RESEARCH ARTICLE

JunB regulates angiogenesis and neurovascular parallel alignment in mouse embryonic skin

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ABSTRACT

Blood vessels and nerve fibers are often closely arranged in parallel throughout the body. Therefore, neurovascular interactions have been suggested to be important for the development of vascular networks. However, the molecular mechanisms and genes regulating this process remain unclear. In the present study, we investigated the genes that are activated in endothelial cells (ECs) following interactions with neurons during vascular development. Microarray analyses of human primary microvascular ECs co-cultured with mouse primary dorsal root ganglion cells showed that JunB is strongly upregulated in ECs by neurovascular interactions. Furthermore, the forced expression of JunB in ECs stimulated a tip-like cell formation and angiogenesis in vitro and induced vascular endothelial growth factor A (VEGFA) and the pro-angiogenic integrin subunit ITGB3 expression. Moreover, in vivo knockdown of JunB in ECs from developing mouse limb skin considerably decreased the parallel alignments of blood vessels and nerve fibers. Taken together, the present data demonstrate for the first time that JunB plays an important role in the formation of embryonic vascular networks. These results contribute to the molecular understanding of neurovascular interactions during embryonic vascular development.

KEY WORDS: JunB, AP-1 transcription factor, Neurovascular interaction, Vascular remodeling

INTRODUCTION

Blood vessels form vast and highly structured networks that pervade all organs in vertebrates and deliver oxygen, nutrition, biologically active substances and immune cells. These highly structured vascular networks are established by endothelial cells (ECs) and the cell–matrix and cell–cell interactions between various cell types, including pericytes, smooth muscle cells and neurons. The ensuing cell–matrix and cell–cell interactions are believed to function as guidance cues for EC differentiation and vascular development for establishing vascular networks. The loss of these guidance cues reportedly leads to abnormal vascularization, which causes several vascular diseases (Dorrell and Friedlander, 2006).

Andreas Vesalius (1514–1564) was the first to describe parallel alignments of blood vessels and nerves throughout the body (reviewed in Carmeliet and Tessier-Lavigne, 2005) and since then, the presence of neurovascular parallelism has been widely recognized. Accordingly, morphological and genetic evidence suggests that interaction between vascular and nervous systems is critical to appropriate patterning and branching of vascular networks (Carmeliet, 2003).

In recent studies, numerous molecular cues have been associated with guidance of EC tip cells and axonal growth cones, and these have been categorized as attractive and repulsive cues for both EC tip cells and axonal growth cones (reviewed in Wälchli et al., 2015). Hence, vascular patterning seems to depend on the combination of attractive and repulsive cues that control sites and times of developmental processes. In particular, arterial-specific ephrin-B2 (EFNB2) and venous-specific EphB4 regulate the establishment of the arterial–venous plexus and form distinct boundaries between these vessels through repulsive effects (Klein, 2004; Pasquale, 2005; Poliakov et al., 2004). In contrast, attraction between arteries and veins has been associated with apelin and its receptor APJ (also known as APLNR) signaling (Kidoya et al., 2015). Moreover, axon–artery interactions have been reported, and behaviors of endothelial tip cells and axonal growth cones are reportedly regulated by direct interactions with the ECM (Gerhardt et al., 2003; Ruhrberg et al., 2002). Moreover, endothelial tip cell selection is reportedly regulated by laminin–integrin signaling, which induces the expression ofDll4 in ECs (Estrach et al., 2011; Stenzel et al., 2011).

During neurovascular patterning and parallelism, local signals from peripheral nerves reportedly control arterial differentiation and patterning of vascular networks in embryonic mouse limb skin (Mukouyama et al., 2002). Moreover, peripheral nerve-derived VEGFA promotes arterial blood vessel differentiation via NRPI-mediated positive feedback (Mukouyama et al., 2005), although altered vascular patterning did not affect nerve patterns (Bates et al., 2003). Hence, cues for vascular patterning that are mediated by neurovascular interactions (i.e. interaction between the nervous and vascular system) and may only operate from nerves to ECs.

Recently, neurovascular alignment was shown to be dependent on the activity ofCxcl12–Cxc4 chemokine signaling. Specifically, peripheral neuron-derived Cxcl12 stimulates EC migration as an attractive cue for the recruitment and alignment of distant vessels with nerves (Li et al., 2013). Hence, several of the neuron-derived factors that are involved in neurovascular interactions have been described, although the ensuing EC-mediated intracellular signaling pathways that lead to neurovascular parallelism have not yet been fully elucidated.
In this study, we investigated EC gene regulatory networks that are triggered by neurovascular interactions during embryonic vascular network formation, and identified JunB as a novel angiogenic regulator of blood vessel patterning and the alignment with nerve fibers during vascular development.

RESULTS

Neurovascular interactions in developing embryonic mouse limb skin

Initially, we characterized interactions of ECs with nerve fibers at various stages of mouse embryonic limb skin development, and examined vascular and nerve network formation from embryonic day (E)9.5 to E17.5 using whole-mount immunohistochemical staining (Fig. 1A). In a previous study of neurovascular remodeling, angiogenesis was observed in the primary capillary plexus on E9.5 and E13.5, and peripheral nerve migration was observed on E15.5–17.5 (Mukouyama et al., 2002). In the present study, nerve fibers innervated tissues at E13.5, when remodeling of the pre-existing primary capillary commenced. Subsequently, neurovascular juxtapositional alignments were established (Fig. 1A), and following nerve fiber innervation at E13.5, the total branch lengths of blood vessels were increased (Fig. 1B) with decreases in individual branch lengths of blood vessels (Fig. 1C). These data indicate that following nerve fiber innervation, vascular growth and branch compaction progress, and mature vascular networks are formed until E17.5. In association with these vascular developmental stages, the numbers of neurovascular cell–cell contacts increased transiently at E15.5, and then decreased by E17.5 (Fig. 1D). Moreover, the numbers of neurovascular parallel branches increased from E13.5 to E17.5 (Fig. 1E), suggesting that neurovascular juxtapositional alignment is triggered by direct contact of ECs with neurons (Fig. 1A–E).

To investigate the molecular mechanisms underlying neurovascular juxtapositional alignment, we analyzed gene regulation in ECs during neurovascular interactions with co-cultured dorsal root ganglion cells (DRGs). Neurons produced elongated axons in the surrounding spaces, and sufficient numbers of ECs were concomitantly attached to neuronal axons (Fig. 1F).

After microarray analyses of gene expression in the ECs, we selected 69 candidate genes that had a >1.2-fold expression change...
Candidate genes were then categorized and compared with a list of genes reportedly involved in angiogenesis (Fig. 1G), and 59% of these were matched with angiogenesis-related genes (Fig. 1G, indicated with asterisks). In agreement, gene ontology (GO) analyses demonstrated a high representation of angiogenesis and vascular development GO categories among candidate genes (Table S2). Among these, CXCR4 was increased by the presence of neurovascular interactions (Fig. 1G), and was induced by neurovascular interactions as with a CXCL12 receptor in a previous study (Li et al., 2013). Neuron-derived VEGFA reportedly stimulates arterial differentiation of ECs via neurovascular interaction (Mukouyama et al., 2005). In agreement with this, the arterial differentiation marker EFNB2 was upregulated during neurovascular interactions in the present study (Fig. 1G), and strong VEGFA induction in ECs was observed (Fig. 1G), potentially offering synergetic contributions to arterial differentiation and angiogenesis. Furthermore, ITGB3, which encodes the β3 subunit of integrin αVβ3 and is known to be involved in angiogenesis (Davis and Senger, 2005; Hayashi et al., 2008), was also significantly upregulated (Fig. 1G).

In further analyses of central gene regulators in ECs during neurovascular juxtapositional alignment, the AP-1 transcription factor JunB was the most strongly and consistently upregulated transcription factor (Fig. 2A,B). Consistent with this finding, JunB has been associated with cell lineage specification and differentiation in several cell types, including in hematopoietic stem cells (Santaguida et al., 2009), T cells (Son et al., 2011), osteoblasts (Guo et al., 2014) and skeletal muscle cells (Raffaello et al., 2010). Moreover, mice that are homozygous null for JunB die between E8.5 and E10 due to impaired placental development, and although cell proliferation is normal in JunB-null embryos, retarded embryo growth may reflect failure to establish maternal circulation (Schorpp-Kistner et al., 1999). Thus, in further experiments, we examined the roles of JunB in vascular network formation and neurovascular parallelism.

**JunB expression in ECs during embryonic vascular development and in vitro angiogenesis**

Herein, we determined JunB expression in ECs during co-culture with DRGs and confirmed induction by neurovascular interactions by performing reverse transcription followed by quantitative real-time PCR (RT-qPCR) (Fig. 2A). In addition, we confirmed the expression of other AP-1 family members, including JUN, JUND, FOS, FOSL1, FOSL2, ATF2, ATF3 and ATF7, which reportedly form heterodimers with JunB (van Dam and Castellazzi, 2001). No upregulation among the AP-1 family, except JunB, was observed in ECs co-cultured with DRGs (Fig. 2A). In agreement with the RT-qPCR results, JunB promoter activity was upregulated by neurovascular interactions in luciferase reporter assays, as determined using by a NanoLuc luciferase construct containing the human JunB promoter (Fig. 2B). JunB expression in DRGs was unchanged by co-culture with ECs (Fig. 2C). Moreover, whole-mount immunohistochemical analyses of mouse embryonic limb skin demonstrated that JunB is expressed in ECs, particularly in those attached to neurons, in contrast to Fos staining (Fig. 2D).
In agreement with these results, in vitro 3D angiogenesis assays showed strong JunB induction in tip cell-like ECs at invading angiogenic frontiers (Fig. 2D).

**JunB expression in ECs affects EC shapes and the formation of tip cell-like protrusions, and promotes angiogenesis in vitro**

To elucidate JunB functions in ECs, we tested the effects of JunB overexpression and suppression using shRNA gene silencing methods. In these experiments, basal JunB expression in ECs was not detectable under these conditions (Fig. 3A, left panel). Subsequently, knockdown of JunB using an shJunB vector was confirmed in JunB-overexpressing HEK293 cells (Fig. 3A, right panel). Overexpression and suppression of JunB strongly affected EC morphology (Fig. 3B). Specifically, JunB-overexpressing ECs exhibited elongated cell shapes with tip cell-like filopodia and ruffling membrane formations, whereas JunB-suppressed ECs showed polygonal and less-elongated cell shapes (Fig. 3C). Annexin V staining confirmed that JunB knockdown does not induce apoptosis (Fig. S2). In addition, cell widths were significantly decreased following JunB overexpression and were increased by JunB suppression (Fig. 3C,D). Accordingly, spheroid outgrowth assays showed that the formation of tip cell-like ECs was dependent on JunB expression (Fig. 3E). Mixing spheroid outgrowth assays using control cells and JunB-overexpressing cells with different fluorescent markers showed that JunB-overexpressing ECs preferentially produce tip-like cells compared to control cells (Fig. 3F). Moreover, in vitro 3D angiogenesis assays showed that overexpression of JunB significantly increased the numbers of cells that migrated into collagen matrices compared with those transduced with the vector control, and JunB knockdown suppressed EC migration into collagen matrices (Fig. 3G). Taken together, these data show that JunB controls EC cell elongation and cell motility, and positively regulates angiogenesis in collagen matrices. In contrast, tube formation on Matrigel surfaces was indistinguishable between JunB-overexpressing and JunB-suppressed ECs, with no significant differences in tube lengths or numbers of branches (data not shown), suggesting that JunB-mediated angiogenesis is accompanied by cell migration and degradation of 3D matrices.

**In vivo JunB knockdown in ECs from developing mouse limb skin abrogates neurovascular alignment**

To validate our in vitro data, we examined the effects of in vivo JunB knockdown in ECs from embryonic limb skin vasculature. Because JunB knockout (KO) mice die by E9.5 due to placental malformation (Schorpp-Kistner et al., 1999), analyses of the roles of JunB in vascular network formation at E9.5–17.5 cannot be achieved in both conventional and EC-specific conditional JunB KO mice. Therefore, we developed a novel strategy for developmental stage-, position- and cell-specific JunB knockdown using a combination of in utero lentivirus injections and Cre-mediated cell-specific gene knockdown.

These experiments were performed using a shJunB lentivirus vector (Fig. S4A, upper panel) that expresses shRNA and GFP in all infected cells, allowing positive identification of lentivirus-infected cells according to GFP expression. In addition, we administered an EC-specific shJunB lentivirus vector (EC-spe-shJunB; Fig. S4A, lower panel), which contains a loxp-STOP-loxp stuffer sequence between the EF1 promoter and the GFP-miR-E-backbone-shRNA-JunB-coding sequence (Fallmann et al., 2013; see Materials and Methods section). This EC-spe-shJunB vector expresses shRNA and GFP only in Cre-expressing cells, and when injected into VE-cadherin-Cre transgenic mice, the loxp-STOP-loxp stuffer sequence is removed by Cre-mediated recombination only in cells expressing the vascular EC marker VE-cadherin.

During nerve and vascular development in embryonic mouse limb skin, the capillary primary plexus is formed at around E9.5, and peripheral nerves (PNs) migrate onto the capillary plexus at around E13.5. After innervations of PNs onto the capillary plexus, mature vascular networks are formed through neurovascular interactions until E17.5. Accordingly, we administered lentiviruses via intra-uterine injections into embryo limbs at E12.5, immediately before the onset of neurovascular interactions, and examined limb skin vasculature after embryo growth until E17.5 (Fig. S4B). Following injection of shJunB lentiviruses (Fig. S4A, upper panel) into mice, lentivirus-infected and shRNA-expressing cells were identified as GFP positive (Fig. S4C). In subsequent whole-mount immunohistochemical analyses, mosaic patterns of shJunB expressing and GFP-positive cells were observed in CD31-positive ECs (CD31 is also known as PECAM1) and in other cell types (Fig. S4D). GFP-negative and CD31-positive ECs were also observed in the fields (Fig. S4E). Thus, these mosaic patterns of infection were used to analyze EC-specific JunB functions in comparison with GFP-negative (non-infected) ECs.

We used EC-ttdTomato mice for lentivirus injection. EC-ttdTomato mice were generated by crossbreeding of VE-cadherin-Cre mice with B6.Cg-Gt(ROSA)26Sor<tm9(CAG-ttdTomato)Hze/J mice. EC-ttdTomato mice expressed ttdTomato fluorescent protein specifically in CD31-positive ECs (Fig. S1), allowing identification of vascular ECs according to ttdTomato expression (Fig. 4A). Subsequently, we analyzed blood vessel formation and neurovascular alignment of limb skin vasculature in shJunB-lentivirus-infected embryos (Fig. 4). Although no significant differences in lengths of non-parallel branches were observed between GFP-positive and -negative blood vessels (Fig. 4B), lengths of GFP-positive (JunB knockdown) parallel branches were significantly less than those of non-GFP parallel branches (Fig. 4C). These results indicate that parallel neurovascular alignment was inhibited in shJunB-expressing vasculature. In agreement, the numbers of parallel GFP-positive JunB knockdown branches were decreased in these animals (see data points in Fig. 4C). Non-target control shRNA injection did not show any effect on branch length or the proportion of parallel branches, similar to no injection (data not shown).

In further studies, we compared the number of parallel branches to the total number of branches between GFP-positive and -negative vessels, and found that percentage of parallel branches as a proportion the total number of branches was significantly decreased in GFP-positive (JunB knockdown) vessels (Fig. 4D). In further analyses of neurovascular parallelisms in larger blood vessels (>6.6 µm in diameter), the percentage of parallel branches in GFP-positive (JunB knockdown) vessels were dramatically decreased to 20%, whereas those in GFP-negative vessels were 60% of the total number of branches (Fig. 4E). Similar results were obtained in mice injected with EC-specific shRNA (EC-spe-shJunB; Fig. 4F–H), indicating that downregulation of JunB expression disrupts the balance of EC cell migration and differentiation that is necessary for the development of vascularization and the morphogenesis required for neurovascular juxtaposition.

**Correlations of EC gene regulation with JunB overexpression and neurovascular interactions**

To investigate pathways downstream of JunB, we performed microarray gene expression analyses in JunB-overexpressing ECs,
Fig. 3. JunB promotes tip cell-like EC formation and *in vitro* 3D-collagen angiogenesis. (A) JunB was overexpressed or suppressed in ECs through lentivirus vectors, and expression was confirmed by western blotting with an anti-JunB antibody; Cont, vector control; JunBOE, JunB overexpression; shCont, non-target shRNA control vector; shJunB, JunB-targeted shRNA. Suppression of JunB by shJunB was confirmed in JunB-overexpressing HEK293 cells (right panel). (B–D) ECs were transduced with lentiviruses expressing human JunB or shJunB and morphological changes were monitored. (B) Phase contrast images. (C) Longitudinal and lateral lengths of cells were measured and plotted. The bar indicates the regression line as calculated by the software R. (D) Quantification of the lateral lengths of JunB-overexpressing and JunB-suppressed ECs (*n* = 5, three independent experiments). (E) EC spheroid sprouting assays. Overexpression of JunB in ECs stimulated the formation of angiogenic tip cell-like protrusions in 3D-collagen angiogenesis assays, whereas suppression of JunB expression inhibited the formation of tip cells. Results are mean ± s.d. (three independent experiments). (F) EC spheroid sprouting assays of control and JunB-overexpressing ECs. Mixed cultures with equal amounts of mCherry-expressing control ECs (Cont, in red) and GFP-expressing control ECs (Cont, in green), or GFP-expressing-JunB-overexpressing ECs (JunBOE, in green) were subjected to the tip-cell assay, and the ratio of the number of tip cells for the green cells to the red cells was quantified (right panel, mean ± s.d., *P* < 0.01 mCherry-expressing-EC with GFP-expressing-EC vs mCherry-expressing-EC with JunBOE-EC). (G) *In vitro* 3D angiogenesis assays represented in inverted images of DAPI staining. White lines indicate the surface of collagen matrices and dotted lines indicate average migration distance of ECs; overexpression of JunB significantly stimulated angiogenesis into 3D collagen matrices and suppression of JunB resulted in dramatic inhibition of 3D angiogenesis. (*n* = 5, three independent experiments). For the plots in D and G, the box represents the 25–75th percentiles, and the median is indicated. The whiskers show the maximum and minimum values (three independent experiments). Scale bars: 100 µm (B,E,F).
and analyzed the expression of the 69 genes that were identified in neurovascular interaction experiments (Fig. 1G). Among these 69 genes, 34 gene expression changes were consistent with those accompanying neurovascular interactions (Fig. 5A), and were likely downstream of JunB signaling pathways that are triggered by direct neurovascular interactions.

Among candidate molecules, the integrin subunit β3 (encoded by ITGB3) was previously shown to promote angiogenesis (Davis and Senger, 2005; Hayashi et al., 2008). In agreement, the present analyses showed a significant upregulation of ITGB3 in JunB-overexpressing ECs (Fig. 5B), indicating that ITGB3 expression is activated by JunB following neurovascular interactions.

Accordingly, we also observed the induction of VEGFA, EFNB2 and HES1 in JunB-overexpressing ECs (Fig. 5B). In addition, ITGB3 knockdown in JunB-overexpressing cells significantly reduced angiogenesis activity in 3D collagen matrices (Fig. 6E,F). The integrin αVβ3-specific inhibitor Cyclo (RGDfC) also caused significant suppression of JunB-induced angiogenesis (Fig. 6F).

**Involvement of JunB in known neurovascular interaction signaling pathways**

Finally, we investigated relationships between JunB activation and other known signaling pathways during neurovascular interactions. A previous study showed that Cxcl12 is secreted by peripheral...
neurons and Schwann cells, and stimulates angiogenesis and vascular remodeling via the Cxcl12 receptor Cxcr4 during the formation of embryonic vascular networks (Li et al., 2013). Thus, we examined the involvement of JunB in Cxcl12-induced 3D angiogenesis in vitro (Koh et al., 2008) as described in Fig. 3D. In these experiments, the presence of Cxcl12 significantly activated angiogenesis as previously reported (Fig. 6A), but did not induce JunB expression in EC cultures (Fig. 6B), suggesting that JunB is not regulated downstream of the Cxcl12 pathway.

Subsequently, we determined whether neurovascular interactions in this system were mediated by soluble factors or by direct cell–cell interactions using analyses of the effects of conditioned medium from DRGs on JunB expression in ECs. However, JunB expression was not affected by the addition of DRG conditioned medium in RT-qPCR (Fig. 6C) or JunB promoter assays (Fig. 6D). These results indicate that induction of JunB during neurovascular interactions of ECs is directly triggered by cell–cell or cell–ECM interaction.

DISCUSSION

In the present study, we demonstrated that JunB is induced in ECs by neurovascular interactions, and that it regulates neurovascular juxtapositional alignments during embryonic vascular development. The present whole-mount immunohistochemical analyses showed that neurovascular interactions started at E13.5, and neurovascular juxtapositional alignments were established during E13.5–17.5 (Fig. 1). Moreover, neurovascular juxtapositional alignments were triggered by the direct contact of ECs with neurons (Fig. 1A). Thus, to model in vivo neurovascular interactions and investigate the underlying molecular mechanisms, we analyzed changes in EC gene expression during co-culture with DRG neurons. The ensuing microarray analyses identified 69 genes that were significantly regulated under these conditions, and JunB was the most differentially expressed transcription factor, indicating that it had roles in neurovascular interactions during vascular network formation (Figs 1G and 2A).

Further whole-mount immunohistochemistry analyses of mouse embryonic limb skin confirmed increased expression of JunB in ECs that were attached to neurons at E13.5 and E17.5 (Fig. 2C), and strong JunB induction was observed in tip cell-like ECs at invading angiogenic frontiers in the in vitro 3D angiogenesis assays (Fig. 2D). Additionally, EC morphologies were found to be dependent on JunB expression levels (Fig. 3), indicating that JunB controls EC shape and motility. Accordingly, in vitro 3D angiogenesis was dependent on JunB expression levels (Fig. 3), further indicating that JunB positively regulates angiogenesis.

In accordance with the present in vitro data, in vivo JunB knockdown in the ECs of embryonic limb skin vasculature just before the onset of neurovascular interactions significantly decreased the numbers and lengths of parallel branches with neurons (Fig. 4). Moreover, the percentage of parallel branches as a proportion of the total branches was significantly decreased in JunB-knockdown vasculature (Fig. 4E). Taken together, these data demonstrate the necessity of JunB for blood vessel formation and neurovascular juxtaposition.

Further microarray analyses showed that interactions with DRGs induced arterial differentiation of ECs, as indicated by upregulation of EFNB2 (Fig. 1G), suggesting that neuron-derived VEGFA induces arterial differentiation of ECs, as reported previously (Li et al., 2013). In the present study, neurovascular interactions induced VEGFA expression in ECs (Fig. 1G), indicating self-activation of arterial differentiation and angiogenesis following neurovascular interactions. VEGF induction was also observed in JunB-overexpressing ECs (Fig. 5), further implicating JunB in the induction of VEGFA in ECs during neurovascular interactions.
Li et al. reported that neurovascular interactions activate VEGFA and Cxcl12 production in neurons, and that neuron-derived VEGFA induces EC differentiation, whereas Cxcl12 induces angiogenesis and vascular remodeling (Li et al., 2013). Similarly, the present data show that neurovascular interactions activate VEGFA expression in ECs (Fig. 1G), likely contributing synergistically to EC differentiation, vascular remodeling and activation of angiogenesis. In addition, VEGFA was upregulated in JunB-overexpressing ECs (Fig. 5), suggesting that VEGFA is induced by JunB during neurovascular interactions.

Fig. 6. JunB functions in known pathways of neurovascular interaction induced angiogenesis. (A) Effects of Cxcl12 on 3D-collagen angiogenesis (n=5). (B) RT-qPCR expression analysis of JunB following Cxcl12-induced angiogenesis. (C,D) Effects of conditioned medium from DRG cell cultures on JunB expression (C) and JunB promoter activity (D) in ECs; soluble factors in DRG conditioned medium did not activate JunB expression in ECs. (E,F) Effects of ITGB3 knockdown in JunB-overexpressing ECs; soluble factors in DRG conditioned medium did not activate JunB expression in ECs. (E) RT-qPCR expression analysis of JunB following Cxcl2 induction. (F) 3D angiogenesis (A) Effects of Cxcl12 on 3D-collagen angiogenesis (n=5). (B) RT-qPCR expression analysis of JunB following Cxcl12-induced angiogenesis. (C,D) Effects of conditioned medium from DRG cell cultures on JunB expression (C) and JunB promoter activity (D) in ECs; soluble factors in DRG conditioned medium did not activate JunB expression in ECs. (E,F) Effects of ITGB3 knockdown in JunB-overexpressing ECs; soluble factors in DRG conditioned medium did not activate JunB expression in ECs. (E) RT-qPCR expression analysis of JunB following Cxcl2 induction. (F) 3D angiogenesis.

Roles for JunB in vascular development have been reported previously, and JunB is known to activate Foxo1 by inducing miR-182, contributing to lymphatic vascular development in zebrafish. In agreement, loss of JunB in zebrafish resulted in the formation of parachordal lymphangioblasts (Kiesow et al., 2015), and Foxo1 expression reportedly effected EC migration, sprouting and tube formation (Potente et al., 2005). However, the present microarray analysis showed no upregulation of Foxo1 during neurovascular interactions (data not shown), suggesting that the JunB–miR-182–Foxo1 axis is not involved in JunB-mediated neurovascular juxtapositional alignment.

Gene expression analyses of JunB-overexpressing ECs co-identified about half of those that were regulated by neurovascular interactions (Fig. 5), indicating considerable involvement of JunB signaling in neurovascular interactions. Integrins play important roles in cell adhesion and migration (Desgrosellier and Cheresh, 2010), and ECs express several integrin heterodimers, including αVβ3, α5β1 and αβ5 (Plow et al., 2000). In particular, integrin αVβ3 is a critical angiogenic regulator for neovascularization that acts by co-operating with VEGFR2 (Somanath et al., 2009). Integrin αVβ3 is expressed at lower levels in quiescent ECs, but it is significantly upregulated in angiogenesis (Leu et al., 2002). Functional blocking of αVβ3 results in defects pertaining to neovascularization without affecting the normal pre-existing vasculature (Brooks et al., 1994a,b). Thus, integrin αVβ3 is a key regulator of angiogenesis. In the present experiment, we observed the upregulation of ITGB3, which encodes the β3 subunit of integrin αVβ3, during neurovascular interaction (Fig. 1G). We also observed the upregulation of ITGB3 in JunB-overexpressing ECs (Fig. 5), indicating that the upregulation of ITGB3 during neurovascular interaction is mediated by JunB. Moreover, ITGB3 knockdown and integrin αVβ3-specific inhibition significantly suppressed JunB-induced 3D angiogenesis (Fig. 5D), suggesting that integrin αVβ3 is one of the downstream effector molecules in JunB signaling. The notch signaling protein HES1 reportedly regulates vascular remodeling and arterial specification of ECs during the development of brain vasculature (Kitagawa et al., 2013). HES1 was reportedly regulated by JunB via direct binding to the HES1 promoter (Santaguida et al., 2009). These results suggest that vascular remodeling and formation of neurovascular parallelism are mediated in part by integrin and/or notch signaling pathways.

Neuron-derived Cxcl12 is known to stimulate angiogenesis and vascular remodeling following binding of its receptor Cxcr4 during the formation of embryonic vascular networks (Li et al., 2013). However, the present data do not indicate that JunB is regulated downstream of the Cxcl12 pathway during neurovascular interactions (Fig. 6A,B), and instead suggest angiogenic roles for JunB that are independent of the Cxcl12 pathway under these conditions. Cxcl12 was upregulated in ECs upon JunB overexpression (Fig. 5B), and was downregulated by neurovascular interaction in ECs (Fig. 1G). We need to clarify the mechanisms for these differences in gene regulation of Cxcl12 between ECs and neurons in future studies.
In summary, we present several lines of evidence for a novel mechanism involving the induction of the transcription factor JunB during neurovascular interactions, and subsequent regulation of angiogenesis toward arterial differentiation and neurovascular juxtapositional alignment. Specifically, neurovascular interactions induced JunB expression in ECs of the primary capillary plexus, and the resulting activation of pro-angiogenic genes, such as VEGFA and ITGB3, led to the morphological changes of ECs, with formation of tip cell-like cells and migration toward nerve fibers, and establishment of mature neurovascular aligned blood vessels. Although the present experiments reveal novel aspects of neurovascular interactions during the formation of vascular networks, further studies are necessary to confirm the relationships between JunB and other signaling pathways known to be associated with neurovascular interactions, and to define the mechanisms of JunB induction. Nonetheless, the present study provides clues for future studies regarding the regulatory mechanisms involved in embryonic vascular development.

MATERIALS AND METHODS

Cell culture and animals

Human primary microvascular ECs (HMVECs) were isolated from newborn foreskins (LifeLine Cell Technology, Frederick, MD) and were maintained in HuMedia-E2B medium supplemented with 5% (v/v) fetal bovine serum (FBS), 5 ng/ml basic fibroblast growth factor (bFGF), 10 ng/ml heparin, 10 ng/ml epidermal growth factor (EGF), 1 μg/ml hydrocortisone, and 39.3 μg/ml dibutyryl cAMP according to the manufacturer’s instructions (Kunbo, Tokyo Japan). Tielomerase immortalized HMVECs (TIME cells; #CRL4025, ATCC, Manassas, VA) were maintained in M131 medium supplemented with MVGF growth supplements (InVitrogen, Carlsbad, CA).

Dorsal root ganglion cells (DRGs) were isolated from C57BL/6 mice on postnatal day 3 and were dissociated for 10 min at 37°C using 1-mg/ml collagenase and 5-mg/ml Dispase in HBSS, and were then cultured on poly-D-lysine-coated plates. Cultures were grown in neurobasal medium supplemented with B27 and 10-ng/ml NGF (BD Biosciences, Franklin Lakes, NJ), 100-ng/ml penicillin, and 100-U/ml streptomycin. Prior to EC and DRG co-culture experiments, DRGs were grown in six-well plates, and detached HMVEC were inoculated onto DRG cultures in EC medium at a EC:DRG ratio of 10:1. Co-cultures were then incubated for 8 h and RNA was extracted.

To prepare lentiviral constructs, the coding region of human JunB (RefSeq NM_002229) was amplified by RT-PCR using total RNA from HMVECs and was cloned into pSH vector (System Biosciences, Palo Alto, CA), and the sequences were then verified. shRNA vectors were constructed in the pSH vector (System Biosciences) using synthetic oligonucleotides against human and mouse JunB antisense sequences (shJunB1, 5′-AGGCTCTGTTCTTACCATGTTATGTCG-3′ or shJunB2, 5′-TTGAGGCTTCCACCTGTTCTCAGGGC-3′) and human ITGB3 antisense sequences (shITGB3-1, 5′-TACTGGATACTGAGCCAGCTATCCTC-3′ or shITGB3-2, 5′-AAACAGGTGCATTAAATGAGTATGCC-3′) with loop 5′-TCAAGAG-3′. A synthetic DNA containing the non-target sequence 5′-GCATACAGAGCTAACATCGAGTACT-3′ carrying the same loop was used as the negative control (sh-Vec). Tissue-specific JunB knockdown in mice was achieved by using the lentivirus (EC-spe-shJunB) containing the modified miR30- (miR-E-) miRNA backbone shRNA (Fellmann et al., 2013) with loop-stop-loop stuffer sequence. In brief, the mouse JunB antisense sequence 5′-TTTACGAAGTTGTAGTGTCG-3′ with a 5′-TAATGAAA-GCCACAGATGTA-3′ loop for shRNA-JunB was inserted into the miR-E- microRNA backbone (miR-E-shJunB). miR-E-shJunB was cloned into the pCDH vector downstream of the GFP open reading frame (ORF). The loop-stop-loop fragment was then inserted between EF1 promoter and the GFP transcriptional start site to allow induction of GFP and shRNA expression upon Cre-mediated recombination in Cre expressing cells. Lentivirus particles were then produced in HEK293T cells (System Biosciences) following transfection of the above constructs, the psPAX2 packaging vector and the pMD2.G envelope vector [deposited by Didier Trono, Laboratory of Virology and Genetics, École Polytechnique Fédérale de Lausanne, Switzerland; Addgene plasmid #12259 and #12259 respectively, Addgene, MA]. Supernatants from 48 h culture media were then collected and lentiviral particles were concentrated using PEG-it solution (System Biosciences). Lentivirus infectious units (IU) were determined according to the number of GFP-positive ECs as assessed with serial dilutions of lentivirus from cells at 24 h post transduction.

Microarray analysis

Total RNA was extracted using TRIzol reagent (Invitrogen) and purified using RNeasy Mini kits (Qiagen, Valencia, CA). RNA quality was assessed according to 28S/18S ratios of rRNA bands on electrophoresis gels under denaturing conditions. Subsequently, 100-ng aliquots of total RNA were labeled according to the manufacturer’s instructions for the GeneChip WT Sense Target Labeling kit (Affymetrix, Santa Clara, CA). Fragmented and labeled cDNAs were then hybridized onto Affymetrix GeneChip Human Gene 1.0 ST or human U133 plus 2.0 arrays. Arrays were then washed and stained using the GeneChip Fluidics Station 450 and scanned using a 3000 7G GeneChip Scanner (Affymetrix). All arrays passed the quality control criteria of the Expression Console software (Affymetrix). Raw data CEL files were then normalized using the RMA algorithm and the data exported using Expression Console or Gene Spring software (Agilent Technologies, Santa Clara, CA). Genes with a ≥1.2-fold change in transcription in two independent experiments were selected, and non-annotated genes and functionally unknown genes were excluded. Gene ontology (GO) analyses of genes with significant transcriptional changes during neurovascular interactions were performed using the human reference list in the PANTHER v10.0 Enrichment analysis program provided by the Gene Ontology consortium (http://pantherdb.org/).

RT-qPCR

Total RNA was extracted from cells as described above. Subsequently, 1-ng aliquots of total RNA samples were reverse transcribed using PrimeScript RT reagent Kit (TaKaRa, Otsu, Japan). qPCR was performed using the resulting cDNAs with an initial denaturation at 95°C for 30 s, followed by 45 cycles of 95°C for 5 s and 60°C for 30 s using the SYBR Green Premix (Takara) with the primers listed in Table S1 in a StepOnePlus Realtime-PCR System (Applied Biosystems, CA).

Western blotting

Cells were suspended in Laemmli-sample buffer containing 5% 2-mercaptoethanol and were sonicated for 30 s and heated at 98°C for 5 min. The resulting protein samples were subjected to SDS-PAGE using 4–12% polyacrylamide gels (Wako, Osaka Japan). After electrophoresis, proteins were transferred on PVDF membranes (Invitrogen) and blocked...
with 5% skimmed milk in Tris-buffered saline. Anti-JunB (G53, cat. no. 3746, 1:1000; Cell Signaling Technology, Danvers, MA) and anti-β-actin (cat. no. A2228, 1:4000; Sigma-Aldrich) antibodies were used as primary antibodies, and anti-rabbit IgG-HRP (Amasham GE, MA) was used as a secondary antibody. Protein signals were detected using ECL Plus Chemiluminescent Substrate (Thermo Scientific, Rockford, IL).

**In vitro 3D collagen matrix angiogenesis assays**

Angiogenic activities of ECs were analyzed using three-dimensional (3D) collagen matrix angiogenesis assays as described previously (Koh et al., 2008). Briefly, type I collagen from rat tails (Millipore, Billerica, MA) was reconstituted in M199 medium supplemented with 1-μM phosphoglucone isomerase (SIP; Avanti Polar Lipids, AL) with or without 200 ng/ml recombinant human Cxcl12 (R&D Systems, Minneapolis, MN) and was then placed in the bottom chamber of a µ-Slide (ibidi, Martinsried, Germany) and was transformed into a gel by incubation at 37°C in a CO2 incubator. Detached ECs were then counted and re-suspended in M199 medium containing reduced serum supplement II at a dilution of 1:250, 40 ng/ml FGF-2, 50 μg/ml ascorbic acid and 50 ng/ml 12-O-tetradecanoylphorbol-13-acetate (TPA). To analyze the involvement of integrin αβ3 in JunB-induced 3D collagen angiogenesis, Cyclo (RGDFC) peptide (AnaSpec, CA), an inhibitor for integrin αβ3-mediated cell attachment, was added in collagen gel matrices at a concentration of 5 μM. Subsequently, 2×10^4 cells were plated on the upper layer of the µ-Slide and were incubated in a CO2 incubator for 48 h. Cells were fixed and then stained with anti-human CD31 (WM59, cat no. 303101, 1:200; BioLegend), anti-JunB (ab28838, 1:100; Abcam, Cambridge, MA) antibodies and DAPI (Invitrogen). Alexa Fluor conjugates of anti-rat, anti-mouse, and anti-rabbit IgG (Invitrogen) were then used as secondary antibodies and series of fluorescent Z-stack images of dermal tissue vasculature and nerve fiber networks were generated by using a LSM 710 confocal microscope (Carl Zeiss, Germany) and angiogenic activities were assessed according to numbers of ECs that had migrated to a depth >100 μm.

**EC spheroid sprouting assays**

Thin Matrigel (BD Biosciences) matrices were formed by adding 10 μl of Matrigel to the bottom chambers of µ-Slide Angiogenesis plates (ibidi). Subsequently, detached ECs were inoculated at high density (4×10^4 cells/well) in HuMedia-EB2 medium supplemented 1% FBS and were incubated until multicellular spheroids formed. After 24 h culture, spheroid cells were visualized using a ZEISS Axiosvert A1 microscope (Carl Zeiss) at 10× magnification, and the numbers of migrated cells per 20 surface cells were counted on at least six spheroids.

**Analyses of human JunB promoter activity**

A human JunB promoter region containing 2 kb of sequence upstream of the start site was PCR amplified using genomic DNA from HMVECs and the following primers: 5′-GAAGATCTAATTTCTGGCAGACATGTCT-3′ and 5′-CCCAAGCTTCGCTGCGGTGACCGGACTGG-3′, and were cloned into pNL1.2 Nano-Luc-PEST vectors (Promega). JunB promoter-Nano-Luc-PEST vectors were then transfected into pCDH vector or the shJunB vector, both containing a GFP marker, were detached and re-suspended in binding buffer containing 5 μl of Annexin-V--biotin solution and incubated for 5 min. After washing, the cells were incubated with avidin--APC. Annexin-V-positive and GFP-expressing cells were analyzed by confocal microscopy (Carl Zeiss).

**Whole-mount immunohistochemistry**

Whole-mount immunohistochemistry analyses of mouse limb skin from embryos was performed according to a previously described procedure (Li and Mukouyama, 2011) with some modifications. Briefly, mouse limbs from E9.5, E13.5 and E17.5 embryos were dissected and fixed in 4% paraformaldehyde (PFA) overnight with gentle agitation. After three washes with phosphate-buffered saline (PBS) containing 0.2% Triton X-100 (PBS-T), dermal tissues were separated from muscles and bones, and skin vasculature and neural fibers in dermal tissues were blocked with 10% goat serum in PBS-T, and were then stained with anti-mouse-CD31 (MEC13.3, 1:200; BD Biosciences), anti-Neurofilament M (MAB1621, 1:200; Millipore), anti-JunB (ab28838 and ab12878, 1:100 and 1:250, respectively; Abcam), e/luor-660-conjugated-anti-B tubulin class III (2G10-TB3, cat. no. 50-4510, 1:250 eBioscience, San Diego, CA) and DAPI (Invitrogen). Alexa Fluor conjugates of anti-rat, anti-mouse, and anti-rabbit IgG (Invitrogen) were then used as secondary antibodies and series of fluorescent Z-stack images of dermal tissue vasculature and nerve fiber networks were generated by using a LSM 710 confocal microscope (Carl Zeiss).

**Statistical and image analyses**

Data are expressed as mean±s.d. or s.e.m. Statistical analyses were performed using the R package (version 3.3.0; http://www.r-project.org). Pairwise differences between homoscedastic samples were identified using Student’s t-tests, and those between heteroscedastic samples were identified using Welch’s t-test. Image analysis and quantification were performed with the Fiji package of ImageJ (http://fiji.sc/), and Z-stacks were flattened through a maximum intensity projections. Vasculature formation analyses were performed by counting numbers of neurovascular contacts and blood vessel branches, and measuring branch lengths according to the criteria described in Fig. S2. The images presented are representative of the analyzed data.

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**Competing interests**

The authors declare no competing or financial interests.

**Author contributions**

Y. Yoshitomi and H.Y. conceived and designed the experiments; Y.Yoshitomi and H.Y. performed the majority of the experiments and analyzed the data with some contributions from Y.I., T.I., H.S. and Y. Yoshitake. N.K. and T.H. assisted with DRG contributions from Y.I., T.I., H.S. and Y. Yoshitake. N.K. and T.H. assisted with DRG experimental assistance.

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**Data availability**

The JunB overexpression effect on microvascular endothelial cell culture microarray data are available in Gene Expression Omnibus (GEO) under accession number GSE93616. Microarray data for gene expression in microvascular endothelial cells co-cultured with dorsal root ganglion cells are available under accession number GSE93696.

**Supplementary information**

Supplementary information available online at http://jcs.biologists.org/lookup/doi/10.1242/jcs.196303.supplemental
References
Supplemental information

**Fig. S1**

Expression of CD31 in tdTomato positive cells isolated from EC-tdTomato mice

Cells were dissociated from EC-tdTomato mouse lungs and analyzed using FACS with an APC conjugated anti-CD31 antibody. Expression of the vascular endothelial cell marker CD31 was observed in tdTomato positive cells only, indicating that tdTomato expression was limited in vascular ECs.
Annexin-V staining in shJunB-expressing ECs

ECs infected with shRNA lentiviruses were detached and stained with Annexin-V-biotin, followed by APC-avidin staining. Annexin-V-positive and GFP-positive cells (lentivirus-infected cells) were counted and quantified using the particle analyzer in Fiji Image J software. Sh-Cont is the control non-targeting shRNA vector. shJunB-1 and shJunB-2 are described in the Experimental procedures.

For apoptosis controls, ECs were treated with 1 µg/mL of Mitomycin C (+Mitomycin C) or exposed to UV radiation for 30 sec followed by 4 h culture (+UV) before cell detachment.
Criteria for analysis

Fig. S3

Criteria for analyses of vasculature in sh-JunB lentivirus-injected mouse limb skin

Vessels and nerves were identified using neurofilament staining and Td-Tomato expression, respectively. Vessels expressing JunB shRNA were identified by EGFP expression and are indicated with green lines. Branches were defined as vessels between adjacent branch points, and lengths of branches (branch lengths) were calculated by counting pixels between adjacent branch points (upper right panel). The blood vessel branches localized within a 20-pixel distance from neurons with an angle of less than 45° were counted as parallel. Parallel branches are indicated with blue lines.
Fig. S4

In vivo JunB knockdown strategies using in utero injections of shRNA-JunB lentivirus into developing mouse limbs

(a) Schematic representation of lentiviral vector constructs for conventional shJunB and for EC-specific expression of shJunB by VE-cadherin-Cre mediated transcriptional activation (EC-spe-shJunB). (b) Schematic experimental schedule for in utero lentivirus injections; developmental stages of blood vessels and peripheral nerve fibers in mouse skin are indicated. In utero lentiviral injections were performed at E12.5 immediately before peripheral nerve system (PNS) migration into limb skin, and limb tissues were harvested at E17.5 and for analyses of vasculatures as described in the Experimental Procedures. (c) Lentivirus-infected and shRNA-transduced cells can be identified by GFP expression. (d) Representative images of limb skin vasculature from in utero shJunB lentivirus injected EC-tdTomato mice (see Cell culture and animals in Experimental Procedures).