DEVELOPMENT AND DISEASE

The meso-angioblast: a multipotent, self-renewing cell that originates from the dorsal aorta and differentiates into most mesodermal tissues

Maria G. Minasi[1,2,*], Mara Riminucci[3,*], Luciana De Angelis[2,*], Ugo Borello[1], Barbara Berarducci[1], Anna Innocenzi[1], Arianna Caprioli[4], Dario Sirabella[2], Marta Baiocchi[2,5], Ruggero De Maria[5], Renata Boratto[6], Thierry Jaffredo[4], Vania Broccoli[1], Paolo Bianco[7,†] and Giulio Cossu[1,2,†]

[1]Stem Cell Research Institute, Dibit, H. S. Raffaele, Via Olgettina 58, 20132 Milano, Italy
[2]Dipartimento di Istologia ed Embriologia Medica, Universita' di Roma 'La Sapienza' Via Scarpa 14, 00161 Roma, Italy
[3]Dipartimento di Medicina Sperimentale, Università de L'Aquila, 67100 L'Aquila, Italy
[4]Institut d'Embryologie Cellulaire et Moléculaire du CNRS et du College de France; 49 bis, Avenue de la Belle Gabrielle, Nogent sur Marne, France
[5]Laboratorio di Ematologia Oncologia, Istituto Superiore di Sanità, Viale Elena 297, 00161 Roma, Italy
[6]Dipartimento di Medicina Sperimentale, Università di Pavia, Via Forlanini 8, 27100 Pavia, Italy
[7]Dipartimento di Medicina Sperimentale e Patologia, Universita' degli Studi di Roma 'La Sapienza', Viale Regina Elena 324, 00161 Roma, Italy
*These authors contributed equally to this work
†Authors for correspondence (e-mail: p.bianco@flashnet.it and cossu.giulio@hsr.it)

SUMMARY

We have previously reported the origin of a class of skeletal myogenic cells from explants of dorsal aorta. This finding disagrees with the known origin of all skeletal muscle from somites and has therefore led us to investigate the in vivo origin of these cells and, moreover, whether their fate is restricted to skeletal muscle, as observed in vitro under the experimental conditions used. To address these issues, we grafted quail or mouse embryonic aorta into host chick embryos. Donor cells, initially incorporated into the host vessels, were later integrated into mesodermal tissues, including blood, cartilage, bone, smooth, skeletal and cardiac muscle. When expanded on a feeder layer of embryonic fibroblasts, the clonal progeny of a single cell from the mouse dorsal aorta acquired unlimited lifespan, expressed hemo-angioblastic markers (CD34, Flk1 and Kit) at both early and late passages, and maintained multipotency in culture or when transplanted into a chick embryo. We conclude that these newly identified vessel-associated stem cells, the meso-angioblasts, participate in postembryonic development of the mesoderm, and we speculate that postnatal mesodermal stem cells may be derived from a vascular developmental origin.

Key words: Stem cells, Endothelium, Pericyte, Angiogenesis, Chimera, Mesoderm, Histogenesis, Mouse, Quail

INTRODUCTION

Our current appreciation of stem cells plasticity has changed dramatically in the last years, following the report that the adult bone marrow contains progenitors that can be incorporated into skeletal muscle (Ferrari et al., 1998; Gussoni et al., 1999), liver (Paterson et al., 1999; Lagasse et al., 2000) and the central nervous system (Kopen et al., 1999; Mezey et al., 2000; Brazelton et al., 2000). Conversely, the CNS contains stem cells that can differentiate into hematopoietic (Bjorson et al., 1999) or skeletal muscle (Galli et al., 2000). Therefore, several adult tissues contain progenitors that, under specific conditions, may give rise to different embryologically unrelated derivatives; this multipotency is hard to reconcile with the fate decisions that

are necessary to allocate cells irreversibly to a given tissue anlage during embryogenesis.

Prompted by this apparent paradox, we investigated the origin of myogenic cells during fetal and perinatal development and reported the unexpected origin of a subset of myogenic progenitors from the dorsal aorta and not from somites, canonical source of all skeletal muscle of the body (De Angelis et al., 1999). Interestingly, in the same year Jackson et al. (Jackson et al., 1999) reported that adult skeletal muscle contain progenitors that were capable of repopulating the hematopoietic system of a mouse. Thus, an unpredictable relationship appears to exist between progenitors of different systems, with an apparent common root into the hemo-angioblastic system. Based on these data, we proposed that during tissue histogenesis, when vessels penetrate into

developing tissues, vessel-associated progenitors, which possibly originate from a common ancestor (a 'meso-angioblast', rather than an hemo-angioblast) would leave the vessel and adopt the fate of the tissue where the vessel has entered (Bianco and Cossu, 1999). This fate choice would depend upon local signals emanating from differentiating cells of that tissue and would be functionally different from fate choices dictated by embryonic signaling centers such as the notochord or Hensen's node, even though the same molecules may be involved. Some of the vessel-associated progenitors may remain undifferentiated and capable of differentiating later in life during postnatal growth or regeneration. Because of their origin, they would remain capable of differentiating into other tissue types when naturally or experimentally relocated into a different tissue.

To prove this hypothesis, we transplanted quail or mouse embryonic aorta into chick embryos and searched for donor cells in the chimera at the fetal stage. We now report that progenitor cells associated with the donor vessel are distributed in most mesoderm tissues that we have analyzed, including tissues distant from the transplantation site such as the myocardium. This capacity was associated with both the endothelial and sub-endothelial component, although endothelial cells possibly contributed to mesoderm tissues to a greater extent. Furthermore we show that aorta-derived cells generate a clonal progeny that can be indefinitely expanded in vitro, while maintaining multipotency in vitro and in vivo upon transplantation into a chick embryo. These features define vessel-associated progenitors as true stem cells, that in the fetal stage of development are distributed to tissue anlagen through angiogenesis and through the circulation. They contribute to peri-natal tissue growth and may represent the ancestors of postnatal stem cells.

MATERIALS AND METHODS

Transgenic mouse lines

Experiments were performed using embryos derived from wild type or from MLC1/3F-*nlacZ* (Kelly et al., 1995) transgenic mice.

Transplantation studies

Quail (*Coturnis coturnis japonica*) donor embryos were incubated at 38°C up to stages HH16 to 18 (E3). The aorta was dissected from surrounding tissues as described (Cormier et al., 1986). Donor mouse embryos were collected at E9.5 (22-24 somites). The dorsal aorta, which is adjacent to the segmental plate, was isolated after pancreatin treatment as described previously (De Angelis et al., 1999). In some experiments, the aorta was dissected from the AGM region of older embryos (Medviski and Dzierzak, 1996). Contamination by adjacent somitic tissue was tested by RT-PCR on randomly selected samples.

Fragments of quail aorta containing approximately 10^4 quail cells were cut with a micro-scalpel and transplanted into a slit that was made in the proximal region of chick wing buds of similarly aged embryos. Tissue fragments or isolated mouse cells (approximately 10^4 cells), dissociated from the dissected aorta, were transplanted into a slit made between neural tube and somite, at the inter-somitic levels of E2 (HH 12 to 14) chick embryo. This turned out to be the latest possible stage for transplantation of mouse cells (Fontaine-Perus, 2000). In fact, when mouse cells were transplanted into the wing bud of E3 chick embryos, they did not spread throughout the host tissue and later were expelled as a coherent cluster from the chick wing (not

shown). Before transplantation, mouse tissues and cells were labeled with DiI as described previously (Tajbakhsh et al., 1994).

Flow cytometry

In some experiments, the aorta was digested for 30 minutes with 0.1% collagenase and isolated cells were incubated at 4°C for 1 hour with anti-Quek1, anti-QH1 (quail) or anti-PECAM (mouse) monoclonal antibodies (supernatant diluted 1:10), and were washed and reacted with FITC-conjugated anti-mouse IG (Cappel, 1:300). After three final washes, cells were separated on a FACS scan using a Consort-30 software. Forward and 90°C side-scatter were used to identify and gate positive and negative fractions. Background subtraction using an unrelated antibody was performed for each sample. Approximately 10^4 cells for each fraction were centrifuged and then implanted as pellets into recipient chick.

In other experiments, both control GFP-labeled A4 cells and GFP-labeled A4 cells that had been co-cultured with unlabeled bone marrow from wild-type adult mice (see below) were incubated with anti murine CD45, Ter 119, Mac-3, CD11b, Gr-1 monoclonal antibodies (all conjugated with Phyco-erythrin from Pharmingen), at 4°C for 1 hour, washed three times and then separated on a FACS scan as described above.

Cell culture and clonal selection

Quail or mouse embryo fibroblasts were isolated from trypsin digestion of E3 quail and E10 mouse embryos and passed twice in culture before being transplanted as described above or used as feeder layers (after Mitomycin C treatment: 2.5 µg/ml for 2 hours in complete medium).

Mouse dorsal aorta from E9.5 embryos was grown as an explant culture as described elsewhere (De Angelis et al., 1999). After 5 days the explant was dissociated into a single cell suspension, labeled with DiI as described (Tajbakhsh et al., 1994). After labeling, an aliquot of cells was stained with Hoechst to confirm that more than 95% of the cell population had been labeled. The cell suspension was plated at limiting dilution on a feeder layer of Mitomycin C-treated primary embryonic mouse fibroblasts, or C3H 10T1/2 or STO fibroblast in 96 multiwell plates in complete medium. After 6 hours, the plates were scored under an inverted fluorescence microscope and only wells containing 1 DiI labeled cell were considered for further analysis. After 1 week, clones appeared in approximately 2-4% of the wells. When the clones had grown to approximately 10^3 cells they were passed twice on feeder layers and thereafter on gelatin-coated dishes. Among different feeder layers, STO fibroblasts consistently gave the largest number of clones and were used in all successive experiments. To date, the clonal isolates have been grown in culture for more than 50 passages with a doubling time of about 12 hours. They have been sub-cloned with a cloning efficiency of approximately 10%. Attempts to expand the clones without feeder layers and with various growth factors (e.g. bFGF, PDGFbb, VEGF, IGFI, EGF, LIF, IL3) in different concentrations and combinations have been unsuccessful. Among the different clones initially characterized, clones A4 and B13 were used for the in vitro and in vivo differentiation experiments described here. Other clones are shown in the RT-PCR analysis.

In vitro differentiation of A4 and other clonal isolates from dorsal aorta

Cells were treated with 1 ng/ml of BMP 2 in complete medium for 5 days (BMP2 was added every other days), then fixed and stained for alkaline phosphatase or analyzed for the expression of osteogenic markers by RT-PCR. Alternatively, cells were treated with 10 ng/ml of dexamethasone under the same conditions, and then analyzed for adipocyte morphology and expression of adipogenic markers. To induce osteoclastic differentiation, A4 cells were treated with 10^{-8} M 1,25 (OH)$_2$ vitamin D3 for 5 days. For co-culture experiments, cells were infected for 4 hours with third generation lentiviral vector
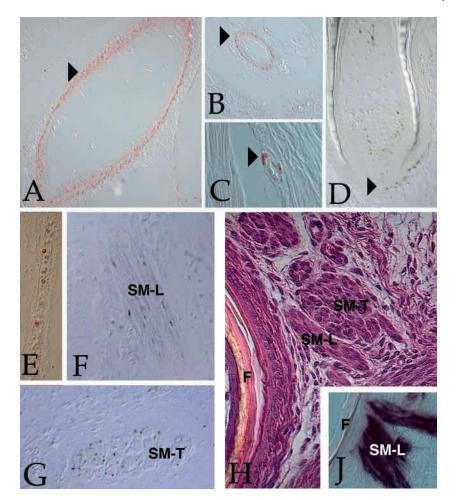
Fig. 1. Immunoperoxidase staining with QCPN antibody of chick-quail chimeras sacrificed at E19-E20. Regular arrays of quail nuclei (dark brown, arrowheads) are detected in the vessel wall of blood vessels of large (A), intermediate (B) and small (C) sizes. Donor nuclei are detected in pre-capillary arterioles, adjacent the base of a feather bud (arrowhead) and inside the bud (D), as well as circulating cells in the vessel lumen (E). Quail nuclei are detected in cutaneous smooth muscle in quail-chick chimeras, as seen in longitudinal (SM-L in F) and transverse (SM-T in G) sections. (H) Hematoxylin and Eosin staining of a similar section showing morphology of bundles of smooth muscle associated with feather buds (F), immunostained for α-smooth actin (J).

pRRLsin.PPT-PGK.GFPpre-expressing green fluorescent protein (GFP) as described elsewhere (Follenzi et al., 2000). Approximately 80-90% of the infected population expressed GFP in the cytoplasm. GFP+ cells were cultured with a fourfold excess of unlabeled C2C12 myoblasts or rat neonatal cardiocytes, or with a 10-fold excess of adult bone marrow cells in Dexter-like conditions as described previously (Dexter and Testa, 1976). After different periods, cultures were analyzed for co-expression of GFP and tissue-specific markers (MyHC, cardiac troponin 1 and CD45) by either immunofluorescence or FACS analysis.

Immunocytochemistry

The following antibodies were used in this work: QCPN monoclonal antibody (which recognizes quail but not chick nuclei) was obtained from the Developmental Studies Hybridoma bank under contract number NO1-HD-2-3144 from the NICHD; anti-Quek1 and anti-QH1 monoclonals (which recognize the quail VEGF receptor and endothelial cells, respectively) were kindly donated by Anne Eichmann (Eichmann et al., 1997) and by Luc Pardanaud (Pardanaud et al., 1987) (Institut d'Embryologie, Nogent sur Marne, France); anti-myosin heavy chain polyclonal antibody (which recognizes all vertebrate sarcomeric myosins) (Tajbakhsh et al., 1994) was produced in the laboratory; anti-cardiac troponin 1 monoclonal antibody (Di Lisi et al., 1998) was donated by Stefano Schiaffino, University of Padua; anti-α smooth muscle actin monoclonal was from Signet, Dedham, MA; PE-conjugated anti CD-45, anto-Ter119, anti-Mac-3, anti CD11b and anti-Gr-1 were from Pharmingen, BD; anti-PECAM rat monoclonal was a generous gift from A. Vecchi (Istituto Mario Negri, Milan, Italy). Single and double immunolabeling, and alkaline phosphatase cytochemistry on paraffin wax-embedded or cryostat

sections of chick chimeras were performed as described elsewhere (Tajbakhsh et al., 1994; Bianco et al., 1993).
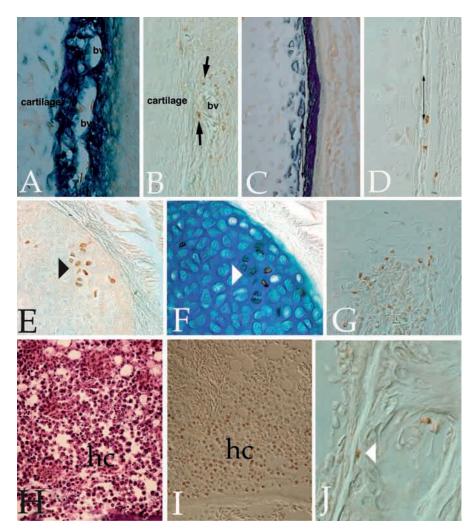
RT-PCR

RT-PCR was performed as described previously (Ferrari et al., 1997). Oligos used for amplification of the following genes were: VE-Cad (227 bp) (5′ gga tgc aga ggc tca cag ag 3′ and 3′ ctg gcg gtt cac gtt gga ct 5′); Flk1 (270 bp) (5′ tct gtg gtt ctg cgt gga ga 3′ and 3′ gta tca ttt cca acc acc ct 5′); CD34 (300 bp) (5′ ttg act tct gca acc acg ga 3′ and 3′ tag atg gca ggc tgg act tc 5′); Myf5 (132 bp) (5′ gag ctg ctg agg gaa cag gtg gag a 3′ and 3′ gtt ctt tcg gga cca gac agg gct g 5′); MyoD (144 bp) (5′ cac tac agt ggc gac tca gac gcg 3′ and 3′ cct gga ctc gcg cac cgc ctc act 5′); Cbfa1 (387 bp) (5′ gag ggc aca agt tct atc tgg a 3′ and 3′ c tct agt agc gcc tgg tgg 5′); Kit (400 bp) (5′ ggc tca taa atg gca tgc tc 3′ and 5′ ctt cca ttg tac ttc ata cat g 3′); Nkx 2.5 (310 bp) (5′ tca aag aca tcc tga acc tgg 3′ and 5′ ctt tgtc cag ctc cac tgc ct 3′); Mef2d (517 bp) (5′ aag gga tga tgt cac cag gga 3′ and 3′ atg ggg agg aaa aag att cag 5′); Oct4 (310 bp) (5′ ctc gaa cca cat cct tct ct 3′ and 3′ ggc gtt ctc ttt gga aag gtg ttc 5′); and the calcitonin receptor (5′ ggc ctt cac agc ctt cag gta c3′ and 3′cca aag acg tga gtc ggt cgt5′).

RESULTS

Donor cells from the dorsal aorta are efficiently integrated into the host developing vasculature

The aorta was isolated from day 3 donor quail embryos and dissected free of adjacent embryonic structures as described (Cormier et al., 1986). Fragments containing approximately

Fig. 2. Immunoperoxidase staining with QCPN antibody of chick-quail chimeras sacrificed at E19-20. (A,B) Detection of quail nuclei in the osteogenic perichondrium at leading edge of periosteal ossification. (A) Alkaline phosphatase staining, ALP; (B) QCPN immunostaining. Note the marked reactivity for ALP of multiple cell layers in the richly vascular osteogenic perichondrium, but not in the adjacent fibroblastic layer. QCPN immunoreactive cells reside within the ALP-positive layer and cluster around thin-walled blood vessels (bv). Arrows in B indicate quail nuclei. (C,D) More advanced stage of bone deposition, marked by a recognizable bony collar (arrows). C, ALP staining; D, QCPN immunostaining. Note the presence of quail nuclei within the boundaries of the ALP-positive osteogenic layer of the perichondrium/periosteum. (E,F) Quail chondrocytes are observed in superficial (sub-perichondral) regions of hyaline cartilage (E, QCPN immunostaining; F, QCPN immunostaining/Alcian Blue), and in chondrocytes surrounding the marrow cavity and vascular canals (G). Quail nuclei also occur frequently in hematopoietic cells (hc) in the bone marrow (H,I). Adjacent HE stained (H) and QCPN immunostained (I) sections depicting the hematopoietic tissue in the marrow cavity of a long bone. (J) QCPN-immunoreactive osteocyte (arrowhead) residing in a typical osteocytic lacuna in well-formed bone.

10^4 cells were transplanted into a slit, made in the proximal region of wing buds of similarly aged chick embryos. At this developmental stage, the aorta is comprised of an inner endothelial layer and one outer coat of mesodermal cells whose origin and mode of organization into the vascular wall have remained elusive (Takagi and Kawase, 1967). The floor of the aorta also contains hematopoietic progenitors (Jaffredo et al., 1998).
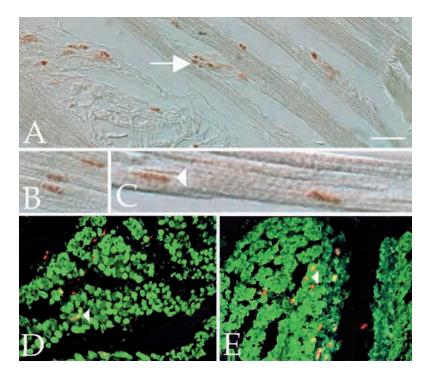
At different periods after the transplantation, the wings of the host embryos were dissected, processed for histology and serially sectioned. Every third section was stained with the QCPN antibody, which selectively recognizes quail nuclei. Five days post-transplantation, several thousand (a total of 3.3, 2.5 and 2.7×10^3) quail donor nuclei were detected in the wing of three separate chimeras. When the chimeras were sacrificed shortly before hatching, 6×10^4 to 10^5 cells with quail donor nuclei were detected in the wing of seven chimeras.

Upon histological analysis of the chimeric tissues, the most prominent finding was a substantial contribution of donor cells to vascular walls and to hematopoiesis, consistent with the known presence of hematopoietic progenitors within the dorsal aorta. The colonization of vascular branches of the host by donor cells was easily appreciated at 5 days post-transplantation (E8), and was extremely prominent in chimeras that were sacrificed shortly before hatching (E19-E20). Donor cells formed regular coats within the smooth muscle layer of arteries of large (Fig. 1A), and small size (Fig. 1B). Arteries branching into peripheral vascular networks were found to give rise to a system of precapillary and capillary vessels with pericytes of donor origin. This was particularly apparent in skeletal muscle (Fig. 1C). When immunostained for α-smooth actin, donor cells were mostly found within the muscular wall (data not shown). Circulating cells of donor origin were also easily detected (Fig. 1E). We concluded from these observation that cells from the transplanted aorta associated in a regulated fashion with the developing vasculature of the host tissues, and participated in their subsequent growth and peripheral branching within tissues, giving rise to a chimeric vascular network.

The dorsal aorta contains progenitors for multiple mesodermal tissues

In chimeras sacrificed at E19, differentiated cellular elements of tegumentary, skeletal and muscular tissues of donor origin were identified in close topographical association with the detected chimeric microvascular districts. Quail cells were detected within feather buds (Fig. 1D), dermis and feather-associated smooth muscle bundles (Fig. 1F-J). In developing bone, quail donor cells were found within the osteogenic periosteum/perichondrium (positive for alkaline phosphatase,

Fig. 3. Immunoperoxidase staining with QCPN antibody of chick-quail chimeras sacrificed at E19-E20. Quail nuclei (dark brown) are detected within and around the wall of intramuscular blood vessels (arrow in A) and also among and sometimes adjacent to developing muscle fibers. Quail nuclei are also seen inside cross-striated muscle fibers (B,C). (D,E) Double immunofluorescence staining with QCPN antibody reveals quail nuclei (red) in skeletal (D) and cardiac (E) muscle cells that express sarcomeric myosin (green). Quail nuclei appear red in the interstitium and orange when inside myosin-positive cells. Scale bar: 25 µm.

and comprising immediate progenitors of bone forming cells (Owen, 1976; Gehron Robey et al., 1992; Bianco et al., 1993), especially at the leading (metaphyseal) edge of periosteal ossification (Fig. 2A-D), which coincides spatially with the site of active longitudinal rudiment growth. Here, donor nuclei were arranged around the growing tips of intra-periosteal vessels. Several osteocytes, representing the terminal stage of differentiation in the osteogenic lineage, were also found to

have originated from donor cells (arrowhead in Fig. 2J). Clusters of quail cells were observed within the cartilaginous rudiments of forming skeletal segments, and were mainly located near the marrow cavity, in the immediate surroundings of epiphyseal vascular canals (Fig. 2G), which are thought to contain chondrogenic mesenchymal progenitors (Lutfi, 1970; Holmbeck, 1999). Many quail donor cells were detected in the developing bone marrow (Fig. 2H,I) but also in the surrounding
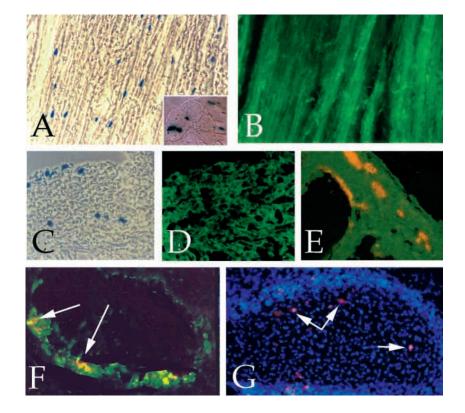

Fig. 4. X-gal staining of mouse-chick chimeras sacrificed at E19. Mouse nuclei that have activated the MLC1/3F-*nlacZ* reporter gene nuclei (blue in A) are detected inside myosin-positive muscle fibers (A,B). Higher magnification in the inset in A shows *lacZ*-positive nuclei inside fibers. Donor *lacZ*-positive cells are also detected in myosin-positive cardiocytes (C,D) of mouse-chick chimeras sacrificed at E19. Mouse donor cells that were sorted for PECAM and labeled with DiI were similarly detected in the host chick heart (E), blood vessels (F) and cartilage (G): E, merged DiI (red) and myosin heavy chain (green) fluorescence images; F, merged DiI (red) and desmin (green) fluorescence images; and G, merged DiI (red) and DAPI (blue) fluorescence images.
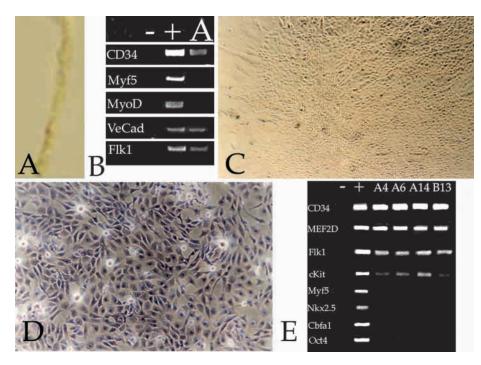
Fig. 5. (A) Morphology of the embryonic dorsal aorta isolated from E9.5 mouse embryos after pancreatin digestion. Note the absence of remnants of adjacent embryonic structures. (B) RT-PCR revealed that endothelial and hematopoietic markers (VE-Cad, Flk1 and CD34), but not myogenic markers (Myf5 and MyoD), were expressed in dissected aorta (A). Total embryo extract was used as a positive control (+). Negative control (no RNA) is shown in the first lane (–). (C) Phase contrast morphology of a clone from embryonic aorta growing on an STO feeder layer. (D) Phase contrast morphology of one typical clone (A4) from embryonic aorta after five passages in vitro. (E) RT-PCR of the messages expressed by several cell lines (A4, A6, A14 and B13) from the dorsal aorta after 5 passages in vitro. Note expression of hemo-angioblastic but not of tissue specific markers such as Myf5 or Nkx2.5.

cartilage or underneath the perichondrium in areas of fully differentiated hyaline cartilage (arrowhead in Fig. 2E,F). To our knowledge this is the first demonstration that cells located within hyaline cartilage are derived from the vasculature.

Fig. 3A shows quail cells residing within the wall of intramuscular blood vessels (arrow) as well as outside of them and within the muscle tissue. Fully differentiated striated muscle fibers of donor origin were easily identified in the same tissue areas where chimeric vessels were identified (Fig. 3B,C). Double immunofluorescence with anti-myosin heavy chain antibody confirmed that QCPN-positive nuclei were indeed present inside differentiated muscle fibers (Fig. 3D).

Cells associated with the embryonic vasculature may enter the circulation and colonize distant organs. This raised the possibility in our transplantation experiments donor cells could contribute to organs distant from the transplantation site. To test this possibility, we sectioned different organs of E19 chimeras. The liver, the brain and the kidney did not contain quail nuclei in the parenchyma, at least not in the chimeric organs examined. By contrast, quail nuclei were incorporated into chick myocardium, and were present inside myosin-positive cardiac cells (Fig. 3E).

Multipotency is associated with both endothelial and sub-endothelial cells of the vascular wall

To investigate whether these aorta-associated mesoderm progenitor(s) originated from either the endothelial layer or its abluminal coating of primitive mesenchymal cells, quail aortas were similarly isolated, dissociated by collagenase treatment and sorted on the basis of reactivity with anti-Quek1 or with anti-QH1 antibodies. The first recognizes the quail homologue of the VEGF receptor 2 (Flk1/KDR) tyrosine kinase (Eichmann et al., 1997), while the second labels specifically quail blood and endothelial cells (Pardanaud et al., 1987). FACS-sorted, positive and negative cells were collected by centrifugation and the resulting pellet was transplanted as

described. Although there was some variability between the different chimeras (two for each antigen), both positive and negative cells did generate developing skeletal tissues, smooth and skeletal muscle fibers (data not shown). Contribution of QH1$^+$ cells was particularly abundant to smooth muscle (particularly in the smooth muscle of the aortic outflow tract) and to a minor extent to skeletal and cardiac muscle and bone. QH1$^-$ cells contributed to a lesser extent to skeletogenic tissues. However, both Quek1$^+$ and Quek1$^-$ cells contributed mainly to blood and secondarily to all the other mesodermal tissue (data not shown). From these data, we concluded that progenitors contributing to the growth of diverse mesodermal tissues are associated with both the endothelial and the perithelial compartment of the embryonic aorta. The different potency of QH1$^+$ versus Quek$^+$ cells may reflect the existence of different subpopulations of endothelial progenitors with different endothelial antigen repertoires, or simply experimental variability, although the issue remains to be investigated further.

Multipotent progenitors are associated with mouse dorsal aorta

To test whether multipotent progenitors would also exist in mammalian embryos, we dissected the dorsal aorta from either the unsegmented, caudal mesoderm of day E9.5 or from the AGM (aorta-gonade-mesonephron) region of day E10.5 mouse embryos. In order to look specifically at the myogenic potential of aorta-derived cells, we initially used transgenic mouse embryos that express the *lacZ* reporter gene (under the transcriptional control of the myosin light chain 1/3F promoter/enhancer) only in striated muscle (Kelly et al., 1995). Wild-type embryos were, by contrast, used to follow the fate of DiI-labeled aorta derived cells in tissues other than striated muscle. Mouse embryonic aorta was dissected free of other embryonic structures such as somites or notochord (Fig. 5A). Histological analysis confirmed that at these developmental

stages the mouse aorta comprises an inner endothelial layer and one outer coat of loosely arranged mesodermal cells, much like the quail aorta. RT-PCR analysis of dissected mouse aortae demonstrated the expression of VE-cadherin, Flk1 and CD34, but not Myf5 or MyoD, ruling out contamination by adjacent paraxial mesoderm (Fig. 5B).

When fragments of the mouse aorta were transplanted in the wing bud of E3 chick embryos, no mouse tissue was detected in the surviving embryos. In one case, we observed at E7 a group of mouse cells protruding out of the host wing, probably in the process of being expelled (data not shown). For this reason, in further experiments the aortas were transplanted between the neural tube and somites at the brachial level of E2 chick embryos. At E19-E20, in three out of 10 surviving chimeras, β-gal-positive nuclei were detected within myosin-positive skeletal muscle fibers (Fig. 4A,B). The inset in Fig. 4A shows a high-magnification transverse section, demonstrating that β-gal-positive nuclei were indeed inside muscle fibers. As the *lacZ* reporter gene is also expressed in cardiocytes, we sectioned the heart of these chimeras. Fig. 4C,D shows the presence of several β-gal-positive nuclei inside the cardiocytes of the chick embryo.

To investigate the potential contribution of aortic cells to tissues other than skeletal and cardiac muscle, cells were dissociated from the aorta isolated from E10 wild-type mouse embryos and separated by FACS on the basis of reactivity to an anti-PECAM antibody that recognizes endothelial and early hematopoietic cells. PECAM$^+$ and PECAM$^-$ cells were labeled with DiI and separately transplanted between the neural tube and somites at the brachial level of E2 chick embryos. At E14, surviving embryos were sacrificed and cryostat sectioned. Areas colonized by donor cells were identified by DiI fluorescence and the presence of mouse nuclei confirmed by DAPI staining. The results of this experiment showed that PECAM$^+$ cells were detected in the vessel walls, connective tissue, perichondrium and cartilage, as well as in cardiac, smooth and skeletal muscle (Fig. 4E-H and data not shown). PECAM-negative cells were found less frequently and were localized mainly in connective tissue and smooth muscle and, to a minor extent, in cardiac muscle. At the stage examined (E14), bone tissue is just beginning to form and we did not detect donor cells in it.

Multipotency is not ubiquitous among embryonic cells

To test whether multipotency is indeed restricted to the cells of the vessel wall or is rather shared by all embryonic mesodermal cells, we carried out control transplantation using quail and mouse embryo fibroblasts (QEF, MEF). QEF and MEF were established in culture (as no embryonic structure is devoid of vessels) and then pellets of 10^4 cells were transplanted into chick embryos as described above. In order to make donor cells detectable in the resulting chimeric tissues, cells were labeled with DiI prior to transplantation. Analysis of quail-chick or mouse-chick chimeras sacrificed before hatching, consistently revealed the presence of donor nuclei only in the connective tissue at the site of transplantation (data not shown).

Aorta-derived multipotent progenitors are indeed bona fide stem cells

The operational definition of a stem cell requires evidence for
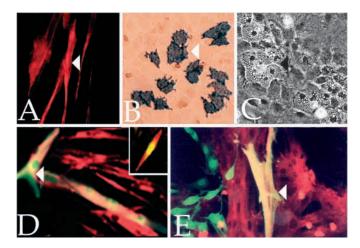

Fig. 6. In vitro differentiation of the aorta derived cell line A4 into different cell types. (A) Smooth muscle cells (SMA positive, arrowheads). (B) Osteoblasts (ALP positive, arrowheads) are detected after treatment with 1 ng/ml BMP2; (C) Adipocytes are detected after treatment with 10 ng/ml of dexamethasone. (D) Skeletal myotubes are detected after co-culture of GFP-labeled A4 cells with C2C12 myoblasts. GFP-positive cells appear green, myocytes and myotubes expressing myosin heavy chains appear red, and cells expressing both appear yellow in the merged image (arrowhead). A mononucleated, differerentiated, GFP-positive myocyte is shown in the inset in D. (E) Cardiocytes are detected after co-culture of GFP-labeled A4 cell with rat neonatal cardiocytes. GFP-positive cells appear green, myocardiocytes expressing cardiac specific troponin 1 appear red and cells expressing both appear yellow in the merged image (arrowhead).

extended self-renewal accompanied by a permanent capacity to generate one or more differentiated cell types. To test whether the population of multipotent progenitors from dorsal aorta may contain stem cells, we cloned them by limiting dilution on a feeder layer of mytomycin C-treated STO fibroblasts. DiI labeling allowed unambiguous detection of individual cells in each well. After 1 week, colonies (on average two to four per 96 multiwell) appeared that were composed of highly refractile cells surrounded by cells with a typical cobblestone morphology (Fig. 5C). After the second passage, most of the clones continued to grow independently of feeder layer and have been continuously in culture for more than 1 year. The cells had an average division time of 12 hours, maintained the morphology shown in Fig. 5D and showed no apparent sign of senescence. Immediately after isolation or after prolonged expansion in vitro virtually all of the clones expressed CD34, Kit, Flk1, MEF2D but not tissue-specific transcription factors, such as Myf5, Nkx2.5 or Cbfa1, nor ES-specific transcription factors, such as Oct4 (Fig. 5E). We thus asked whether cells lines derived from the aorta express and eventually maintain the same multipotency expressed by freshly isolated cells. To answer this, we cultured the cells with specific cytokines, known to activate specific differentiation pathways, or with differentiating cells in vitro, assuming that this situation would mimic the micro-environment where angiogenesis occurs in fetal tissues in vivo. Fig. 6A shows that cells of clone A4 (as well as of the other clones tested) contained approximately 10% of α-smooth muscle actin-positive cells. Subcloning of clone A4 revealed that each sub-

clone contained approximately 10% of SMA-positive cells; BrdU labeling experiments (non shown) indicated that these cells divided less frequently than the general population. As they remain in a constant proportion of the total population, the simplest explanation is that smooth muscle cells are constantly generated but divide more slowly and thus do not overgrow the total population. When treated with BMP2, A4 cells expressed markers of osteoblasts (alkaline phosphatase, bone sialoprotein and Cbfa1; Fig. 6B and data not shown), none of which was expressed at detectable levels in the untreated cells, whereas they acquired a typical adipocyte morphology when treated with dexamethasone (Fig. 6C). Alternatively, the cells were labeled with lentiviral vectors encoding GFP and co-cultured with unlabeled skeletal myoblasts. After 5 days, the presence of double-labeled (GFP and MyHC) cells indicated that a fraction of the clonal cell lines had differentiated into skeletal muscle cells (Fig. 6D). Much as in embryonic myogenesis, differentiation appeared to be independent from fusion, as occasional mononucleated, double labeled cells could be observed (inset in Fig. 6D). When the same cells were co-cultured with primary rat neonatal cardiocytes, the presence of double labeled cells (GFP and cardiac specific troponin 1) indicated that a fraction of the clonal cell line had differentiated into myocardiocytes (Fig. 6D). Indeed, clusters of beating GFP-positive cells could frequently be observed (not shown). Furthermore, the cell population contained a small fraction (3-5%) of CD45-positive cells. When GFP-labeled A4 cells were co-cultured with a 10-fold excess of freshly isolated total bone marrow cells in a Dexter-like type of culture, more than 70% of the GFP-positive cells expressed CD45 (Fig. 7A). Furthermore, a lower but significant percentage of A4 cells expressed macrophage-monocyte markers such as Mac3 and CD11b, both of which were dramatically upregulated after Dexter-like culture (Fig. 7A). In addition, when treated in vitro with $1,25 (OH)_2$ vitamin D_3, A4 cells expressed the osteoclastic markers TRAP and calcitonin receptor (Fig. 7B,C) and some of the cells also acquired a multinucleated osteoclast-like morphology (Fig. 7B). However, markers of mature erytrocytes and granulocytes were not expressed and were upregulated only in a tiny fraction of the cell population under these culture conditions, which do not promote maturation of these lineages. Fig. 7D-G show the macrophage and myeloid morphology of GFP-labeled cells, after Giemsa staining.

To test multipotency in vivo, both A4 and B13 clones, previously transduced with a lentiviral vector expressing GFP protein, were implanted as a pellet into a chick embryo as described above for freshly isolated cells. Both clones implanted at early or late passages gave consistently similar results. Fig. 8A shows a whole mount of an E15 chimera where A4 GFP-positive cells appear to distribute along several directions following vascular branches. Upon sectioning GFP-positive cells were found in the smooth muscle coat of larger vessel (Fig. 8B) or around capillaries (Fig. 8C) where a single double labeled (yellow) cell is indicated. Occasionally fluorescent cells could be identified inside small vessels as circulating cells (inset in Fig. 8C). They accumulated in the dermis but not in the epidermis (arrowhead in Fig. 8D). Labeled cells were also found in myocardium (Fig. 8E) in the smooth muscle of the gut (Fig. 8F), in the cartilage, in the ossifying perichondrium and inside blood vessels (arrowhead,
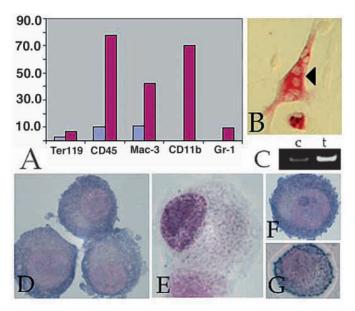

Fig. 7. (A) Histogram showing percentages of GFP-labeled, A4 cells (blue bars) or of GFP-labeled A4 cells co-cultured with wild-type adult bone marrow in a Dexter-type culture (red bars) after FACS sorting for expression of Ter119, CD45, Mac3, CD11b and Gr1. (B) A multinucleated (arrowhead), and another TRAP-positive cell are shown from a culture treated with $1,25 (OH)_2$ vitamin D3. (C) RT-PCR for the expression of the calcitonin receptor in control cells (c) and cells treated (t) as described above. (D-G) Morphology of GFP-positive cells, stained with Giemsa, revealing predominantly blast (D,F), macrophage (E) and immature myeloid (G) morphologies.

asterisk and arrow, respectively, in Fig. 8G) as well as in newly formed skeletal muscle fibers (Fig. 8H).

DISCUSSION

Cells from the embryonic vasculature are incorporated into mesodermal fetal tissue

The data we report indicate that when quail or mouse cells, which are derived from the embryonic aorta, are grafted into a chick embryo, they initially associate with the vasculature of the host, and generate chimeric microvascular districts in a broad range of tissues, including muscle, teguments and skeleton. In the same tissues, fully differentiated cells of donor origin, including chondrocytes, bone cells, smooth and striated muscle cells, are generated during the fetal period of development. In addition, differentiated cells of donor origin are found in blood and hematopoietic tissue. While the origin of blood cells from cells residing within the wall of the embryonic aorta (specifically the AGM region) is well accounted for by current understanding of the origin of definitive hematopoiesis (Jaffredo et al., 1998), the ability of vascular wall cells to give rise also to a broad range of extravascular mesodermal tissues is a novel finding. Tissue-specific differentiation of donor cells within non-hematopoietic mesodermal tissues was confirmed in our experiments by immune detection of tissue-specific antigens in donor cells within a given tissue, and, in the case of bone, cartilage and muscle, by unequivocal morphological hallmarks such as cross

striation and location within osteocytic or chondrocytic lacunae. Tissue-specific differentiation was under all circumstances orthodox. For example, we never detected a myosin-positive cell within bone tissue, or vice-versa, suggesting that this process of terminal differentiation is not random but somehow dictated by the local environment and guided by surrounding cells. This is in agreement with a recent report showing that neural stem cells undergo differentiation in many different cell types when injected into gastrulating mouse and chick embryos (Clarke et al., 2000). In this case, cells always differentiated according to the tissue were they had been placed by the morphogenetic events of the host.

Skeletal tissues

The contribution of donor cells to skeletal tissues is of particular interest in view of the long-surmised – and as yet never directly demonstrated – role of the local vasculature in the processes of chondrogenesis and osteogenesis. Cartilage cells are known to originate from local mesenchymal progenitors associated with the perichondrium, and with the loose perivascular mesenchyme residing within epiphyseal vascular canals (Lutfi, 1970). The latter develop from the perichondrium, and are thought to bring into the interior of the epiphyses mesenchymal progenitors recruitable for osteo-chondrogenesis. Lack of development of vascular canals in the membrane type 1-matrix metallo-proteinase-deficient mice induce not only a marked delay in epiphyseal ossification, but also a shut down of longitudinal growth at metaphyseal growth plates (Holmbeck et al., 1999). We have observed fully differentiated chondrocytes of donor origin both underneath the perichondrium and in the immediate surroundings of the vascular canals, thus providing the most direct evidence so far for the origin of cartilage cells from vascular canal-associated tissues. In bone, a link between vascularity and osteogenesis has been indicated or implied by a number of observations. For example, blockade of angiogenesis in the growth plate arrests endochondral ossification (Gerber et al., 1999). At the leading edge of periosteal ossification, where the bone collar is extended longitudinally, bone cells differentiate de novo and active angiogenesis occurs. In the chick-quail chimeras examined, this site was abundantly replenished with donor cells, regularly arranged around vascular sprouts. Earlier studies have directly demonstrated a rather precise maturational sequence whereby osteoprogenitor cells residing in the periosteum (recognized by expression of ALP activity and incorporation of thymidine or BrdU) progress to osteoblasts that are competent to deposit bone matrix, and ultimately to osteocytes (Owen, 1976; Gehron Robey et al., 1992; Bianco et al., 1993) residing within a bone-encased lacunar space (an osteocytic lacuna). The process of osteocyte formation is so unique to bone cells that detection of osteocytes of donor origin is regarded as a golden standard in transplantation studies aimed at proving osteogenic differentiation (Hou et al., 1999; Nilsson et al., 1999). Our data unequivocally show immature and mature bone cells, including osteocytes, of donor origin that follow transplantation of embryonic aorta.

Skeletal and cardiac muscle

Donor cells were also unequivocally demonstrated in skeletal muscle fibers with characteristic cross-striation and the
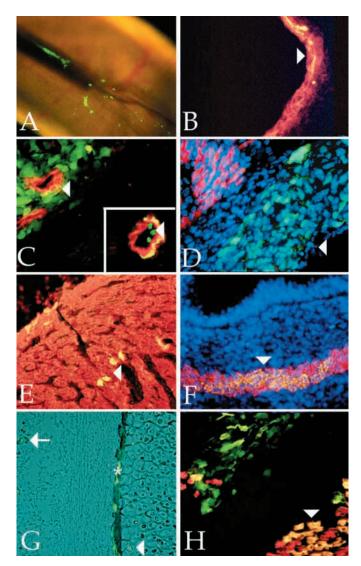

Fig. 8. Chick-mouse chimeras (sacrificed at E14) that had been transplanted with aorta-derived clonal cell line A4, at passage 25. (A) Whole-mount fluorescence of the transplant site, showing a cluster of GFP+ donor cells. (B) Section through a large vessel showing donor cells integrated into a large vessel wall (arrowhead) stained for smooth α-actin. (C) A cluster of donor cells outside a small vessel, with one cell (arrowhead) double expressing GFP and SMA (yellow in the merged image). Inset: GFP circulating cells inside a small vessel (arrowhead). (D) Donor cells accumulate in the dermis but not in the epidermis (arrowhead). Merged image: GFP (green), Hoechst (blue) and myosin heavy chains (red) of an adjacent muscle primordium containing no donor cells. (E) Section through the myocardium showing several donor cells (yellow) in the myocardium (arrowhead) and in the sub-epicardium. (F) Section through the intestine showing donor cells in the smooth muscle layer (arrowhead) stained for SMA. (G) Section through axial tissues showing donor cells in the cartilage adjacent to the perichondrium (arrowhead), in the perichondrium (asterisk) and in the blood vessel (arrow), but not in the intervening muscle fibers. (H) Section through a muscle primordium, showing donor cells (green) intermixed with myosin heavy chain-positive muscle fibers (red). Arrowhead indicates double-labeled fibers.

appropriate profile of muscle-specific antigens upon immunohistological analysis. In muscle, as in other tissues, donor nuclei in myofibers were constantly associated with chimeric intramuscular microvessels, suggesting a migratory process from the vessel to the surrounding muscle fibers. If a fraction of these cells are derived from the vessels, as their location would suggest, they may transiently co-express early endothelial and myogenic markers, as it occurs in cells that can be cloned from the embryonic aorta (De Angelis et al., 1999). This is consistent with a recent report, showing that blood-borne progenitors activate Pax7 when entering a myogenic program in postnatal muscle: in the absence of Pax7, formation of muscle satellite cells is blocked, while the number of hematopoietic progenitors in muscle is increased (Seale et al., 2000). In agreement with this study, some of mouse or quail, vessel-derived, donor cells are found adjacent to muscle fibers, in the typical position of satellite cells, suggesting that a fraction of resident satellite cells in adult muscle are blood borne. However, further work is needed to test whether this is the case or rather all satellite cells derive from somites as a previous study had suggested (Armand et al., 1983).

Distant from the transplantation site, donor cells of both quail and mouse origin, were detected in the host heart; as elsewhere, donor cells were found in blood vessels, in interstitial tissues and inside the myocardium as differentiated cardiomyocytes, expressing sarcomeric genes and a striated muscle-specific transgene. Donor cells must have reached the heart through the general circulation, in agreement with the notion that, during angiogenesis, growing endothelial cells may detach from the vessel inner surface, possibly as a consequence of mitotic activity, and home elsewhere at a site where active vessel growth is occurring. There, as at the original site of transplantation, cells may leave the vessel again, this time outside of it, and enter surrounding mesenchyme where differentiation into local tissue eventually occurs. When labeled with DiI, mouse donor cells were found indeed both in the host endocardium and in the surrounding myocardium.

Both endothelial and perithelial cells are incorporated into mesodermal tissue

Based on the prevailing view of early events in vasculogenesis, endothelial (Flk+, QH1+) and subendothelial (Flk−, QH1−) cells may represent distinct lineages. Growing capillary sprouts may recruit a subendothelial coat of prospective pericytes from the surrounding tissues (Hirschi and D'Amore, 1996) using a precise PDGFB/PDGFRβ ligand-receptor expression loop (Linddahl et al., 1997; Benjamin et al., 1998; Hellstrom et al., 1999). It is conceivable that a similar loop dictates the recruitment of subendothelial cells in the embryonic aorta as well. Our results showing that both endothelial and subendothelial subsets of embryonic aortic cells, as defined by appropriate sorting, are capable of contributing donor cells to extravascular mesodermal tissues elicits two possible interpretations: (1) the two separate lineages show multipotency; (2) multipotent perithelial cells may be generated from endothelial progenitors. With respect to the first possibility, an abundant literature has contended a role as progenitor cells for postnatal microvascular pericytes (subendothelial cells). Some evidence has been provided for their osteogenic and chondrogenic capacity upon ex vivo manipulation (Doherty et al., 1998), and to a limited extent in

in vivo experiments (Diaz-Flores et al., 1991; Diaz-Flores et al., 1992). Thus, the ability of embryonic subendothelial cells to generate skeletal and non-skeletal mesodermal tissues agrees well with these data. Conversely, the ability of endothelial cells to give rise to the same tissues has never been shown. A notable exception, which would support the second possibility, is to be found in recent studies indicating the ability of Flk1+ cells to give rise to both pericytes and smooth muscle cells in the vessel wall (De Ruiter et al., 1997; Yamashita et al., 2000). Besides supporting our own observation of donor-derived smooth muscle cells in different districts (blood vessel walls, skin), these data provide an important hint on the possible common origin of all mesodermal progenitors residing in the vessel wall.

It is, however, important to stress that, as shown by our control transplantation of cultured mouse or quail embryonic fibroblasts, multipotency is not shared by embryonic mesenchymal cells at large, even upon heterotopic transplantation. Indeed, the lack of multipotency of embryonic fibroblasts is consistent with known fundamental laws of embryology, whereby multipotency is restricted to specific cell populations defined by specific temporal and spatial constraints, such as cells from the inner cell mass, germ cells, neural crest cells and the recently described VENT population (Anderson, 2000; Erickson and Weston, 1999; Gardner and Brook, 1997; Labosky et al., 1994; Le Douarin and Ziller, 1993; Smith, 1998). Likewise, heterotopic transplantation per se does not convey plasticity to embryonic cells, as illustrated by the outcome of transplantation of myoblasts, which regularly generate muscle fibers only (Di Mario et al., 1993). Thus, our data identify a novel class of multipotent mesodermal progenitors, associated with the embryonic aorta, and capable of feeding into multiple lineage in postembryonic, late fetal development.

Pathways to final destination of vessel-associated progenitors

The close contiguity of chimeric microvessels and differentiated elements of donor origin that we observed in a variety of tissues, would suggest that angiogenesis itself represents a pathway whereby donor cells that were initially incorporated into growing vessels were delivered to growing tissues. Growth of both endothelial cells and pericytes occurs during angiogenesis, and it is thus conceivable that subsets of either cell compartment would enter a locally cued differentiation pathway during tissue growth. However, contribution of donor cells to the host myocardium, distant from the transplantation site, indicates that donor cells can also circulate. It is known that endothelial cells can circulate, both in the embryo and postnatally, in conjunction with phases of active angiogenesis, and it has been maintained that at least a subset of circulating endothelial cells may behave as progenitors of new endothelial cells at distant sites (Asahara et al., 1999). Thus, it is interesting to note that some circulating endothelial cells may in fact be multipotent, as our data would suggest.

Are vessel associate progenitors true stem cells?

To be unequivocally defined as 'stem cells' the progenitor cells associated with the embryonic vessels should be able to self-renew under appropriate conditions, as do embryonic or neural

stem cells. Furthermore a single self-renewing cell should be able to generate one or more types of differentiated cells continuously. While heterospecific transplantation of a single cell is not technically feasible, it is possible to expand in vitro the progeny of a single founder cell and challenge its multipotency in vitro and in vivo. Indeed, when grown on a feeder layer of embryonic fibroblasts, cells from the aorta generate clones that become immortal and grow in vitro for more than 1 year. Upon continuous sub-culturing, all the clones retain the same morphology, and the expression of 'hemo-angioblastic' markers such as CD34, Flk1 and Kit. More importantly, they retain the capacity to differentiate into most mesoderm cell types, including hematopoietic and solid phase mesodermal tissue lineages, both in vitro and in vivo. By these criteria, we can define the aorta-associated progenitors (which we propose to call 'meso-angioblasts') as a separate and novel kind of stem cell. Based on the developmental time frame during which these cells exist, and on their location and differentiation potential as probed to date, this novel kind of stem cell is distinct from both embryonic stem cells (ES), transiently present in the embryo in the inner cell mass of the blastocyst, and adult stem cells, present in postnatal life. Like ES cells, meso-angioblasts can be grown indefinitely in vitro, are resistant to retroviral transduction (data not shown) and are multipotent, but, conversely, do not express Oct4 and appear to contribute mostly to mesoderm-derived tissues. We do not currently know whether the inability to contribute to ectoderm- or endoderm-derived tissues is intrinsic to these cells or simply reflects inadequate experimental conditions in vitro or physical barriers in vivo (a few quail cells in the chick epidermis were indeed detected).

Are the meso-angioblasts the ancestor of (some) postnatal stem cells?

Current data have repeatedly highlighted the existence, in a variety of tissues, of progenitor cells with a striking degree of phenotypic plasticity, including, in some cases, the ability to differentiate across the boundaries defined by germ layers and dependent derivatives. Recently, multipotent cells have been isolated from adult tissues by virtue of their dye exclusion capacity (SP cells) (Gussoni et al., 1999; Jackson et al., 1999) or their late adhering properties (LP cells) (Lee at al., 2000). Furthermore, we have recently succeeded in isolating similar clonal cell lines from postnatal vessels, although their frequency appears very low (R. Tonlorenzi and G. C., unpublished). Very recently, multipotent adult progenitor cells have been identified in the non hemopoietic compartment of adult bone marrow, which express early endothelial markers (Reynes et al., 2002). Although the molecular characterization of these classes of stem cells is still in progress, it is intriguing that multipotency correlates with expression of angioblastic markers and suggests a common origin in the developing vasculature. In the same context, it is interesting that, although the actual microanatomical niches for stem cells in a variety of tissues is unknown, marrow stromal stem cells, admittedly a noted member of the extended family of somatic stem cells, may be associated with microvascular districts in the marrow (Bianco and Gehron Robey, 2000). The angiogenic potential of postnatal microvascular districts unfolds during organ growth, and then remains dormant within steady-state tissues, unless elicited again during repair or regeneration events, or in neoplasia. Under all these circumstances, active growth of both the endothelial and subendothelial compartments of microvessels occurs. We speculate that progenitors of mesodermal tissues, delivered through fetal angiogenesis, may be activated during such postnatal events. If so, microvasculature and angiogenesis might represent, respectively, the progenitor cell niches and the dynamic events during which progenitors are generated in a variety of postnatal tissues. Testing this hypothesis will be the object of future experiments.

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