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Sensory Nerves Determine the Pattern of Arterial Differentiation and Blood Vessel Branching in the Skin

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Summary

Nerves and blood vessels are branched structures, but whether their branching patterns are established independently or coordinately is not clear. Here we show that arteries, but not veins, are specifically aligned with peripheral nerves in embryonic mouse limb skin. Mutations that eliminate peripheral sensory nerves or Schwann cells prevent proper arteriogenesis, while those that disorganize the nerves maintain the alignment of arteries with misrouted axons. In vitro, sensory neurons or Schwann cells can induce arterial marker expression in isolated embryonic endothelial cells, and VEGF$^{164/120}$ is necessary and sufficient to mediate this induction. These data suggest that peripheral nerves provide a template that determines the organotypic pattern of blood vessel branching and arterial differentiation in the skin, via local secretion of VEGF.

Introduction

The blood vessels and capillaries of the circulatory system display an intricate pattern of branching that serves to maximize the vascular surface area in contact with surrounding tissue. This branching pattern is established during a remodeling process called angiogenesis that follows the initial assembly of primitive capillaries from individual endothelial cells by vasculogenesis (reviewed in Risau and Flamme, 1995). In recent years, a great deal has been learned about the molecular control of angiogenesis (reviewed in Folkman and D'Amore, 1996; Gale and Yancopoulos, 1999; Yancopoulos et al., 2000). What is not clear, however, is whether the pattern of peripheral blood vessel branching is self-organized by endothelial cells, or whether it is determined by structures extrinsic to the cardiovascular system.

Like blood vessels, peripheral nerves are highly branched structures. In adult peripheral tissues, nerves often run along larger blood vessels, reflecting their need for oxygen and nutrients, as well as their physiological control of vaso-constriction and -dilation (reviewed in Burnstock and Ralevic, 1994). This intimate neurovascular association and functional interaction raises the question of whether the branching patterns of these two systems are ontogenetically and/or mechanistically related (reviewed in Shima and Mailhos, 2000). On the one hand, the branching pattern of blood vessels could determine that of nerves, or vice versa. On the other hand, the branching of both systems could be independently controlled by common cues located in surrounding tissue.

Evidence that the patterning of nerve and blood vessel branching is interdependent is extremely limited. Early studies in embryonic chick limb skin using silver staining to reveal the pattern of nerves and India ink injections to reveal that of blood vessels indicated that major nerve fibers and large vessels are in reproducible alignment (Martin and Lewis, 1989). This alignment evidently does not reflect blood vessel-mediated axon guidance, because the initial pattern of nerve fiber arborization is topographically unrelated to the pattern of blood vessel branching at the time the nerves first invade the skin (Martin and Lewis, 1989).

The question of whether nerves are required to establish the branching pattern of blood vessels was addressed by using UV irradiation to locally ablate the peripheral nervous system (PNS) in chick embryos (Martin and Lewis, 1989). The trajectory of large vessels in such aneurogenic limbs was surprisingly unperturbed by this manipulation, suggesting that the spatial organization of nerve fibers and blood vessels is independently controlled by common signals in the overlying skin. It was not clear, however, whether the pattern of smaller-diameter vessel branching was affected by the absence of nerves, because this pattern is less well defined and therefore perturbations in it are more difficult to detect (Martin and Lewis, 1989).

Here we have investigated the influence of the nervous system on blood vessel development in the embryonic mouse limb skin, using genetic methods to eliminate or disorganize peripheral nerves. To assay the effects of such manipulations on the development of blood vessels, we have examined the expression of artery-specific markers, such as ephrinB2 (Adams et al., 1999; Gerety et al., 1999; Wang et al., 1998). We find that arteries are preferentially aligned with nerves and follow their branching pattern, while veins are not. In mutant embryos lacking sensory nerves, arteries fail to properly differentiate, while in those containing disorganized nerves the trajectory of blood vessel branching is altered to follow the nerve. In vitro functional assays and correlated in vivo data suggest that the expression of arterial markers in nerve-associated vessels may be controlled by local secretion of VEGF from sensory nerve fibers, Schwann cells, or both. These data suggest that local signals provided by organized tissue subcomponents, such as nerve fibers, may provide a template that deter-

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mines the organotypic patterning of arteriogenesis and angiogenic remodeling.

Results

Peripheral Nerves Associate Preferentially with Arterial Vessels in Limb Skin

In embryonic chick limb skin, some blood vessels are associated with nerves, while others are not (Martin and Lewis, 1989). We sought to determine whether this heterogeneity reflects random variation or, instead, the deterministic behavior of an identifiable subset of blood vessels. As a first step, we examined the relationship of arteries and veins to nerves in mouse limb skin, using lacZ reporter strains to detect expression of ephrinB2 (arteries) or EphB4 (veins) (Gerety et al., 1999; Shin et al., 2001; Wang et al., 1998), together with antibodies to neurofilament (2H3) and PECAM-1, a pan-endothelial marker. Whole-mount immunohistochemical analysis in embryonic limb skin at E15.5 revealed that peripheral nerves associated preferentially with arterial vessels (Figures 1A–1F and 1M, arrows and open arrowheads; see Supplemental Figure S1 at http://www.cell.com/cgi/content/full/109/6/693/DC1). By contrast, no specific association between peripheral nerves and veins was observed (Figures 1G–1L and 1N, open arrows and arrowheads). The association of arterial vessels with peripheral nerves did not appear to reflect a direct, intimate attachment, but rather a looser alignment (Figures 1C and 1M, arrows and open arrowheads).

Blood Vessel/Nerve Association Coincides with the Induction of Arterial Marker Expression

The observed alignment of arteries and peripheral nerves raised the question of whether arterial differentiation follows or precedes nerve association. To distinguish between these possibilities, we analyzed the time course of blood vessel/nerve association in relation to the appearance of the arterial markers ephrinB2, Neuropilin1 (NP1), and Connexin40 (CX40). NP1 is a coreceptor for VEGF[164] (Kawasaki et al., 1999; Soker et al., 1998) and is preferentially expressed by arteries in chick (Moyon et al., 2001) as well as in mouse embryos (Y.M., D.S., and D.J.A., unpublished data). CX40 is a gap junction protein (van Kempen and Jongsma, 1999) that is specifically expressed in the dorsal aorta as well as in smaller arterial vessels in mouse embryos (D.S., Y.M., and D.J.A., unpublished data).

A primary capillary plexus is established in the mouse limb by E11. Within 24 hr, peripheral nerves have invaded the skin (data not shown). At E13.5, no association between nerves and blood vessels was yet evident (Figure 2A), and no vascular expression of the three arterial markers was detectable (Figures 2D, 2J, and 2P). However, NP1 expression was observed in the nerves (Figure 2J, arrowheads), reflecting its role in axon guidance (reviewed in Kolodkin, 1998). By E14.5, vascular remodeling had occurred, and many vessels were associated with nerves (Figures 2B and 2H, arrows), which had downregulated NP1. At this stage, arterial markers were first detectable in some nerve-associated vessels (Figures 2E, 2K, and 2Q, arrows). Some smaller vessels associated with nerves did not yet express arterial markers (Figures 2B, 2E, 2H, 2K, 2N, and 2Q, open arrowheads). By E15.5, however, most nerve-associated large- and medium-sized vessels expressed ephrinB2 (Figures 2C and 2F, arrows), and many expressed NP1

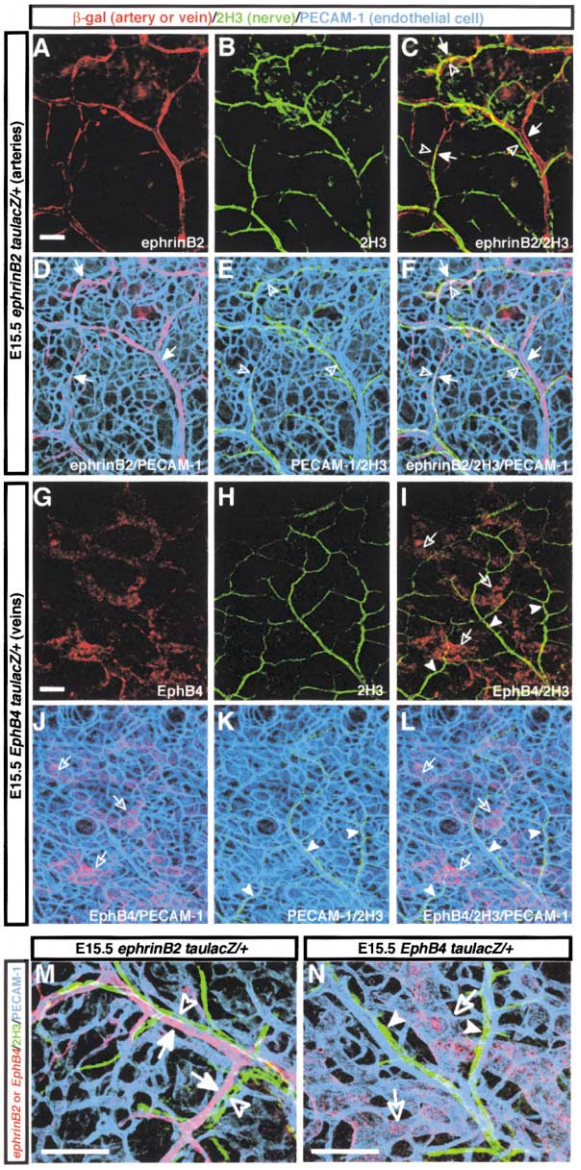

Figure 1. Arteries Are Specifically Aligned with Peripheral Nerves in Limb Skin

Forelimb skin of E15.5 *ephrinB2*$^{taulacZ/+}$ (A–F and M) or *EphB4*$^{taulacZ/+}$ (G–L and N) heterozygous embryos is shown. Whole-mount triple immunofluorescence confocal microscopy with antibodies to the pan-endothelial marker PECAM-1 (blue), neurofilament (2H3; green), and β-galactosidase (β-gal, red) reveals that 2H3^{+} peripheral nerves (C and F, open arrowheads) follow ephrinB2^{taulacZ}/PECAM-1-expressing arterial vessels (C and F, arrows) in *ephrinB2*$^{taulacZ/+}$ heterozygous embryos. In contrast, there is no association between 2H3^{+} peripheral nerves (I and L, arrowheads) and EphB4^{taulacZ}/PECAM-1-expressing venous vessels (I and L, open arrows) in *EphB4*^{taulacZ} heterozygous embryos.

(M and N) Higher-magnification view of limb skin from E15.5 *ephrinB2*$^{taulacZ/+}$ or *EphB4*$^{taulacZ/+}$ embryos. Note that arteries are aligned, but not tightly associated, with nerves. Scale bars are 100 μm.

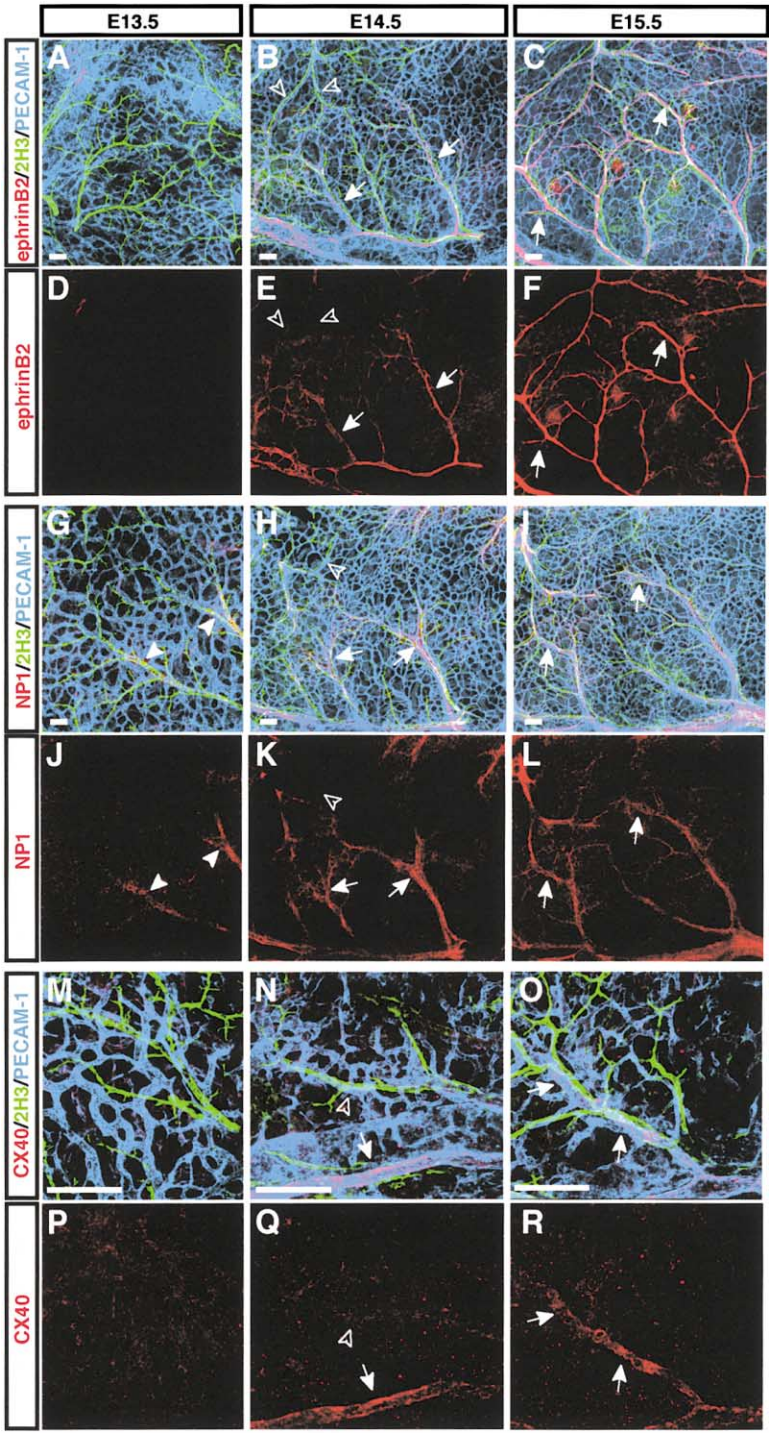

Figure 2. Nerve Association Precedes Arterial Differentiation

Whole-mount triple immunofluorescence confocal microscopy was performed with antibodies to the arterial markers ephrinB2 (indicated by β-gal staining) (A–F, red), NP1 (G–L, red), or CX40 (M–R, red) together with antibodies to PECAM-1 (A–C, G–I, and M–O, blue) and neurofilament (2H3) (A–C, G–I, and M–O, green) at the indicated developmental ages. Note that an association between $2H3^+$ peripheral nerves and arterial vessels was observed at E14.5 to E15.5 but not E13.5. NP1 expression at E13.5 is seen in peripheral nerves but not in blood vessels (G and J, arrowheads). Scale bars are 100 μm.

and CX40 (Figures 2I, 2L, 2O, and 2R, arrows). Taken together, these data suggest that arterial markers do not become expressed until after blood vessels associate with nerves in limb skin.

Blood Vessel/Nerve Association Precedes the Migration and/or Differentiation of Smooth Muscle Cells

Association of smooth muscle cells with endothelial cells is an important step in vascular remodeling (reviewed in Folkman and D'Amore, 1996). We were interested to know whether smooth muscle association with arteries occurs concomitantly with, or following, nerve association and arterial differentiation of endothelial cells. To address this question, we examined the location of smooth muscle cells in the limb skin from E13.5 through E15.5. At E13.5, αSMA^+ smooth muscle cells not yet associated with blood vessels (or had associated but still remained undifferentiated; Figures 3A and 3D). By E14.5, vascular αSMA^+ cells were detectable, but

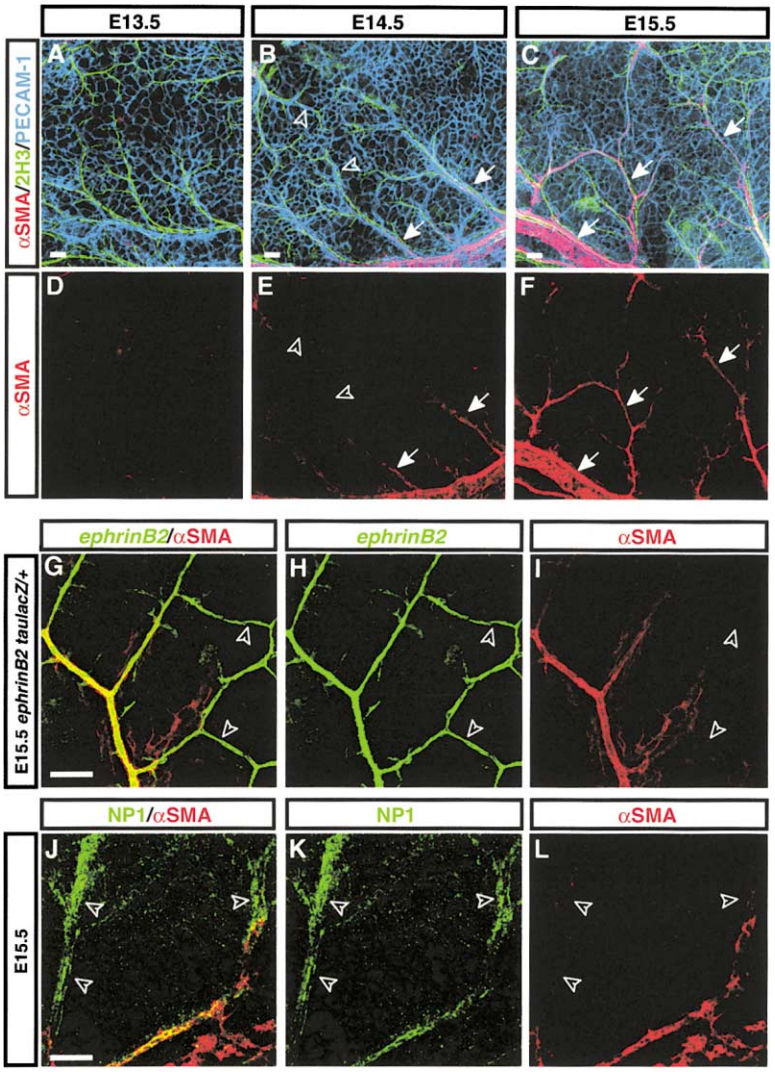

Figure 3. Blood Vessel/Nerve Association and Arterial Marker Expression Precedes Smooth Muscle Cell Differentiation

(A–F) Whole-mount triple immunofluorescence confocal microscopy was performed with antibodies to the smooth muscle cell marker, αSMA (A–F, red), in addition to PECAM-1 (A–C, blue) and 2H3 (A–C, green), at the indicated developmental ages. Note that by E15.5, αSMA^+ smooth muscle cell coverage occurred around $PECAM\text{-}1^+$ vessels adjacent to $2H3^+$ nerves (arrows). By E14.5, blood vessel/nerve association could be seen before smooth muscle cell coverage (B and E, open arrowheads).

(G–L) Arterial marker expression such as ephrinB2 (indicated by β-gal staining) (G and H, green) and NP1 (J and K, green) is detectable before αSMA^+ smooth muscle cell coverage (G, I, J, and L, red, open arrowheads). Scale bars are 100 μm.

only in larger nerve-associated vessels (Figures 3B and 3E, arrows). However, not all nerve-associated vessels had αSMA^+ cells at this time point (Figures 3B and 3E, open arrowheads). By E15.5, αSMA^+ cell coverage had extended further into the capillary network, on smaller branches as well (Figures 3C and 3F, arrows). However, the expression of ephrinB2 and NP1 at this stage appeared to extend further into the smaller vessel branches than did that of αSMA (Figures 3G–3I and 3J–3L, open arrowheads). These results indicate that smooth muscle cells associate with vessels (or differentiate) only after such vessels have become aligned with nerves and begun to express arterial markers. Taken together, these observations suggest that arteriogenesis—the molecular differentiation of arterial endothelial cells and associated smooth muscle cells—is initiated in smaller vessels only after they associate with nerves.

Defective Arteriogenesis in the Absence of Peripheral Nerves

To determine whether peripheral nerves are required for arteriogenesis, we took advantage of mutations that cause severe defects in peripheral nerve development. *Neurogenin1/Neurogenin2 (Ngn1/Ngn2)* double homozygous mutants lack peripheral sensory nerves (Ma et al., 1999), whereas *Phox2b* homozygous mutants lack peripheral autonomic nerves (Pattyn et al., 1999). A comparison of the expression of the sensory markers substance P, IB4 lectin, and trkA with the sympathetic markers tyrosine hydroxylase and dopamine β hydroxylase revealed that only sensory nerves were present in the limb skin between E13.5 and E15.5 (data not shown). Consistent with these data, *Phox2b* homozygous mutants showed no peripheral nerve defects in the limb skin at these stages (data not shown). In contrast, $Ngn1^{-/-}$; $Ngn2^{-/-}$ embryos showed a virtually complete absence of peripheral nerves and associated Schwann cells in the limb skin, as determined by staining for neurofilament and the glial marker Brain Fatty Acid Binding Protein (BFABP), respectively (Figures 4A and 4B, open arrowheads; Kurtz et al., 1994).

In the absence of peripheral sensory nerves, some vascular remodeling of large and small vessels occurred (Figure 4C versus 4D, arrows). However, the progressive branching pattern was disrupted so that large-diameter vessels branched directly into small-diameter vessels with fewer intermediate-size branches. In addition to this altered morphology, the expression of arterial markers

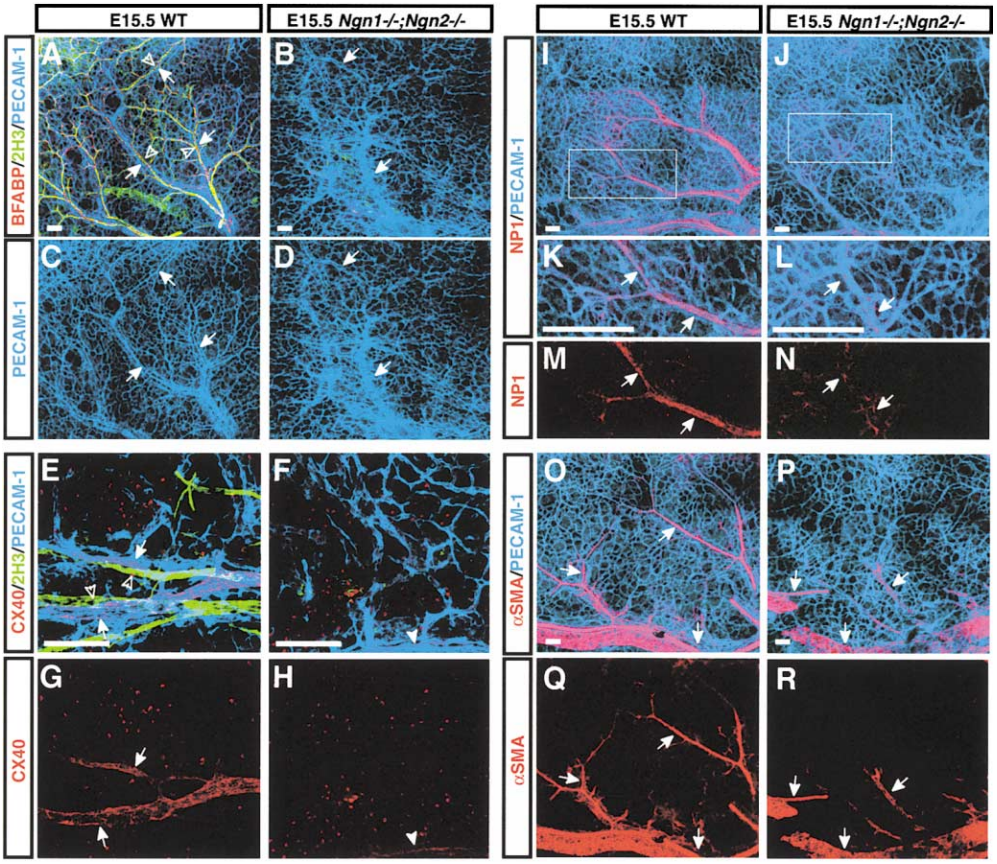

Figure 4. Defective Arterial Differentiation in the Absence of Peripheral Nerves

(A–D) Whole-mount triple immunofluorescence confocal microscopy was performed with antibodies to the Schwann cell marker, BFABP (A and B, red), as well as PECAM-1 (A–D, blue) and 2H3 (A and B, green), in *Ngn1*$^{-/-}$*; Ngn2*$^{-/-}$ double homozygous mutants (B and D) or wild-type littermates (A and C) at E15.5. Note that *Ngn1*$^{-/-}$*; Ngn2*$^{-/-}$ mutants have no peripheral nerves and associated BFABP^{+} Schwann cells (A versus B, open arrowheads). In addition, defective patterning of vascular remodeling is seen in *Ngn1*$^{-/-}$*; Ngn2*$^{-/-}$ mutants (C versus D, arrows).

(E–H) Triple labeling for CX40 (E–H, red), PECAM-1 (E and F, blue), and 2H3 (E and F, green) in *Ngn1*$^{-/-}$*; Ngn2*$^{-/-}$ mutants (F and H) or wild-type littermates (E and G) at E15.5.

(I–N) Double labeling with NP1 (I–N, red) and PECAM-1 (I–L, blue) in *Ngn1*$^{-/-}$*; Ngn2*$^{-/-}$ mutants (J, L, and N) or wild-type littermates (I, K, and M) at E15.5. Close-up (K–N) shows the boxed region in (I) and (J). The expression of arterial markers such as CX40 and NP1 was downregulated in *Ngn1*$^{-/-}$*; Ngn2*$^{-/-}$ mutants (E versus F, G versus H, K versus L, and M versus N, red, arrows).

(O–R) Double labeling for αSMA (O–R, red) and PECAM-1 (O and P, blue) in *Ngn1*$^{-/-}$*; Ngn2*$^{-/-}$ mutant (P and R) or wild-type littermates (O and Q) at E15.5. αSMA^{+} cells are associated with larger vessels but are reduced in smaller branched vessels in *Ngn1*$^{-/-}$*; Ngn2*$^{-/-}$ mutants (O versus P, Q versus R, arrows). Scale bars are 100 μm.

such as CX40, NP1, and *ephrinB2* was greatly reduced in *Ngn1, 2* double mutant embryos (Figures 4E–4N, arrows; see Supplemental Figures S2A and S2B at http://www.cell.com/cgi/content/full/109/6/693/DC1). This reduction of arterial marker expression was most apparent in smaller-diameter vessels (5–15 μm), while larger-diameter vessels (30–100 μm) appeared unaffected (Supplemental Figure S2B, arrows). In addition, there appeared to be a loss of αSMA^{+} cells (or lack of αSMA expression) in smaller vessels in the mutant embryos (Figure 4O versus 4P, Figure 4Q versus 4R, arrows). Taken together, these data suggest that arteriogenesis, particularly of smaller-diameter vessels, is deficient in mouse limbs lacking peripheral nerves.

Nerves Direct the Patterning of Vascular Remodeling and Arteriogenesis

The preceding observations raised the question of whether peripheral nerves are simply required for proper arterial differentiation, or whether they actually direct the patterning of vascular remodeling and arteriogenesis. Because there is no clear anatomical frame of reference to compare the pattern of blood vessel branching in the complete absence of peripheral nerves, we sought a mutation that disrupted the organization of peripheral nerves without completely eliminating them.

Such a phenotype is caused by a mutation in the *Semaphorin3A* gene (*Sema3A*), which encodes an axon guidance molecule (reviewed in Kolodkin, 1998). In *Sema3A* mutants, the pattern of peripheral nerve growth is disorganized (Taniguchi et al., 1997; see Supplemental Figures S3A, S3B, S3D and S3E), and the axons appear more fasciculated and less finely branched (Figure 5A versus 5B, 2H3 staining [green]). Strikingly, despite this disorganization, smaller-diameter blood vessels were still aligned with the disorganized peripheral nerves in *Sema3A*$^{-/-}$ limb skin at E15.5 (Figures 5B and 5E, arrows and open arrowheads). In addition, these nerve-associ-

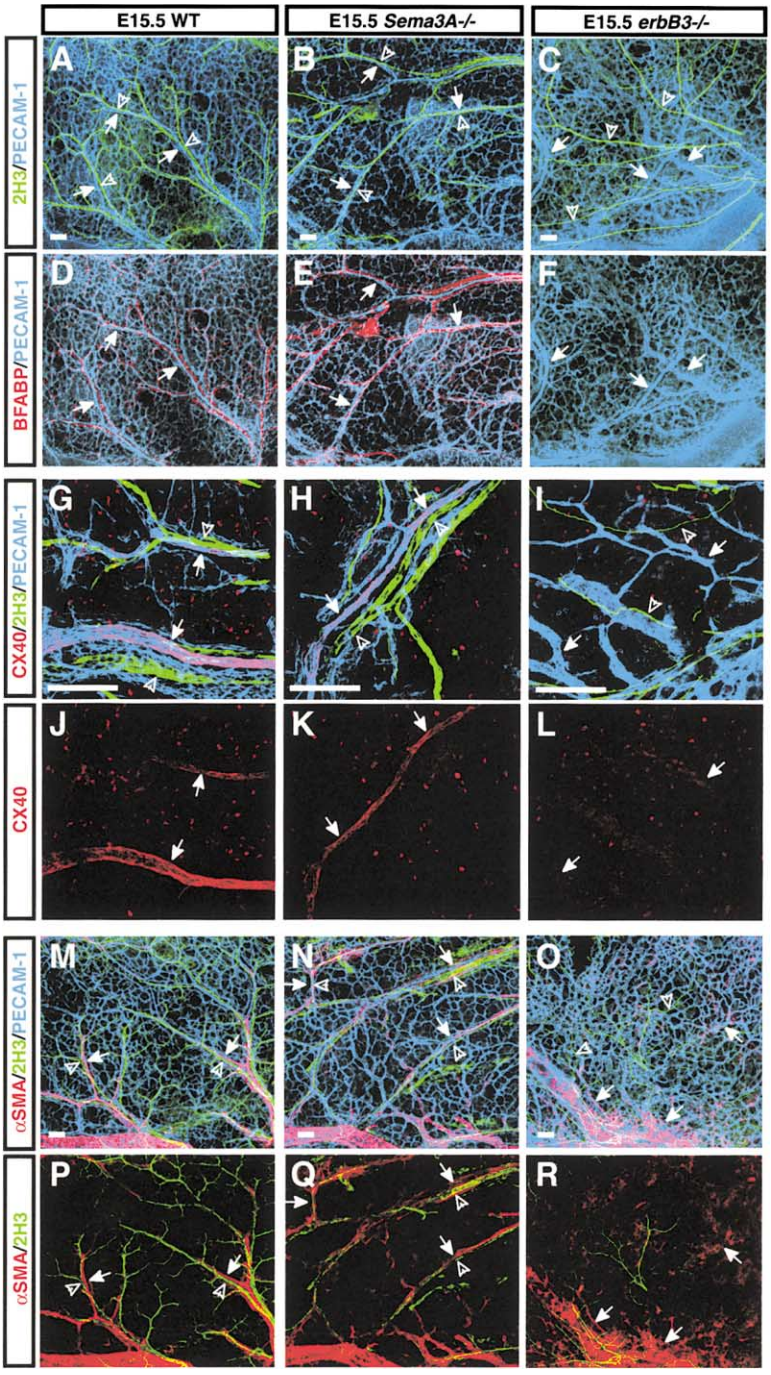

Figure 5. Peripheral Nerves Pattern Arteriogenesis and Blood Vessel Branching in an *erbB3*-Dependent Manner

(A–F) Whole-mount triple immunofluorescence confocal microscopy was performed with antibodies to BFABP (D–F, red) in addition to PECAM-1 (A–F, blue, arrows) and 2H3 (A–C, green, open arrowheads) in *Sema3A*$^{-/-}$ homozygous mutants (B and E), *erbB3*$^{-/-}$ homozygous mutants (C and F), or wild-type littermates (A and D) at E15.5. *Sema3A*$^{-/-}$ mutants contain a normal complement of BFABP^{+} Schwann cells (D versus E) while *erbB3* mutants lack them completely (F). Arteries are still aligned with disorganized nerves in *Sema3A*$^{-/-}$ mutants (B and E, arrows and open arrowheads) but not in *erbB3*$^{-/-}$ mutants (C and F, arrows and open arrowheads).

(G–L) Triple labeling with CX40 (G–L, red, arrows), PECAM-1 (G–I, blue), and 2H3 (G–I, green, open arrowheads) in *Sema3A*$^{-/-}$ mutants (H and K), *erbB3*$^{-/-}$ mutants (I and L), or wild-type littermates (G and J) at E15.5. The expression of CX40 was downregulated in *erbB3*$^{-/-}$ mutants (J and K versus L, arrows). Similar results were obtained with NP1 (not shown). Note that the nerves of *erbB3*$^{-/-}$ mutants are not as tightly bundled as those in wild-type and *Sema3A*$^{-/-}$ mutants.

(M–R) Triple labeling with αSMA (M–R, red, arrows), PECAM-1 (M–O, blue), and 2H3 (M–R, green, open arrowheads) in *Sema3A*$^{-/-}$ mutants (N and Q), *erbB3*$^{-/-}$ mutants (O and R), or wild-type littermates (M and P) at E15.5. αSMA^{+} cell association is observed around large vessels but in fewer of the smaller branched vessels in *erbB3*$^{-/-}$ mutants as compared to wild-type and *Sema3A*$^{-/-}$ mutants (M and N versus O, P and Q versus R, red, arrows). Relative scale bars are shown in (A)–(C), (G)–(I), and (M)–(O). Scale bars are 100 μm.

ated vessels still expressed arterial markers such as Cx40 (Figures 5H and 5K, arrows and open arrowheads) and NP1 (data not shown) and were covered with αSMA^{+} cells (Figures 5N and 5Q, arrows and open arrowheads). These data suggest that peripheral nerves are not simply necessary for arteriogenesis but also direct the patterning of arterial differentiation and vascular remodeling in limb skin.

Schwann Cells Are Required for Arterial Differentiation and Blood Vessel-Nerve Alignment

A question raised by the foregoing observations is whether the nerve dependence of arteriogenesis reflects a requirement for axons, their associated Schwann cells, or both. This could not be determined in *Sema3A*$^{-/-}$ mice, because Schwann cells remain associated with peripheral nerves in this mutant (Figures 5D and 5E; BFABP). To address this question, we used *erbB3*$^{-/-}$ mice. ErbB3 is a coreceptor for the axon-derived signal Neuregulin-1, which is expressed by Schwann cells and is essential for their development and survival (reviewed in Garratt et al., 2000). In *erbB3*$^{-/-}$ mice (Riethmacher et al., 1997), there is a complete absence of peripheral nerve-associated Schwann cells (Figure 5D versus 5F; BFABP), and the pattern of axon growth is disorganized (Figure 5A versus 5C; 2H3, open arrowheads; see Sup-

plemental Figures S3C and S3F at http://www.cell.com/cgi/content/full/109/6/693/DC1).

In contrast to *Sema3A* mutants, association of blood vessels with peripheral nerves was greatly reduced in *erbB3*$^{-/-}$ limb skin at E15.5 (Figure 5A versus 5C, arrows and open arrowheads). To quantify this, we measured the fractional length of nerve fibers associated with blood vessels. There was no statistically significant difference in this parameter between *Sema3A*$^{-/-}$ mutants (95.4% $\pm$ 4.0%) and wild-type littermates (89.7% $\pm$ 4.1%) (see Supplemental Figure S3G at http://www.cell.com/cgi/content/full/109/6/693/DC1). In contrast, the amount of alignment was greatly reduced in *erbB3*$^{-/-}$ mutants (14.9% $\pm$ 12.0%). In addition, there was a clear reduction in the expression of the arterial markers CX40 (Figures 5G and 5J versus 5I and 5L, arrows), NP1 (data not shown), and *ephrinB2* (see Supplemental Figure S2C) in *erbB3*$^{-/-}$ mutants, like that in *Ngn1, 2* double mutants (cf. Figures 4G and 4H), though somewhat less severe. Similarly, there was a pronounced but less extensive deficiency of αSMA^{+} cells associated with smaller-diameter blood vessels in *erbB3*$^{-/-}$ mice (Figures 5M and 5P versus 5O and 5R, arrows). Similar results were obtained in *erbB2* mutant embryos in which the early cardiac defect is genetically rescued by an α myosin heavy-chain (α-MHC) promoter-driven erbB2 cDNA (data not shown), embryos which exhibit a similar peripheral nerve phenotype as do those lacking *erbB3* (Morris et al., 1999).

These data suggest that both the patterning of vascular remodeling along peripheral nerves and the induction of arterial differentiation are defective in *erbB3*$^{-/-}$ mice and are associated with a lack of Schwann cells in peripheral nerve. The absence of Schwann cells may cause this vascular defect, or the defect may instead reflect an autonomous requirement for *erbB3* function in endothelial cells. As a first step to addressing this issue, we examined the expression of *erbB* family members in limb endothelial cells. PECAM-1-positive endothelial cells were isolated from E15.5 limb skin by fluorescence-activated cell sorting (FACS) and were analyzed for expression of *erbB2, erbB3*, and *erbB4* mRNAs by RT-PCR. No *erbB3* or *erbB4* and only very weak *erbB2* expression were detectable in these isolated endothelial cells (data not shown). These data suggest that the requirement for *erbB3* in peripheral arterial differentiation is unlikely to reflect an autonomous requirement in endothelial cells, and more likely reflects the absence of Schwann cells. Confirmation of this will, however, require a specific knockout of this gene in Schwann cells.

VEGF Is Expressed in Peripheral Nerves

We next sought to identify candidate signals that might mediate the influence of peripheral nerves on arteriogenesis in order to determine whether these signals are provided by Schwann cells or are provided by axons in a Schwann cell-dependent manner. We initially focused on vascular endothelial growth factor (VEGF) because it is known to be involved in a number of different aspects of blood vessel development, including both vasculogenesis and angiogenesis in vivo (Carmeliet et al., 1996; reviewed in Ferrara, 2000; Ferrara et al., 1996; Fong et al., 1995; Shalaby et al., 1995).

We first examined the expression of VEGF in the limb skin using anti-VEGF antibodies. Double-labeling with antibodies to VEGF and the neuron-specific marker βIII tubulin (TuJ1) revealed that the growth factor was expressed in nerves at a relatively higher level than in surrounding mesenchymal tissue (Figures 6A and 6B, arrows versus open arrowheads). To confirm the expression of VEGF in peripheral nerve by an independent method and to analyze the contribution of neurons and Schwann cells to this expression, we performed RT-PCR experiments on acutely isolated peripheral neurons and glia. Sensory neurons isolated from E14.5 dorsal root sensory ganglia (DRG) by FACS using the lectin IB4 expressed the 120, 144, and 164 but not the 188 isoforms of VEGF (Figure 6D, in vivo). By contrast, Schwann cells isolated from limb skin by FACS using anti-p75^{LNTR} at the same age expressed only the 120 isoform of VEGF (Figure 6D, in vivo). These results indicate that both sensory neurons and glia contribute to the expression of multiple isoforms of VEGF in peripheral nerves in vivo.

VEGF Induces Arterial Differentiation In Vitro

We next examined whether VEGF can induce arterial marker expression in isolated primary embryonic endothelial cells in vitro. To do this, we isolated ephrinB2-negative endothelial cells from E10.5 *ephrinB2*$^{taulacZ/+}$ heterozygous embryos by two color FACS, using anti-PECAM-1 antibody and a fluorescent β-galactosidase substrate, FDG (Nolan et al., 1988), and gating on the PECAM-1^{+}, lacZ^{-} population (Figure 6E, left, R1). Cytospin analysis of the sorted cells performed directly after FACS confirmed that freshly isolated cells from the R1 fraction did not express β-galactosidase as detected by X-gal staining (Figure 6E, right, R1 versus R2) but were positive for PECAM-1 (data not shown).

To determine whether VEGF can induce ephrinB2 expression, we cultured the isolated ephrinB2-negative endothelial cells (Figure 6E, R1) for 2 days on collagen type IV-coated plates in the presence of basic FGF (bFGF) to support survival. Under these conditions, only $\sim$10% of endothelial cells spontaneously expressed ephrinB2 (Figure 6F, left). By contrast, both VEGF^{120} and VEGF^{164} strongly enhanced ephrinB2 expression, by 4- to 5-fold over control, at concentrations as low as 2.5 pM (Figure 6F, left). However, the proportion of ephrinB2-positive cells never exceeded 50% even at higher concentrations of VEGF. Increased expression of NP1 and CX40 was also detected by immunohistochemical staining (data not shown). We also tested a number of other candidate growth factors for their ability to promote ephrinB2 expression. BMP2, PDGF, IGF-I, NGF, NT-3, BDNF, GGF, and Sonic Hedgehog (Shh), like bFGF, did not substantially upregulate the arterial marker at any concentration tested (data not shown). However, TGFβ1 and Activin-A did increase expression of ephrinB2 to a similar extent as did VEGF, but only at concentrations two orders of magnitude higher than the minimum effective dose of VEGF (250 versus 2.5 pM, respectively). Soluble VEGFR2(Flk1)-Fc, a potent VEGF antagonist (reviewed in Ferrara and Davis-Smyth, 1997), blocked VEGF- but not TGFβ1-mediated induction of ephrinB2, while conversely anti-TGFβ antibody blocked the TGFβ1 but not the VEGF effect (see Supplemental Figures S4A

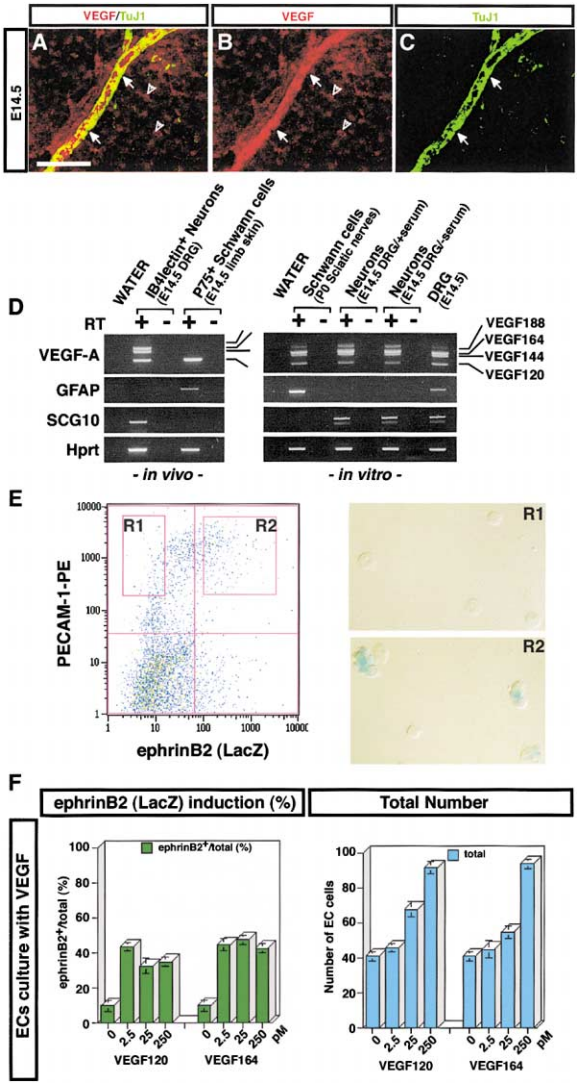

Figure 6. VEGF Is Expressed in Peripheral Nerve and Induces Arterial Differentiation In Vitro

(A–C) VEGF expression in embryonic limb skin at E14.5. Whole-mount double immunofluorescence confocal microscopy was performed with antibodies to VEGF (A and B, red) and the neuron-specific marker βIII tubulin (TuJ1) (A and C, green). Note that VEGF is highly expressed in nerves (A–C, arrows) as compared to its expression in surrounding cells (A and B, open arrowheads). Scale bars, 100 μm.

(D) RT-PCR analysis of VEGF-A isoforms (VEGF-188, -164, -144, and -120) expression in freshly isolated IB4 lectin$^+$ DRG neurons and P75$^+$ limb skin Schwann cells (in vivo), and in primary DRG neurons and sciatic nerve-derived Schwann cells (in vitro). The acutely isolated DRG neurons express VEGF-164, -144, and -120, whereas the Schwann cells express only VEGF-120. In contrast, both cultured DRG neurons and Schwann cells express all isoforms of VEGF. There is no difference in neuronal VEGF expression under serum- or nonserum-containing culture conditions. GFAP and SCG10 were used as Schwann cell and neuronal markers, respectively. Messenger RNA from E14.5 DRG was used as a positive control.

(E) FACS profile of cells derived from E10.5 *ephrinB2*$^{taulacZ/+}$ heterozygous embryos and labeled with anti-PECAM-PE antibody and FACS-gal. PECAM$^+$, ephrinB2$^-$ (R1) and PECAM$^+$, ephrinB2$^+$ (R2) sorted cells were stained using X-gal immediately after isolation (right).

(F) PECAM$^+$, ephrinB2$^-$ endothelial cells (ECs) (E, fraction R1) were cultured in various concentrations of VEGF^{120} or VEGF^{164} for 2 days, and were double-stained with X-gal and anti-PECAM antibody. Both the percentage of ECs expressing ephrinB2-lacZ (left) and the total number of ECs (right) were measured. Bars represent mean ± SEM. Note that maximal induction of ephrinB2 expression (5-fold) is achieved at 2.5 pM VEGF$^{120/164}$, at which concentration there is no net change in the total number of ECs.

and S4C at http://www.cell.com/cgi/content/full/109/6/693/DC1).

It was important to distinguish whether VEGF increased the proportion of ephrinB2-positive cells by selection or by induction of expression. Although the two VEGF isoforms tested each promoted cell proliferation and/or survival at higher concentrations (Figure 6F, right), at the lowest concentration that induced ephrinB2 expression there was no net change in total cell number, while the percentage of ephrinB2-positive cells was increased about 5-fold (Figure 6F, 2.5 pM, left versus right). This lack of a change in net cell number did not reflect increased proliferation balanced by increased cell death, because only ~5% of the cells died in the presence or absence of VEGF under these conditions (data not shown). Finally, at high concentrations of VEGF that caused a >2-fold increase in cell number (250 pM), there was no further increase in the proportion of ephrinB2-positive cells (Figure 6F, 250 pM). Taken together, these data suggest that the increase in the proportion of ephrinB2-positive cells caused by VEGF$^{164/120}$ cannot be explained by increased cell survival or cell proliferation, but rather reflects induction of expression.

Neurons and Schwann Cells Induce EphrinB2 Expression via VEGF In Vitro

The expression of VEGF in peripheral nerve, the requirement of the nerve for arteriogenesis in vivo, and the ability of VEGF to induce ephrinB2 expression in vitro together suggested that neurons and/or Schwann cells might induce ephrinB2 expression via VEGF. To directly test this, we cocultured isolated ephrinB2-negative endothelial cells with purified DRG sensory neurons or Schwann cells (see Experimental Procedures). The purity of these neuronal or glial cultures was over 95% as assessed by immunostaining for the glial marker GFAP and the neuronal marker peripherin (Figures 7A–7D), consistent with RT-PCR analysis of neuronal and glial marker mRNAs (Figure 6D, in vitro, GFAP and SCG10). The cultured sensory neurons expressed a similar complement of VEGF isoforms as they did in vivo, except that VEGF^{188} was weakly expressed (Figure 6D). However, although freshly isolated Schwann cells expressed only VEGF^{120}, when they were cultured in the absence of neurons they expressed the same VEGF isoforms as did DRG neurons (Figure 6D, in vitro). The induction of other VEGF isoforms in these cultured glia may be caused either by factors in the culture medium or by the removal of an inhibitory influence normally exerted on Schwann cells by axons in vivo.

When such purified Schwann cells or sensory neurons were cocultured for 2 days with isolated ephrinB2-negative endothelial cells, both neural cell types induced endothelial ephrinB2 expression in ~60% of the cells (Figures 7E and 7G). As was the case for purified VEGF,

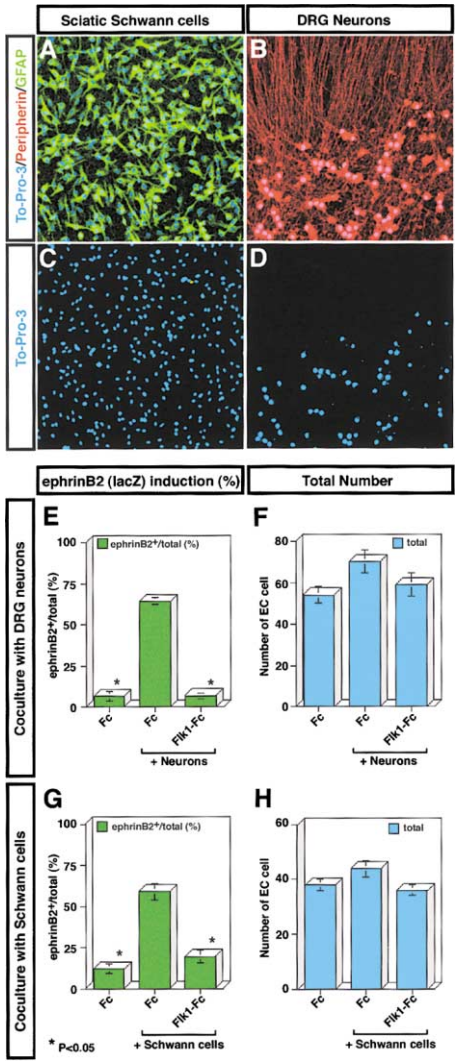

Figure 7. Purified Sensory Neurons or Schwann Cells Promote Arterial Differentiation In Vitro via VEGF

(A–D) Primary cultures of sciatic nerve-derived Schwann cells or DRG neurons were triple-labeled with antibodies to the Schwann cell marker GFAP (A and B, green) and the neuronal marker peripherin (A and B, red) in combination with the nuclear dye To-Pro-3 (A–D, blue). More than 95% of the cells in these cultures were GFAP$^+$, peripherin$^-$ Schwann cells or peripherin$^+$, GFAP$^-$ neurons, respectively. The same culture preparations were used for RT-PCR analysis (Figure 6D, in vitro).

(E–H) PECAM$^+$, ephrinB2$^-$ endothelial cells were cocultured with DRG neurons or sciatic nerve-derived Schwann cells (A–D) for 2 days in the presence or absence of Flk1-Fc (soluble VEGFR2) protein, and were double-stained with X-gal and anti-PECAM antibody. Quantification was performed as in Figure 6. Note that both neurons and Schwann cells promote ephrinB2 expression to a similar extent as did VEGF (Figure 6), without changing total EC number. The effect of the neural cells is blocked by Flk1-Fc and is therefore VEGF dependent. Bars represent mean ± SEM. Asterisk (E and G) indicates statistically significant difference ($p < 0.05$) according to Student's t test.

this increase in ephrinB2 expression reflected induction and not simply increased cell survival or proliferation, since the total number of endothelial cells did not increase appreciably in these cocultures (Figures 7F and 7H). Soluble VEGFR2 (Flk1)-Fc protein was able to block this induction (Figures 7E–7H), suggesting that the effect of both neural cell types on ephrinB2 expression is mediated by VEGF (Figure 6D, in vitro). In contrast, anti-TGFβ antibody had no effect (data not shown). These data indicate that neurons and Schwann cells can induce ephrinB2 in primary embryonic endothelial cells in vitro and that they do so through secretion of one or more isoforms of VEGF.

Schwann Cells Are Required for VEGF Expression in Peripheral Nerves

The foregoing data provided evidence that peripheral nerves can induce arterial marker expression in cultured endothelial cells via local secretion of VEGF. As a first step toward testing this hypothesis in vivo, we asked whether VEGF expression in peripheral nerve was affected in *erbB3*$^{-/-}$ mutants. As shown earlier, in these mutants peripheral nerves are present in the skin, but blood vessels do not remodel in association with them or express arterial markers. Strikingly, in *erbB3*$^{-/-}$ limbs at E15.5, little or no VEGF expression was detected in peripheral nerves (Figures 8A, 8D, and 8G versus 8C, 8F, and 8I, arrows). However, VEGF expression in surrounding nonneural tissues appeared unaffected (Figures 8A and 8D versus 8C and 8F, open arrowheads). By contrast, nerve bundles of similar size expressed VEGF in *Sema3A*$^{-/-}$ embryos, in which nerve-associated vascular remodeling and arterial differentiation do occur (Figures 8B, 8E, and 8H, arrows). These data indicate that the absence of Schwann cells and arteriogenesis in *erbB3*$^{-/-}$ mutants is correlated with a specific reduction in nerve-associated VEGF expression. In contrast, in *Sema3A*$^{-/-}$ mutant where peripheral nerves are disorganized but Schwann cells are present, VEGF expression in the nerve is maintained and nerve-associated endothelial cell remodeling and arterial differentiation also occurred.

Discussion

Peripheral nerves are known to associate with subsets of blood vessels (Martin and Lewis, 1989), but the significance of this association for vascular development has been unclear. Using several recently described artery-specific markers, we now show that arterial blood vessels are specifically aligned with peripheral sensory nerves in embryonic limb skin. Several lines of evidence suggest that this association reflects a requirement for the nerve to induce arterial differentiation. First, in mutants lacking peripheral sensory axons and/or Schwann cells, arterial differentiation does not occur and remodeling appears abnormal. Second, in mutants containing disorganized peripheral nerve fibers, arteries continue to follow the altered nerve branching patterns. Third, purified peripheral sensory neurons or Schwann cells can induce arterial marker expression in cultured endothelial cells. The fact that this induction can be blocked by a specific VEGF antagonist and mimicked by purified VEGF^{120} or VEGF^{164}, taken together with the relatively high level of VEGF expression in peripheral nerve in vivo, further suggests that the effect of the nerve to promote

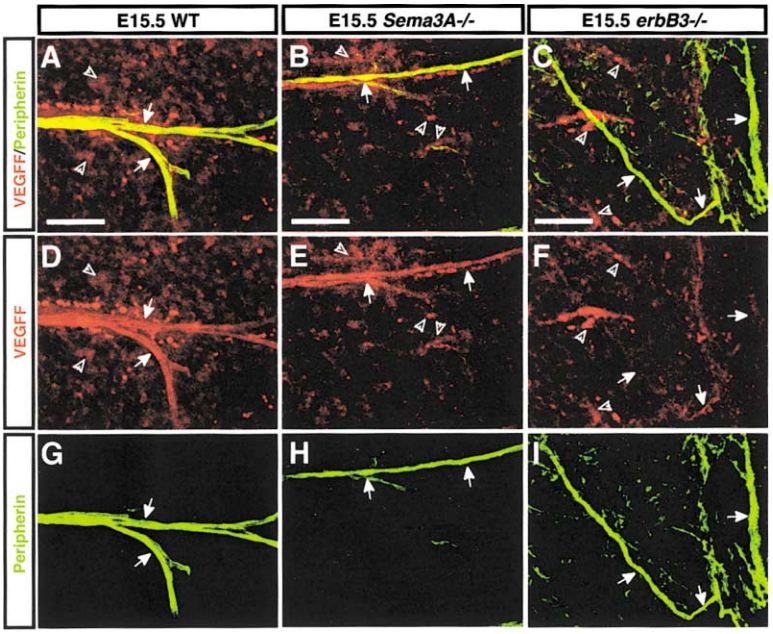

Figure 8. Requirement of *erbB3* for Neuronal VEGF Expression

Whole-mount double immunofluorescence confocal microscopy was performed with antibodies to VEGF (A–F, red) and peripherin (A–C and G–I, green) in *Sema3A*$^{-/-}$ mutants (B, E, and H), *erbB3*$^{-/-}$ mutants (C, F, and I), or wild-type littermates (A, D, and G) at E15.5. No or only weak VEGF expression was detected in nerves in *erbB3*$^{-/-}$ mutants as compared to its expression in wild-type and *Sema3A*$^{-/-}$ mutants (A and B versus C, D and E versus F, arrows). VEGF expression in nonneural elements was retained in *erbB3*$^{-/-}$ mutants (D–F, open arrowheads and data not shown). The reduction of VEGF expression in peripheral nerves of *erbB3*$^{-/-}$ embryos is correlated with the absence of Schwann cells from these nerves (cf. Figures 5D and 5F). Scale bars are 100 μm.

arteriogenesis may be mediated by local secretion of VEGF.

Arterial Differentiation Occurs in Association with Peripheral Nerves

The use of arterial markers has revealed that blood vessel-nerve association in the skin is not random but reflects an invariant alignment of arterial vessels with nerves. Our genetic data indicate that this nerve association is essential for the differentiation of arteries and guides the patterning of arteriogenesis. Consistent with the chick studies (Martin and Lewis, 1989), we observe that the arterial differentiation of the major, large-diameter vessels in the skin is relatively less affected by the absence of nerves. Nevertheless, it remains unclear whether the subset of vessels whose arterial differentiation is nerve dependent is selected simply by proximity or is rather prespecified prior to nerve association. In other systems such as the yolk sac and tumor vessels, arterial differentiation occurs in the absence of any innervation. Thus, association with nerves is not a general requirement for arterial differentiation. However, similar signals as released by the nerve to promote arterial differentiation in the limb could be provided by other tissues in different developmental contexts.

Peripheral Nerves Promote Arterial Marker Expression via VEGF

The data presented here suggest that peripheral nerves may promote blood vessel association and arteriogenesis by local secretion of VEGF in vivo. The in vitro data suggest that this growth factor could be provided by axons, by Schwann cells, or by both. In *erbB3*$^{-/-}$ embryos, which lack Schwann cells but not sensory axons at E15.5, expression of VEGF in peripheral nerve is greatly attenuated, and expression of arterial markers does not occur in association with such axons. These data suggest either that Schwann cells themselves are the principal source of VEGF, or that axons are the principal source but their expression of the growth factor requires the presence of Schwann cells. In support of the latter explanation, sensory neurons express a greater diversity of VEGF isoforms in vivo than do Schwann cells. Resolution of this issue, as well as a direct demonstration that nerve-derived VEGF is required for arterial differentiation in vivo, will require sensory neuron- and Schwann cell-specific knockouts of *VEGF*.

Although VEGF expression is detected in nerves at a higher level than in surrounding tissue, the growth factor is nevertheless expressed by such nonneural elements in the skin. This fact may explain why some remodeling, and weak expression of arterial markers, occurs even in the absence of nerves or Schwann cells. Similarly, the fact that VEGF is also expressed at relatively higher levels by larger-diameter arteries may explain why the expression of arterial markers by these vessels seems less affected by the absence of nerves. In the presence of nerves, however, local secretion of VEGF by the nerves may act to pattern the arterial differentiation of the smaller-diameter vessels. Whether this VEGF acts via a concentration gradient or is immobilized via the extracellular matrix remains to be determined.

VEGF as a Candidate Arterial Differentiation Factor

We find that VEGF^{164} and VEGF^{120} are sufficient to promote expression of arterial markers in isolated primary embryonic endothelial cells in vitro. The vivo relevance of this observation is supported by the correlative genetic data presented here, as well as by the recent observation that mutant mice lacking the 164 and 120, but not the 188, isoforms of VEGF show defects in ephrinB2 expression in retinal vessels vivo (Stalmans et al., 2002). Recent work has implicated Alk-1, a receptor for certain TGFβ superfamily ligands (Urness et al., 2000), as a potential determinant of arterial identity in vivo in mouse.

Indeed, we observed that TGFβ and Activin-A are able to induce arterial differentiation in vitro at high concentrations. Importantly, however, the effect of isolated neurons or Schwann cells to induce ephrinB2 expression was not blocked by a neutralizing anti-TGFβ antibody. Notch-Delta signaling has also recently been implicated in the choice between arterial and venous fates in zebrafish (Lawson et al., 2001; Zhong et al., 2001; reviewed in Thurston and Yancopoulos, 2001. Whether it is involved in the induction of arterial differentiation by VEGF remains to be determined.

Interestingly, the proportion of endothelial cells that expressed ephrinB2 in vitro, even at the highest concentration of VEGF tested, was never more than 50%. The same was true when ephrinB2 was induced by coculture with neurons or Schwann cells. One interpretation of these observations is that the ephrinB2-negative population isolated for these experiments may be heterogeneous and consist of both determined but undifferentiated arterial precursors as well as other cells not yet determined for an arterial fate, in roughly equal proportions. If so, then VEGF might suffice to trigger overt arterial differentiation in the former, but not the latter, endothelial cells. Indeed, recent lineage-tracing studies in zebrafish have revealed that angioblasts are at least specified for (although not necessarily committed to) arterial or venous identities prior to vasculogenesis of the dorsal aorta and cardinal veins (Zhong et al., 2001). In that scenario, VEGF would be a permissive inducing signal rather than an instructive determinant of arterial identity. Alternatively, VEGF might indeed be an instructive arterial determinant, but the proportion of cells expressing ephrinB2 in response to VEGF might be limited by local cell-cell interactions occurring within our cultures.

Local Control of Vascular Patterning

Why should peripheral nerves direct the differentiation of arteries and the pattern of angiogenic remodeling? In addition to supplying oxygen and nutrients, arterial vessels express neurotrophic factors such as NGF (Scarisbrick et al., 1993), NT3 (Francis et al., 1999), and BDNF (Donovan et al., 2000). The expression of such neurotrophic factors in blood vessels is thought to be important to maintain the survival of growing axons before they arrive at their peripheral targets, which provide an independent source of neurotrophins (reviewed in Lewin and Barde, 1996). Thus, nerves may promote blood vessel association and arterial differentiation shortly after their arrival in the periphery in order to ensure access to a local source of survival factors during subsequent growth.

The ontogenetic interdependence of arteries and nerves also makes sense in terms of the later functional interactions between the nervous and circulatory systems. For example, peripheral autonomic nerves release catecholamines to activate β-adrenergic receptors on arterial smooth muscle cells (Zukowska-Grojec et al., 1993), while sensory nerves regulate injury-induced inflammation through the release of neuropeptides (reviewed in Baluk, 1997). An early association between arteries and axons could facilitate the later development of such functional interactions. In this way, reciprocal signaling between nerves and blood vessels during development may serve to orchestrate their mutually dependent functions in both space and time. More generally, our results raise the possibility that there may be organotypic patterns of arterial branching in various tissues, which are adapted to their unique physiology. If so, then local signals provided by organized tissue subcomponents, such as nerve fibers or other structures, may provide a template to pattern arteriogenesis in these organs.

Experimental Procedures

Whole-Mount Immunohistochemistry of Limb Skin

Staining was performed essentially as described previously (Gerety et al., 1999; Wang et al., 1998). Details of the procedure are available in the Supplemental Experimental Procedures at http://www.cell.com/cgi/content/full/109/6/693/DC1).

Whole-Mount In Situ Hybridization

Whole-mount in situ hybridization was carried out essentially as described previously (Wang et al., 1998). E15.5 limbs were hybridized with a cRNA probe against the ephrinB2 extracellular domain.

LacZ and Immunohistochemistry in Culture

Staining was performed essentially as described previously (Gerety et al., 1999; Wang et al., 1998). Details of the procedure are available in the Supplemental Experimental Procedures.

Flow Cytometry

To isolate ephrinB2-negative endothelial cells, E10.5 embryos were dissociated by digestion with type 3 collagenase (Worthington) and deoxyribonuclease type 1 (DNase 1; Sigma). The cells were labeled with PE-conjugated anti-PECAM antibody (BD Pharmingen) and then loaded with fluorescein di-β-D-galactoside (FDG) by osmotic shock (1 min at 37°C) according to the manufacturer's instructions (Molecular Probe). All sorts and analyses were performed on a FACS Vantage dual laser flow cytometer (BD Biosciences). Details of the procedure are available in the Supplemental Experimental Procedures.

Gene-Specific RT-PCR Analysis

Total RNA was isolated from whole E14.5 DRGs and FACS-isolated cells from E14.5 DRGs or limb skin, as well as from cultured DRG neurons and sciatic nerve-derived Schwann cells using the Micro RNA Isolation Kit (Stratagene), digested with DNase 1 (GIBCO-BRL) and reverse-transcribed with Superscript First-Strand Synthesis System for RT-PCR (GIBCO-BRL). VEGF, GFAP, SCG10, and Hprt genes were amplified using PLATINUM Taq DNA polymerase (GIBCO-BRL) for 45 cycles. The PCR products were fractionated by electrophoresis. The sequences of the PCR primers are available in the Supplemental Experimental Procedures at http://www.cell.com/cgi/content/full/109/6/693/DC1.

Culture Methods

Primary embryonic mouse endothelial cells isolated by FACS were cultured on poly-D-lysine- (Biomedical Technologies Inc), laminin- (BD Biosciences), and collagen type IV-coated plates (BD Biosciences). The culture medium contained EMB-2 (Clonetics) with 15% FBS (Hyclone Laboratories), Penicillin/Streptomycin (BioWhittaker), and 10 ng/ml bFGF (R&D). $VEGF^{120}$, $VEGF^{164}$, TGFβ1, IGF1, PDGF, NT3, BDNF, Activin-A, Flk1-Fc, Flt1-Fc, and anti-TGFβ blocking antibody were purchased from R&D. BMP2 was a gift from the Genentics Institute. Rat Schwann cells were isolated from sciatic nerves of P0 neonatal rats as described (Brockes et al., 1979). Rat sensory neurons were isolated from DRG of E14.5–E16.5 rat embryos as described (Murphy et al., 1996). Most cultures were incubated for 2 days in a reduced oxygen environment to more closely approximate physiological oxygen levels (Studer et al., 2000). Similar results were observed under standard conditions (see Supplemental Figures S5E and S5F at http://www.cell.com/cgi/content/full/109/6/693/DC1).

Details of the procedure are available in the Supplemental Experimental Procedures.

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