimeric vessel in the limb, two cells that appeared hematopoietic by morphological criteria were observed, indicating that chimeric vessels participated in embryonic blood circulation (Fig. 7G, arrows).

DISCUSSION

Blood vessel assembly is crucial to embryonic development, but how this process occurs is not well understood.

Our analysis of marked angioblasts shows that numerous vessels of the trunk and the limbs assemble via vasculogenesis. Moreover, it is highly likely that most analyzed vascular beds have been colonized by migratory angioblasts derived from somites, suggesting that vascular patterning cues direct angioblast migration.

Angioblasts were marked by Dil injection of somites in otherwise unperturbed embryos, and they subsequently migrated significant distances to colonize vascular beds of the trunk and limbs. Labeled endothelial cells were found at most sites colonized by somite graft-derived vascular cells including the dorsal aorta, the perineural vascular plexus, the limb, and the cardinal vein. This result indicates that the colonization of host vessels by graft-derived angioblasts from avian (Wilting *et al.*, 1995; Pardanaud *et al.*, 1996) or mouse (this study) somites reflects processes that normally occur during embryonic development.

To determine whether vascular patterning cues are species-restricted and to take advantage of the ability to genetically mark all graft vascular cells, we analyzed mouse–avian chimeras. Mammalian presomitic mesoderm contains vascular precursor cells that differentiate and migrate extensively in the avian host. The initial location of vascular precursor cells in the murine grafts was not precisely identified, but graft labeling prior to placement in the host showed that most graft vascular cells derive from cells initially interior in the graft. The finding that all quadrants of the avian somite and the cells surrounding the somitocoele contribute to host vasculature is consistent with a predominantly internal localization of vascular precursor cells (Wilting *et al.*, 1995). Attempts to identify presomitic or somitic cells that express the mouse vascular markers *flk-1* or PECAM-1 have been largely negative (N. Harmaty and V.L.B., unpublished results), and the *flk-1*+/- grafts contained no β -galactosidase-positive cells at transplantation. Vascular marker expression in avian mesoderm is complex. Expression of Quek1, the quail homologue of *flk-1*, was restricted to the dorsolateral half of the developing somite (Eichmann *et al.*, 1995), although expression of QH1, the quail angioblast marker, was observed in individual cells throughout the somite (Pardanaud *et al.*, 1996). Mesodermal regions of quail embryos that contained no QH1-positive cells were grafted ectopically and contributed to the vasculature of the chick host (Noden, 1989). Our results suggest that either mouse presomitic vascular precursor cells do not express *flk-1* or PECAM-1, or the vascular fate of the precursor cells is not determined at transplantation.

Mammalian vascular cells, like their avian counterparts, appear to be highly migratory and invasive (Noden, 1989; Poole and Coffin, 1989; Pardanaud and Dieterlen-Lievre, 1993). Murine graft-derived vascular cells were frequently seen posterior, lateral, and medial to the graft site, often at large distances from the body of the graft. Several lines of evidence strongly suggest that mammalian vascular cells recognize avian vascular patterning cues. Mouse vascular graft cells were primarily restricted to the graft side, indi-

cating that restrictions to midline crossing operative in quail–chick chimeras (Wilting *et al.*, 1995; Klessinger and Christ, 1996) also apply to mammalian vascular cells. Chimeric embryos had a specific and reproducible pattern of colonization of host vessels and vascular beds from mouse presomitic mesoderm grafts (Fig. 8). Mouse vascular cells were seen in the perineural vascular plexus, the limb, the embryonic kidney region, the cardinal vein, and in vessels connected to the dorsal aorta. Similar results were reported in avian somite graft experiments (Wilting *et al.*, 1995; Pardanaud *et al.*, 1996), indicating that mammalian and avian vascular cells share the capacity to colonize these vascular beds. It may be that angioblasts are fate-restricted to develop into specific vascular structures, but this seems unlikely. Both avian (Noden, 1989; Poole and Coffin, 1989) and mouse (V.L.B., unpublished results) grafts placed at ectopic sites contributed to surrounding vasculature, supporting the idea that angioblasts respond to environmental vascular patterning signals.

The perineural vascular plexus forms around the neural tube. Mouse graft-derived vascular cells were found in the perineural vascular plexus of all analyzed chimeric embryos, even when the grafts were displaced ventrolaterally and at some distance from the neural tube. When ventrolateral portions of avian somites were grafted orthotopically, graft-derived cells contributed to the perineural vascular plexus, indicating that avian vascular cells migrated through the medial somite to reach the perineural area (Wilting *et al.*, 1995). Our results show that mouse vascular cells, like their avian counterparts, can migrate some distance from the body of the graft prior to contributing to the host perineural vascular plexus, presumably in response to host-generated vascular migratory signals. Mouse vascular cells also consistently migrated large distances into the limb and formed an extensive network in some chimeric embryos.

One major difference is that avian somitic grafts had cells that contributed to the endothelium of the dorsal aorta (Wilting *et al.*, 1995; Pardanaud *et al.*, 1996), while mouse graft-derived cells were seen in the endothelium of the dorsal aorta only rarely. The difference in graft contribution to the dorsal aorta may be a result of surgical technique, although, in a small number of quail–chick chimeras we generated and analyzed, quail cells were observed in the dorsal aorta (unpublished results). The difference may also reflect species-specific differences in vascular development. The dorsal aorta has already formed at the graft site at the time of surgery (Coffin and Poole, 1988), so perhaps vascular remodeling signals are species-restricted. In contrast, mouse graft-derived vascular cells were consistently found in the cardinal vein. The difference between colonization of the dorsal aorta and the cardinal vein may result from differences in the timing of vessel development, since the cardinal vein has not yet formed in the graft region at transplantation (Coffin and Poole, 1991). Alternatively, the graft vascular precursor cells may have a restricted potential at transplantation.

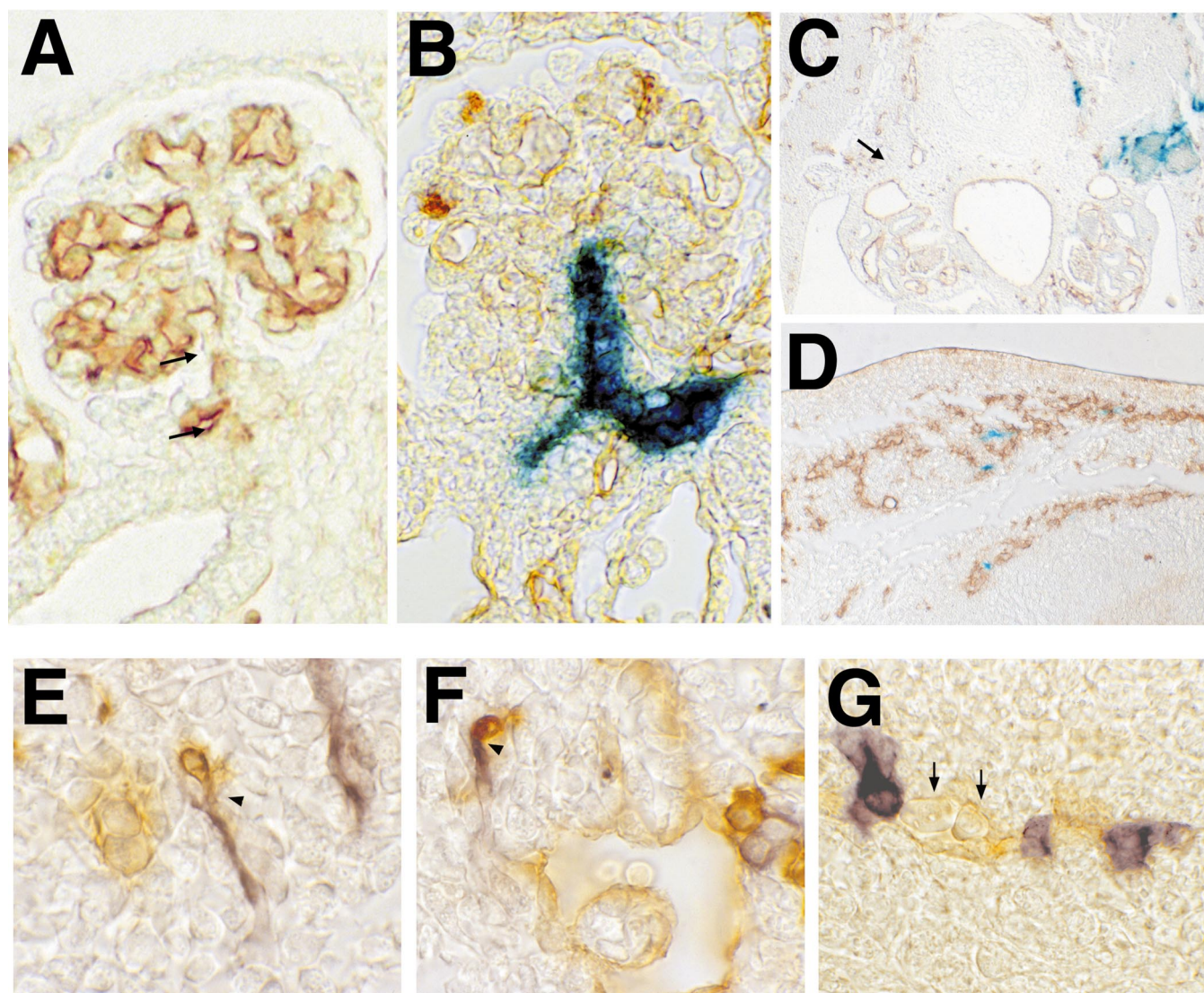


FIG. 7. Colocalization of graft and host vasculature. Transverse sections of mouse–quail chimeras with *flk-1*^{+/-} grafts (A–D) or wild-type grafts (E–G). Embryos with *flk-1*^{+/-} grafts were stained for β -galactosidase (blue), embryos with wild-type grafts were stained for PECAM-1 (purple), and all chimeras were stained with QH1 (brown). (A) Kidney region of nongrafted (control) side of a chimera. Arrows, QH1-positive quail vasculature in the kidney rudiment. (B) Kidney region of a chimera on the grafted side. β -Galactosidase-positive graft-derived vasculature is similar to vessels on the control side. (C) Ectopic region of β -galactosidase-positive cells on the grafted side of a chimeric embryo, with no corresponding region of quail vasculature on the contralateral side of the embryo (arrow). (D) β -Galactosidase-positive mouse vasculature present among QH1-positive host vessels in the limb. (E, F) Association of graft cells with host vascular cells in the limb. Arrowheads, QH1-positive (brown) host cells adjacent to PECAM-1-positive (purple) graft cells are closely juxtaposed in a chimeric vessel. (G) Vessel in the limb made up of PECAM-1-positive (purple) graft cells and QH1-positive (brown) host vascular cells. The arrows point to two host-derived presumptive hematopoietic cells.

It is crucial to understand how embryonic blood vessels are assembled, because different cells are the targets of vascular patterning cues in each process—angioblasts during vasculogenesis and endothelial cells during angiogenesis. We took advantage of the fact that excellent vascular markers are available for both mouse and quail vascular cells to visualize all cells of chimeric blood vessels. In many

of these chimeric vessels, the cells of each species were interdigitated to produce finely chimeric structures. This type of chimerism strongly indicates that these vessels were assembled by a vasculogenic process, since vessels formed by sprouting angiogenesis would be expected to be nonchimeric, or only chimeric in large patches if the parent vessel was chimeric. The avian cardinal vein is known to

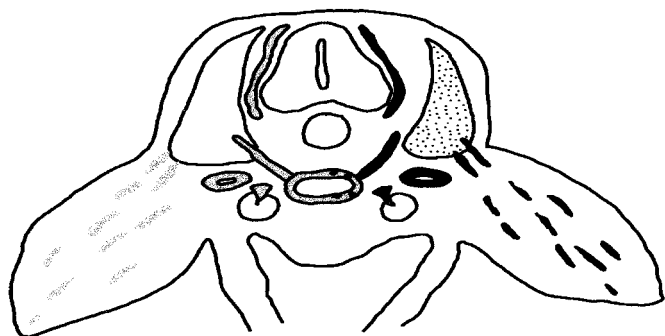


FIG. 8. Summary of mouse graft vascular development in the avian environment. Mouse grafts (stippled) incorporated and developed in the avian host. Mouse vascular cells were detected in several regions of the chimeric embryo, including the limb, the perineural vascular plexus, the cardinal vein, the vessels connected to the dorsal aorta, and the kidney rudiment (black filled areas). Host vessels are gray.

develop by vasculogenesis (Poole and Coffin, 1991), and our data confirm that vasculogenesis contributes to cardinal vein assembly. The finely chimeric vessels found in the perineural vascular plexus and in the limb indicate that vasculogenic processes contribute to the formation of these vascular beds as well, as suggested by earlier studies (Noden, 1989; Wilms *et al.*, 1991; Brand-Saberi *et al.*, 1995). Moreover, both vascular beds were some distance from the body of the graft, and endothelial sprouts connecting the graft proper and the vascular bed were seen only rarely in the limb and never in the perineural area. Thus, mouse vascular cells most likely migrated to the perineural and limb areas and assembled by vasculogenesis type II.

It is highly likely that angiogenesis also contributed to vascular development in some areas. Chimeric embryos had limb vessels that appeared nonchimeric and derived from either host or graft. While these vessels could have formed by vasculogenesis, their composition is consistent with formation by sprouting angiogenesis, as previously suggested (Wilms *et al.*, 1991). Larger patches of mouse graft-derived cells were seen in vessels that connected to the dorsal aorta, including presumptive intersomitic vessels. Since mouse cells were not consistently seen in the dorsal aorta, the graft vessels could not have developed solely by sprouting angiogenesis from the dorsal aorta. The patchy composition of these vessels suggests that they formed by a combination of vasculogenic and angiogenic processes. Finally, the pattern of graft colonization of the kidney suggested a process of sprouting angiogenesis of graft-derived vascular cells with a host contribution by vasculogenesis, which correlates with recent studies showing that both vasculogenesis and angiogenesis contribute to kidney vascularization (Sariola *et al.*, 1984; Robert *et al.*, 1998).

Mouse graft-derived angioblasts migrate extensively,

then participate in avian vascular development. They do so in patterns similar to those of endogenous somite-derived angioblasts. They participate in both extensive vasculogenesis and in angiogenesis, and in the limb and perineural vascular beds it is highly likely that vessels assemble in part by vasculogenesis type II. The reproducible pattern of colonization of the avian host shows that mammalian vascular cells can respond to avian vascular patterning signals. The extensive graft contribution by vasculogenesis at sites distant from the graft in the trunk and limbs suggests that mammalian angioblasts are the targets of host migratory cues. The mouse-avian chimeras can now be used to dissect the signaling pathways involved in vascular migration and patterning, by analyzing mutant grafts and by modulating signaling in the host.

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