Spatiotemporal Distribution of Neurovascular Alignment in Remodeling Adult Rat Mesentery Microvascular Networks

Peter C. Stapor · Walter L. Murfee

Department of Biomedical Engineering, Tulane University, New Orleans, La., USA

Key Words

Angiogenesis · Microcirculation · Neurogenesis ·
Neurovascular alignment · Peripheral nerves

Abstract

An emerging area of microvascular research focuses on the links between neural and vascular patterning. However, the functional dependence between vascular and neural growth in adult tissues remains underinvestigated. The objective of this study was to determine the spatial and temporal coordination between vascular and neural networks over a time course of adult microvascular growth. Mesentery tissues from adult male Wistar rats were harvested prior to stimulation, and 2, 10 and 30 days after angiogenesis stimulated by mast cell degranulation. Tissues were immunolabeled for PECAM (endothelial cell marker) and class III β -tubulin (peripheral nerve marker). Neurovascular alignment was quantified per vessel category: arterioles ($>20\ \mu\text{m}$), pre-capillary arterioles ($10\text{--}20\ \mu\text{m}$), post-capillary venules ($10\text{--}20\ \mu\text{m}$), venules ($>20\ \mu\text{m}$), capillaries ($<10\ \mu\text{m}$) and capillary sprouts. Neurovascular alignment along pre-capillary arterioles, capillaries, post-capillary venules and venules was decreased compared to unstimulated levels on days 2 and 10. These decreases inversely correlated with increases in vessel density per vessel category. By day 30, alignment either returned to unstimulated levels or was increased compared to day 10.

These results suggest that neurovascular alignment arises after microvascular network growth and is present along arterioles, venules and even capillaries.

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Introduction

Neurovascular alignment is critical for microvascular function. During development, peripheral vascular and neural networks exhibit similar anatomical structures and recent work has highlighted the common molecular signals involved in the growth of both systems [1]. Overlapping signaling molecules include ephrins and their Eph receptors, Slits and their Robo receptors, semaphorins and their plexin and/or neuropilin receptors, and netrins and their DCC and Unc5 receptors [2–4]. While the functional relationships between vascular and nerve growth are apparent, how one network influences the pattern of the other and the extent of neurovascular alignment along an angiogenic microvascular network remain underinvestigated.

Neural networks are capable of influencing microvascular patterning and, conversely, microvascular networks can direct neural growth. During development in the avian forelimb, neurovascular alignment arises as major vessels and nerves independently follow stereo-

typed pathways in cases of either vascular or neural lead growth [5–7]. Neuronal precursors preexisting in the adult brain are able to induce angiogenesis and endothelial cell tube formation [8]. Neurons can also secrete VEGF to influence capillary sprouting and arterial differentiation [9, 10]. In the adult retina, preexisting glial cells were observed to guide capillary sprout formation [11]. Angiogenesis has also been shown to precede neurovascular alignment. For example, the initial capillary plexus in embryonic mouse limb skin is established before neural in-growth [10, 12]. In adult wound healing and skin graft models, innervation follows vascular patterns established during tissue vascularization [13, 14]. While these studies have provided valuable information regarding the potential patterning relationships between the two systems, insight into their interdependence during the angiogenic process can be gained by examining the extent of neurovascular alignment across a remodeling adult microvascular network.

The objective of this study was to determine the spatial and temporal coordination between vascular and neural networks over a time course of adult microvascular growth. We examined nerve alignment with vessels across the hierarchy of rat mesenteric microvascular networks after stimulation using an inflammatory model of angiogenesis. The rat mesentery was selected because it offers a unique two-dimensional view of intact microvascular networks down to the single cell level. The results suggest that sympathetic nerves align with microvascular arterioles, capillaries and venules in unstimulated networks, and that microvascular growth precedes neural growth.

Materials and Methods

Mast Cell Degranulation Model of Angiogenesis

All experiments were performed in accordance with the guidelines of the Tulane University Care and Use Committee. Single 2-ml doses of compound 48/80 were injected intraperitoneally over the time course of 3 days (twice a day and once on the final day) in increasing concentrations (200, 400, 600, 800 and 1,000 $\mu\text{g}/\text{ml}$ in saline) into 350- to 450-gram adult male Wistar rats, according to a previously established protocol [15]. Mesenteric windows were harvested and processed for immunohistochemistry in the unstimulated group ($n = 4$ animals), and in the compound 48/80-stimulated groups 2 ($n = 4$ animals), 10 ($n = 4$ animals) and 30 days after stimulation ($n = 4$ animals). A mesenteric window is defined as the thin translucent membrane in-between the artery/vein pairs feeding the small intestine. Three end time points after 48/80 stimulus were included in the study to capture the initial time course of network growth. From each of the 4 animals per group, 3 randomly selected networks from ran-

domly selected tissues were analyzed ($n = 12$ tissues per time point). This particular model was selected because it produces a robust angiogenic response over the hierarchy of mesenteric microvascular networks within a relatively short time period.

Immunohistochemistry

Tissues were fixed in methanol at -20°C for 30 min, and then labeled with antibodies against PECAM (CD31) and class III β -tubulin, or class III β -tubulin and tyrosine hydroxylase (TH). PECAM + class III β -tubulin labeling protocol: (1) 36-hour incubation at 4°C with 1:200 mouse monoclonal class III β -tubulin antibody (2G10; Abcam, Cambridge, Mass., USA); (2) 36-hour incubation at 4°C with 1:100 goat anti-mouse CY2-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, Pa., USA) and 5% normal goat serum (Jackson ImmunoResearch Laboratories), diluted in antibody buffer; (3) 1-hour incubation at room temperature with 5% normal mouse serum (Jackson ImmunoResearch Laboratories); (4) 1-hour incubation at room temperature with 1:200 mouse monoclonal biotinylated CD31 antibody (BD Pharmingen, San Diego, Calif., USA), and (5) 1-hour incubation with CY3-conjugated streptavidin secondary antibody buffer (Jackson ImmunoResearch Laboratories). Class III β -tubulin + TH labeling protocol: (1) 36-hour incubation at 4°C with 1:200 rabbit polyclonal TH antibody, and 1:200 mouse monoclonal class III β -tubulin antibody diluted in antibody buffer (0.1% saponin in PBS + 2% BSA), and (2) 36-hour incubation at 4°C with 1:100 goat anti-rabbit CY2-conjugated antibody and 1:100 goat anti-mouse CY3-conjugated secondary antibody (Jackson ImmunoResearch Laboratories) diluted in antibody buffer.

Quantification of Vascular Remodeling and Neurovascular Alignment along Vessel Hierarchy

Vascular densities and neurovascular alignment was quantified per network ($n = 4$ rats; 12 networks per time point). A network was defined as having a feeding arteriole, draining venule and multiple branches. Five-by-five image areas of the networks were captured using a $10\times$ objective (Dry; NA = 0.3) on an inverted microscope (Olympus IX70) coupled with a PixelFly camera. Higher magnification images (fig. 1, 2, 4, 7) were acquired using a $20\times$ (Oil; NA = 0.8) or $60\times$ objective (Oil; NA = 1.4) and an inverted microscope (Olympus IX70) coupled with a Photometrics CoolSNAP EZ camera. The number of vessel segments per image for arterioles ($>20\ \mu\text{m}$), pre-capillary arterioles ($10\text{--}20\ \mu\text{m}$), capillaries, post-capillary venules ($10\text{--}20\ \mu\text{m}$), venules ($>20\ \mu\text{m}$) and capillary sprouts were counted on every 3rd image using the Java-based NIH image processing software ImageJ version 1.43 u. Vessel types were identified by endothelial cell morphology, vessel diameter and location within the network. Blood vessels and nerves were considered aligned when they were within $10\ \mu\text{m}$ of each other for at least $50\ \mu\text{m}$. The number of aligned vessel segments per vessel category was counted at each time point.

Alternative Models of Angiogenesis

All experiments were performed in accordance with the guidelines of the Tulane University Care and Use Committee. For chronic hypoxia stimulation of angiogenesis, animals were placed in a chamber and maintained at 10% oxygen for 3 days. The temperature, humidity and carbon dioxide levels were monitored and maintained at normal levels. The mesentery exteriorization model entails exposing 8 mesenteric tissues outside the peritoneal cav-

Fig. 1. Examples of neurovascular alignment in adult rat mesentery microvascular networks immunolabeled for PECAM (red, endothelial cells) and class III β -tubulin (green, nerves; colors are shown in the online version). Neurovascular alignment is observable in unstimulated tissues along arterioles (**a**), venules (**b**) and capillary sprouts (**c**). A = Arteriole; V = venule; c = capillary; arrows point to nerves aligned with vessels. Scale bars = 50 μ m.

ity for 20 min. Three days after exteriorization, tissues were harvested. Both models have previously been documented as angiogenic stimuli [15]. Tissues were immunolabeled for PECAM and class III β -tubulin.

Statistical Analysis

Network remodeling and neurovascular alignment metrics were compared across the time course of angiogenesis after compound 48/80 stimulation using one-way ANOVA followed by Tukey's pairwise comparison test (SigmaStat). Statistical significance was accepted for $p < 0.05$. Values are presented as means $\pm$ SEM.

Results

Neurovascular alignment in unstimulated adult male rat mesenteric microvascular networks was observed along arterioles, venules and capillaries (fig. 1). Along

capillary sprouts, examples of nerve alignment were also noted (fig. 2). Nerves both led and lagged endothelial tip cells. Alignment was most frequent in proximal regions of the network along feeding arterioles and draining venules (fig. 3a). Ten days after 48/80 stimulation, networks displayed a dramatic increase in vessel density (fig. 3b), yet the increase in new vessels was not accompanied by apparent nerve growth or neurovascular alignment. By day 30, stimulated mesenteric tissues displayed increased vascular density, neural density and neurovascular alignment across the hierarchy of the remodeled vascular network (fig. 3c). Nerves were identified based on morphology and class III β -tubulin-positive labeling. Class III β -tubulin-positive nerves positively labeled for TH indicating that they are sympathetic nerves (fig. 4). As evidence that nerves were not sensory, they did not label positively for substance P (online suppl. fig. S1; see www.

Fig. 2. Example of neurovascular alignment along a capillary sprout (S). Immunolabeling for PECAM (**a, d**) identifies endothelial cells and class III β -tubulin (**b, e**) identifies nerves. Arrows indicate nerve location along the sprout. **a–c** Individually labeled

and merged images of nerve extension past the endothelial tip cell. **d–f** Higher magnification images of the sprout tip. Scale bars = 20 (**a–c**) and 10 μ m (**d–f**).

karger.com/doi/10.1159/000336714 for all online suppl. material). Nerve identification using class III β -tubulin was additionally confirmed by immunohistochemical labeling with PGP9.5, NG2 and neurofilament (data not shown).

Microvascular network growth after 48/80 stimulation is supported by the quantification of vessel segment density per vessel type across the time course of initial network growth (fig. 5). Angiogenesis, characterized by sprouts per vascular area, peaked 2 days after stimulation. By day 30, capillary sprouting returned to normal levels compared to the unstimulated tissues (fig. 5f). Large arteriole ($>20 \mu$ m in diameter) density increased 2

days after stimulation, but was similar to the unstimulated density on days 10 and 30 (fig. 5a, d). The density of large venules ($>20 \mu$ m in diameter) increased by day 2 and returned to the unstimulated level by day 30. The dramatic network growth can be attributed to increased pre-capillary arteriole (10–20 μ m), capillary, and post-capillary venule (10–20 μ m) densities over the 30-day time course. Segment densities were increased by day 10 and remained elevated by day 30 (fig. 5b, c, e).

The increases in vessel densities 10 days after stimulation were temporally correlated with decreased neurovascular alignment per vessel type. The percentage of vessels aligned with nerves was determined as the num-

Fig. 3. Representative montages of adult rat mesentery window microvascular networks unstimulated (**a**), and 10 (**b**) and 30 days (**c**) after compound 48/80 stimulation immunolabeled for PECAM (red, endothelial cells) and class III β -tubulin (green, nerves; colors are shown in the online version). **a** In unstimulated tissues, PECAM and class III β -tubulin labeling identified aligned vessels and nerves (arrows). **b** Vascular density increased by day 10, while neurovascular alignment decreased in the network. **c** By day 30 after stimulation, neurogenesis occurred and neurovascular alignment either increased compared to day 10 or returned to unstimulated levels. Scale bars = 200 μ m.

Fig. 4. Representative images of nerves (arrows) in unstimulated adult rat mesentery tissue immunolabeled with class III β -tubulin (red) and TH (green; colors are shown in the online version). TH identifies the class III β -tubulin-positive neurons as sympathetic nerves. Scale bars = 20 μ m.

Fig. 5. Quantification of vessel segment density in unstimulated microvascular networks (Un), and networks 2, 10 and 30 days (D) after compound 48/80 stimulation. Vessel densities represented by vessel segment per area (mm^2) were measured for six vessel categories: arterioles $>20 \mu\text{m}$ (a), pre-capillary arterioles 10–20

μm (b), capillaries (c), venules $>20 \mu\text{m}$ (d), post-capillary venules 10–20 μm (e) and sprouts (f). Statistical comparisons were made using a one-way ANOVA followed by Tukey's pairwise comparison test. ^a $p < 0.05$ vs. unstimulated tissues, ^b $p < 0.05$ vs. the previous time point. Values are means $\pm$ SEM.

ber of vessel segments with nerves aligned divided by the total number of vessel segments. Ninety percent of large arterioles ($>20 \mu\text{m}$ in diameter) were aligned with nerves in unstimulated tissue. No significant difference was seen across time points (fig. 6a). The percentage of small arterioles (10–20 μm) aligned with nerves decreased from 78 to 45% by day 2, and to 21% by day 10. By day 30, 44% of small arterioles were aligned with nerves, yet this percentage was still less than the unstimulated level (fig. 6b). The inverse temporal correlation between vessel growth and nerve alignment over 10 days followed by a return toward unstimulated alignment levels was also characteristic for capillaries, post-capillary venules (10–20 μm) and larger venules ($>20 \mu\text{m}$ in diameter). Capillary align-

ment with nerves was reduced 2 and 10 days after stimulation from 45 to 11 and 5%, respectively. Alignment along capillaries increased from 5% on day 10 to 24% by day 30, but still had not returned to alignment percentages observed in unstimulated tissues (fig. 6c). The percentages of large and small venules aligned with nerves decreased by day 10 from 58 and 48 to 24 and 11%, respectively. By day 30, venule alignment percentages returned to statistically similar levels as unstimulated networks (fig. 6d, e). The percentage of sprouts aligned with nerves was significantly reduced 2 and 10 days after 48/80 stimulation compared to the unstimulated group and returned to the unstimulated levels by day 30 (fig. 6f).

Fig. 6. Quantification of the percentage of vessel segments aligned with nerves in unstimulated microvascular networks (Un), and networks 2, 10 and 30 days (D) after compound 48/80 stimulation. Neurovascular alignment percentages were determined as the percentage of segments with a nerve aligned out of the total number of vessel segments per vessel category: arterioles >20 μm (a),

pre-capillary arterioles 10–20 μm (b), capillaries (c), venules >20 μm (d), post-capillary venules 10–20 μm (e) and sprouts (f). Statistical comparisons were made using a one-way ANOVA followed by Tukey's pairwise comparison test. ^a $p < 0.05$ vs. unstimulated tissues, ^b $p < 0.05$ vs. the previous time point. Values are means ± SEM.

In order to confirm that neural alignment lagging vascular growth during angiogenesis was not model specific, we also harvested mesenteric tissues after two alternative stimuli: chronic hypoxia and mesentery exteriorization. For both scenarios, angiogenic networks were characterized by high capillary density and vessel tortuosity and lacked obvious neural/vascular alignment along smaller vessels. These qualitative observations from hypoxia and exteriorization models support the quantitative data on days 2 and 10 presented for the mast cell degranulation model (fig. 7). The chronic hypoxia model represents a more physiological stimulus and is relevant to tissue ischemia scenarios. The exteriorization model represents an alternative inflammatory stimulus [15].

Discussion

The primary finding of this study is that neurovascular alignment follows microvascular growth in adult rat mesentery microvascular networks. Neurovascular alignment has previously been observed in the rat mesentery down to pre-capillary arterioles and post-capillary venules [16]. We show that alignment is also present throughout the network down to the capillary level indicating that nerves do associate with blood vessels along all microvascular vessel types (fig. 6). After 48/80 stimulation, mesenteric microvascular network growth is characterized by a dramatic increase in angiogenesis and subsequent increases in the densities of arterioles, capillaries

Fig. 7. Representative montages of mesentery window microvascular networks stimulated by chronic hypoxia (**a**) and mesentery exteriorization (**b**). Neurovascular alignment was prominent in proximal regions of microvascular networks (arrows). Distal re-

gions of angiogenic microvascular networks were void of neurovascular alignment. Scale bars = 100 μ m. A = Arteriole; V = venule; c = capillary.

and venules. Initial network growth is then followed by neurovascular alignment.

The link between neural and vascular patterning has recently emerged as a new area of microvascular research. At the molecular level, the link is supported by shared signaling mechanisms, including Ephs/ephrins, semaphorins/neuropilins and plexins and netrins/DCC and Unc5 receptors, as well as multiple growth factors [1, 3, 17]. However, the spatial relationships between both systems in adult tissues and during angiogenesis remain unclear. In embryonic mouse limb skin, neural-derived VEGF directs arterialization through the neuropilin 1 receptor after a capillary bed has been established [10]. VEGF derived from arteries also coordinates growth of motor neurons and sympathetic innervation [18, 19]. All together, the coordinated growth mechanisms involved in neurovascular alignment are just beginning to be understood. Insight can be gained by examining the spatial interactions between the two systems across different vessel types within adult microvascular networks. In 2005, Bearden and Segal [20] showed that somatic motor nerves in adult mouse skeletal muscle align with arterioles 15–60 μ m in diameter. These observations are in agreement with our results that nerves align with arterioles in rat mesenteric microvascular networks. We also show that nerves are capable of aligning with microvessels across the hierarchy of a network, including venules and capillaries.

In adult tissue denervation and sympathectomy models, nerves use vessels as guides while reinnervation occurs [18, 19]. During remodeling associated with skin and neuronal grafts, neovascularization precedes neural growth [13, 14, 21]. Our results provide additional support for neurovascular alignment trailing angiogenesis and add a valuable view of these dynamics across the hierarchy of an intact microvascular network.

In a study by Furness and Marshall [22], sympathetic nerves in adult rat mesenteric networks have been shown to influence vessel diameter. Paravascular nerve stimulation affected the diameters of terminal arterioles and larger arterioles, but not pre-capillary arterioles. On the venule side, only venules >30 μ m were affected by paravascular nerve stimulation. These results and our observations of nerves present along a percentage of smaller arterioles and venules in unstimulated tissue motivate the need for future investigations aimed at determining whether nerve presence necessarily correlates with neuronal function. Our characterization of neurovascular alignment over the time course of microvascular network growth also motivates future studies to determine when new nerves become functional.

The rat mesentery represents a model to study the interaction of multiple cell types involved in microvascular remodeling [15, 23]. It allows for observation across the entire hierarchy of intact microvascular and neural networks down to the cellular and sub-cellular level at dif-

ferent time points during microvascular remodeling. Unstimulated tissues display nominal levels of angiogenesis intrinsic to physiological vascular network expansion within mesenteric windows. In this study, we stimulated rapid network growth via compound 48/80 injections. Compound 48/80 stimulation produces a robust angiogenic response through mast cell activation and histamine release, followed by further network remodeling [24, 25]. The initial increase in capillary sprouting by day 2 corresponds with subsequent density increases for pre-capillary arterioles, capillaries, post-capillary venules and venules at day 10. As evident by increases in vascular density upstream and downstream of the capillary level, 48/80-stimulated angiogenesis is followed by arteriogenesis. Over the 30-day time course of network growth, the densities for the largest arterioles and venules transiently increases. By day 30 for venules and by day 10 for arterioles, vessel density is not statistically different from unstimulated levels. A possible explanation for these transient increases is vessel dilation, which is a characteristic of angiogenesis [26]. During angiogenesis, which peaks on day 2 in our model, dilation of smaller vessels could cause them to be counted as larger vessels. At later time points (i.e. day 30) when angiogenesis has returned to unstimulated levels, these vessels might then be classified as smaller vessels again.

The relative distribution of nerve alignment along growing networks implicates a role for nerves in the vessel maturation process rather than initial growth. To examine this potential correlation between perivascular cell and nerve recruitment, we additionally examined nerve and perivascular cell presence 10 days after compound 48/80 stimulation (online suppl. fig. S2). The majority of the α -smooth muscle actin-positive vessels were not associated with nerves. These observations indicate that the recruitment of α -smooth muscle actin-positive cells is not dependent on innervation. The presence of perivascular cells prior to nerve alignment is consistent with a role for perivascular cells in nerve recruitment and their ability to secrete nerve growth factor, a potent neurotrophic factor, which promotes sympathetic innervation of the microvasculature [27, 28].

We selected diameter categories for our study in order to capture potential differences in nerve/blood vessel alignment in different regions of a network. In particular, we focused on the capillary level, the pre- or post-capillary level, or larger vessels. Relative vessel diameter binning is justified because we used a consistent fixation method. However, tissue fixation could influence nominal diameter measurements. In our laboratory, we have

shown that methanol fixation could indeed influence vessel diameter (data not shown). Feeding mesenteric arterioles in the methanol fixation group were smaller when compared to vessels fixed with paraformaldehyde. The diameters of venules fixed in methanol were significantly smaller than those that were either unfixed or fixed with paraformaldehyde. In the context of our results, the potential effect of decreased diameter measurements due to methanol fixation would have caused larger vessels to be potentially counted in the pre-/post-capillary vessel category. Given that in general larger vessels are more aligned with nerves, this potential shift in vessel count would have caused an apparent increase in alignment in the smaller vessel category. Our main result is that nerve alignment lags vessel growth for these vessels, and, thus, the influence of any potential vessel shift can be assumed to be negligible.

In summary, the present study shows that neurovascular alignment arises after microvascular network growth along arterioles, venules and even capillaries, and suggests that neurogenesis lags behind angiogenesis in the adult rat mesentery. Current research on neurovascular patterning focuses on determining the molecular cues and cell dynamics which lead to neurovascular alignment. The results from this study offer insight into when and where neurovascular alignment occurs during a time course of microvascular network growth. Defining the related functional interactions and mechanisms in the adult will have important implications for tissue engineering, regenerative medicine and neurodegenerative-associated vascular diseases.

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References

- 1 Larrivee B, Freitas C, Suchting S, Brunet I, Eichmann A: Guidance of vascular development: lessons from the nervous system. *Circ Res* 2009;104:428–441.
- 2 Carmeliet P, Tessier-Lavigne M: Common mechanisms of nerve and blood vessel wiring. *Nature* 2005;436:193–200.
- 3 Park JA, Choi KS, Kim SY, Kim KW: Coordinated interaction of the vascular and nervous systems: from molecule- to cell-based approaches. *Biochem Biophys Res Commun* 2003;311:247–253.

- 4 Weinstein BM: Vessels and nerves: marching to the same tune. *Cell* 2005;120:299–302.
- 5 Bates D, Taylor GI, Newgreen DF: The pattern of neurovascular development in the forelimb of the quail embryo. *Dev Biol* 2002; 249:300–320.
- 6 Bates D, Taylor GI, Minichiello J, Farlie P, Cichowitz A, Watson N, Klagsbrun M, Mamluk R, Newgreen DF: Neurovascular congruence results from a shared patterning mechanism that utilizes Semaphorin3A and Neuropilin-1. *Dev Biol* 2003;255:77–98.
- 7 Martin P, Lewis J: Origins of the neurovascular bundle: interactions between developing nerves and blood vessels in embryonic chick skin. *Int J Dev Biol* 1989;33:379–387.
- 8 Ogunshola OO, Stewart WB, Mihalcik V, Solli T, Madri JA, Ment LR: Neuronal VEGF expression correlates with angiogenesis in postnatal developing rat brain. *Brain Res Dev Brain Res* 2000;119:139–153.
- 9 Ogunshola OO, Antic A, Donoghue MJ, Fan SY, Kim H, Stewart WB, Madri JA, Ment LR: Paracrine and autocrine functions of neuronal vascular endothelial growth factor (VEGF) in the central nervous system. *J Biol Chem* 2002;277:11410–11415.
- 10 Mukouyama YS, Gerber HP, Ferrara N, Gu C, Anderson DJ: Peripheral nerve-derived VEGF promotes arterial differentiation via neuropilin 1-mediated positive feedback. *Development* 2005;132:941–952.
- 11 Gerhardt H, Golding M, Fruttiger M, Ruhrberg C, Lundkvist A, Abramsson A, Jeltsch M, Mitchell C, Alitalo K, Shima D, Betsholtz C: VEGF guides angiogenic sprouting utilizing endothelial tip cell filopodia. *J Cell Biol* 2003;161:1163–1177.
- 12 Mukouyama YS, Shin D, Britsch S, Taniguchi M, Anderson DJ: Sensory nerves determine the pattern of arterial differentiation and blood vessel branching in the skin. *Cell* 2002;109:693–705.
- 13 Ferretti A, Boschi E, Stefani A, Spiga S, Romanelli M, Lemmi M, Giovannetti A, Longoni B, Mosca F: Angiogenesis and nerve regeneration in a model of human skin equivalent transplant. *Life Sci* 2003;73:1985–1994.
- 14 Hobson MI, Brown R, Green CJ, Terenghi G: Inter-relationships between angiogenesis and nerve regeneration: a histochemical study. *Br J Plast Surg* 1997;50:125–131.
- 15 Murfee WL, Rehorn MR, Peirce SM, Skalak TC: Perivascular cells along venules upregulate NG2 expression during microvascular remodeling. *Microcirculation* 2006;13:261–273.
- 16 Furness JB: Arrangement of blood vessels and their relation with adrenergic nerves in the rat mesentery. *J Anat* 1973;115:347–364.
- 17 Sandvig A, Berry M, Barrett LB, Butt A, Logan A: Myelin-, reactive glia-, and scar-derived CNS axon growth inhibitors: expression, receptor signaling, and correlation with axon regeneration. *Glia* 2004;46:225–251.
- 18 Marko SB, Damon DH: VEGF promotes vascular sympathetic innervation. *Am J Physiol Heart Circ Physiol* 2008;294:H2646–H2652.
- 19 Bearden SE, Segal SS: Microvessels promote motor nerve survival and regeneration through local VEGF release following ectopic reattachment. *Microcirculation* 2004;11: 633–644.
- 20 Bearden SE, Segal SS: Neurovascular alignment in adult mouse skeletal muscles. *Microcirculation* 2005;12:161–167.
- 21 Gu XH, Terenghi G, Kangesu T, Navsaria HA, Springall DR, Leigh IM, Green CJ, Polak JM: Regeneration pattern of blood vessels and nerves in cultured keratinocyte grafts assessed by confocal laser scanning microscopy. *Br J Dermatol* 1995;132:376–383.
- 22 Furness JB, Marshall JM: Correlation of the directly observed responses of mesenteric vessels of the rat to nerve stimulation and noradrenaline with the distribution of adrenergic nerves. *J Physiol* 1974;239:75–88.
- 23 Ponce AM, Price RJ: Angiogenic stimulus determines the positioning of pericytes within capillary sprouts in vivo. *Microvasc Res* 2003;65:45–48.
- 24 Franzen L, Ghassemifar R, Malcherek P: Experimental mast cell activation improves connective tissue repair in the perforated rat mesentery. *Agents Actions* 1991;33:371–377.
- 25 Norrby K, Jakobsson A, Sörbo J: Mast-cell-mediated angiogenesis: a novel experimental model using the rat mesentery. *Virchows Arch B Cell Pathol Incl Mol Pathol* 1986;52: 195–206.
- 26 Zawicki DF, Jain RK, Schmid-Schoenbein GW, Chien S: Dynamics of neovascularization in normal tissue. *Microvasc Res* 1981;21: 27–47.
- 27 Creedon D, Tuttle JB: Nerve growth factor synthesis in vascular smooth muscle. *Hypertension* 1991;18:730–741.
- 28 Zettler C, Rush RA: Elevated concentrations of nerve growth factor in heart and mesenteric arteries of spontaneously hypertensive rats. *Brain Res* 1993;614:15–20.