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FGF2-dependent neovascularization of subcutaneous Matrigel plugs is initiated by bone marrow-derived pericytes and macrophages

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Vessel-like networks are quickly formed in subcutaneous FGF2-supplemented Matrigel plugs by two cell types: NG2$^+$ pericytes and F4/80$^+$ macrophages. Although not detected in these networks until 7 days after plug implantation, the appearance of CD31$^+$ endothelial cells marks the onset of vessel perfusion and the establishment of mature vessel morphology, with endothelial cells invested tightly by pericytes and more loosely by macrophages. Evidence that mature vessels develop from pericyte/macrophage networks comes from experiments in which 5-day plugs are transplanted into EGFP$^+$ recipients and allowed to mature. Fewer than 5% of pericytes in mature vessels are EGFP$^+$ in this paradigm, demonstrating their presence in the networks prior to plug transplantation. Endothelial cells represent the major vascular cell type recruited during later stages of vessel maturation. Bone marrow transplantation using EGFP$^+$ donors establishes that almost all macrophages and more than half of the pericytes in Matrigel vessels are derived from the bone marrow. By contrast, only 10% of endothelial cells exhibit a bone marrow origin. The vasculogenic, rather than angiogenic, nature of this neovascularization process is unique in that it is initiated by pericyte and macrophage progenitors, with endothelial cell recruitment occurring as a later step in the maturation process.

KEY WORDS: Vasculogenesis, Pericyte, Macrophage, Endothelial cell, Bone marrow progenitor

INTRODUCTION

Progenitor cells from the adult bone marrow, peripheral circulation and local tissues make substantial contributions to microvascularization, especially during tumor growth and other types of pathological vascularization. The participation of these progenitors often gives the neovascularization process more of a vasculogenic than angiogenic flavor (Hirschi and Majesky, 2004; Tepper et al., 2005). As it frequently involves stimulation by combinations of factors not encountered during normal development, pathological neovascularization may not be a uniform stereotyped process. Instead, it may be a highly plastic and mosaic process involving both vasculogenic and angiogenic mechanisms, depending on the specific environment.

Although most studies on neovascular plasticity have focused on the recruitment of endothelial progenitors from bone marrow and other progenitor sources (Asahara et al., 1999; Lyden et al., 2001; Bailey et al., 2004), a smaller number of cases have demonstrated a bone marrow origin for microvascular pericytes (De Palma et al., 2003; Rajantie et al., 2004; Song et al., 2005; Ozerdem et al., 2005). Compared with their endothelial partners, pericytes are a rather poorly understood cell type (Sims, 2000; Betsholtz et al., 2005; Bergers and Song, 2005). The crucial importance of these cells in the formation of functional microvessels is demonstrated by the pathological phenotypes of mice with impaired pericyte development (Hellstrom et al., 2001; Lindahl et al., 1997; Enge et al., 2002; Ozerdem and Stallcup, 2004). Pericyte function has mostly been attributed to participation in relatively late events associated with microvessel development, such as stabilization of maturing blood vessels, formation of permeability barriers and regulation of blood flow (Allt and Lawrenson, 2001; Gerhardt and Betsholtz, 2003; Betsholtz et al., 2005; Bergers and Song, 2005). However, the use of early pericyte markers such as the NG2 proteoglycan and PDGFβ-receptor (PDGFβR) demonstrates that pericytes are often present during the initial stages of neovascularization and may even be involved in initiating microvascular development (Schlingemann et al., 1990; Nehls et al., 1992; Wesseling et al., 1995; Redmer et al., 2001; Gerhardt and Betsholtz, 2003; Ozerdem et al., 2001; Ozerdem et al., 2002; Ozerdem and Stallcup, 2003; Song et al., 2005; Virgintino et al., 2007). Accordingly, the relationship between pericytes and their endothelial partners spans the entire life of microvessels, from earliest development to later maintenance and repair.

Primarily recognized as phagocytes and for their role in inflammation, macrophages represent another class of bone marrow-derived cells that play an important early role in neovascularization (Beck et al., 1983; Hume et al., 1984; Sunderkotter et al., 1994; Takakura et al., 2000). The functional role of macrophages in blood vessel development is demonstrated by reduced neovascularization in models where macrophage recruitment is blocked (Lyden et al., 2001; Luttun et al., 2002; Sakurai et al., 2003; Grunewald et al., 2006). As a rich source of cytokines and proteases, macrophages mediate both recruitment and extracellular matrix degradation as means of promoting the influx of vascular cells (Murdoch et al., 2004; Moldovan and Moldovan, 2005; Lamagna et al., 2006). More surprising is the possible role of macrophages as structural components during blood vessel assembly. Macrophages represent a major population of cells that invade subcutaneous Matrigel plugs supplemented with fibroblast growth factor 2 (FGF2), contributing prominently to the formation of functionally perfused vessels (Schmeisser et al., 2001; Anghelina et al., 2004; Anghelina et al., 2006).

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In this report, we also use subcutaneous FGF2-containing Matrigel plugs to document the initiation of new vessel formation by populations of bone marrow-derived pericytes and macrophages. These two cell types rapidly form vessel-like networks in the absence of cells expressing the endothelial marker CD31. These structurally simple early networks give rise to more complex, functionally perfused structures containing $CD31^+$ endothelial cells, $NG2^+$ pericytes and $F4/80^+$ macrophages.

MATERIALS AND METHODS

Antibodies

Polyclonal antibodies against NG2 proteoglycan and PDGFβR have been previously described (Ozerdem et al., 2001; Ozerdem et al., 2002). Rat monoclonal antibodies against mouse CD31, CD34 and Sca-1 (also known as Ly6a – Mouse Genome Informatics) were purchased from BD Biosciences (La Jolla, CA). Goat antibody against CD45 was obtained from R&D Systems (Minneapolis, MN). Rat monoclonal antibody against F4/80 was purchased from BioSource International (Camarillo, CA), and rabbit anti-desmin was from Dako. Hamster anti-CD31 was obtained from Chemicon (Temecula, CA). Cy5 or FITC-conjugated second antibodies were obtained from Jackson ImmunoResearch (West Grove, PA). Other secondary antibodies were purchased from Molecular Probes (Eugene, OR). Secondary antibodies were from goat, except when goat anti-CD45 was used. In that case all secondary antibodies were from donkey.

Animals

C57Bl/6 mice and C57Bl/6 mice expressing EGFP under control of the β-actin promoter (β-actin/EGFP; Jackson Laboratories) were maintained in the Burnham Institute Vivarium (fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care). All animal procedures were performed in accordance with Office of Laboratory Animal Welfare regulations and were approved by Burnham Institute Animal Care and Use Committee review prior to execution.

Matrigel plugs

Aliquots (0.4 ml) of growth factor-reduced Matrigel (BD Biosciences) containing 200 ng FGF2 (R&D Systems, Minneapolis, MN) and 60 U/ml heparin (Sigma-Aldrich, St Louis, MO) were prepared on ice. Mice receiving subcutaneous Matrigel plugs were anesthetized by intraperitoneal injection of Avertin (0.015 ml/g body weight). Abdominal and inguinal areas were shaved and swabbed with 70% ethanol. Matrigel aliquots were injected bilaterally into the inguinal areas and allowed to gel at body temperature.

At desired time points (5-14 days), mice were euthanized by CO_2 asphyxiation for plug excision. Plugs were fixed for 8 hours in 4% paraformaldehyde, cryoprotected overnight in 20% (wt/vol) sucrose and frozen in OCT embedding compound (Tissue-Tek). Sections (30 μm) were prepared using a Reichert cryostat microtome.

Bone marrow transplantation

Donor mice (usually β-actin/EGFP) were euthanized by CO_2 asphyxiation. Dissected femurs and tibiae were flushed with 1 ml of PBS containing 2% fetal calf serum (FCS) and 5 mM EDTA (PBS/FCS/EDTA). Bone marrow cells were washed once with PBS/FCS/EDTA prior to red blood cell lysis on ice for 5 minutes with ACK buffer (150 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA, pH 7.4). Typically, $5\text{-}7\times10^7$ cells were collected from a single donor.

Immediately before injection into recipient mice, cells were washed three times with PBS/FCS/EDTA, passed through a 62 μm nylon filter (Small Parts, Miami Lakes, FL) and washed twice with Ringer's solution.

Recipient mice were gamma irradiated (two 5 Gy doses administered 3 hours apart) using a ^{137}Cs Gammacell-40 Exactor irradiator. Animals were immediately reconstituted via retro-orbital injection of 5×10^5 bone marrow cells in 100 μl of Ringer's solution, and were maintained on antibiotic water (neomycin sulphate, 1.1 g/l and Polymyxin B sulphate, 455 mg/l) for 6 weeks to allow hematopoietic re-establishment. Retro-orbital blood samples (or bone marrow samples) were taken from each recipient, and the extent of EGFP engraftment was determined by flow cytometric analysis. Animals exhibiting at least 75% engraftment were used for establishment of Matrigel plugs.

FITC-dextran angiography

Mice carrying FGF2-supplemented Matrigel plugs were anesthetized with Avertin and injected via the tail vein with 200 μl of 50 mg/ml fluorescein isothiocyanate (FITC)-dextran (2×10^6 M_r, Sigma-Aldrich). After a 10-minute incubation, mice were euthanized by cervical dislocation while still under anesthesia, and Matrigel plugs were removed for histological analysis.

Immunostaining and confocal microscopy

Immunolabeling of sections and analysis by confocal microscopy were performed as previously described (Ozerdem et al., 2001; Ozerdem et al., 2002). A BioRad inverted Radiance 2100 Multiphoton Confocal Microscope was used to obtain serial 1-1.5 μm optical sections across the entire 30 μm thickness of the histological specimens. This confocal system provides for analysis of four fluorochromes, allowing us to perform quadruple labeling with DAPI and three antibodies, or with DAPI, two antibodies, and either FITC-dextran or the EGFP transgene. Overlaid serial optical sections (*z*-stacks) were analyzed using Volocity 4D Rendering software (Version 3.7) for unambiguous determination of the spatial relationship between cells in vessel-like structures.

Matrigel plug transplantation

Mice were euthanized by CO_2 asphyxiation 5 days after establishment of Matrigel plugs. Plugs were excised, washed twice in cold sterile PBS, and incubated on ice for 45 minutes in sterile PBS containing 200 ng FGF2. β-Actin/EGFP mice were anesthetized with Avertin, and their left abdominal areas were shaved and swabbed with 70% ethanol. A 1 cm incision was made on the left side of the abdomen, and a subcutaneous pouch was formed by blunt dissection. The donor plug was inserted into this skin pouch, which was closed with two or three sutures. After 14 days the transplanted plugs were processed for immunostaining and confocal microscopy.

Analytical flow cytometry

Peripheral blood or bone marrow samples taken from EGFP-reconstituted mice were collected in PBS containing 2% FCS and treated in ACK buffer to lyse red blood cells (see bone marrow transplantation). After passage through a 62 μm nylon filter, at least 10^4 white blood cells or bone marrow cells were analyzed for EGFP expression using a FACSCanto instrument (BD Biosciences).

Flow cytometry to immunophenotype vascular cell types in Matrigel plugs was performed after mincing the plugs and treatment at 37°C for 1 hour in PBS containing 0.2% collagenase 1A (Sigma-Aldrich), 200 U/ml DNase I (Sigma-Aldrich), 10 mM $MgCl_2$, and 10% FCS. The mixture was repeatedly passed through a 21-gauge needle to facilitate cell dissociation. After washing three times in PBS containing 10% FCS, cells were passed through a 62 μm filter, labeled with the desired antibody combinations, and analyzed on the FACSCanto instrument (at least 5000 cells analyzed per trial). The fluorescent membrane-permeant DNA-stain LDS-751 (1 μg/ml, Invitrogen, Carlsbad, CA) was used to identify nucleated cells and exclude debris from the analysis.

RESULTS

In contrast to the complexity of most types of pathological vascularization, the subcutaneous Matrigel plug assay (Passaniti et al., 1992; Malinda, 1996) offers the potential for studying neovascularization in response to a specific initial stimulus. We have used FGF2, a factor with documented ability to initiate neovascularization not only in Matrigel plugs (Anghelina et al., 2004; Anghelina et al., 2006), but also in other models such as corneal angiogenesis and the chick embryo chorioallantoic membrane assay (Auerbach et al., 2003; Kenyon et al., 1996; Ribatti and Presta, 2002). We used immunocytochemical and flow cytometric techniques to characterize the cellular composition of developing Matrigel vessels.

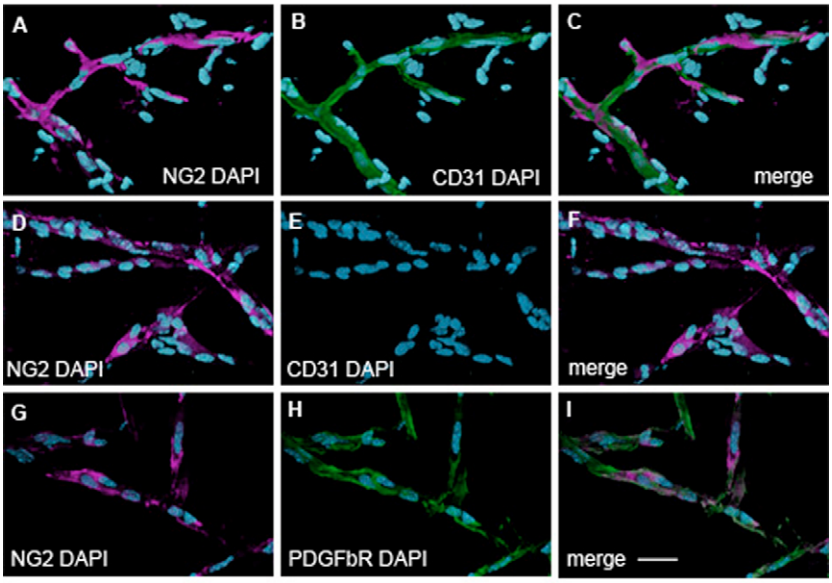

Fig. 1. Early presence of pericytes in Matrigel vessels. Matrigel plugs were removed for analysis after 9 days (**A-C**) or 5 days (**D-I**). In A-F, endothelial cells were labeled for CD31 (green), while pericytes were labeled for NG2 (magenta). In G-I, pericytes were labeled for both NG2 (magenta) and PDGFβR (green). Cell nuclei were stained using DAPI (blue). At 5 days, the vessel-like networks contain $NG2^+PDGF\beta R^+$ pericytes in the absence of CD31-positive endothelial cells. At 9 days, both types of cells are present in the structures. All panels represent *z*-stacks of confocal images. Scale bar: 40 μm.

Cells in early vessel-like structures have a pericytic rather than endothelial phenotype

Although cellular invasion occurs in unsupplemented Matrigel implants, organized cellular structures are rarely seen in these controls (data not shown). By contrast, Fig. 1A-C shows that 9 days following initial establishment, FGF2-supplemented Matrigel plugs contain vessel-like structures composed of $CD31^+$ endothelial cells (green) closely invested by $NG2^+$ pericytes (magenta), the cellular arrangement typical of blood vessels in normal tissues. Remarkably, at earlier time points the plugs contain cellular networks devoid of $CD31^+$ endothelial cells, but composed of a high percentage of $NG2^+$ cells (Fig. 1D-F). Most of these $NG2^+$ cells also express additional pericyte markers such as desmin (not shown) and PDGFβR (Fig. 1G-I). Confocal sectioning through these pericyte networks reveals that 79% of $NG2^+$ cells are $PDGF\beta R^+$, and verifies the absence of $CD31^+$ cells through the entire thickness of the specimen.

The timing of the transition from pericyte networks to vessel-like structures containing both pericytes and endothelial cells was established by the analysis of 10 FGF2-containing Matrigel plugs at each of four time points. After 5 days of vascularization, 8 out of 10 plugs contained $NG2^+$ $CD31^-$ vessel-like structures. Networks containing $CD31^+$ endothelial cells were never present at this early time point. After 7 days we observed $NG2^+$ $CD31^-$ networks in 9 out of the 10 plugs. Occasional structures with $CD31^+$ endothelial cells were found at this intermediate time point. After 9 and 14 days the majority of vessel-like structures in all plugs contained both $CD31^+$ endothelial cells and $NG2^+$ pericytes. Nevertheless, some $NG2^+$ $CD31^-$ structures could still be observed at these later time points.

Vessel functionality requires the incorporation of CD31-positive endothelial cells

Along with documenting the transition of $NG2^+$ $CD31^-$ networks in early plugs to $NG2^+$ $CD31^+$ vessel-like structures at later time points, we also used FITC-dextran perfusion to determine whether either of these structures represent functional vessels perfused by the circulation. Fig. 2A-D shows an $NG2^+$ $CD31^-$ pericyte network from a 5-day Matrigel plug. The absence of FITC-dextran indicates that this structure is not yet perfused by the circulation. The use of DAPI to label cell nuclei, along with construction of *z*-stacks of confocal sections, provides assurance that we have not overlooked CD31 or FITC-dextran labeling at any level of the structure. Examination of 80 $NG2^+CD31^-$ pericyte networks in a total of five 5-day Matrigel plugs failed to reveal a single case of FITC-dextran perfusion.

By contrast, samples from a 9-day plug exhibit clear evidence of perfusion of $NG2^+CD31^+$ vessels by FITC-dextran (Fig. 2E-H). Examination of 80 $NG2^+CD31^+$ structures in a total of five 9-day Matrigel plugs identified more than 90% of them as perfused vessels. These findings indicate that the Matrigel vessels do not become functional until the early pericyte networks acquire an endothelial lining.

Cells in early networks express pericyte, myeloid, and progenitor cell markers

To further characterize the cell populations that comprise early vessel-like structures, we analyzed 85 early vessel-like structures (five plugs) and 80 mature vessels (five plugs) for expression of CD34 and Sca-1, which are present on many types of immature precursor cells. DAPI-staining of nuclei and careful use of the Volocity 4D program to analyze *z*-stack confocal images showed unambiguously that CD34 and Sca-1 are co-expressed with NG2 on individual cells that invade Matrigel plugs during the first 5 days after implantation, suggestive of a progenitor cell origin. The co-expression of NG2 with CD34 (Fig. 3A-D) and Sca-1 (Fig. 3E-H) is also maintained in early pericyte networks. These immunocytochemical findings are reinforced by parallel experiments in which we dissociated 5-day Matrigel plugs and used flow cytometry to identify the cell populations that were present. Fig. 4A and Table 1 show that about 70% of the cells in early plugs are characterized by an $NG2^+CD34^+$ phenotype. Probably representing a substantial overlap with these cells, ~50% of the population exhibits an $NG2^+CD31^-$ phenotype. Emphasizing the scarcity of cells with an endothelial phenotype, only 4% of the total population has an $NG2^-CD31^+$ phenotype. These studies also identify interesting subpopulations of cells in which NG2 and CD34 do not overlap (7-21% $NG2^-CD34^+$ and 5-8% $NG2^+CD34^-$), possibly representing, respectively, endothelial precursors and pericytes derived from local sources.

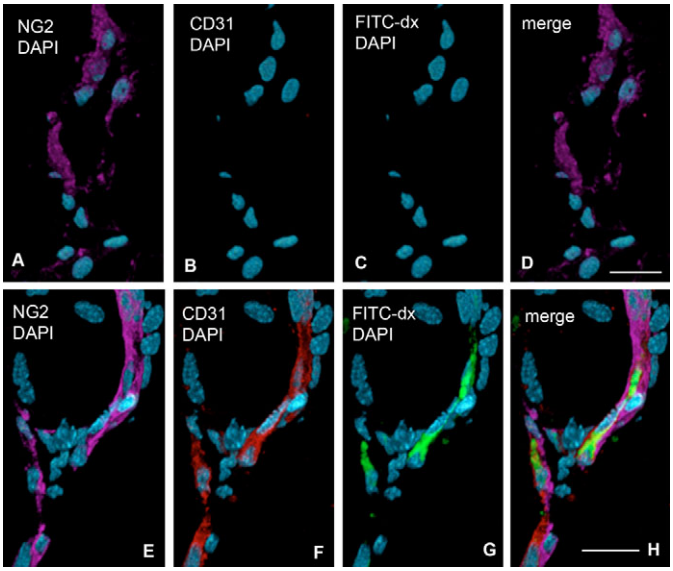

Fig. 2. Functional perfusion of FGF2-induced Matrigel vessels. Mice containing Matrigel plugs were utilized on day 5 (**A-D**) and day 9 (**E-H**) for FITC-dextran (FITC-dx; green) perfusion to identify vessels connected to the circulation. Sections prepared from the plugs were stained for DAPI (blue), CD31 (red) and NG2 (magenta). At day 5, prior to the appearance of CD31$^+$ endothelial cells, FITC-dextran is not present in the cellular networks. At day 9, FITC-dextran perfusion is apparent in vessels containing both pericytes and endothelial cells. All panels represent *z*-stacks of confocal images. Scale bars: 40 μm.

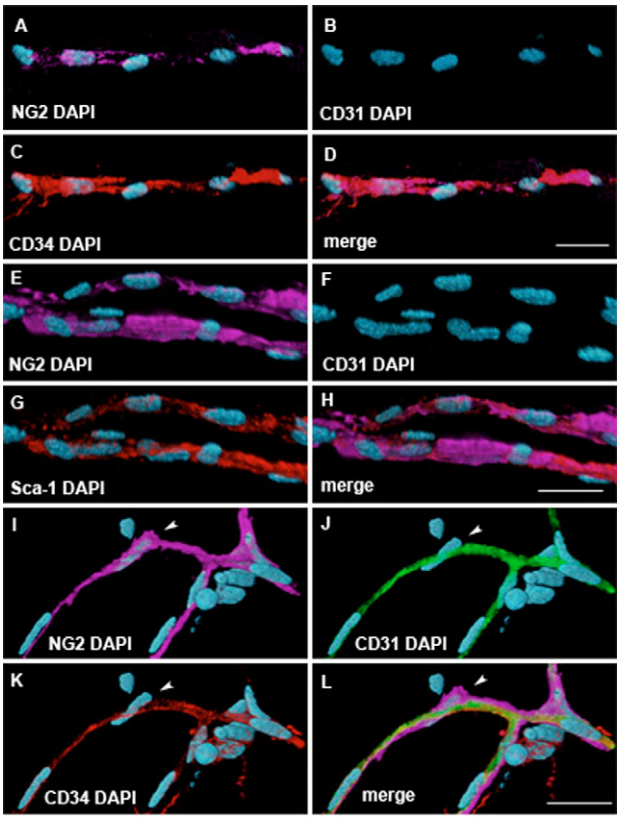

Fig. 3. CD34 and Sca-1-positive cells in Matrigel vessels. Sections from 5 day (**A-H**) and 9 day (**I-L**) Matrigel plugs were stained for DAPI (blue), NG2 (magenta), CD34 (red), Sca-1 (red) and CD31 (green). Prior to the appearance of CD31$^+$ endothelial cells, many cells in the vessel-like networks co-express NG2 and the markers CD34 and Sca-1. In older plugs, CD34 is co-localized with CD31 on endothelial cells. A CD31$^-$ CD34$^-$ pericyte is marked with an arrowhead. All panels are *z*-stacks of confocal images. Scale bars: 40 μm.

In contrast to early pericyte networks in which CD34 is co-expressed with NG2, immunocytochemical studies of mature vessels (Fig. 3I-L) establish that the CD34 marker is found on CD31$^+$ endothelial cells rather than on NG2$^+$ pericytes (arrowheads). The co-expression of CD34 and CD31 supports the concept that mature endothelial cells arise from CD34$^+$ hematopoietic progenitors. An alternative interpretation of the pericyte-to-endothelial shift in CD34 expression might be that endothelial cells are derived from pericyte progenitors.

Because perivascular cells with macrophage-like phenotypes have been found in close association with developing vasculature (Anghelina et al., 2006; De Palma et al., 2003; De Palma et al., 2005; Grunewald et al., 2006), we also examined Matrigel vessels for expression of the macrophage marker F4/80. Fig. 5A-D shows that the relationship between F4/80 and NG2 labeling is complex in early vascular structures, with some cells co-expressing both markers (arrow), and others expressing just NG2 (arrowhead) or F4/80 (asterisk). Use of the Volocity 4D software and *z*-stacks of confocal images to analyze 1200 individual cells in 79 early vessel-like structures reveals that 23% of cells express both NG2 and F4/80, whereas 20% express only NG2 and 30% express F4/80 alone (Table 2A). The remaining 27% of cells express neither marker. Analysis of 1050 individual cells in 73 mature vessels reveals that macrophage expression of NG2 is downregulated as vessels develop, with fewer than 10% of F4/80$^+$ cells expressing low levels of NG2. Thus, NG2$^-$F4/80$^+$ cells are the predominant macrophage species associated with mature vessels. Furthermore, the detailed spatial relationship between macrophages, pericytes and endothelial cells reflects the differences in the respective functions of these cell types. CD31$^+$ endothelial cells form the innermost layer of the structure (Fig. 5E-H), invested closely by NG2$^+$ F4/80$^-$ pericytes (arrowhead). NG2$^-$ F4/80$^+$ macrophages (arrows) are less intimately associated with the endothelium. We were unable to find any examples of NG2$^-$ F4/80$^+$ macrophages in direct contact with CD31$^+$ endothelial cells, whereas at least 95% of NG2$^+$F4/80$^-$ pericytes exhibit this type of intimate relationship with the endothelium.

Bone marrow origin of vascular cells

The finding that many NG2$^+$ cells are F4/80$^+$ macrophages is consistent with a bone marrow origin for this NG2$^+$ population. The presence of CD34 and Sca-1 on NG2$^+$ pericyte progenitors suggests that these cells might also originate from the bone marrow, as indicated by several recent reports (De Palma et al., 2003; Rajantie et al., 2004; Ozerdem et al., 2005; Song et al., 2005; Lamagna and Bergers, 2006). Interestingly, flow cytometric analysis of adult mouse bone marrow reveals that NG2$^+$CD34$^+$ cells represent roughly 2% of the total bone marrow population (Fig. 4B). Similar data were obtained for NG2/Sca-1 sorting (not shown). These findings suggest the possibility that NG2$^+$CD34$^+$Sca-1$^+$ bone marrow cells are the source of the initial NG2$^+$CD34$^+$Sca-1$^+$ pericyte-like population found in Matrigel vessels. To directly address the bone marrow origin of cells in developing Matrigel vasculature, we applied a bone marrow transplantation approach. Wild-type mice were gamma-irradiated and then reconstituted using

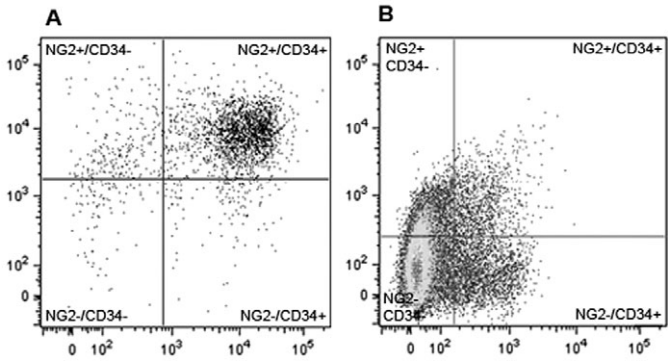

Fig. 4. Flow cytometric analysis of NG2-positive, CD34-positive populations. (**A**,**B**) Flow cytometry was used to examine NG2 and CD34 expression by (A) cells recovered from dissociated 5-day Matrigel plugs and (B) cells in total adult bone marrow. In A, 8.2% of the Matrigel population expressed NG2 alone, 6.6% expressed CD34 alone and 74.2% expressed both markers. In B, 5.3% of the total bone marrow population expressed NG2 alone, 8.1% expressed CD34 alone and 1.7% expressed both markers. In control experiments with second antibodies alone, fewer than 0.5% of cells were scored as positive.

bone marrow cells from EGFP$^+$ donors. After a 6-week recovery period, Matrigel plugs were established in animals with white blood cell populations that were at least 75% EGFP$^+$. In many experiments the engraftment level approached 90%. Although not routinely feasible owing to the terminal nature of the procedure, we found in three trials that EGFP engraftment levels in the bone marrow were also in the 80-90% range.

As anticipated from their marker phenotypes, large numbers of cells in early vessel-like structures are EGFP$^+$, indicative of their bone marrow origin. Fig. 6A-D illustrates EGFP expression by NG2$^+$CD34$^+$ cells in 5-day vessel-like networks, while Fig. 6E-H demonstrates the presence of EGFP in F4/80$^+$ cells. Table 2B shows that 77% of NG2$^-$F4/80$^+$ macrophages express EGFP. With regard to the NG2$^+$ populations, 65% of NG2$^+$ F4/80$^+$ macrophages express EGFP, while 46% of NG2$^+$ F4/80$^-$ pericytes are EGFP$^+$. The 77% level of EGFP expression in NG2$^-$F4/80$^+$ macrophages lies within the 75-90% engraftment range of our transplantation experiments, consistent with a bone marrow origin for essentially all cells in this population. Moreover, both groups of NG2$^+$ cells (macrophages and pericytes) are at least partly derived from the bone marrow. However, the fact that EGFP expression in NG2$^+$F4/80$^-$ cells falls well short of the 75-90% engraftment level indicates that a significant portion of the pericyte population may arise from non-bone marrow sources.

Table 1. Flow cytometric quantitation of cell types in early Matrigel plugs

	Abundance	
Cell phenotype	Experiment 1	Experiment 2
NG2$^+$ CD34$^+$	74.2%	66.7%
NG2$^+$ CD31$^-$	51.9%	44.6%
NG2$^-$ CD31$^+$	4.7%	3.2%
NG2$^+$ CD34$^-$	8.2%	5.3%
NG2$^-$ CD34$^+$	6.6%	21.4%

Five-day FGF2-containing Matrigel plugs were used for flow cytometric analysis of vascular cells expressing various combinations of markers. Values represent the abundance of the respective cell types as a percentage of the total cell population. The results of two independent trials are shown.

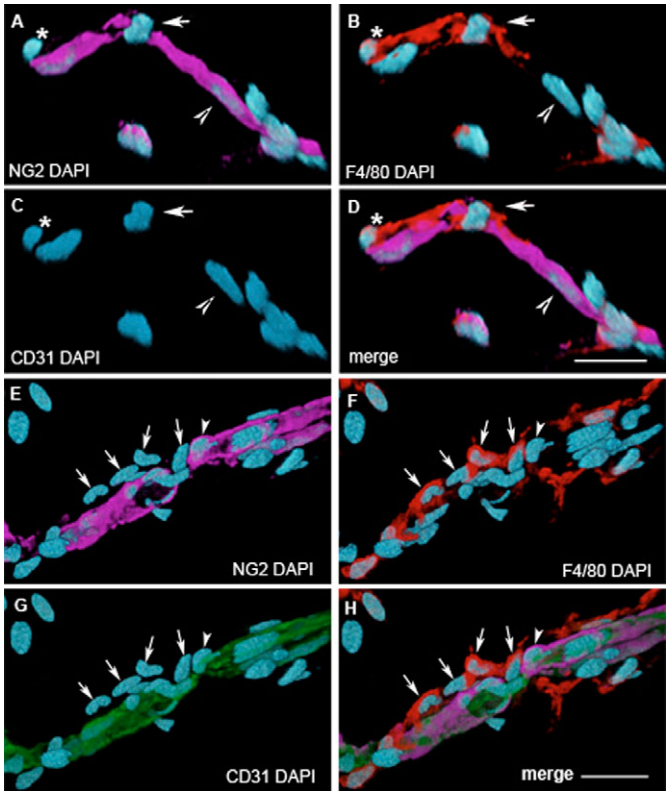

Fig. 5. Contribution of macrophages to Matrigel vascularization. Sections of 5 day (**A-D**) and 9 day (**E-H**) Matrigel plugs were stained for DAPI (blue), NG2 (magenta), CD31 (green) and F4/80 (red) to investigate the presence of macrophages in developing vessels. At day 5, NG2$^+$F4/80$^-$ pericytes are present (arrowhead). Some F4/80$^+$ macrophages also express NG2 (arrow), while others are NG2$^-$ (asterisk). At day 9, NG2 and F4/80 are expressed on non-overlapping populations of pericytes and macrophages, respectively. At this stage, pericytes (arrowhead) exhibit a more intimate physical relationship with endothelial cells than macrophages do (arrows). All panels represent *z*-stacks of confocal images. Sale bars: 40 μm.

We also examined the distribution of the EGFP tag in mature vessels in older Matrigel plugs. Fig. 6I-L shows examples of EGFP expression in both NG2$^+$ and CD31$^+$ cells in mature 9-day vessels. Volocity 4D-assisted analysis of 1200 individual cells in 76 mature vessels (four FGF2-containing Matrigel plugs) reveals EGFP expression in 40% of pericytes (arrowheads) but in only 10% of endothelial cells (arrow). Thus, the bone marrow derived pericytes in early vascular networks persist at about the same level in more mature vessels (40% vs 46%), indicative of a developmental relationship between the immature and mature structures. However, the relatively low percentage of EGFP$^+$ endothelial cells in mature vessels shows that these cells are primarily derived from non-bone marrow sources, and further indicates that bone marrow-derived pericyte progenitors are unlikely to be a major source of vascular endothelial cells.

Although the transplantation experiments confirm the bone marrow origin of a substantial number of NG2$^+$ pericytes, they do not allow us to determine whether these cells arise from hematopoietic or stromal progenitors. To further address this issue, we characterized NG2$^+$ and F4/80$^+$ cells in early Matrigel networks for expression of CD45, a general marker for cells of the

Table 2. Quantitation of NG2$^+$ and F4/80$^+$ cells in early Matrigel plugs

A

	Expression of NG2 and F4/80
Cell phenotype	Abundance
NG2$^+$F4/80$^+$	23%
NG2$^+$F4/80$^-$	20%
NG2$^-$F4/80$^+$	30%
NG2$^-$F4/80$^-$	27%

Five-day FGF2-containing Matrigel plugs were used to examine NG2 and F4/80 expression in 79 immature vessel-like networks. A total of 1200 individual cells were examined by confocal microscopy to obtain the abundance data.

B

	Expression of EGFP in NG2$^+$ and F4/80$^+$ cells
Cell phenotype	Abundance of EGFP$^+$ cells
NG2$^-$F4/80$^+$	77%
NG2$^+$F4/80$^+$	65%
NG2$^+$F4/80$^-$	46%

Five-day FGF2-containing Matrigel plugs in EGFP$^+$ bone marrow-transplanted mice were used to examine EGFP, NG2 and F4/80 expression in 75 immature vessel-like networks. A total of 1100 individual cells were examined by confocal microscopy to obtain the abundance data.

hematopoietic lineage (Dahlke et al., 2004; Sakhinia et al., 2006). Fig. 7A-D shows that many cells co-express all three markers (NG2, CD45 and F4/80), while some cells express just NG2 and CD45 (arrow), and still others express only NG2 (arrowheads). In this scheme, the NG2$^+$F4/80$^+$CD45$^+$ cells are likely to be macrophages, whereas NG2$^+$CD45$^+$F4/80$^-$ and NG2$^+$CD45$^-$F4/80$^-$ cells may represent pericytes of hematopoietic and non-hematopoietic origin, respectively. In mature Matrigel vessels, CD45 expression is retained by F4/80$^+$ macrophages, but is lost by NG2$^+$ pericytes (not shown). This phenomenon resembles the loss of the CD34 marker by maturing pericytes.

Development of mature vessels from cellular networks present in early Matrigel plugs

To further test the hypothesis that mature vessels in late Matrigel plugs develop from early immature vessel-like networks, we devised a plug transplantation scheme. FGF2-containing Matrigel was injected into a C57Bl/6 mouse (mouse 1). After 5 days, a time at which the Matrigel contains only early vessel-like networks, the plugs were removed from mouse 1 and transplanted under the skin of a C57Bl/6 EGFP$^+$ mouse (mouse 2). Transplanted plugs were left in mouse 2 for 14 days to ensure complete vascularization, and then studied by confocal microscopy. If early vessel-like networks seen in 5-day old Matrigel plugs develop directly into the functional vessels seen in older Matrigel plugs, a large number of cells in mature vessels of transplanted plugs should be wild type (the phenotype of mouse 1) and not EGFP$^+$ (the phenotype of mouse 2).

Indeed, analysis of 120 CD31$^+$ mature vessels in four transplanted plugs reveals that 70% of these structures are almost completely devoid of EGFP$^+$ cells, confirming the origin of these vessels from cellular networks that existed at the time of transplantation. Single cell analysis of the 30% of vessels containing significant numbers of EGFP$^+$ cells reveals two striking features (Fig. 7E-H). First, more than 95% of all pericytes (arrowheads) in these structures are EGFP$^-$, therefore originating from mouse 1. This is consistent with our conclusion that a new population of pericytes is not required for maturation of immature networks to functional vessels. Second, only

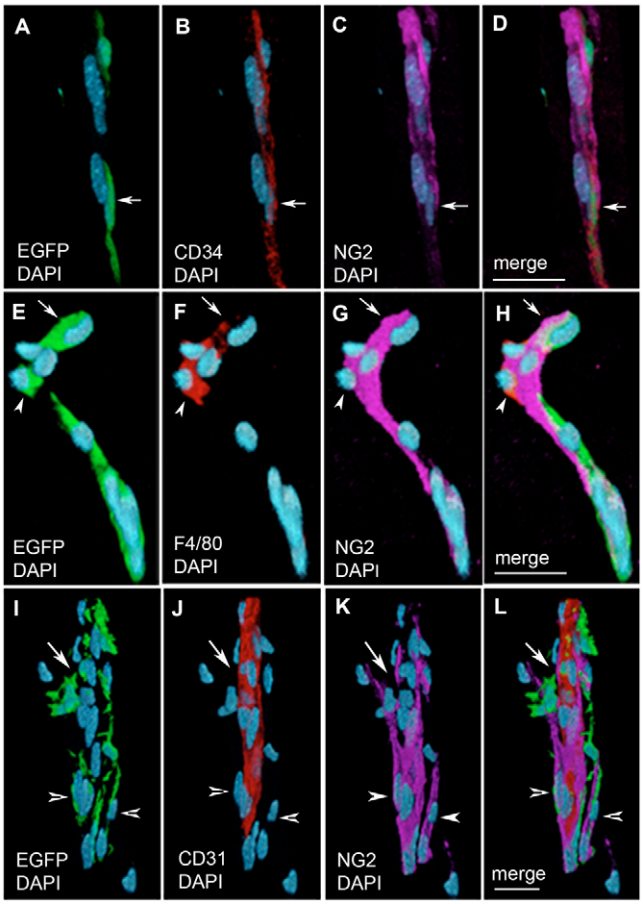

Fig. 6. Bone marrow origin of vascular cells. Matrigel plugs were established in irradiated mice that had been reconstituted using bone marrow cells from an EGFP$^+$ donor. At day 5 (**A-D**) sections were analyzed by staining for NG2 (magenta), CD34 (red) and DAPI (blue). The EGFP marker (green) is seen in cells that co-express NG2 and CD34 (arrows). Also at day 5 (**E-H**), NG2 (magenta), F4/80 (red), EGFP (green) and DAPI (blue) labeling were compared. The EGFP label is apparent in both populations of macrophages: F4/80$^+$NG2$^+$ (arrows) and F4/80$^+$NG2$^-$ (arrowheads). At day 9 (**I-L**), sections were analyzed for NG2 (magenta), CD31 (red), EGFP (green) and DAPI (blue). The EGFP marker is frequently found in NG2$^+$ cells (arrowheads), and more rarely in CD31$^+$ cells (arrows). All panels represent *z*-stacks of confocal images. Scale bars: 40 μm.

about 50% of the CD31$^+$ endothelial cells (arrows) in these vessels are EGFP$^+$ and thus are derived from mouse 2. The EGFP$^-$ population must have been present in the Matrigel plug at the time of its removal from mouse 1.

DISCUSSION

A striking observation in our work with pathological vascularization models was the early presence of pericytes in vascularizing tissues and the apparent ability of these cells to form pericyte networks in the absence of endothelial cells (Ozerdem et al., 2002; Ozerdem and Stallcup, 2003). Our current follow-up work uses FGF2-supplemented Matrigel plugs to document the absence of CD31$^+$ endothelial cells during early stages of vessel formation. Our results, facilitated by the use of Volocity 4D software to analyze *z*-stacks of confocal images, allow unambiguous identification of the cells that initiate neovascularization in this model. Developing vessel-like

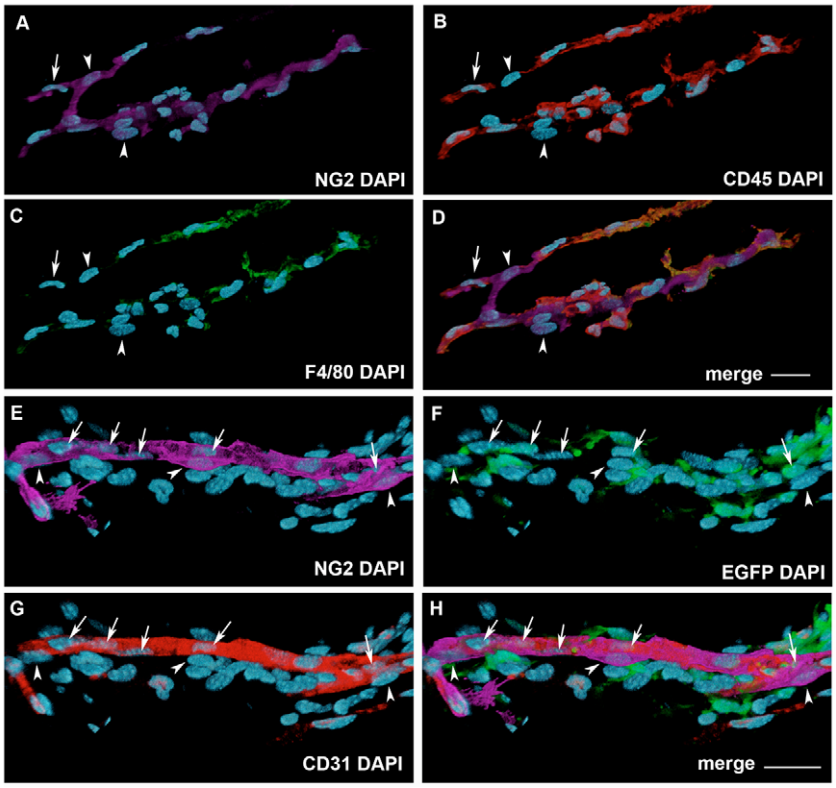

Fig. 7. Additional details concerning pericyte origin. (**A-D**) Expression of CD45 by pericytes and macrophages. Sections of 5 day Matrigel plugs were stained for DAPI (blue), CD45 (red), F4/80 (green) and NG2 (magenta). Although many NG2$^+$ cells express both CD45 and F4/80, others express only CD45 (arrow) and still others lack both CD45 and F4/80 (arrowheads). All panels represent *z*-stacks of confocal images. Scale bar: 40 μm. (**E-H**) Mature Matrigel vessels are derived from immature cellular networks. Five-day Matrigel plugs were transplanted into EGFP$^+$ recipients and allowed to continue their vascularization for 14 additional days. Sections were analyzed by staining for NG2 (magenta), CD31 (red) and DAPI (blue). The majority of vessels in the transplanted plugs were negative for EGFP (green), demonstrating that the cellular components of the vessels were already present prior to transplantation. In other vessels such as the one shown here, the EGFP label is absent from NG2$^+$ pericytes (arrowheads) and present in some but not all CD31$^+$ endothelial cells (arrows). Thus, only a subpopulation of the endothelial cells are recruited subsequent to transplantation. All panels represent *z*-stacks of confocal images. Scale bar: 40 μm.

networks are initially assembled by cells whose marker profiles identify them as pericytes (NG2$^+$PDGFβR$^+$F4/80$^-$) and macrophages (NG2$^+$F4/80$^+$ or NG2$^-$F4/80$^+$). The precocious role of pericytes in Matrigel vascularization echoes results from other systems in which the use of nascent pericyte markers has revealed early pericyte participation in the neovascularization process (Schlingemann et al., 1990; Nehls et al., 1992; Wesseling et al., 1995; Redmer et al., 2001; Ozerdem and Stallcup, 2003; Virgintino et al., 2007).

Previous reports of macrophage contribution to Matrigel vascularization (Schmeisser et al., 2001; Anghelina et al., 2004; Anghelina et al., 2006) are confirmed in our studies by the early participation of cells positive for F4/80, one of the most specific markers for this class of cells (Hume et al., 1984; Inoue et al., 2005; Anghelina et al., 2006). In tumor progression, wound healing, arthritis, atherosclerosis and choroidal angiogenesis models, the functional importance of macrophages has been established by the ability of macrophage depletion/suppression strategies to block neovascularization (Lyden et al., 2001; Luttun et al., 2002; Sakurai et al., 2003; Grunewald et al., 2006). Our Matrigel studies identify two populations of macrophage-like cells (NG2$^-$F4/80$^+$ and NG2$^+$F4/80$^+$) in early vessel-like networks. The decline of the NG2$^+$F4/80$^+$ population with vessel maturation suggests that this could be a transitional monocyte population that gives rise to NG2$^-$F4/80$^+$ macrophages. Transient NG2 expression by activated macrophages has been reported previously (Bu et al., 2001; Jones et al., 2002; de Castro et al., 2005). Alternatively, the possible derivation of macrophages from pericytes is supported by reports that microvascular pericytes in the central nervous system can express macrophage markers and perform macrophage-like functions (Balabanov et al., 1996; Rucker et al., 2000). Significantly, the NG2$^-$F4/80$^+$ macrophage population is clearly distinct from the NG2$^+$F4/80$^-$ pericyte population not only by its marker profile, but also by its spatial relationship to endothelial cells. Whereas pericytes are intimately apposed to luminal endothelial cells in mature vessels, macrophages are more loosely associated with vessels, lacking direct contact with the vascular endothelium.

Anghelina et al. (Anghelina et al., 2006) used perfusion with India ink to demonstrate the functionality of vessels in long-term Matrigel plugs. Significantly, FITC-dextran perfusion shows that early pericyte/macrophage networks are not functionally connected to the circulation. Functional perfusion is not achieved until primitive networks develop a structure in which abluminal NG2$^+$ mural cells acquire a lining of CD31$^+$ endothelial cells, reflecting the cooperation that is required between endothelial and perivascular cells throughout the lifetime of microvessels.

A second key feature of our work is the demonstration of a bone marrow origin for substantial proportions of both pericytes and macrophages that comprise the early vessel-like networks. This is hardly surprising in the case of macrophages, which are well-known as bone marrow-derived myeloid cells. However, only recently has the bone marrow origin of pericytes been noted (De Palma et al., 2003; Ozerdem et al., 2005; Song et al., 2005; Lamagna and Bergers, 2006). Taken together, our results with NG2, CD34, Sca-1, and F4/80 labeling indicate that NG2$^+$CD34$^+$Sca-1$^+$ pericytes and NG2$^+$F4/80$^+$ macrophages in early Matrigel networks are derived from distinct progenitor populations. However, an additional approach was needed to determine the origin of pericyte progenitors, as CD34 and Sca-1 are expressed not only by cells of hematopoietic origin, but also by other progenitor populations residing in adipose tissue, hair follicles, mammary gland, and smooth, skeletal and cardiac muscle (Lawson et al., 2007; Inoue et al., 2007; Wang et al., 2006; Xiao et al., 2007; Ning et al., 2006). We therefore used bone marrow transplantation from EGFP$^+$ donors to directly demonstrate the bone marrow derivation of both NG2$^+$F4/80$^-$ pericytes and F4/80$^+$ macrophages. Virtually all macrophages (both NG2$^+$F4/80$^+$ and NG2$^-$F4/80$^+$) and at least half of the pericytes in early Matrigel vessels arise from EGFP$^+$ bone marrow progenitors.

The latter observation suggests that Matrigel vessels may contain pericytes of both bone marrow and non-bone marrow origin. Specifically, in transplantation experiments where the EGFP engraftment level was greater than 75%, only 46% of $NG2^+F4/80^-$ pericytes were $EGFP^+$. A caveat here is that, even though engraftment levels in the bone marrow were also in the 80-90% range, we cannot conclusively rule out the possible origin of $NG2^+EGFP^-$ pericytes from a small, radio-resistant population of $EGFP^-$ host bone marrow progenitors that persist following reconstitution. Nevertheless, EGFP-independent data in support of non-bone marrow-derived pericytes comes from our flow cytometry experiments. Although 70% of cells in early Matrigel plugs are $NG2^+CD34^+$, consistent with bone marrow derivation, 7% of the plug population is $NG2^+CD34^-$. This latter population may therefore arise from non-bone marrow sources in the local environment. The derivation of pericytes from non-bone marrow sources is consistent with reports that pericytes and smooth muscle cell progenitors can be recruited from pre-existing vasculature and from local tissues (Hirschi and Majesky, 2004; Majka et al., 2003; McKinney-Freeman et al., 2003).

The fact that some $NG2^+$ pericytes express the general hematopoietic marker CD45 whereas others do not further suggests that pericytes may arise from both hematopoietic and non-hematopoietic sources. Although one possible non-hematopoietic source is the bone marrow stroma, our observation of both $EGFP^+$ and $EGFP^-$ pericytes in the transplantation experiments leaves open the possibility of non-hematopoietic pericyte origins outside the bone marrow. Additional experiments are planned to elucidate more clearly this diversity in the origin of pericytes.

In understanding the maturation of immature vascular networks to functional vessels, a crucial observation from the bone marrow transplantation work is that 40% of pericytes in mature Matrigel vessels continue to express the EGFP tag. This value is not significantly different from the 46% of $EGFP^+$ pericytes found in immature cellular networks, suggesting that bone marrow-derived pericytes persist through the process of vessel maturation. This indicates that mature vessels develop directly from the immature vascular networks seen in early plugs, rather than being assembled via a separate process. These ideas are confirmed by experiments in which Matrigel plugs containing immature vessels were transplanted into $EGFP^+$ recipients for further development. At least 95% of the pericytes in the mature vessels of transplanted plugs are negative for EGFP, showing that these cells were present in the plug prior to its transplantation, that a new supply of perivascular cells is not needed for development of mature vessels, and that mature vessels are the developmental descendents of immature vascular networks assembled at early time points.

In contrast to the substantial number of bone marrow-derived pericytes, only 10% of $CD31^+$ endothelial cells in mature Matrigel vessels are found to be $EGFP^+$ in bone marrow transplantation experiments. That bone marrow is not the major source of vascular endothelial cells in Matrigel vessels is highly reminiscent of results obtained in four recent studies of tumor vascularization (Gothert et al., 2004; De Palma et al., 2003; Rajantie et al., 2004; Ozerdem et al., 2005), none of which found evidence for significant numbers of bone marrow-derived endothelial cells. In future experiments it will be important to determine the extent to which endothelial cell replacement/renewal by cells from the local environment contributes to the low number of bone marrow-derived endothelial cells seen in these experiments. Although it is clear in our own experiments with Matrigel plugs transplanted into $EGFP^+$ recipients that replacement of pericytes occurs infrequently (fewer than 5% become $EGFP^+$), a significant number of $CD31^+EGFP^+$ endothelial cells are recruited to developing vessels. However, as $CD31^+$ cells were not present in the plugs at the time of transplantation, it is not clear whether this endothelial cell recruitment represents a replacement event or the initial establishment of the vascular endothelium.

The low numbers of bone marrow-derived endothelial cells in mature Matrigel vessels raises other issues about the origins of these cells. The experiments with early Matrigel plugs transplanted into $EGFP^+$ recipients indicate that many $CD31^+$ endothelial cells in mature vessels are $EGFP^-$, and thus were present in the plug at the time of its transplantation. As we see very few cells that express CD31 in early plugs, these $CD31^+EGFP^-$ cells must arise from $CD31^-$ progenitors. It is therefore of interest (1) that $CD31^+$ endothelial cells in mature vessels express the progenitor marker CD34, and (2) that flow cytometry identifies up to 20% of the cells in early plugs as having an $NG2^-CD34^+$ phenotype. It seems possible that this $NG2^-CD34^+$ population comprises endothelial progenitors derived from the circulation or local environment, consistent with the finding that endothelial cells continue to be recruited from the local environment during vessel maturation. Preadipocytes represent one cell type reported to have the capability of transdifferentiation to endothelial cells (Planat-Benard et al., 2004). Although macrophages are also reported to be capable of generating endothelial cells (Schmeisser et al., 2001; Rehman et al., 2003; Bailey et al., 2006), the low percentage of $EGFP^+$ endothelial cells in our bone marrow transplantation experiments suggests that this mechanism does not play a major role in Matrigel vascularization. The low number of $EGFP^+$ endothelial cells also does not support the hypothesis that $CD34^+$ endothelial cells might be derived from the much larger number of $EGFP^+NG2^+CD34^+$ pericyte progenitors. Our failure to identify cells with a transitional $NG2^+CD31^+CD34^+$ phenotype further discounts this hypothesis.

Our studies indicate that postnatal neovascularization, even in response to a single initiating factor (FGF2), can be a mosaic process in which perivascular cells and endothelial cells are recruited by several different mechanisms and from more than one source. These phenomena are also observed in other systems, including corneal vascularization, which resembles the Matrigel model in its use of a single initial stimulus. Using FGF2 in the corneal model, we identified mosaic vessels in which some vessel segments contained both pericytes and endothelial cells, whereas other segments were composed exclusively of pericytes (Ozerdem et al., 2002). More recent studies demonstrate the mosaic nature of vascular cell recruitment in this model (Ozerdem et al., 2005). Virtually all endothelial cells were recruited, not from bone marrow, but from pre-existing limbal vessels. By contrast, roughly half of pericytes were bone marrow derived, the other half arising from limbal capillaries. In these respects, the Matrigel and corneal models offer similar examples of vascular mosaicism.

The process by which vessels develop in FGF2-containing Matrigel plugs is more reminiscent of vasculogenesis than angiogenesis, as mature vessels appear to develop, not by sprouting from pre-existing vessels, but from a primitive vascular plexus established in the plug by progenitor cells. The additional interesting twists to this story are (1) that the vascular progenitors in this case are mural rather than endothelial in nature, and (2) that endothelial cells appear as later additions needed to complete the maturation of the vascular network. This sequence of events differs substantially from more traditional models of neovascularization in which endothelial tubes form and are thereafter stabilized by the recruitment of mural cells (Risau and Flamme, 1995; Risau, 1997).

The pattern of vascularization produced by FGF2 may be due to effects of the growth factor that extend beyond stimulation of endothelial cells (Anghelina et al., 2006). For example, FGF2 is implicated in macrophage recruitment (Tang et al., 2005; Numata et al., 2006) and in upregulating PDGFβR expression by pericytes (Cao et al., 2003; Kano et al., 2005), rendering them more sensitive to PDGF-BB-dependent recruitment and maturation. Significantly, PDGF-BB is one of many crucial factors produced by macrophages (Saraban and Kufe, 1988; Nagaoka et al., 1990). Such an FGF2-dependent collaboration between macrophages and pericytes may determine the sequence of events observed in our Matrigel vascularization studies.

Mosaicism may be especially common in cases of pathological neovascularization, during which tissues can be exposed to combinations of stimuli not experienced during the course of normal development. In future experiments, it will be of great interest to use the Matrigel model to examine the sequence of events that occur in response to other factors such as VEGF and PDGF. As these factors are known to be more directly responsible for endothelial cell and pericyte recruitment, respectively, they may produce vessels with different degrees of mosaicism than seen with FGF2. The Matrigel model has also been used effectively to study interactive/synergistic effects between factors (Kano et al., 2005), a situation that more accurately reflects the conditions present during most types of pathological vascularization. These types of studies will be crucial for deeper understanding of the mechanisms that regulate vascularization under pathological conditions.

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