Perivascular cells expressing annexin A5 define a novel mesenchymal stem cell-like population with the capacity to differentiate into multiple mesenchymal lineages

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Summary

The annexin A5 gene (*Anxa5*) was recently found to be expressed in the developing and adult vascular system as well as the skeletal system. In this paper, the expression of an *Anxa5-lacZ* fusion gene was used to define the onset of expression in the vasculature and to characterize these *Anxa5-lacZ*-expressing vasculature-associated cells. After blastocyst implantation, *Anxa5-lacZ*-positive cells were first detected in extra-embryonic tissues and in angioblast progenitors forming the primary vascular plexus. Later, expression is highly restricted to perivascular cells in most blood vessels resembling pericytes or vascular smooth muscle cells. Viable *Anxa5-lacZ*+ perivascular cells were isolated from embryos as well as adult brain meninges by specific staining with fluorescent X-gal substrates and cell-sorting. These purified *lacZ*+ cells specifically express known markers of pericytes, but also markers characteristic for stem cell populations. In vitro and in vivo differentiation experiments show that this cell pool expresses early markers of chondrogenesis, is capable of forming a calcified matrix and differentiates into adipocytes. Hence, Anxa5 expression in perivascular cells from mouse defines a novel population of cells with a distinct developmental potential.

Key words: Annexin A5, Perivascular cells, Pericytes, Adult stem cells

Introduction

Formation of the embryonic vascular system happens before nutrition by diffusion runs into its limitation, and represents the first organ system in the early embryo (Carmeliet, 2003; Noden, 1989). Vasculogenesis, the process of de novo formation of vascular structures, is characterized by the differentiation of mesodermal derived angioblast precursor cells into endothelial cells forming the primary capillary plexus (Risau, 1997; Risau and Flamme, 1995). Subsequently, during angiogenesis this early vascular system undergoes a period of pruning, remodeling and maturation. Additionally, it takes two cell types to generate functional blood vessels, endothelial cells and perivascular cells (Gerhardt and Betsholtz, 2003). Perivascular cells (PVC) can be classified as pericytes in capillaries or vascular smooth muscle cells (vSMC) in larger vessels, depending on their morphology and location. Both may represent subtypes of a continuum of related cell types.

PVCs are essential for the development of functional vessel walls and contribute to the structural integrity and contractility of vessels. However, developmental origins of PVCs remain unclear. Different speculations were proposed, such as derivation from mesenchymal and epicardial cells or neural crest (Gerhardt and Betsholtz, 2003), but also trans-differentiation of endothelial cells is discussed (DeRuiter et al., 1997). Recent data support the idea of a common progenitor of perivascular and endothelial cells – the FLK1-positive angioblast (Ema et al., 2003; Yamashita et al., 2000).

Pericytes were originally defined by their morphology and close contact to endothelial cells located within a shared basement membrane (Sims, 1986; Tilton, 1991). They represent a heterogeneous population of cells involved in normal and pathological angiogenesis, such as diabetic microangiopathy (Cogan et al., 1961; Hirschi and D'Amore, 1996), atherosclerosis (Bostrom et al., 1993) or cancer (Wesseling et al., 1995). Recruitment and coverage of vessels with pericytes is essential for the development of vasculature, as has been shown by genetic ablation of PDGFB signaling. In mice with disrupted signaling, the expansion and spreading of pericytes is impaired and leads to perinatal lethality due to leakage and hemorrhage (Leveen et al., 1994; Lindahl et al., 1997; Soriano, 1994). Presently, no 'pan-specific' marker is available that defines the pericyte phenotype unambiguously (Gerhardt and Betsholtz, 2003). Common markers such as smooth muscle actin (Nehls and Drenckhahn, 1991), NG2-

proteoglycan/HMW-MMA (Ozerdem et al., 2001), PDGFRβ receptor (Lindahl et al., 1997) and the regulator of G-protein-signaling RGS5 (Bondjers et al., 2003) define subsets of pericytes in a time- and tissue-dependent manner. Recently, the promoter-trap transgene XlacZ4 revealed a specific co-expression in vSMCs and pericytes (Tidhar et al., 2001).

Previous studies have shown that PVCs can differentiate into various cell types such as adipocytes, chondrocytes, fibroblasts and macrophages (Canfield et al., 2000; Diaz-Flores et al., 1992). These cells may therefore reflect in some aspects the phenotype of mesenchymal stem cells (MSC) originally isolated from bone marrow stroma (Caplan, 1991). Owing to general difficulties with isolation and characterization of pericytes, it is not yet possible to purify these cells from mouse. Therefore, detailed analysis of pericytes and PVCs in the mouse system is still pending.

Annexin A5 represents a typical member of the annexin family, characterized by the ability to bind to phospholipid membranes in the presence of Ca^{2+} (Moss et al., 1991). Although numerous functions of annexin A5 have been described in vitro, its in vivo role still remains unclear (Brachvogel et al., 2003). Expression analysis of the annexin A5 gene (*Anxa5*) by the use of an *Anxa5-lacZ* fusion gene in vivo showed that this gene is expressed in vasculature-associated cells and at later stages (E13.5-P1) in mesenchymal condensations, chondrocytes as well as all skeletal elements (Brachvogel et al., 2001).

In this report, we used the *Anxa5-lacZ* promoter trap first to describe the onset of *Anxa5-lacZ* expression early in development and second to characterize the *Anxa5-lacZ*+ cells of different developmental stages in vitro and in vivo. Surprisingly, *Anxa5-lacZ* expression is initially detected in cells associated with the primary vascular plexus. Later, expression is restricted to cells associated with endothelial cells. By using fluorescence-activated cell sorting (FACS) we were able to purify and characterize *Anxa5-lacZ*+ PVCs for the first time from mouse. The purified cell populations revealed unique expression profiles of markers characteristic for pericytes and mesenchymal stem cells. Additionally, these isolated PVCs have a capacity for differentiation into mesenchymal cell lineages. In the future, this will enable us to define the developmental ontogeny and plasticity of the heterogeneous, poorly characterized perivascular cell population.

Materials and methods

Mouse strains
Ablation of the annexin A5 gene by homologous recombination and generation of an *Anxa5-lacZ* fusion gene has been described recently. Genotyping of mice was carried out as described (Brachvogel et al., 2003). Experiments were performed with mice displaying mixed genetic background of C57/BL6x129/SvJ. Immunodeficient NOD/SCID mice were purchased from Taconic M&B, Denmark.

Immunohistochemistry
Embryos and tissues were processed for frozen sections and immunostaining as described previously (Ringelmann et al., 1999). Primary antibodies detecting smooth muscle actin, desmin (Sigma), NG2-proteoglycan (Chemicon), PECAM/CD31, Sca-1, CD34 (Pharmingen), PPARγ2 (Affinity BioReagents), collagen I (Sigma), collagen II (Lab Vision), collagen IX (generously provided by Vic

Duance, Cardiff) and collagen VI (kindly provided by R. Timpl, Martinsried) were used with corresponding secondary antibodies conjugated with Cy2, Cy3 or Cy5 (Jackson). Nuclei were stained by DAPI according to suppliers protocol (Sigma). Immunolabeled sections were stained for β-galactosidase activity at room temperature in X-gal solution (1 mg/ml) for 16-24 hours (Sigma) according to Hogan et al. (Hogan et al., 1994). Sections were stained with oil Red O and Hematoxylin as described earlier (Nuttall et al., 1998).

Detection of β-galactosidase activity
Whole-mount staining of embryos was performed after fixation in 0.2% glutaraldehyde for 10 minutes and stained in X-gal solution at room temperature for 24 hours. Treated embryos were dehydrated by increasing concentrations of ethanol and embedded in paraffin wax according to standard procedures. Embryos were cut into 9 µm sections (Leica RM 205), dried for 1 hour at 60°C and de-waxed in xylene. Specimens were counterstained in eosin for morphological assessment.

Purification of *lacZ*-expressing cells
Cells were isolated from heterozygous embryos of different stages (E8.5-E16.5) or adult brain meninges (dura, arachnoid and pia mater). In embryos, the heart was removed. Residual embryos and meninges were digested with 0.4% collagenase II (Worthington) for 60 minutes at 37°C, followed by 2% trypsin (Sigma)/100 U DNAse I (Roche) for 30 minutes at 37°C. Subsequently, cell suspensions were filtered through a 100 µm nylon mesh (Becton Dickinson). Suspensions were stained for β-galactosidase activity as described (Miles et al., 1997). Briefly, 1×10^6 cells were suspended in 20 µl PBS and added to 20 µl of 2 mM fluorescein-di-(β-D-galactopyranoside) FDG (Sigma). Cells were incubated at 37°C for 75 seconds and subsequently 500 µl of ice-cold PBS were added. Cells were stored on ice for 3 hours. The suspension was stained with propidium iodide (PI, 1 µg/ml) to label dead cells during flow cytometry. *lacZ*+ cells were detected by the FITC channel, besides FSC/SSC and PI. Cells were sorted by fluorescence-activated cell sorting (MoFlow, Cytomation) and viable PI⁻/*lacZ*+ cells were collected. Cells derived from wild-type embryos or adult brain meninges were used as negative control.

In vitro culture of sorted cells
Purified *lacZ*+ cells from adult brains or embryos were plated on gelatin-coated 24-well plates (5×10^4 cells/well) in proliferation medium [MCDB-131, 5% FCS, EGF (10 ng/ml), bFGF (2 ng/ml), insulin (5 µg/ml), PDGF-BB (5 ng/ml) (Gibco)]. Medium was changed every second day. Aggregate cultures of *lacZ*+ cells were performed as described (Johnstone et al., 1998). Briefly, 2×10^5 of confluent cells were centrifuged in a 15 ml polystyrene tube and incubated in chondrogenic [DMEM, 10% FCS, 100 nM dexamethason, insulin-transferrin-sodium selenite media supplement, 5.35 µg/ml linoleic acid, 1.25 µg/ml bovine serum albumin (Sigma), 100 ng/ml hBMP2 (Sigma)] or osteogenic medium [DMEM, 10% FCS, 100 nM dexamethason, 10 mM glycerophosphate, 10 mM $CaCl_2$, 250 µM ascorbate-2-phosphate (Sigma)] at 37°C and 5% CO_2. Cell aggregates were cultured in tubes for 14 or 21 days and medium was changed at 3-day intervals.

Ultrastructural analysis
Tissue specimens were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 24 hours at 4°C, postfixed in 1% buffered osmium tetroxide and processed for embedding in epoxy resin (EPON 812). Sections (2 µm) were analyzed by transmission electron microscopy (LEO 906E, Oberkochen; Germany).

Regeneration of muscle after crush injury
Regeneration studies of tibialis anterior muscle (TA) were carried out as described (Mitchell et al., 1992). Briefly, mice (15-20 weeks old) were anesthetized and a transversal crush injury was induced.

Immediately after damaging the muscle, 25 µl of FDG-sorted PVCs from meninges of 2-month-old mice (5×10^4 cells/25 µl) were injected into damaged region with a 25 µl syringe. The wound was closed with 6-0 suture-silk; the mice were allowed to recover in a warmed environment and placed in standard cages. Mice were sacrificed 13 days after injury and the treated muscle was processed for frozen sections.

Regeneration of bone marrow by *Anxa5-lacZ*+ PVCs

The *Anxa5-lacZ*+ mutant was generated on a C57BL/6×129SvJ background, displaying the Ly5.2 marker on the surface of bone marrow cells (BMCs). *lacZ*+ PVCs of adult meninges were isolated from heterozygous animals (2-4 months of age). Supporting BMCs were isolated from C57BL/6-Ly-5.1 mice. Supporting BMCs (9×10^5 cells) were mixed with *Anxa5-lacZ*-positive PVCs (1×10^5 cells) and injected (100 µl) into the tail vein of C57BL/6-Ly-5.1 recipient, that had been given an irradiation dose of 900 Rad (Kawada and Ogawa, 2001). As control, BMCs from *Anxa5-lacZ*+-Ly-5.2 were mixed with supporting BMCs from C57BL/6-Ly-5.1 and injected into irradiated C57BL/6-Ly-5.1 recipients. Peripheral blood was isolated 4 weeks after transplantation from the retro-orbital plexus of anaesthetized recipients and C57BL/6-Ly-5.1 mice. Cells (10^6) were stained with PE-conjugated Ly5.1 or FITC-conjugated Ly5.2 antibodies (Pharmingen) and incubated on ice for 30 minutes. Cells were washed three times in PBS/5% FCS and stained for dead cells with PI. Analysis was performed by flow cytometry detecting PE- and FITC-specific fluorescence.

Expression analysis

Total RNA of embryos or sorted cells was isolated by Trizol purification (Chomczynski and Sacchi, 1987) and concentrated by ethanol precipitation. RNA was reverse transcribed (Roche) and mRNAs of specific genes were detected by PCR using optimized primers: Flk1 (270 bp; X70842, positions 315-584), Kit (404 bp; AY536431, positions 1292-1695), Sca1 (409 bp; NM_010738, positions 35-443), CD34 (303 bp; BC066820, positions 122-424), VE-cadherin (230 bp; NM_009868, positions 52-281), Pax7 (492 bp; NM_011039, positions 1043-1534), Myod1 (130 bp; NM_010866, positions 762-891), PDGFRβ (451 bp; BC055311, positions 3290-3740), NG2 (236 bp, AF352400, positions 4290-4525), Anxa5 (300 bp; MUSANXV, positions 209-508) and Gapdh (443 bp; XM_284895, positions 532-974).

Results

Anxa5 is expressed during early stages of vasculogenesis

An *Anxa5-lacZ* promoter trap mutant was previously generated to inactivate the murine annexin A5 gene (*Anxa5*) in mice. Initial studies showed that this fusion gene is expressed in cells associated with the vascular network of embryos (E10.5-E14.5) (Brachvogel et al., 2001). To define the onset of expression during development, we analyzed Anxa5-*lacZ*+ expression during early embryogenesis (E5.5-E9.5). The first expression of Anxa5-*lacZ*+ was detected in the ectoplacental cone of embryos at E5.5, persisting during development (Fig. 1). As soon as vasculogenesis starts (~E7.5), *lacZ*-activity was detected in the developing capillary network of the yolk sac
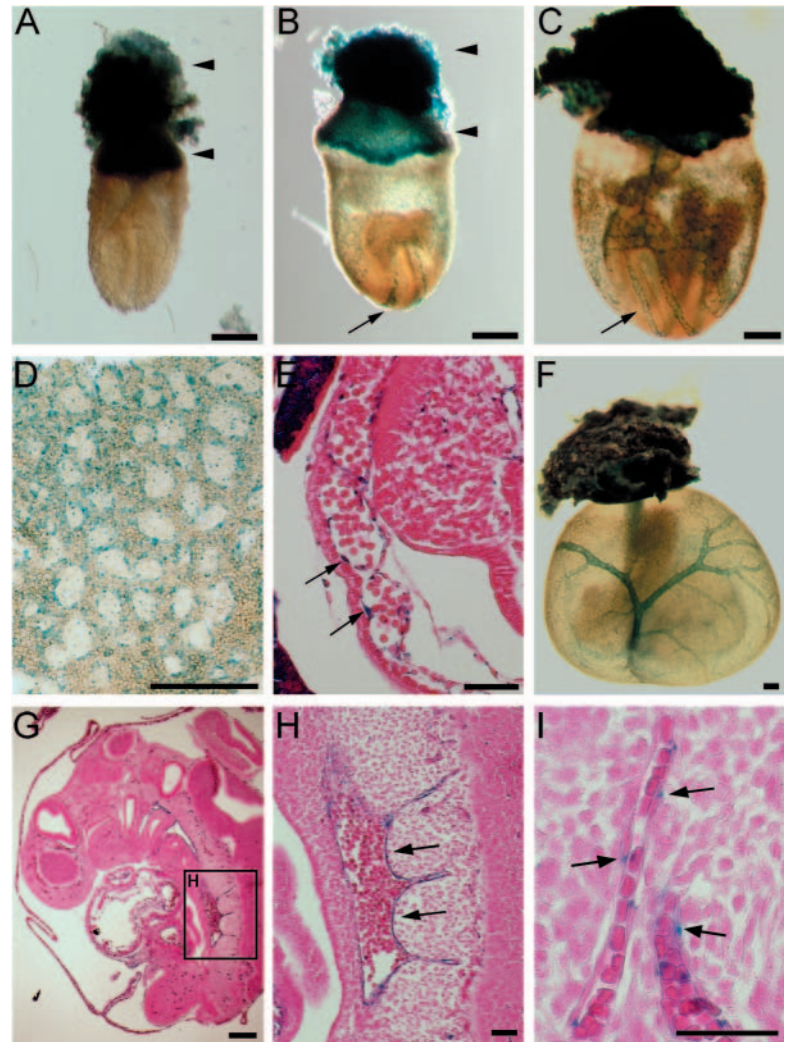

Fig. 1. Expression of Anxa5 in early embryonic development is restricted to the primary capillary plexus, dorsal aorta and extra-embryonic tissues. Whole-mount embryos heterozygous for *Anxa5-lacZ* fusion gene were stained for β-galactosidase activity at E7.0 (A), E7.5 (B), E8.5 (C,D) and E9.0 (F). Sections of E8.5 (E) and E9.0 (G-I) embryos were counterstained with Eosin after X-gal staining. Strong expression is detected in the ectoplacental cone (A,B, arrowheads). Expression of *Anxa5-lacZ* reporter in lining angioblasts of the visceral yolk sac mesoderm (D; E, arrows), dorsal aorta (B,C,H arrows) and in intersegmental vessels (I, arrows) is marked. Scale bar: 50 µm in E,H,I; 200 µm in A-D,F,G.

and dorsal aorta (Fig. 1B). At E8.5 the intensity of staining increased and all elements of the primary capillary plexus in the yolk sac were stained (Fig. 1C,D). Interestingly, no staining was observed in the endoderm of the yolk sac and in hematopoietic precursors of blood islands (Fig. 1D,E). The *lacZ* staining was clearly restricted to lining angioblasts of yolk sac mesoderm.

At E9.5, after initial remodeling of the vascular plexus, the reporter gene was still active in blood vessels of yolk sac (Fig. 1F-I). Additionally, staining of the intra-embryonic vasculature persisted at this time point and again, only cells closely associated with the dorsal aorta and intersegmental vessels express *Anxa5-lacZ* (Fig. 1H,I). No blood cells were stained. Therefore, the onset and persistence of *Anxa5-lacZ* expression
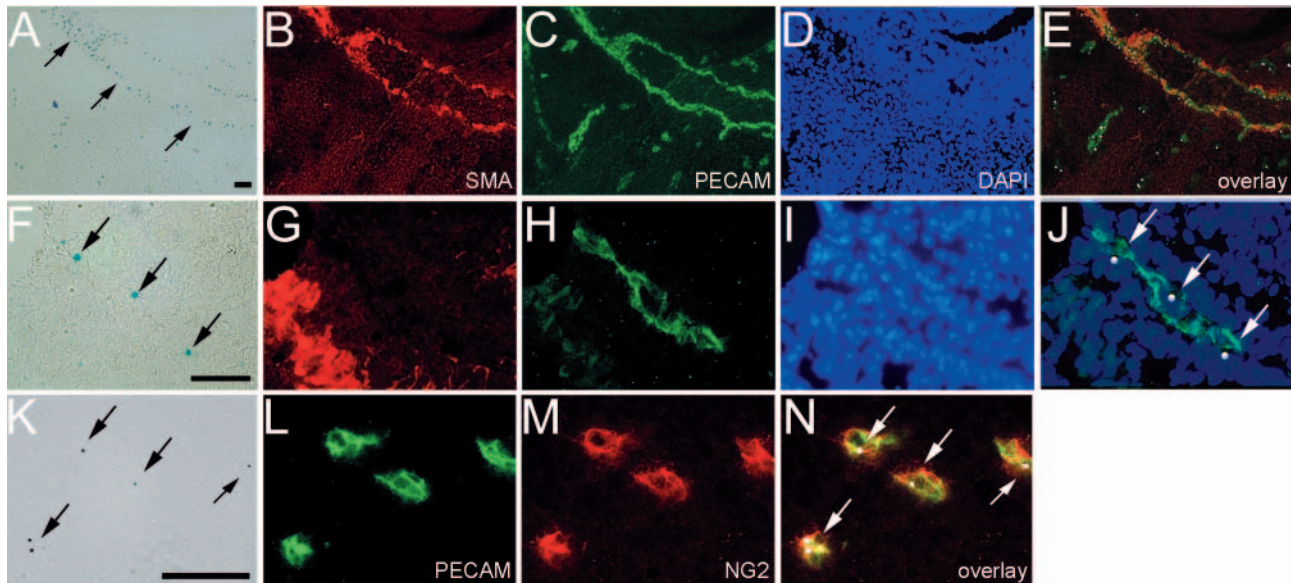
Fig. 2. *Anxa5-lacZ* reporter is specifically expressed in the vasculature of E10.5 embryos. Sagittal sections (A-D,F-I) of heterozygous embryos were stained for β-galactosidase activity (A,F) followed by immunodetection of SMA (B,G), PECAM (C,H) and nuclear staining by DAPI (D,I). In the overlays (E,J), X-gal deposits are indicated by white spots. *Anxa5-lacZ*^{+} cells are restricted to SMA[+]/PECAM[+] dorsal aorta and PECAM[+] capillaries (E). At higher magnification, only a subset of PECAM-associated cells was positive for *lacZ*, as shown by DAPI staining (J, arrows). (K-N) *Anxa5-lacZ*^{+} cells are located in regions positive for the pericyte-specific marker NG2-proteoglycan in embryonic brain at E13.5. Transverse sections of capillaries were stained for β-galactosidase activity (K, arrows). Positive areas appear black because of the monochrome character of the camera system. In parallel, PECAM (L) and NG2-proteoglycan (M) were detected by immunostaining. After merging, colocalization of PECAM and NG2 appears orange (N). X-gal deposits are shown as white spots (N, arrows). Scale bar: 50 μm.

reflect the formation and development of primary vasculature in yolk sac and embryo.

Anxa5-lacZ expression defines a population of perivascular cells

Vascular elements expand and remodel during angiogenesis. At this stage, vasculature is characterized by differentiated endothelial cells as well as perivascular cells (PVCs). Initially, PVCs are loosely attached to the endothelial network and not separated by a common basement membrane. To define the location of *Anxa5-lacZ* expression, we combined the detection of the β-galactosidase activity (β-gal) with specific immunostaining for platelet endothelial cell adhesion molecule 1 (PECAM/CD31) and smooth muscle actin (SMA) antibodies (Fig. 2). Whereas PECAM marks specifically the endothelial layer, SMA detects most vSMCs and pericytes (DeRuiter et al., 1997). In E10.5 embryos, it is clearly visible that *lacZ*-reporter expression colocalizes with the expression of SMA and PECAM in the dorsal aorta (Fig. 2A-E). Additionally, smaller vessels expressing *Anxa5-lacZ* stained for PECAM, but were negative for SMA (Fig. 2E). Labeling of nuclei by DAPI staining in the region of intersegmental vessels revealed that only a subset of cells associated with PECAM-positive endothelial cells are expressing *Anxa5-lacZ* (Fig. 2F-J). This is also indicated in capillaries of heterozygous E10.5 embryos co-stained with a PECAM-specific antibody and X-gal (see Fig. 3A). By merging phase contrast and fluorescence images, it became obvious that PECAM^{+} cells covered the inside lumen of the capillary, whereas only some cells adjacent to the endothelial layer were positive for Anxa5-*lacZ* (black arrows). *Anxa5-lacZ*-expression was also seen in small capillaries of the

brain from heterozygous embryos at E13.5 (Fig. 2K-N). In transverse sections, the deposits of the X-gal product are found in cells next to the endothelial cells of capillaries in colocalization with NG2-proteoglycan, as indicated by staining with specific antibodies (Fig. 2M). The vessel character was confirmed by immunostaining for PECAM (Fig. 2L). These data prove that only perivascular cells express the *Anxa5-lacZ* marker at E10.5 and E13.5; lining endothelial cells do not.

Anxa5 expression represents a characteristic marker for perivascular cells

Owing to the variable expression of markers in PVCs, their specificity varies depending on the tissue- and development-related context (Gerhardt and Betsholtz, 2003). Therefore, staining for SMA does not represent an unequivocal marker for PVCs and their precursors. By contrast, electron microscopy allows the identification of cells by their localization within vessel structure. Specific staining for β-gal activity in *Anxa5-lacZ*-expressing cells results in the formation of an unusual cytoplasmatic deposit of the reaction product in expressing cells, represented by an intracytoplasmatic vesicle of unknown origin (data not shown). Hence, positive cells can be identified in electron microcopy by the presence of these electron-dense vesicles (Fig. 3B, arrow). In heterozygous *lacZ*-expressing embryos of stage E10.5 these deposits were exclusively detected in PVCs closely associated with capillaries, but not in endothelial cells. The characteristic common basement membrane of endothelial cells and pericytes of functional blood vessels is not yet visible at these stages and therefore cannot be used for identification. These data support the idea that *Anxa5-lacZ* represents a novel, specific marker for PVCs.
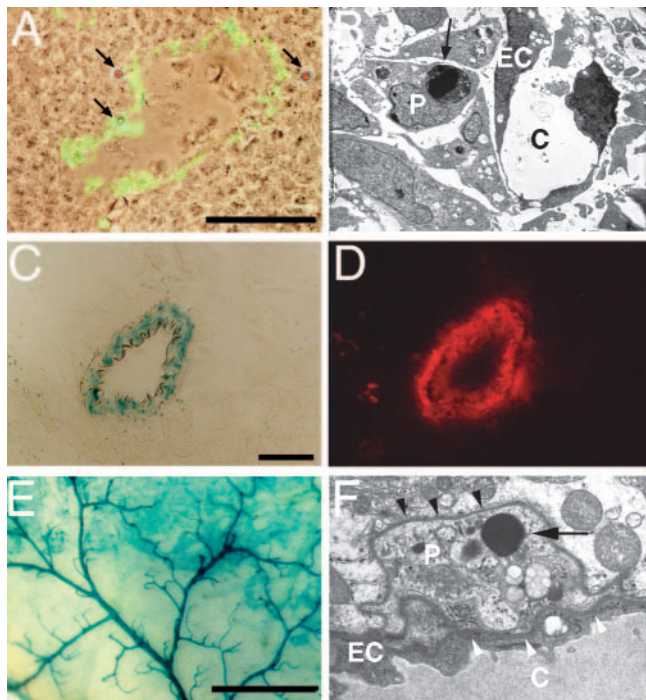
Fig. 3. *Anxa5-lacZ*^{+} cells are found associated with, but are not identical to, the endothelium in capillaries of E10.5 embryos and adult vasculature. (A) Sagittal sections of heterozygous embryos at E10.5 were stained for *lacZ* activity and immunostained for PECAM. After merging, *Anxa5-lacZ*[+] regions appear red (arrows). *Anxa5-lacZ*^{+} cells are located parallel to the lining endothelial cells. (B) Ultrastuctural localization of *Anxa5-lacZ*[+] expression in heterozygous embryos at E10.5 by electron microscopy. An electron-dense deposit of X-gal product is detected (arrow) exclusively in PVCs. (C,D) Sections from adult kidney tested for *lacZ* activity (C) and SMA expression (D) showed colocalization in the vascular walls of large blood vessels. The relative intensity of fluorescence was reduced by X-gal staining. (E) Expression of the *Anxa5-lacZ* reporter in brain of adult mice is found in the pial vasculature. (F) X-gal staining (arrow) is restricted to cytoplasmic vesicles in pericytes, characterized by the common basement membrane (arrowhead) with endothelial cells. Capillaries (C), endothelial cells (EC) and pericytes (P) are marked (B,F). Scale bars: 50 μm (A,C); 1 mm (E).

basement membrane with the underlying endothelial cell (Fig. 3F).

Purification of perivascular *Anxa5-lacZ*-positive cells by cell-sorting

Pericytes had been isolated from various organs (brain, retina and lung) of only some species but not from murine tissues yet (Doherty and Canfield, 1999; Schor and Canfield, 1998). Therefore, we used the expression of the *Anxa5-lacZ* reporter for detection and purification of these cells by cell sorting (Fig. 4). Initially, heterozygous embryos at stage E12.5 were used, which express the *Anxa5-lacZ* reporter only in vasculature and heart (Brachvogel et al., 2001). After removal of the heart, *Anxa5-lacZ*^{+} cells exclusively represent cells closely associated with vascular elements. After dissociation of embryos, viable cells were stained in vivo with the *lacZ* substrate fluorescein-di-β-D-galactopyranoside (FDG) (Miles et al., 1997). Damaged cells were excluded by propidium iodide (PI) staining. FDG-positive cells were virtually absent in wild-type controls (Fig. 4B). By contrast, a population of cells exhibiting a strong fluorescent signal (FDG[+]/PI[−]) was observed in heterozygous embryos (Fig. 4C).

To test the limits of the method, we also isolated FDG^{+} cells from E10.5 and E8.5

Similarly, *Anxa5-lacZ*[+] was also expressed in PVCs of adult mice as high levels of β-gal-activity were detected in smooth muscle layers of larger vessels in many tissues such as muscle, lung, brain and kidney. As an example, kidney was stained for β-gal activity and with a SMA-specific antibody (Fig. 3C,D). Obviously, both stains colocalize and vSMCs express the *Anxa5-lacZ* reporter. Pial vasculature of adult brain was strongly positive for β-galactosidase, as shown in whole mount X-gal staining (Fig. 3E). Electron microscopy was used to localize the *Anxa5-lacZ*^{+} cells in the brain capillaries. Unique electron-dense staining aggregates were only found within pericytes, which are identified in adult tissues by their common
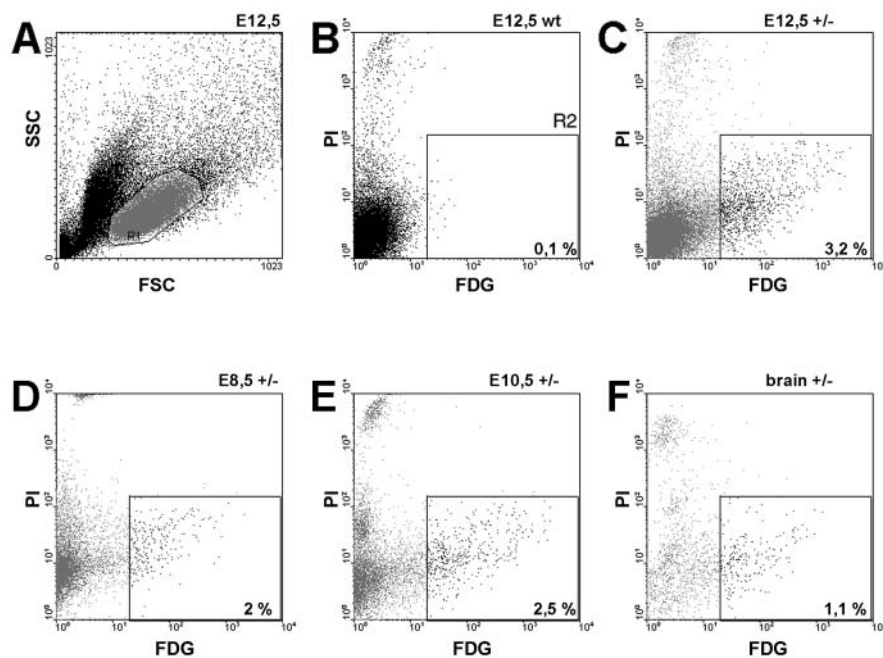

Fig. 4. Detection and purification of *lacZ*^{+} cells by fluorescence-activated cell sorting. Single cell suspensions of heterozygous embryos at E12.5 were stained with the fluorescent *lacZ* substrate FDG and cell death was detected by adding propidium iodide (PI). In FACS analysis, only vital cells in gate R1 (FSC/SSC) were considered for data analysis (A). Cells from wild-type embryos were used as control and almost no events were detected in gate R2 (B). About 3% of the heterozygous cells analyzed, represent vital *Anxa5-lacZ*^{+} cells (C). Similar amounts of *Anxa5-lacZ*^{+} cells were detected in embryos at E8.5 (D) and E10.5 (E) as well as in adult brain meninges (F). The percentages of cells within the gate are depicted, genotypes (wild type, wt; heterozygous, +/–) are indicated.

embryos. Embryos at E10.5 were similarly processed and *Anxa5-lacZ*^{+} cells (2.5%) could be purified from individual embryos (Fig. 4E). By contrast, pools of heterozygous E8.5 embryos had to be used because of the lower total cell numbers (Fig. 4D). Out of five heterozygous embryos (E8.5), only 15,000 cells were isolated with a small subset (~300 cells) representing *Anxa5-lacZ*^{+} cells. Typically, isolations of cells from different embryonic stages (E8.5-E12.5) resulted in a relative yield of 2-4% viable *Anxa5-lacZ*^{+} cells.

This experimental procedure could also be used for isolation of *Anxa5-lacZ*^{+} cells from adult mice (Fig. 4F). Vasculature of the meninges is easily accessible and represents an appropriate source of vascular-associated cells because of the low complexity of this tissue. Therefore, single cell suspensions of isolated meninges of the dura, arachnoid and pia mater were pooled from several *Anxa5-lacZ*[+] mice, stained with FDG and sorted for β-gal activity. About 1% of cells were positive and could be sorted from the pool of vital cells. Typically, about 7000 *Anxa5-lacZ*^{+} cells can be isolated from the meninges of an individual mouse. Depending on the gating stringency, an enrichment of up to 99% *lacZ*-expressing cells was achieved after sorting.

The purification of *Anxa5-lacZ*^{+} cells allowed the analysis of the expression profile of these murine PVCs by qualitative RT-PCR (Fig. 5). Sorted cells from whole embryos at E10.5, isolated brains of E16.5 embryos and brain meninges of adult animals were used for RNA isolation. Total RNA from E10.5 embryos represented the positive control. These RNAs were tested by RT-PCR for the expression of NG2 and PDGFRβ, which represent characteristic markers for pericytes (Gerhardt and Betsholtz, 2003). Purified PVCs were clearly positive for these markers. By contrast, markers for muscle satellite stem cells (Pax7) or differentiating muscle cells (Myod1) were not expressed. Therefore, purified *Anxa5-lacZ*^{+} cells display typical markers of vascular pericytes.

Anxa5-lacZ^{+} cells may represent a stem cell-like population

Surprisingly, sorted cells from E10.5, E16.5 and adult meninges also expressed the stem cell markers Flk1, Kit, Sca1, CD34 and low amounts of VE-cadherin (Fig. 5A-C). None of the sorted cells produced CD45 mRNA, a marker common to hematopoietic cells (Asakura et al., 2002). The expression profiles of sorted *lacZ*^{+} cells from various developmental stages and tissues were highly homologous (Fig. 5). A reproducible, quantitative difference was seen for the relative expression of Sca1 and Kit, with Kit being strongly expressed at E10.5 and E16.5, whereas only low levels were seen in adult PVCs. By contrast, Sca1 was highly expressed in adult PVCs but was detected at low amounts in purified cells from embryos (E10.5, E16.5). These data indicate that the *Anxa5-lacZ* marker defines a homogenous pool of cells from embryonic and adult vasculature, which resembles characteristics of pericytes as well as stem cell populations (Minasi et al., 2002).

Expression profiles indicated that isolated *Anxa5-lacZ*[+] PVCs may represent a pool of cells with stem cell-like character. For testing their potential differentiation capabilities isolated cells were cultured in vitro. Single PVCs displayed a stellate morphology, as it was described for pericytes (Canfield et al., 2000). Subsequently, PVCs started to align and after
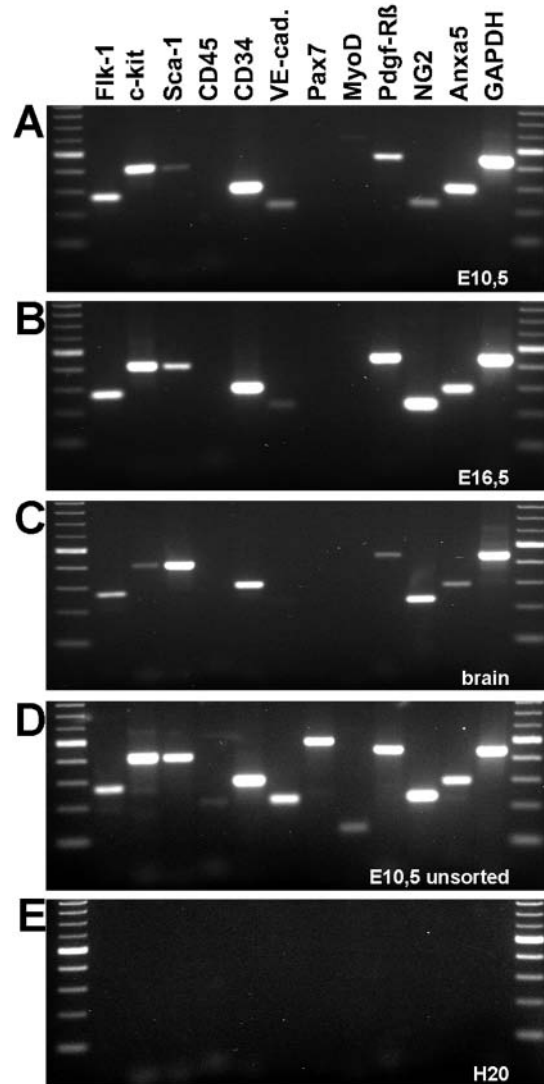

Fig. 5. Expression profiles of isolated *Anxa5-lacZ*^{+} cells. Cells were sorted from heterozygous embryos at E10.5 (A), dissected brains from E16.5 embryos (B), adult brain meninges (C) and RNA was isolated. RNA isolated from total embryos at E10.5 represented the positive control (D). RT-PCR was used to detect the mRNA of specific markers for stem cells (Flk1, Kit, Sca1, CD45, CD34, VE-cadherin), myogenic cells (Myod1) as well as myogenic satellite cells (Pax7) and pericytes (PDGFRβ, NG2). *Anxa5* was tested as a positive control and reactions without mRNA (E) were used as negative controls. GAPDH was used for standardization.

reaching confluence, the formation of multilayered areas is observed (data not shown).

Owing to the ability of isolated bovine pericytes to differentiate into a bone and cartilage-like phenotype (Canfield et al., 2000), confluent layer of PVCs were tested for their capability to differentiate into chondrogenic and osteogenic lineages. In vitro chondrogenesis or osteogenesis was induced by culturing 2×10^5 cells in three dimensional aggregates in the presence of human BMP2 (Johnstone et al., 1998) or ascorbate-2-phosphate (Bianco et al., 1998), respectively. Aggregates were harvested after 14 or 21 days of culture and sections were immunostained for the presence of chondrogenic and

osteogenic-specific markers, reflecting various stages of differentiation, such as collagen I, collagen II, collagen VI, collagen IX and collagen X (Kong et al., 1993; Quarto et al., 1993; Zhang et al., 2003; Zhou et al., 1995). Differentiation to an early chondrogenic phenotype became evident after 14 days as the expression of collagen VI (Fig. 6C,F) as well as of collagen IX (Fig. 6B,E) was clearly detected. By contrast, collagen II as a marker for differentiated chondrocytes was barely detectable even after 21 days in culture when compared with the secondary antibody control (Fig. 6A,D). No signal was found for collagen X as a marker of hypertrophic chondrocytes (not shown). Therefore, isolated PVCs have at least the capacity to undergo some initial steps of differentiation towards chondrogenic cells but do not develop a full chondrocyte phenotype. Three-dimensional aggregates also respond to osteogenic signals in the medium, as indicated by their brittle phenotype after 21 days in culture. Subsequently, sections of these cultures were analyzed for Ca^{2+} deposits by van Kossa and Alizarin Red staining. The accumulation of Ca^{2+} deposits was clearly detectable with both methods (Fig. 6H,I), whereas no staining was observed in aggregate cultures kept in proliferating medium (Fig. 6K,L). Additionally, aggregates cultivated in osteogenic medium expressed collagen I, as shown by immunostaining on sections of these cultures (Fig. 6G,J). Hence, PVCs can organize a mineralizing bone-like matrix in vitro, as shown previously for chondrocytes (Bianco et al., 1998).

Mesenchymal stem cells are capable of differentiating into adipocytes; this is the least understood pathway of mesenchymal stem cells differentiation (Caplan and Bruder, 2001). Confluent layers of isolated PVCs have therefore been cultivated in medium that induces adipogenesis in mesenchymal stem cells or adult trabecular-derived bone cells (Nuttall et al., 1998), but as yet, no increased expression of specific adipogenic markers, such as lipoprotein lipase (LPL) and proliferator-activated receptor $\gamma 2$ (PPAR$\gamma 2$), has been detected by RT-PCR. Additionally, no excessive deposition of lipid droplets can be seen using Oil red O staining (Nuttall et al., 1998) (data not shown). Surprisingly, a differentiation into adipocyte-like cells is detectable in aggregates cultured in chondrogenic medium. A strong staining with Oil Red O, which is indicative of synthesis of
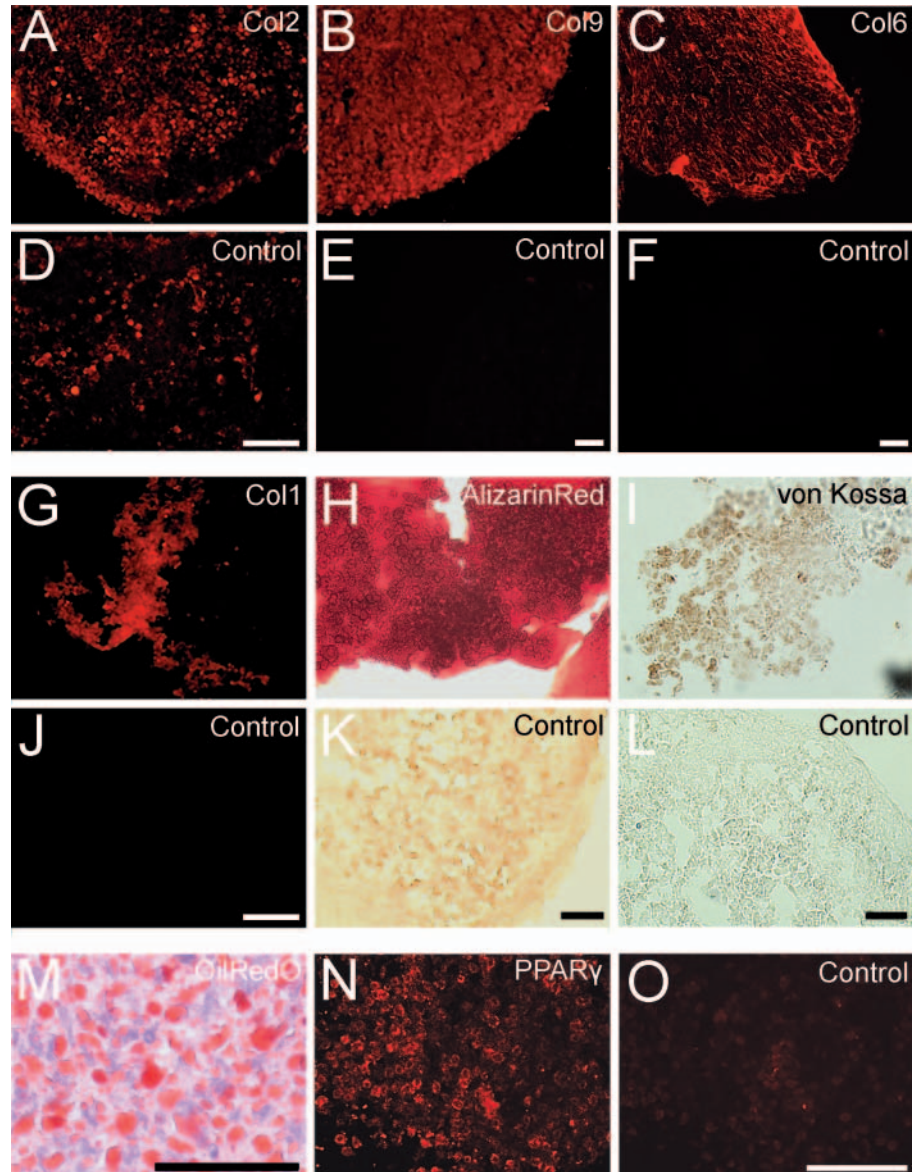

Fig. 6. *Anxa5-lacZ*^{+} cells differentiate into early chondrogenic, osteogenic and adipocyte lineages in vitro. Aggregate cultures of PVCs were cultivated in chondrogenic medium (A-F,M-O). By day 14 (B,C,E,F,M-O) or 21 (A,D), cryosections were performed and strong staining was seen for collagen VI (Col6; C) and collagen IX (Col9; B), whereas collagen II was barely detectable (Col2; A). Aggregate sections were stained for neutral fat deposits using oil Red O staining (red droplets) and Hematoxylin (M) as well as for PPAR$\gamma 2$ (N). Additionally, isolated PVCs were cultivated in osteogenic medium for 21 days (G-L). Ca^{2+} deposits in cryosections of these aggregates were detected by Alizarin Red (H) as well as van Kossa staining (I). Collagen I was detected by immunostaining (G). Aggregates of PVCs were cultivated in proliferating medium as negative control for the staining of Ca^{2+} deposits (K,L). Secondary antibodies were used as negative control for the immunostaining (D-F,J,O). Scale bars: 50 µm.

neutral lipids, is detectable on sections of these aggregate cultures (Fig. 6M). This differentiation event was never observed in cells cultured in confluent monolayer cultures. Additionally, these cells express the adipocyte-specific protein PPAR$\gamma 2$, as shown by immunostaining (Fig. 6N,O) (Chawla et al., 1994). Therefore, we conclude that the cultured *Anxa5-lacZ*[+] population retain the potential to differentiate into early

chondrogenic, osteogenic and adipogenic cells in three-dimensional aggregate cultures. A similar differentiation capacity has been seen previously with isolated bovine pericytes (Canfield et al., 2000).

Anxa5-lacZ⁺ perivascular cells are associated with muscle fibers in regenerating muscle

Isolated *Anxa5-lacZ⁺* cells express typical stem cell markers and differentiate into adipocytes, chondrogenic and osteogenic cells in vitro. To examine their developmental plasticity in vivo, we tested their ability to participate in regeneration processes of skeletal muscle (Asakura et al., 2002). As the expression profiles of purified *Anxa5-lacZ⁺* cells from embryos and adult meninges were similar, we used sorted PVCs from adult meninges. Sorted cells (50,000) were injected into regenerating tibialis anterior (TA) muscle of immunodeficient NOD/SCID mice (Shultz et al., 1995) immediately after a local damage was induced into the muscle by crushing (Mitchell et al., 1992). The muscle was allowed to regenerate for 13 days, isolated and serial cryosections were analyzed by staining with X-gal to track incorporated *Anxa5-lacZ⁺* cells. Isolated PVCs from adult meninges were detectable after 13 days within the zone of regeneration, whereas no stained cells were observable outside the regenerating zone (Fig. 7). At day 13, most of the meninges-derived PVCs were detected in columnar arrangements surrounding regenerated desmin-positive muscle fibers but were excluded from intact or regenerated muscle fibers (Fig. 7B,E). Interestingly, the injected cells were concentrated in areas with high levels of CD34 expression that were not congruent with the PECAM-positive vasculature (Fig. 7C,F). It is assumed that hematopoietic stem cells also express CD34 (Dell'Agnola et al., 2002; Mahmud et al., 2002; Uchida et al., 2001). In addition, some of the injected PVCs could be found in regions of Sca1-expressing cells (not shown), another stem cell marker (Asakura et al., 2002; Howell et al., 2002). These data indicate that injected *Anxa5-lacZ⁺* PVCs are retained and may participate in the regeneration process. Nevertheless, localization does not implicate participation, and future studies will clarify whether these cells have the potential to differentiate into muscle fibers.

Anxa5-lacZ⁺ perivascular cells do not exhibit hematopoietic potential

Adult stem cells have been characterized in many tissues and some populations have revealed the potential for hematopoietic differentiation (De Angelis et al., 1999; Jackson et al., 2002). Consequently, we addressed the issue of whether some of these *Anxa5-lacZ⁺* cells exhibit a capacity for reconstitution of the hematopoietic system, as has been shown for murine muscle fractions (Howell et al., 2002). Therefore, we injected *Anxa5-lacZ⁺* PVCs from adult meninges into lethally irradiated C57BL/6 mice, which expressed the polymorphic allele Ly5.1 on all nucleated hematopoietic cells (Shen et al., 1986). Isolated PVCs (1×10^5) from 14 adult brain meninges of heterozygous, Ly5.2 expressing mice were mixed with bone marrow cells (9×10^5 cells) from C57BL/6-Ly5.1 mice to support the regeneration of the bone marrow. This mixture was injected into the tail vein of lethally irradiated Ly5.1 recipient mice. As a positive control, 1×10^5 bone marrow cells from Ly5.2-positive mice were injected in a mixture with 9×10^5 bone marrow cells from Ly5.1 mice. Four weeks after injection, Ly5.1 and Ly5.2 expression was analyzed in the peripheral blood of bone marrow reconstituted mice (Fig. 8). As expected, no Ly5.2⁺ cells were detectable in untreated C57BL/6-Ly5.1 mice. In control experiments about 18% of blood cells were detected to be Ly5.2 positive (Fig. 8B). No Ly5.2⁺ cells could be detected in the peripheral blood of recipients that received *Anxa5-lacZ⁺*/Ly5.2⁺ PVCs (Fig. 8C). Therefore, purified *Anxa5-lacZ⁺* PVCs do not exhibit the capacity to reconstitute cells of the hematopoietic system in vivo.

Discussion

Perivascular cells (PVCs) are essential for the development of functional vessels and contribute to the structural integrity as well as contractility of vessels. Nevertheless, this cell population is poorly characterized and the developmental origin of PVCs still remains unclear (Gerhardt and Betsholtz, 2003). In this paper, we have isolated and characterized murine *Anxa5-lacZ⁺* PVCs, showing that these cells retain the capacity to differentiate into adipocytes and osteoblastic cells but not into hematopoietic lineages.

It has been suggested that pericytes and vSMC are phenotypic variants of a continuous population of PVCs and have the potential to give rise to each other, but may differentiate to chondrocytes, osteoblasts and adipocytes (Canfield et al., 2000; Nehls and Drenckhahn, 1993). The lack

Fig. 7. Purified PVCs participate in muscle regeneration in vivo. Isolated Anxa5-*lacZ⁺* PVCs of adult brain meninges were injected into regenerating areas of crushed tibialis anterior (TA) muscle of immunodeficient NOD/SCID mice. By day 13, consecutive muscle sections were analyzed for β-galactosidase activity by X-gal staining and expression of desmin (B,E), PECAM (C) as well as CD34 (F) by immunohistochemistry. Injected *Anxa5-lacZ⁺* cells were detected at day 13 in the zone of regeneration (arrows, A,D) in columnar structures associated with intact desmin-positive muscle fibers (B,E). No staining was seen in intact fibers. *lacZ*-expressing cells were found in areas positive for CD34⁺ (D,F) but not in areas positive for PECAM (A,C). Scale bars: 50 μm.
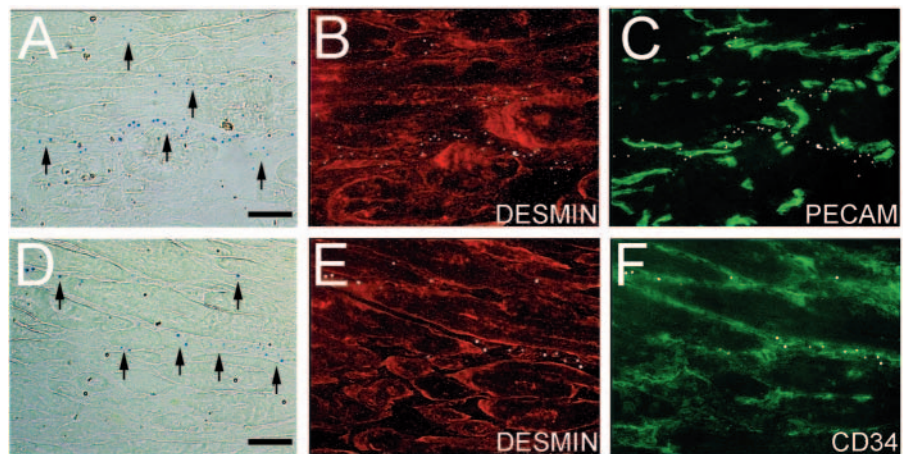
Fig. 8. Purified *Anxa5-lacZ*⁺ PVCs do not have hematopoietic capacity in vivo. Purified *Anxa5-lacZ*⁺ PVCs from adult brain meninges (Ly5.2⁺, 1×10^5 cells) were mixed with bone marrow cells (Ly5.1⁺, 9×10^5) and injected intravenously into recipient mice (Ly5.1⁺) after lethal irradiation. After 28 days, peripheral blood of recipients was analyzed by flow cytometry for the presence of Ly5-markers. (A) No Ly5.2⁺ cells were detectable in blood of the negative control mice, represented by non irradiated wild-type Ly5.1⁺ mice without transplantation. (B) In the positive control, 18% of the blood cells derived from the injected Ly5.2⁺ BMCs. (C) No Ly5.2⁺ cells could be detected after administration of *Anxa5-lacZ*⁺/Ly5.2⁺ donor PVCs.
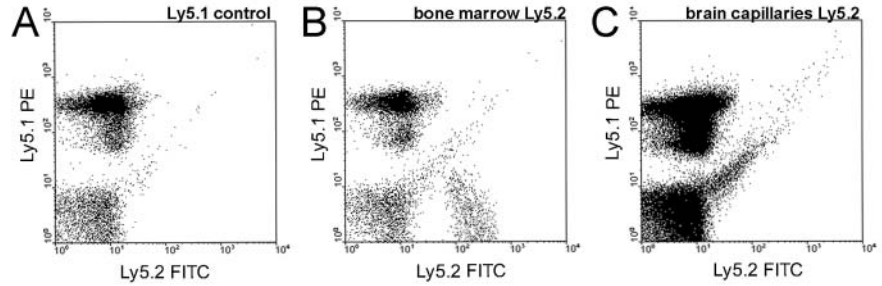
of adequate markers during early development of vasculature causes a major problem for analyzing the role of PVCs in this process. During growth, remodeling and differentiation of the vasculature, expression of specific markers like SMA and NG2-proteoglycan become indicative for PVCs only at later stages (Ozerdem et al., 2001). Three lines of evidence now clearly indicate that *Anxa5-lacZ* expression represents a novel and highly specific marker for these PVCs during development and in the adult. First, *Anxa5-lacZ*⁺ cells are exclusively detected in close proximity to PECAM-positive endothelial cells and show colocalization with the markers SMA or NG2 in many cells. Second, electron microscopy detects deposits of the *lacZ*-product in cells directly contacting the endothelium. This can be seen even in early embryos, where the characteristic common basement membrane is not yet developed, as well as in pericytes of adult blood vessels defined by their typical basement membrane (Fig. 3). The third and most important argument is seen with the expression analysis of isolated *Anxa5-lacZ*⁺ cells. As previously shown, the presence of NG2, SMA and PDFGRβ is highly indicative for pericytes or pericyte-related cell populations (Gerhardt and Betsholtz, 2003). All these markers are also significantly expressed in purified *Anxa5-lacZ*⁺ cells from various sources. The expression profile of these markers was identical among populations isolated from E10.5, E16.5 and even adult meninges, which defines a common conserved expression pattern at different stages of development. These data clearly show that *Anxa5-lacZ*⁺ expression correlate with typical markers of PVCs, even at very early stages of development.

Recently, a transgenic mouse line (XlacZ4) was described to display specific β-galactosidase expression in pericytes (Tidhar et al., 2001). Yet it is not clear which gene is reflected by the XlacZ4 gene-trap. Both the XlacZ4 and *Anxa5-lacZ* reporters define overlapping expression patterns, as they are expressed in PVCs of adult vasculature. It is remarkable that both reporters are not expressed in the microvasculature of the liver, highlighting the specific nature of hepatic microvasculature, especially of the pericyte-related Ito cells (Tidhar et al., 2001). Nevertheless, there are significant differences early in development. Whereas the XlacZ4 reporter is first active in the basilar artery within the head mesenchyme of E10-E10.5 embryos, *Anxa5-lacZ* is already expressed in angioblasts of the primary vascular plexus. At later stages, the *Anxa5-lacZ* reporter is active in mesenchymal condensates giving rise to chondrogenic and osteogenic tissues, whereas XlacZ4 is expressed only in a subpopulation of cartilage cells

in joints of the appendages. Therefore, the expression of both reporter genes is similar at later developmental stages, but *Anxa5-lacZ* expression may define a unique population of vasculature-associated cells also early in development.

During vasculogenesis, the expression of the *Anxa5-lacZ* reporter gene was detected in cells forming the primary capillary plexus in the yolk sac of E7.5 to E8.5 embryos. This staining resembles the angioblast appearing at onset of vasculogenesis. This expression is reflected by Flk1 expression in the purified Anxa5-lacZ⁺ cells (not shown), representing a characteristic marker of angioblasts of the yolk sac mesoderm (Shalaby et al., 1995). Later during angiogenic remodeling, expression of the *Anxa5-lacZ* marker defines the population of PVCs. Many studies have indicated that PVCs are recruited from stromal cells by mutual contacts with endothelial cells (reviewed by Gerhardt and Betsholtz, 2003). Alternatively, trans-differentiation from endothelial cells was discussed to contribute to the PVC cell pool. Flk1⁺ cells were also identified in ES-cell cultures, which can serve as a progenitor for endothelial cells and SMCs in vitro and in vivo (Ema et al., 2003; Yamashita et al., 2000). Therefore, we assume that Anxa5 expression may represent a novel marker for a subset of the mesenchymal stem cell compartment (Dennis and Charbord, 2002) that differentiates into angioblast and later strictly correlates with the transition to PVCs. Although 'it takes two to make blood vessels – endothelial cells and pericytes' (Gerhardt and Betsholtz, 2003), the understanding of PVCs is rather limited in comparison to endothelial cells. Therefore, the purification of *Anxa5-lacZ*⁺ PVCs represents a novel and unique tool with which to characterize this cell type by in vitro and in vivo studies in the mouse system.

The isolated PVCs clearly reflect a pericyte-like phenotype that is indicated by their morphology, by the detection of NG2 and SMA protein in cultured cells, and by their differentiation into adipogenic, early chondrogenic and osteogenic cells. Earlier studies have indicated that bovine derived pericytes, mesenchymal stem cells and adult trabecular-derived bone cells are able to differentiate into chondrogenic cells and adipocytes (Canfield et al., 2000; Nuttall et al., 1998). Interestingly, isolated cell populations were found to express different stem cell markers, such as Flk1, Kit, Sca1 and CD34 (see Fig. 5). This pattern reflects the phenotype of aorta-derived multipotent progenitors (Minasi et al., 2002), as both pools express the 'hemangioblast' markers CD34, Kit and Flk1. Additionally, the stem cell marker Sca1 is expressed. It has been discussed that Sca1⁺-expression in muscle defines a

vascular-associated pool of adult stem cells in skeletal muscle with a high degree of phenotypic plasticity (Asakura et al., 2002; Cao et al., 2003; De Angelis et al., 1999; Tamaki et al., 2002). Surprisingly, this marker was also detected in *Anxa5-lacZ*[+] PVCs from early embryos and was even upregulated in adult meninges. Moreover, Flk1 and VE-cadherin are expressed in purified PVCs, two markers also detected in embryoid body-derived precursor blast colonies of the hematopoietic as well as the endothelial cell lineage (Kennedy et al., 1997). Interestingly, the progeny of cultured ES-cells with hemangioblast potential also express Flk1 and VE-cadherin, but not CD45 (Nishikawa et al., 1998).

In vivo regeneration experiments of muscle clearly show that isolated PVCs are specifically incorporated into columnar structures associated with blood vessels located between muscle fibers, but not integrated into newly formed muscle fibers. This correlates with the finding that *Anxa5-lacZ*^{+} cells do not express the characteristic marker Pax7 for satellite cells, the major myogenic stem cells (Seale et al., 2000) cannot be detected. Therefore, *Anxa5-lacZ*[+] PVCs may not retain myogenic differentiation capacity in vivo. Nevertheless, we cannot exclude the possibility that *Anxa5-lacZ* reporter expression is inactivated in differentiated muscle fibers, as we never detected expression of the reporter gene in skeletal muscle cells.

Multiple stem cell populations have been defined in skeletal muscle. The side population (SP) represents a well characterized cell pool (Goodell et al., 1997), which is found associated to the vasculature and displays hematopoietic potential in vitro (Asakura et al., 2002). Muscle adult stem cells also exhibit hematopoietic capacity in vivo (Gussoni et al., 1999; Howell et al., 2002; Jackson et al., 1999). The markers CD34 and Sca1 are characteristically expressed by the SP cells (Goodell et al., 1997), although the correlation of CD34 expression and stem cell potential still remains unclear (Parmar et al., 2003). Sca1 expression is used as a marker for potential hematopoietic cells between muscle fibers (Asakura et al., 2002). Although these markers are expressed in *Anxa5-lacZ*^{+} PVCs and injected PVCs were found in Sca-1^{+}/CD34^{+} areas in regenerating muscle, we could not define a capacity for hematopoietic differentiation of *Anxa5-lacZ*^{+} PVCs isolated from adult meninges by bone marrow reconstitution experiments.

Interestingly, the *Anxa5-lacZ* reporter is highly active in mesenchymal condensations at E13.5, preceding formation of cartilage and bone during development, but also later in all differentiated skeletal elements (Brachvogel et al., 2001). Additionally, isolated PVCs had the potential to develop into early chondrogenic, osteogenic and adipogenic lineages. Recently, it was shown that the embryonic dorsal aorta of E10.5 embryos contains progenitor cells termed meso-angioblast, which are able to participate in the development of perichondrium and cartilage, connective tissue, smooth muscle and cardiac muscle (Minasi et al., 2002). It became obvious that a mesenchymal stem cell compartment may exist that is represented by a spectrum of related cells with capacity for phenotypic differentiation into stroma cells, adipocytes, chondrocytes or bone cells (Caplan, 1991; Dennis and Charbord, 2002). This model differs from the standard paradigm that postulates hierarchical lineages during development, as in the hematopoietic system, by a high degree of plasticity during lineage progression and transition (Theise et al., 2003). We propose that *Anxa5-lacZ* expression defines a subset of the mesenchymal stem cell compartment and may reflect some aspects of various differentiation pathways during development.

In this paper, we describe for the first time the use of Anxa5 expression for the isolation of a novel cell population resembling perivascular cells (pericytes and vSMCs), from mouse tissues, characterized by the expression of pericyte-specific markers. In future, this unique method will help to define the capacities of the poorly characterized PVCs. Our data show that these cells additionally display markers of mesenchymal stem cells and have the capacity to differentiate into adipocytes and osteoblastic cells. Therefore, expression of Anxa5 defines a novel subset of the mesenchymal stem cell compartment that reflects variable lineage progression and phenotypic plasticity.

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