Peripheral nerve-derived VEGF promotes arterial differentiation via neuropilin 1-mediated positive feedback

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Summary

In developing limb skin, peripheral nerves are required for arterial differentiation, and guide the pattern of arterial branching. In vitro experiments suggest that nerve-derived VEGF may be important for arteriogenesis, but its role in vivo remains unclear. Using a series of nerve-specific Cre lines, we show that VEGF derived from sensory neurons, motoneurons and/or Schwann cells is required for arteriogenesis in vivo. Arteriogenesis also requires endothelial expression of NRP1, an artery-specific co-receptor for VEGF^{164} that is itself induced by VEGF. Our results provide the first evidence that VEGF is necessary for arteriogenesis from a primitive capillary plexus in vivo, and show that in limb skin the nerve is indeed the principal source of this signal. They also suggest a model in which a 'winner-takes-all' competition for VEGF may control arterial differentiation, with the outcome biased by a VEGF^{164}-NRP1 positive-feedback loop. Our results also demonstrate that nerve-vessel alignment is a necessary, but not sufficient, condition for nerve-induced arteriogenesis. Different mechanisms therefore probably underlie these endothelial patterning and differentiation processes.

Key words: VEGF, Neuropilin 1, Arterial differentiation, Mouse

Introduction

Proper vascularization of organs and tissues requires not only angiogenesis and arteriovenous differentiation, but also appropriate patterning of the vascular network. Emerging evidence has suggested that the nervous system may play a role in coordinating these processes (reviewed by Carmeliet, 2003), but precisely how is unclear. Previously, we have found that in embryonic limb skin, arteries but not veins are aligned with peripheral nerves, and their branching pattern follows that of the nerves (Mukouyama et al., 2002). In embryonic mouse mutants lacking such nerves, the primitive capillary plexus forms normally but arterial differentiation fails to occur. Moreover, in mutants where the pattern of nerve branching is disrupted, the arterial branching pattern still follows the trajectory of the disorganized nerves. Thus, in limb skin, the pattern of peripheral nerve branching provides a template that instructively guides the branching of the emerging arterial vascular network.

These observations raised the question of the nature of the signal(s) that control nerve-vessel alignment and arterial differentiation, and their cellular source(s). In culture, VEGF-related factors derived from sensory neurons or Schwann cells can induce arterial differentiation in endothelial cell (EC) precursors (Mukouyama et al., 2002). These studies, however, left unresolved the issue of whether nerve-derived VEGFA is actually required for arterial differentiation in the limb skin in vivo. Furthermore, the lack of an in vitro assay for nerve-vessel alignment made it unclear whether VEGF, or rather some other molecule, is involved in this patterning process.

Two recent studies have provided evidence of a role for VEGFA in promoting arterial differentiation in vivo. In the zebrafish, *Vegf* is essential for formation of the dorsal aorta, the major midline arterial vessel (Lawson et al., 2002). However, this vessel develops by de novo assembly of angioblasts, rather than by remodeling of a capillary network. Therefore, these data do not address whether *Vegf* is required to induce arterial differentiation from a pre-existing capillary plexus, the mechanism by which most small arterial vessels form in higher vertebrates. In mice, transgenic overexpression of *Vegfa* in the heart promoted an increased number of cardiac arterial vessels (Visconti et al., 2002). Although these gain-of-function data suggest that VEGFA can promote or enhance arteriogenesis in vivo, they did not address whether it is actually required for this process during normal development.

The early embryonic lethality of *Vegfa* mutants has made it difficult to address whether nerve-derived VEGFA is required for nerve-vessel alignment and arterial differentiation in vivo (Carmeliet et al., 1996; Ferrara et al., 1996). To circumvent this problem, we have now used several different lines of transgenic mice expressing Cre recombinase, singly or in combination, to specifically delete *Vegfa* in the major cell types comprising

peripheral nerve. In parallel, we have examined the role of neuropilin 1 (NRP1), a co-receptor for VEGF[164] that is specifically expressed in arteries. Our data indicate that nerve-derived VEGFA is indeed required for arterial differentiation in vivo, and further suggest that arteriogenesis may be promoted by an NRP1-mediated positive feedback loop. Surprisingly, nerve-vessel alignment occurs normally in these conditional mutants, despite the defect in arteriogenesis. This suggests that such alignment is mediated either by a residual low level of VEGFA, or by a distinct nerve-derived signal.

Materials and methods

Experimental animals and generation of nerve-*Vegf-A* conditional and endothelial *Nrp1* mutants

The characterization of *Vegf* floxed mice (Gerber et al., 1999), *Nrp1* floxed mice (Gu et al., 2003), *Wnt1-Cre* mice (Danielian et al., 1998), *Isl1-Cre* mice (Srinivas et al., 2001; Yang et al., 2001), *Tie2-Cre* mice (Gu et al., 2003; Kisanuki et al., 2001), *HB9-eGFP* mice (Wichterle et al., 2002) and *R26R* mice (Soriano, 1999) have been reported elsewhere. *Wnt1-Cre; Isl1-Cre; Vegf*$^{flox/flox}$, *Wnt1-Cre;Vegf*$^{flox/flox}$ or *Isl1-Cre;Vegf*$^{flox/flox}$ embryos were produced by mating *Wnt1-Cre; Vegf*$^{flox/+}$ with *Isl1-Cre; Vegf*$^{flox/+}$ mice. Offspring were genotyped by PCR using primers that specifically detect the transgene for *Wnt1-Cre* or *Isl1-Cre* and the floxed *Vegf* allele (see 'recombination assay by genomic PCR'). *Tie2-Cre;Nrp1*$^{flox/-}$ embryos were produced by mating *Nrp1*$^{flox/-}$ (harboring one floxed allele and one *Nrp1* null allele) with *Tie-2-Cre; Nrp1*$^{flox/+}$ mice.

Genomic PCR

Cre-mediated recombination was assayed using genomic DNA essentially as described (Inoue et al., 2002). Ten dorsal root ganglia were isolated from one E15.5 embryo and dissociated with Proteinase K (Sigma). Skin from both limbs was isolated from one E15.5 embryo, and dissociated by digestion with type 3 collagenase (Worthington) and dispase (Gibco BRL). The cells were incubated with rat anti-mouse P75 monoclonal antibody (M. Rao and D.J.A., unpublished) and then goat anti-rat IgG magnetic beads (Dynal). The magnet-selected P75^{+} cells were dissociated with Proteinase K. Genomic DNA was extracted and analyzed for recombination by genomic PCR. using expand Taq DNA polymerase (Roche) for 45 cycles. The PCR products were fractionated by electrophoresis. The sequences of the PCR primers are: VEGF419F (5′-CCT-GGCCCTCAAGTACACCTT-3′) and VEGFc5R2 (5′-ACATCTGC-TGTGCTGTAGGAAG-3′). For genotyping, genomic DNA was isolated from embryonic tail tissue and the presence of Cre transgenes was detected by PCR using PLATINUM Taq DNA polymerase (GibcoBRL) for 28 cycles. The sequence of the PCR primers were VEGF419F (5′-CCTGGCCCTCAAGTACACCTT-3′) and VEGF567R (5′-TCCGTACGACGCATTTCTAG-3′) for floxed *Vegf* allele; Cre5′a (5′-ACCTTCCTCTTCTTCTTGGG-3′) and Wnt1s (5′-TAAGAGGCCTATAAGAGGCG-3′) for *Wnt1-Cre* transgene; IRES-N (5′-GCAAGGTCTGTTGAATGTCGTTGA-3′) and IRES-C (5′-GTACCTTCTGGGCATCCTTCAGC-3′) for *Isl1-Cre* transgene; and Cre1 (5′-GTTCGCAAGAACCTGATGGACA-3′) Cre2 (5′-CTAG-AGCCTGTTTTGCACGTTC-3′) for Cre transgene.

Immunohistochemistry

Staining was performed essentially as described previously (Mukouyama et al., 2002). Embryos or limb skin tissue was fixed with 4% paraformaldehyde/PBS at 4°C overnight, sunk in 30% sucrose/PBS at 4°C and then embedded in OCT compound for frozen sectioning (15 m). Staining was performed using anti-β-galactosidase antibodies (rabbit polyclonal antibody, 5-prime 3-prime, 1:1000, overnight at 4°C or goat polyclonal antibody, Bio Trend, 1:1000, overnight at 4°C) to detect *lacZ* expression; anti-GFP antibody (rabbit polyclonal antibody, Molecular Probes; 1:500, overnight at 4°C); anti-BFABP antibody (rabbit polyclonal antibody, T. Müller, 1:1000, 3 hours at room temperature) to detect glial cells; Alexa green-conjugated HuD antibody (mouse monoclonal antibody, clone 16A11, Molecular Probe; 1:50, 1 hour at room temperature); anti-neuronal class III β-tubulin antibody (mouse monoclonal antibody, clone Tuj1, Covance, 1:500, 1 hour at room temperature) to detect neurons; anti-PECAM-1 antibody (rat monoclonal antibody, clone MEC 13.3, BD Pharmingen, 1:300, overnight at 4°C) to detect endothelial cells; Cy3-conjugated anti-αSMA antibody (mouse monoclonal antibody, clone 1A4, Sigma, 1:500, 1 hour at room temperature) to detect smooth muscle cells; and anti-VEGF antibody (goat polyclonal antibody, R&D, 1:200, overnight at 4°C). For immunofluorescent detection, FITC-, Cy3-, Alexa-488-, Alexa-568-, or Cy5-conjugated secondary antibodies (Jackson, 1:300, Southern Biotechnology Associations, 1:300, and Molecular Probes, 1:250, 1 hour at room temperature) were used. All confocal microscopy was carried out on a Leica SP confocal (Leica).

Whole-mount immunohistochemical staining of limb skin was performed as described previously (Mukouyama et al., 2002). Embryos at E15.5 were dissected, fixed overnight in 4% paraformaldehyde/PBS at 4°C, and dehydrated in 100% methanol at –20°C. Staining was performed using anti-PECAM-1 antibody to detect endothelial cells; anti-β-galactosidase antibody to detect *lacZ* expression; anti-NRP1 antibody (rabbit polyclonal antibody, A.L. Kolodkin, 1:3000, 3 hours at room temperature); and anti-CX40 antibody (rabbit polyclonal antibody, Alpha Diagnostic International, 1:300, overnight at 4°C) as arterial markers; Cy3-conjugated anti-αSMA antibody to detect smooth muscle cells; anti-neurofilament antibody (mouse monoclonal antibody, clone 2H3, Developmental Studies Hybridoma Bank, 1:200, 1 hour at room temperature); anti-peripherin antibody (rabbit polyclonal antibody, Chemicon, 1:1000, 1 hour at room temperature) to detect nerve fibers; anti-BFABP antibody to detect Schwann cells; and anti-VEGF antibody. FITC-, Cy3-, Alexa-488-, Alexa-568- or Cy5-conjugated secondary antibodies were from Jackson, Southern Biotechnology Associations and Molecular Probes. TUNEL labeling was performed according to the manufacturer's protocol (In Situ Cell Death Detection, Roche). The average mean fluorescence (pixels/area) was analyzed using ImageJ software, and statistical significance of samples (n>3) was assessed using Student's t-test.

Whole-mount in situ hybridization of limb skin

Whole-mount in situ hybridization was carried out essentially as described previously (Mukouyama et al., 2002; Wang et al., 1998). E15.5 limbs were hybridized with a cRNA probe against the ephrin B2 (Efnb2 – Mouse Genome Informatics) extracellular domain.

Flow cytometry and culture methods

The isolation and culture of ephrin B2-negative endothelial cells from E10.5 *Efnb2*$^{lacZ/+}$ embryos was performed as described previously (Mukouyama et al., 2002). All sorts and analyses were performed on a FACS Vantage dual laser flow cytometer (BD Biosciences). The culture medium contained EMB-2 (Clonetics) with 15% FBS (Hyclone Laboratories), Penicillin/Streptomycin (BioWhittaker) and 10 ng/ml bFGF (R&D). The freshly isolated endothelial cells were challenged with serial dilutions (1-100 pg/ml) of VEGF[120] or VEGF[164] isoforms (R&D), used at equal mass/volume concentrations according to the manufacturer's technical specifications. Cultures were incubated for 2 days in a reduced oxygen environment to more closely approximate physiological oxygen levels (Mukouyama et al., 2002; Studer et al., 2000). X-gal staining and immunohistochemistry on these cultures were performed as described (Mukouyama et al., 2002).

The ephrin B2-lacZ-positive or negative cells were counted in

PECAM1-positive cells and statistical significance was assessed using the Student's *t*-test.

Results

Peripheral nerve-specific deletion of *Vegfa* requires Cre-mediated recombination in three different cell types

To test directly whether peripheral nerve-derived VEGFA is required for arteriogenesis of nerve-associated vessels in vivo, we sought to conditionally knock out *Vegfa*, specifically in these neural elements. At the stages of development investigated (E13.5-E15.5), peripheral nerves are comprised of Schwann (glial) cells and sensory, but not autonomic, axons (Mukouyama et al., 2002). We have previously shown that both Schwann cells and sensory neurons express mRNAs for various VEGF isoforms, in vivo (Mukouyama et al., 2002). As both of these cell types are neural crest-derived, deletion of *Vegfa* in them can be accomplished by employing a *Wnt1-Cre* driver, which is active in the pre-migratory neural crest (Danielian et al., 1998). We confirmed deletion in both sensory neurons and peripheral glia by Wnt1-Cre, using the *Rosa26* Cre-dependent *lacZ* reporter (*R26R*) line (Soriano, 1999) (Fig. 1A,G,I,K).

Wnt1-Cre does not delete in motoneurons (Fig. 1I, open arrow). However, motor axons are present in the peripheral nerves innervating limb skin at E14.5, as revealed by an enhanced green fluorescent protein (eGFP) reporter driven by the motoneuron-specific *HB9* promoter (see Fig. S1A-E, arrows, in the supplementary material) (Wichterle et al., 2002). To determine whether such motor axons might be an additional source of VEGFA, we carried out an RT-PCR analysis of *Vegfa* mRNA expression on EGFP$^+$ motor neurons isolated by fluorescence-activated cell sorting (FACS), from the spinal cord of *Hb9::EGFP* embryos. This analysis revealed that motor neurons do express mRNAs for all isoforms of VEGFA (see Fig. S1F in the supplementary material). These data therefore indicated that elimination of all nerve-derived sources of *Vegfa* in the limb skin should require deletion of the gene in Schwann cells, sensory and motoneurons.

As there is no single promoter-driven Cre line that

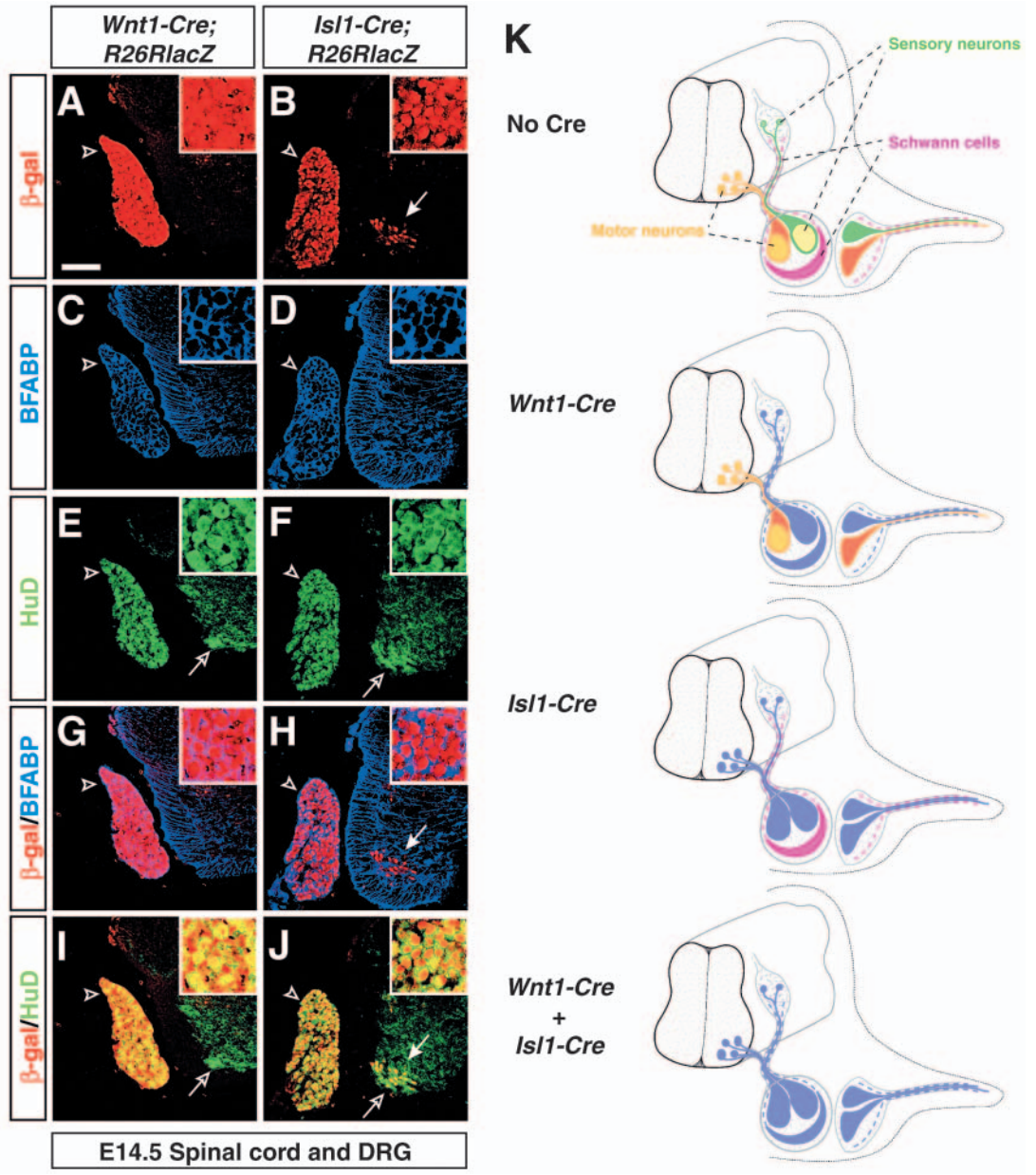

Fig. 1. Activity of *Wnt1-Cre* or *Isl1-Cre* in peripheral nerves. The spinal cord and dorsal root ganglia (DRG) of E14.5 *Rosa26 Cre-dependent lacZ* reporter (*R26R*) line with *Wnt1-Cre* (A,C,E,G,I) or *Isl1-Cre* transgene (B,D,F,H,J) are shown. Triple immunofluorescence confocal microscopy with antibodies to β-galactosidase (β-gal) (red), the glial marker BFABP (blue) and the neuronal marker HuD (green) is shown. Open arrowheads indicate DRG and open arrows indicate motoneurons in the spinal cord. White arrows indicate Cre activation in motoneurons. Insets are higher-magnification details of the DRG. Scale bar: 100 m. (K) Schematic illustrating peripheral nerve-specific Cre activation (blue).

simultaneously and specifically targets all three of these cell types, we combined the Wnt1-Cre driver with an Isl1-Cre driver, which is active in both sensory and motoneurons, but not in Schwann cells (T. M. Jessell, unpublished) (Srinivas et al., 2001; Yang et al., 2001). Analysis of *Isl1-Cre; R26R* embryos using cell type-specific markers confirmed that this Cre driver deletes in both sensory and motoneurons, but not in Schwann cells (Fig. 1B; Fig. 1H, inset; Fig. 1J,K). Thus, the combination of *Wnt1-Cre* and *Isl1-Cre* should suffice to delete the conditional *Vegfa* allele ($Vegf^{flox}$) in sensory neurons, motoneurons and Schwann cells (Fig. 1K; see Fig. S2 in the supplementary material).

Peripheral nerve-derived VEGFA is required for proper arteriogenesis in the limb skin

To create embryos carrying both the *Wnt1-Cre* and *Isl1-Cre*

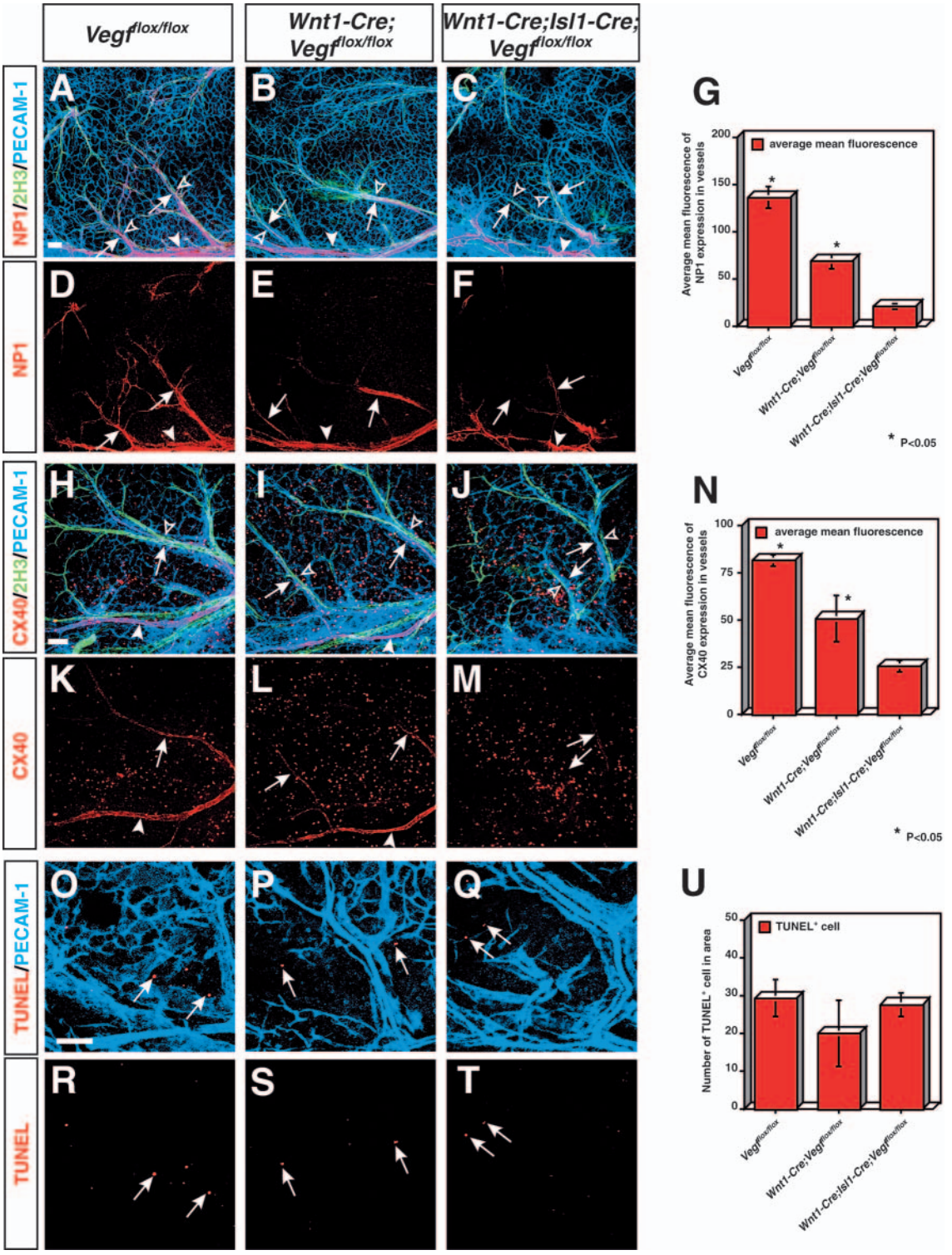

Fig. 2. VEGFA is required for arterial differentiation in limb skin. (A-F,H-M) triple immunofluorescence confocal microscopy using antibodies to either NRP1 (A-F, red, arrows and arrowhead) or CX40 (H-M, red, arrows and arrowhead), together with antibodies to PECAM1 (A-C,H-J, blue) and neurofilament (2H3) (A-C,H-J, green, open arrowheads). Arrowheads indicate large-diameter vessels. (G,N) Quantitative analysis of arterial marker expression in small-diameter vessels. Asterisks indicate statistically significant differences ($P<0.05$) in $Vegf^{flox/flox}$ or *Wnt1-Cre;* $Vegf^{flox/flox}$ compared with *Wnt1-Cre; Isl1-Cre;* $Vegf^{flox/flox}$ (Student's *t*-test). (O-T) Whole-mount double labeling with TUNEL (O-T, red, arrows) and PECAM1 (O-Q, blue) reveals that no significant increase in apoptosis in the absence of nerve-VEGFA , confirmed by quantitative analysis (U). Scale bars: 100 m.

transgenes, as well as both 'floxed' alleles of the *Vegf* gene (Gerber et al., 1999), we generated and then inter-crossed *Wnt1-Cre; Vegf*$^{flox/+}$ with *Isl1-Cre; Vegf*$^{flox/+}$ mice. On average, one in 16 embryos derived from this inter-cross inherited both the *Wnt1-Cre* and *Isl1-Cre* transgenes, as well as both *Vegf* alleles (*Vegf*$^{flox/flox}$). The limb skin of such embryos was subjected to whole-mount analysis of the expression of three independent arterial markers: neuropilin 1 (NRP1), connexin 40 (CX40; GJA5 – Mouse Genome Informatics) and ephrin B2. The expression of all three markers was greatly reduced in small-diameter vessels of embryos of this genotype (Fig. 2D,F,G,K,M, arrows; Fig. 2N; data not shown). The expression of smooth muscle markers was also reduced, albeit to a lesser extent than that of the arterial endothelial markers, in such embryos (data not shown). Embryos carrying either of the individual *Cre* transgenes also showed a clear, but less extensive, reduction of arterial marker expression (*Wnt1-Cre*; Fig. 2E,G,L, arrows; Fig. 2N); this was particularly evident for *Isl1-Cre* (data not shown). TUNEL-labeling experiments indicated that the reduction of arterial marker expression does not reflect a selective death of arterial endothelial cells in these *Vegf* conditional knockout embryos (Fig. 2O-U).

In contrast to the defective arterial differentiation of smaller-diameter vessels, the expression of NRP1 and ephrin B2 was relatively unaffected in the major, large-diameter vessels in the skin (Fig. 2D-F; arrowheads; data not shown). These data are consistent with our previous observation that expression of these arterial markers is not diminished in the large-diameter limb vessels of mutants lacking peripheral nerve fibers or Schwann cells (Mukouyama et al., 2002). Similarly, no reduction in the expression of arterial markers in other large-diameter vessels, such as the carotid and thoracic arteries, was observed in the mutants (see Fig. S3 in the supplementary material).

These data suggested that the arterial differentiation of small-diameter nerve-associated vessels, but not of large-diameter vessels, is compromised in *Wnt1-Cre; Isl1-Cre; Vegf*$^{flox/flox}$ embryos. One possible explanation for this dissociation is that autocrine or paracrine secretion of VEGFA by endothelial or smooth muscle cells in large-diameter vessels may compensate for the absence of nerve-derived VEGFA. In support of this, antibody staining revealed that VEGFA is expressed in both endothelial and smooth muscle cells of large-diameter blood vessels (see Fig. S4E-H in the supplementary material, arrows). Furthermore, expression of mRNA for VEGF^{164} was detected by RT-PCR in endothelial cells that were freshly isolated from E15.5 limb skin (data not shown).

The foregoing data suggested that nerve-derived VEGF is selectively required for arteriogenesis in small-diameter nerve-associated blood vessels within limb skin. To determine whether other aspects of neuro-vascularization might also depend on peripheral-nerve-derived VEGF, we examined blood vessel development in the dorsal root sensory ganglia (DRG) of the *Vegfa* conditional mutants. In control (*Vegf*$^{flox/flox}$) embryos, blood vessels both surround the DRG (Fig. 3C, open arrowhead) and branch into it (Fig. 3C, arrow). By contrast, there was virtually a complete failure of such internal ganglionic vascular branching in the DRG of *Wnt1-Cre; Isl1-Cre; Vegf*$^{lox/lox}$ or *Wnt1-cre; Vegf*$^{lox/lox}$ embryos (Fig. 3B,D,G; data not shown), while the vessels peripheral to the ganglia appeared unaffected (Fig. 3C,D; open arrowheads). These data suggest that VEGFA derived from sensory neurons and/or glia is required for proper vascularization of the DRG, as well as for arteriogenesis in limb skin.

Nerve-blood vessel alignment occurs normally in the absence of nerve-derived VEGFA

In addition to their requirement for arterial differentiation, peripheral nerves instructively guide the pattern of arterial branching in limb skin (Mukouyama et al., 2002). Surprisingly, in contrast to the clear defects in arterial marker expression, no evident perturbation of nerve-blood vessel association was observed in the nerve-derived *Vegfa* conditional mutants (Fig. 4A-I; data not shown). The extent of innervation of the skin, and the coverage of nerves by Schwann cells (detected by expression of BFABP), also appeared to be normal in the mutants (Fig. 4E-H).

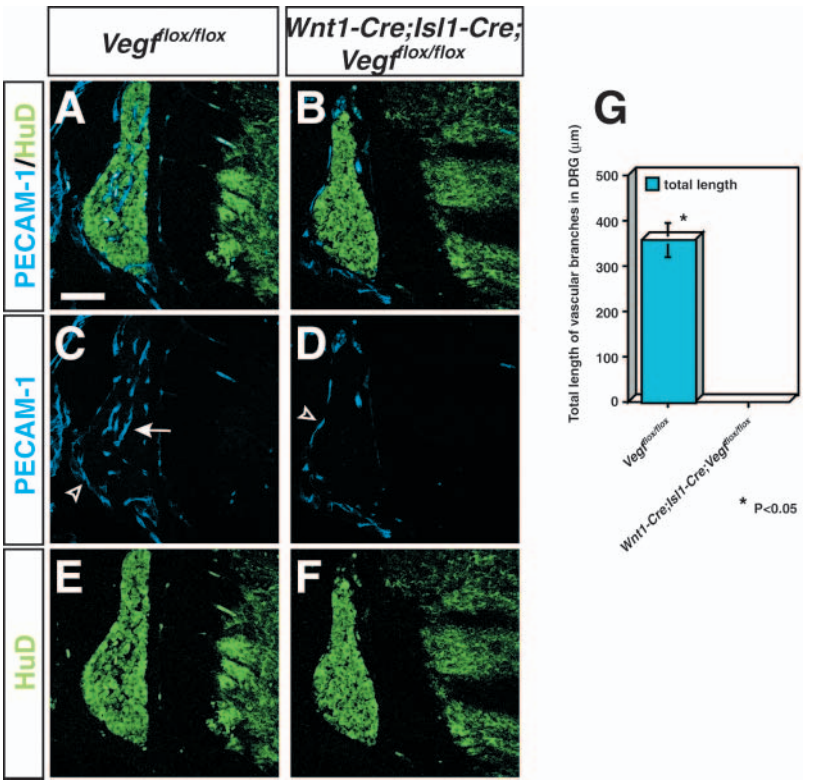

Fig. 3. VEGFA is required for normal DRG vascularization. (A-F) Double immunofluoresence confocal microscopy of E15.5 trunk sections performed using antibodies to PECAM1 (A-D, blue) and HuD (A,B,E,F, green). (G) Quantification of length of PECAM1^{+} vascular branching in DRG. The internal vascularization of the DRG (C, arrow) is disrupted in the nerve-*Vegfa* conditional mutants (D), while vessels peripheral to the DRG appeared unaffected (C,D, arrowheads). Asterisk indicates a statistically significant difference (P<0.05) in *Vegf*$^{flox/flox}$ compared with *Wnt1-Cre; Isl1-Cre; Vegf*$^{flox/flox}$ (Student's t-test). Scale bar: 100 m.

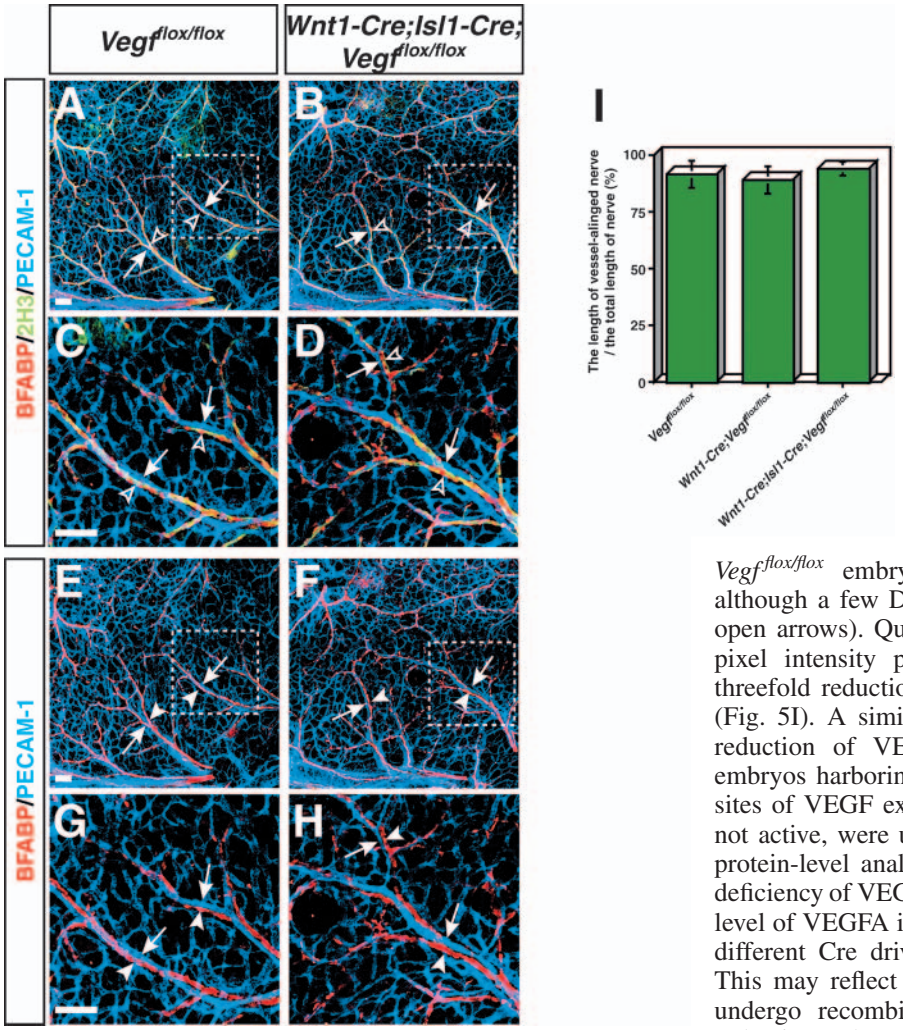

Fig. 4. Nerve-blood vessel alignment is unaffected in the absence of nerve-derived VEGFA. (A-H) Whole-mount triple immunofluorescence confocal microscopy using antibodies to BFABP (A-H, red), PECAM1 (A-H, blue) and neurofilament (2H3) (A-D, green) is shown. Close-up images (C,D,G,H) show the boxed regions in A,B,E,F. Arrows indicate blood vessels, arrowheads the aligned nerves and open arrowheads Schwann cells. Scale bars: 100 m. (I) The alignment of nerves with blood vessels was quantified as the percentage of nerve length aligned with vessels.

Verification of peripheral nerve-specific *Vegfa* deletion

The foregoing experiments indicated that conditional inactivation of *Vegfa* in peripheral nerve caused a failure of arterial differentiation, but not of nerve-vessel alignment. To examine directly the extent of VEGF reduction in the conditional mutants, Cre-dependent excision of exon3 of the floxed *Vegf* allele was first examined by genomic PCR in both the DRG (Fig. 5J) and limb skin (data not shown). This analysis revealed a high degree of recombination in DRG from *Wnt1-Cre, Isl1-Cre; VEGF$^{flox/flox}$* embryos (non-recombined:recombined=30%:70%).

The residual non-recombined *Vegf^{flox}* DNA (Fig. 5J, upper band) may reflect incomplete excision by Cre recombinase in cell types expressing *Wnt1-Cre* and *Isl1-Cre*, or else contaminating non-neural cells (fibroblasts, endothelial cells, smooth muscle cells, etc.) that do not express these Cre drivers. To distinguish these alternatives, we analyzed the level of VEGFA protein expression in such embryos by anti-VEGF antibody staining (Fig. 5A-I). Anti-VEGFA immunoreactivity was detected in both the DRG and spinal cord of control *Vegf$^{flox/flox}$* embryos (Fig. 5A, arrows and arrowheads, respectively). Such immunoreactivity was markedly reduced in both the DRG and spinal cord of *Wnt1-Cre;Isl1-Cre; Vegf$^{flox/flox}$* embryos (Fig. 5C,I; arrows and arrowheads), although a few DRG cells still exhibited staining (Fig. 5C,I; open arrows). Quantification, by measurement of fluorescent pixel intensity per unit area, indicated an approximately threefold reduction in staining levels in the mutant embryos (Fig. 5I). A similar analysis in limb skin revealed a strong reduction of VEGFA expression in peripheral nerves of embryos harboring both *Cre* drivers (data not shown). Other sites of VEGF expression, where Wnt1-Cre and Isl1-Cre are not active, were unaffected. Taken together, these DNA- and protein-level analyses validated the peripheral nerve-specific deficiency of VEGFA. However, there appeared to be a residual level of VEGFA in these neural tissues, despite the use of two different Cre drivers with overlapping cellular specificities. This may reflect the requirement for both *Vegf^{flox}* alleles to undergo recombination in each cell, in order to achieve reduction to homozygosity.

Disruption of arteriogenesis, but not nerve-vessel alignment, by endothelial-specific deletion of the VEGF^{164} co-receptor NRP1

The residual low level of VEGFA in the peripheral nerves of our conditional knockout embryos left open the possibility that such levels were sufficient for the persistence of nerve-vessel alignment in these mutants. To address this issue by an independent approach, we sought to selectively reduce the VEGF sensitivity of endothelial cells. Because deletion of the core VEGFA receptor FLK1 (VEGFR2) in endothelial cells would be expected to compromise their viability (M. Hirashima and J. Rossant, personal communication), we examined embryos with an endothelial cell-specific deletion of the VEGF^{164} co-receptor NRP1 (Kawasaki et al., 1999; Soker et al., 1998).

Such a deletion was achieved by using the endothelial-specific *Tie2*-promoter to drive Cre expression in a genetic background containing a floxed allele of *Nrp1* over a conventional null allele (Gu et al., 2003). In *Tie2-Cre; Nrp1$^{flox/-}$* embryos, expression of arterial markers, including CX40 and ephrin B2, was strongly reduced in limb vessels (compare Fig. 6C with 6D, arrows; Fig. 6E; data not shown). Surprisingly, in these mutants even the large-diameter vessels exhibited a strong reduction of arterial marker expression, as

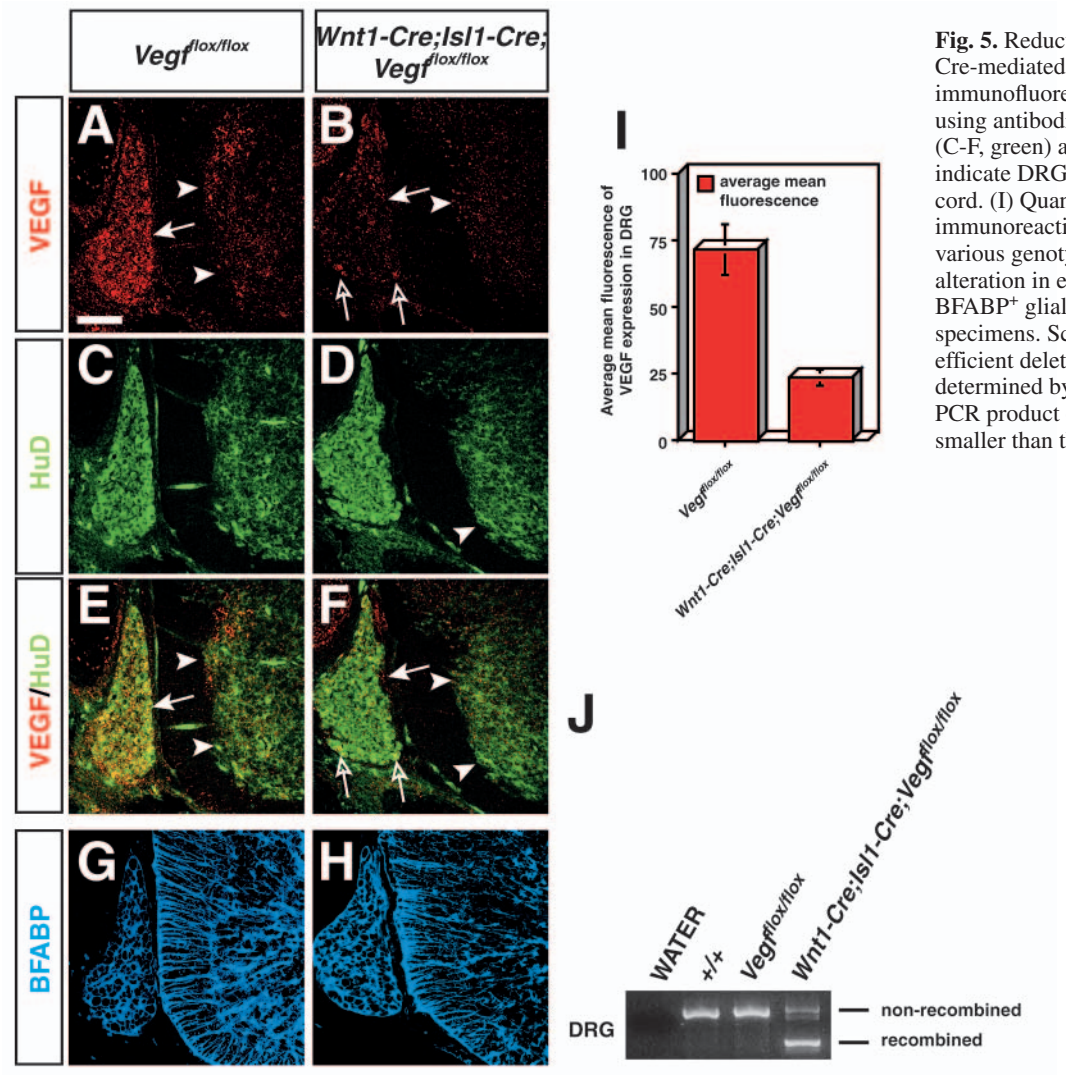

Fig. 5. Reduction of VEGFA expression by Cre-mediated recombination. (A-H) Triple immunofluorescence confocal microscopy using antibodies to VEGF (A,B,E,F, red), HuD (C-F, green) and BFABP (G,H, blue). Arrows indicate DRG; arrowheads indicate spinal cord. (I) Quantification of anti-VEGFA immunoreactivity in DRG in embryos of various genotypes. There was no consistent alteration in either HuD$^+$ neuronal (C,D) or BFABP$^+$ glial (G,H) development in multiple specimens. Scale bars: 100 m. (J) The efficient deletion of the floxed *Vegf* allele was determined by PCR analysis of DRG. The PCR product of the recombined allele is smaller than that of the non-recombined allele.

well as in smooth muscle coverage (compare Fig. 6C with 6D; compare Fig. 6H with 6I, arrowheads). The defects in limb skin arterial differentiation observed in mutants lacking endothelial NRP1 were, therefore, similar but even more pronounced than those observed in embryos deficient in peripheral nerve-derived VEGFA. Despite this more severe failure of arteriogenesis in limb skin, the association of nerves with blood vessels appeared normal in *Tie2-Cre; Nrp1$^{flox/-}$* embryos (Fig. 6B,G, compare arrows with open arrowheads).

NRP1 binding isoforms of VEGFA preferentially induce arterial markers in vitro

The requirement of *Nrp1* for arteriogenesis in vivo is surprising, given that NRP1 is a co-receptor for only the VEGF^{164} isoform, and that peripheral nerve elements express mRNAs for multiple VEGF isoforms (Mukouyama et al., 2002) (see Fig. S1F in the supplementary material). This observation raised the issue of whether VEGF^{164} is more potent than other VEGFA isoforms in its ability to induce arterial marker expression in undifferentiated endothelial cells. To address this, we compared the ability of VEGF^{164} and a non-NRP1-binding isoform, VEGF^{120}, to induce arterial marker expression in vitro. We employed a previously described system in which PECAM1$^+$, β-gal$^-$ embryonic endothelial cells are isolated by flow cytometry from *Efnb2$^{lacZ/+}$* knock-in mice and cultured in bFGF (Mukouyama et al., 2002). Under these conditions, the effects of VEGF in inducing arterial marker expression can be examined in the absence of any confounding effects on cell survival.

In such cultures, both VEGF^{164} and VEGF^{120} induced expression of *Efnb2-lacZ* (Fig. 7A-F). At 100 pg/ml, both isoforms induced ephrin B2 expression to a similar extent (Fig. 7M, left panel). However, at lower concentrations (1-10 pg/ml), VEGF^{164} was at least twice as effective as VEGF^{120} at inducing ephrin B2-*lacZ* expression (Fig. 7M, green bars). There was no effect on cell survival at any of the concentrations tested, for both isoforms (Fig. 7M, right panel). These data suggest that NRP1-binding isoforms of VEGF may preferentially promote arterial differentiation, in comparison with non-NRP1-binding isoforms, at very low concentrations of the growth factor.

We have shown that the artery-specific expression of NRP1 is dependent on nerve-derived VEGFA in vivo (Fig. 2E-G). Moreover, NRP1 itself is required in endothelial cells for arterial differentiation (Fig. 6D), and an NRP1-binding isoform of VEGF preferentially upregulates the arterial marker ephrin B2 in vitro. These data suggest that a positive-feedback loop involving NRP1 and VEGF may contribute to arteriogenesis. To address more directly this possibility, we examined the ability of VEGFA isoforms to upregulate NRP1 expression in vitro. Both VEGF^{120} and VEGF^{164} upregulated expression of NRP1 in cultured embryonic endothelial cells (Fig. 7G-L). The proportion of NRP1$^+$ cells was similar to that of ephrin B2$^+$ cells, in the presence of either VEGF isoform, although direct double-labeling for both markers was precluded by antibody incompatibility. These data demonstrate that NRP1-binding

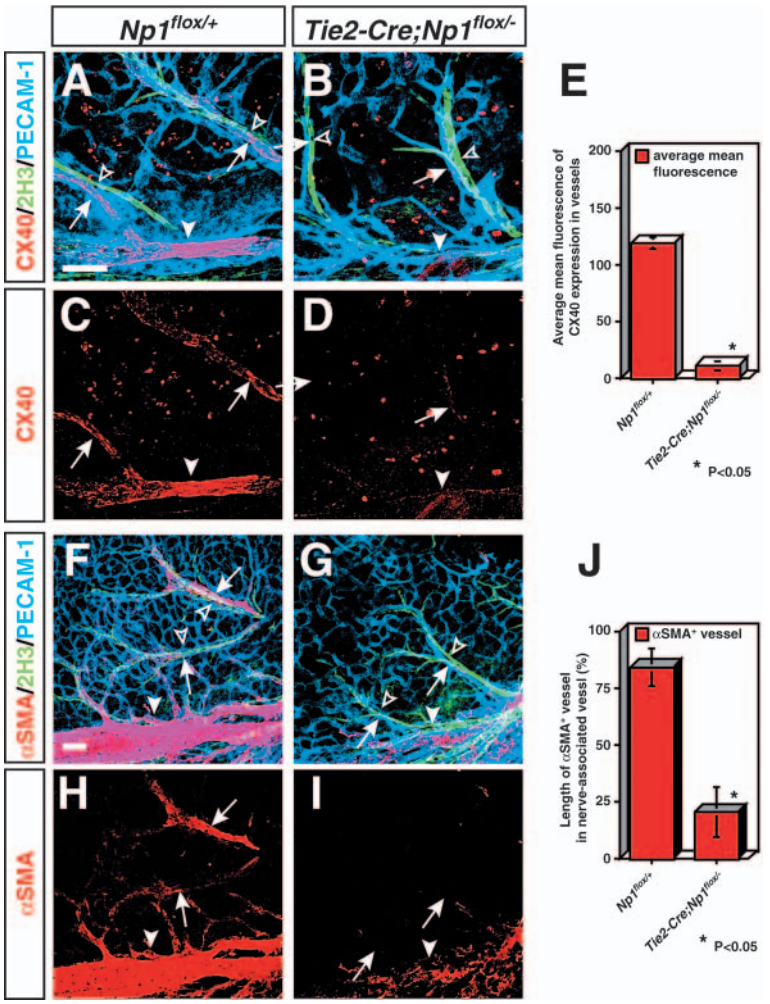

Fig. 6. NRP1 is required for arteriogenesis in limb skin. Analysis of E15.5 limbs from *Tie2-Cre;Nrp1*$^{flox/-}$ mutants or control littermates (*Nrp1*$^{flox/+}$) is shown. (A-D,F-I) Triple immunofluorescence confocal microscopy using antibodies to either CX40 (A-D, red) or αSMA (F-I, red), together with anti-PECAM1 (A,B,F,G, blue) and neurofilament (2H3) (A,B,F,G, green). (E) Quantification of CX40 expression, and (J) of αSMA$^+$ cell coverage in small-diameter vessels. Asterisks indicate statistically significant differences (P<0.05) in *Tie2-Cre;Nrp1*$^{flox/-}$ compared with *Nrp1*$^{flox/+}$ (Student's *t*-test). Nerve-blood vessel alignment in the *Tie2-Cre;Nrp1*$^{flox/-}$ mutants appears normal (C versus D, H versus I, arrows and open arrowheads). Scale bars: 100 m.

VEGFA isoforms can upregulate the expression of NRP1 itself, as well as that of other arterial-specific markers, in embryonic endothelial cells.

Discussion

In this study, we have used a conditional gene inactivation approach to investigate the signaling systems that control nerve-dependent arteriogenesis and blood vessel patterning in developing limb skin. Our data provide the first in vivo evidence that VEGF, which is derived from local tissue sources, is required to induce arteriogenesis during angiogenic remodeling of a primitive capillary network. The results also imply an important role for the heparin-binding isoform of VEGF in arterial differentiation in vivo. Consistent with this, we provide evidence that the NRP1-binding isoform of VEGF is a more potent inducer of arterial markers in vitro, compared with other VEGF isoforms. These data suggest a model in which a 'winner-takes-all' competition for VEGF may control arterial differentiation, with the outcome biased by a VEGF^{164}-NRP1 positive-feedback loop. The requirement for NRP1 may also help to explain how arteriogenesis is restricted to those vessels located in close proximity to the nerve.

Despite their profound effects on arterial differentiation, neither the *Vegfa* nor the *Nrp1* conditional mutants disrupted nerve-vessel alignment. This genetic uncoupling of arteriogenesis and nerve-vessel association suggests that guidance of the vessel branching pattern by the nerve requires either a very low concentration of VEGF, or more likely a second, as yet unidentified, signaling system (Fig. 8A).

Peripheral nerve-derived VEGFA is required for arteriogenesis in the limb skin vasculature in vivo

Previous studies have indicated that VEGF is required for de novo differentiation of the dorsal aorta in zebrafish (Lawson et al., 2002), and is sufficient to increase the density of cardiac arterial vessels when overexpressed in the heart (Visconti et al., 2002). However, these experiments did not address whether VEGF is required for arterial differentiation from a pre-existing capillary network, during normal development in vivo. The present results indicate that nerve-derived VEGFA is required for arteriogenesis in the primitive capillary plexus of murine limb skin. These data provide additional evidence that VEGF functions as an arterial-inducing signal in vivo, in addition to its classical role as a growth and survival factor for endothelial cells (Ferrara, 2000). Interestingly, the degree of arterial differentiation in the nerve-specific *Vegfa* mutants depended upon the extent of *Vegfa* deletion (as observed in different *Wnt1- or Isl1-Cre*-containing genotypes; Fig. 2). This suggests a dose-sensitive requirement for VEGFA in nerve-dependent arteriogenesis, consistent with our in vitro observations (Mukouyama et al., 2002).

Recent data have demonstrated that the endothelial specific Notch ligand Delta-like 4 (Dll4) is essential for arteriogenesis in vivo (Duarte et al., 2004; Gale et al., 2004; Krebs et al., 2004). Studies in zebrafish have suggested that dorsal aorta formation is controlled by VEGF and Notch signaling, acting in series (Lawson et al., 2001; Lawson et al., 2002). In preliminary experiments, we have found that arterial markers are induced in cultured murine embryonic endothelial precursor cells not only by VEGF, as previously reported (Mukouyama et al., 2002), but also by soluble Notch ligands. Interestingly, the ability of VEGF to induce arterial marker expression is blocked by peptide inhibitors of Notch activation, while a soluble VEGF receptor antagonist does not block induction of such markers by Notch ligands (Y.M. and D.J.A., unpublished). These data suggest that in mouse, as in zebrafish, VEGF induces arterial differentiation by acting through the

Notch pathway. How the interaction between these two signaling systems occurs is not yet clear.

What cell type(s) within peripheral nerve are the most important source(s) of VEGFA? We observed the strongest reduction of arterial marker expression when *Vegfa* was deleted in all three neural cell types (*Wnt1-Cre+Isl1-Cre*), and a weaker phenotype using either *Wnt1-Cre* or *Isl1-Cre* alone (Fig. 2 and data not shown). One explanation for this higher expression is that it reflects a requirement to eliminate *Vegfa* expression in all three peripheral nerve cell types, as *Isl1-Cre* is not expressed in Schwann cells, while *Wnt1-Cre* is not expressed in motoneurons. Motoneurons are not the primary source of VEGF in the nerve, however, as arterial marker expression is normal in *Olig1; Olig2* double mutants (Zhou and Anderson, 2002), which lack all spinal motoneurons, but have a normal complement of sensory neurons (Y.M., Q. Zhou and D.J.A., unpublished). A Schwann cell-specific knockout of *Vegfa* is currently not possible, because all available Cre driver lines active in these peripheral glia, including *Po-Cre* (Feltri et al., 1999; Giovannini et al., 2000), desert hedgehog (*Dhh*)-*Cre* (Lindeboom et al., 2003) and *BFABP-Cre* (Anthony et al., 2004), also promote recombination in peripheral neurons (Y.M. and D.J.A., unpublished). This most probably reflects the early expression of these glial promoters in multipotent neural crest stem or progenitor cells (Kim et al., 2003).

A second explanation is that, since both *Wnt1-Cre* and *Isl1-Cre* are expressed in sensory neurons, the stronger phenotype of *Wnt1-Cre*, *Isl1-Cre* embryos reflects a higher level of Cre expression in these cells, leading to more efficient recombination at the *Vegfa* locus. The structure of the conditional *Vegfa* allele makes it impossible to determine whether individual sensory neurons have undergone Cre-mediated recombination in one or both alleles. However, the reduction of VEGFA immunoreactivity in the DRG appeared greater in *Wnt1-Cre; Isl1-Cre* mutants than in *Wnt1-Cre* mutants (data not shown), suggesting a more efficient recombination with both Cre drivers.

NRP1 is important for arteriogenesis

An endothelial-specific knockout of *Nrp1* produced an even more severe defect in arteriogenesis than was observed in the nerve-specific *Vegfa* conditional knockouts. There are several possible explanations for this difference. First, cardiac defects in the endothelial *Nrp1* mutants (Gu et al., 2003) might affect peripheral arteriogenesis via impaired blood flow; by contrast cardiac development in the nerve-*Vegfa* mutants appears to be normal. We think this is unlikely, because dorsal aorta arteriogenesis is normal in conventional *Nrp1*-null mice (Y.M., D.J.A. and H. Fujisawa, unpublished). Second, the *Tie2-Cre; Nrp1* phenotype could reflect a requirement for a ligand of NRP1 other than VEGF^{164}, such as a semaphorin. In avian embryos, implantation of Sema3a protein or of a neutralizing anti-Sema3a antibody, caused a failure of proper vascularization (Bates et al., 2003). Others have reported angiogenesis defects in *Sema3a* mutant mice on a CD-1 genetic background (Serini et al., 2003). However, we observed no defects in limb skin arteriogenesis in *Sema3a*-null mice (Taniguchi et al., 1997), on the C57Bl6 background used here (Mukouyama et al., 2002). Third, the stronger arteriogenesis defects in *Tie2-Cre; Nrp1* embryonic limb skin vasculature may reflect a more complete interruption of VEGF signaling

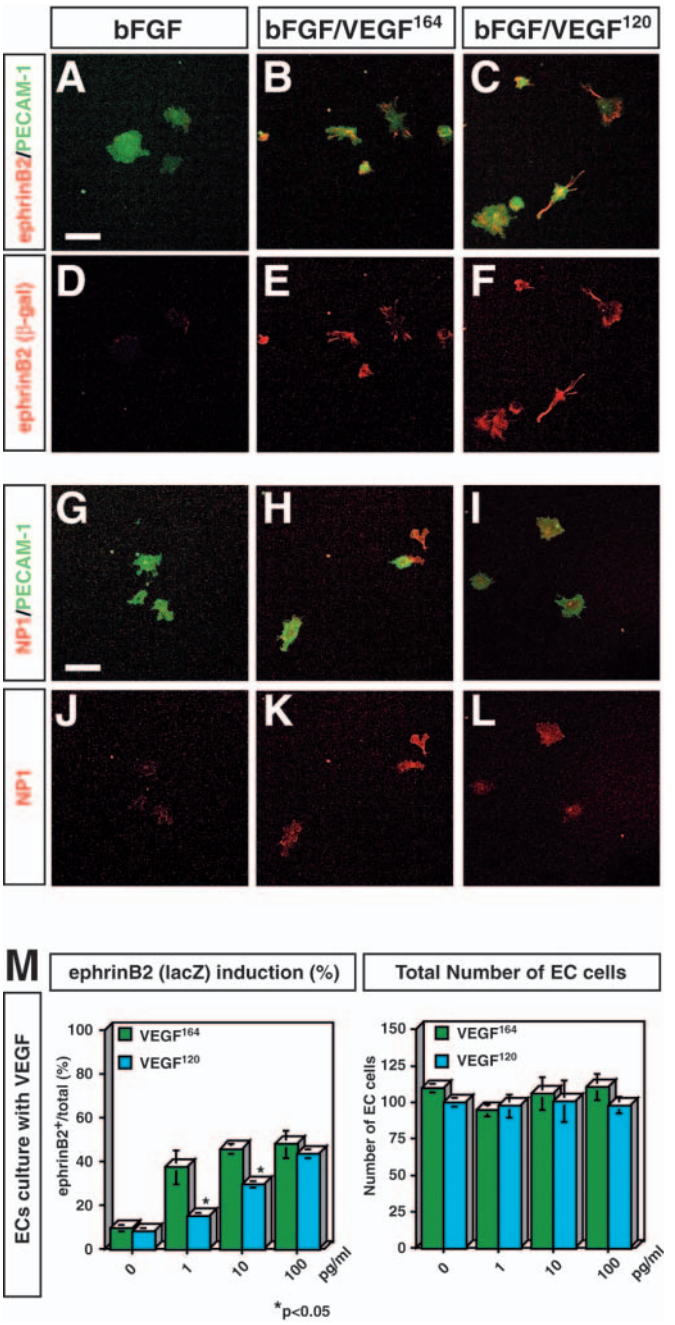

Fig. 7. NRP1 expression and arterial induction by VEGF^{164} in vitro. (A-L) PECAM1^{+}, ephrin B2-β-gal^{-} embryonic endothelial cells isolated by flow cytometry from *Efnb2$^{lacZ/+}$* knock-in embryos were cultured in 100 pg/ml of VEGF^{120} or VEGF^{164} plus 10 ng/ml bFGF for 2 days, and double-labeled with antibodies to either β-gal (A-F, red) or NRP1 (G-L, red) together with PECAM1 (A-C,G-I, green). Scale bars: 100 m. (M) Preferential induction of ephrin B2 by VEGF^{164}. Cells as in A-L were cultured in the indicated concentrations of VEGF^{120} or VEGF^{164} for 2 days, followed by double-labeling with anti-PECAM and X-gal. Both the percentage of ECs expressing ephrin B2-*lacZ* (left) and the total number of ECs (right) were determined. The concentrations of VEGF^{120} were 1.4-fold higher, on a molar basis, then the concentration of VEGF^{164} used at each serial dilution. Bars represent mean±s.e.m. Asterisks indicate statistically significant differences (P<0.05) between VEGF^{164} and VEGF^{120} (Student's t-test).

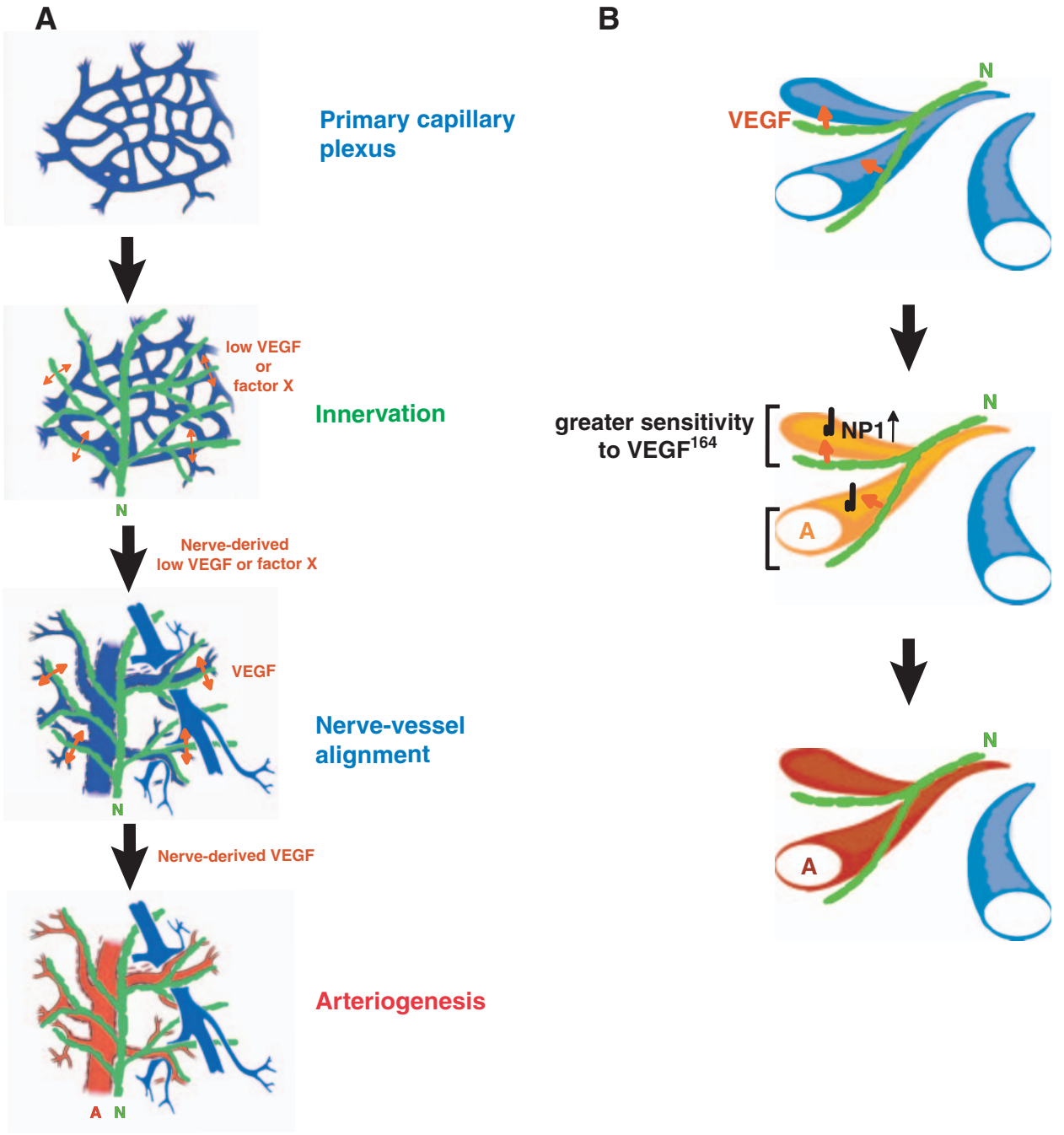

Fig. 8. Schematic models for nerve-mediated arterial differentiation and vascular branching in the limb skin. (A) Proposed sequence of events in vascularization of limb. A low concentration of VEGFA, or a distinct nerve-derived signal ('factor X') promotes nerve-vessel alignment, followed by VEGFA/NP-1-dependent arteriogenesis in nerve-aligned vessels. Modified from Cleaver and Krieg (Cleaver and Krieg, 1999). (B) VEGF promotes arteriogenesis via an NRP1-mediated positive-feedback loop. All vessels (blue) are initially equivalent. Nerve-derived VEGFA promotes arterial differentiation and NRP1 amplifies the VEGFA effect due to increased sensitivity to $VEGF^{164}$ in vessels in close proximity to nerves (N, green). A, artery.

than is obtained in *Wnt1-Cre; Isl1-Cre; Vegf*$^{flox/flox}$ embryos. This could occur either because deletion of a crucial co-receptor in endothelial cells reduces responsiveness to $VEGF^{164}$ derived from all local tissue sources, neural or otherwise, and/or because Cre-mediated recombination of *Nrp1* is required in only one allele to achieve reduction to homozygosity, whereas recombination of both *Vegf*^{flox} alleles is required.

What might be the function(s) of NRP1 in arteriogenesis? Because NRP1 is arterial specific (Moyon et al., 2001; Mukouyama et al., 2002) and is itself induced by VEGF (Y.M., H.-P.G., N.F. and D.J.A., unpublished) (Fig. 7) (Oh et al., 2002), it could mediate a positive-feedback loop, that increases the sensitivity of nascent arterial cells to VEGF. This feedback loop could be initiated by non-NRP1-binding isoforms of VEGF (e.g. $VEGF^{120}$), which we have shown induce NRP1 in

vitro, and maintained or further amplified by the NRP1-binding isoform. These considerations suggest a model in which a 'winner-takes-all' competition for VEGF may control arterial differentiation, with the outcome biased by a $VEGF^{164}$-NRP1 positive-feedback loop (Fig. 8B). In addition, because NRP1 is selective for the heparin-binding isoform of VEGF, it could also function to restrict induction of arterial markers to those vessels in close proximity to nerves.

This model predicts that nerve-dependent arteriogenesis should be compromised in mice selectively lacking the NRP1-binding isoform. But in such mice, e.g. those expressing only $VEGF^{120}$, arterial differentiation is normal in both the retina (Stalmans et al., 2002) and developing limb (Y.M., D.J.A. and P. Carmeliet, unpublished). The levels of $VEGF^{120}$ expression in these mutant mice, however, are similar to the combined levels of $VEGF^{120}$, $VEGF^{164}$ and $VEGF^{188}$ expression in wild-type mice, i.e. almost three-fold higher than normal (Carmeliet et al., 1999). As we have shown in vitro, at higher concentrations (>10 pg/ml), $VEGF^{120}$ is equally effective as $VEGF^{164}$ at inducing arterial differentiation. Therefore the increased levels of $VEGF^{120}$ in $Vegf^{120/120}$ mice may obscure the requirement for the $VEGF^{164}$ isoform in arteriogenesis. Such an explanation would be consistent with the fact that in zebrafish, overexpression of either $Vegf^{121}$ or $Vegf^{165}$ can rescue arterial differentiation blocked by a deficiency of *Shh* signaling (Lawson et al., 2002).

Although a commercial source of both VEGF isoforms was used according to the manufacturer's technical specifications (R&D), we recognize the possibility that these two preparations might have differences in specific activity that could account for their differential ephrin B2-inducing activities in our assay. It is difficult to compare the activities of these two isoforms independently of the presence of NRP1, because both isoforms induce this co-receptor at concentrations well below those at which they promote survival and proliferation. However, using porcine aortic endothelial cells, which do not express NRP1, Soker et al. (Soker et al., 1998) demonstrated that both $VEGF^{121}$ and $VEGF^{165}$ had equal activities in a chemotaxis assay when the cells were reconstituted with exogenous VEGFR2 (Soker et al., 1998). Furthermore the concentrations of $VEGF^{120}$ used in our assay were 1.4-fold higher, on a molar basis, than the concentration of $VEGF^{164}$ used at each serial dilution. The fact that the latter isoform exhibited more potent ephrin B2-inducing activity, under conditions where there was a bias in favor of $VEGF^{120}$, makes it more likely that the difference observed is indeed intrinsic to the two isoforms.

Relationship between arteriogenesis and nerve-vessel alignment

During normal development, arteriogenesis is immediately preceded by nerve-vessel alignment and is dependent on the presence of nerves (Mukouyama et al., 2002). Our results demonstrate that it is possible to genetically uncouple these sequential processes: in mutants that disrupt VEGF signaling from the nerve to the vessels, arteriogenesis is disrupted, while nerve-vessel alignment is apparently unperturbed. The simplest explanation for this uncoupling is that a nerve-derived factor distinct from VEGF mediates the alignment process. However, the residual VEGF detected in the conditional *Vegf* knockout embryos leaves open the possibility that nerve-vessel alignment simply requires a lower threshold level of VEGF, than does arteriogenesis. Nevertheless, the fact that the arteriogenesis defect is even stronger in the endothelial-specific *Nrp1* knockout, while nerve-vessel alignment remains intact, argues against the dual-threshold model. At the very least, for such a model to be tenable, the *Tie2-Cre; Nrp1* data would require that alignment depend only on the non-heparin-binding, more diffusible VEGF isoforms. This is reasonable given that alignment presumably requires action-at-a-distance to attract the vessels to the nerves. However, we did not see any significant defects in nerve-vessel alignment in the limb skin of mutants that lack the $VEGF^{120}$ isoform (Y.M., D.J.A. and P. Carmeliet, unpublished). Therefore, the available data favor the notion that VEGF and a separate, as yet unidentified signal, mediate arteriogenesis and nerve-vessel alignment, respectively.

Is nerve-vessel alignment stochastic or deterministic?

What determines which blood vessels in skin become aligned with nerves and undergo arteriogenesis? One explanation is that the process is stochastic: all vessels are equally capable of becoming aligned with nerves, and the selection of a subset for arteriogenesis is simply determined by their initial proximity to nerves at the time of innervation. A mechanism involving a 'winner-takes-all' competition for limiting amounts of nerve-derived VEGF, biased by the $VEGF^{164}$-NRP1 positive-feedback loop discussed earlier, would be well suited to such a mechanism (Fig. 8B). Alternatively, a subset of vessels may be pre-specified for association with the nerve. For example, if nerves release a signal that recruits vessels to align with them, then a subset of vessels might express higher levels of a receptor for this alignment signal, before the nerves ever arrive. Such a pre-specification mechanism would, however, require precise matching to ensure a sufficient supply of presumptive arterial vessels for developing nerves. The identification of nerve-derived signals for vessel alignment may help to distinguish between these alternatives.

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Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/132/5/941/DC1

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