Ninjurin1 Is a Novel Factor to Regulate Angiogenesis Through the Function of Pericytes

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Background: Capillary pericytes (cPCs), the mural cells of microvessels, play an important role in the formation and maintenance of microvessels; however, little is known about the mechanisms of how cPCs regulate angiogenesis. To identify factors that modulate cPC function, genes whose levels were altered in cPCs during neovessel formation were identified through a microarray screen.

Methods and Results: Ninjurin1 (nerve injury-induced protein, Ninj1) was selected as a candidate factor for angiogenesis regulation. Ninj1 was expressed in capillary cells including endothelial cells (cECs) and was expressed at a higher level in cPCs. Hypoxia induced the gene expression of Ninj1 in addition of vascular endothelial growth factor (VEGF) in cPCs. When cPCs were co-incubated with a thoracic aorta in a three-dimensional Matrigel system, the length of the EC-tubes sprouting from the aorta was increased. Small interfering RNA-mediated downregulation of Ninj1 in cPCs enhanced these cPCs-mediated angiogenic effects, whereas overexpression of Ninj1 attenuated their effects. The production of angiogenic growth factors, such as VEGF and angiopoietin 1, by cPCs was enhanced by the downregulation of Ninj1, and reduced by the overexpression of Ninj1.

Conclusions: Ninj1 is a novel regulator for the angiogenic effect of PCs. Specifically, Ninj1 negatively regulates the formation of neovessels, that is, the EC-tube, by reducing the trophic effects of cPCs.

Key Words: Angiogenesis; Endothelial cells; Ninjurin1; Pericytes

Inadequate vessel formation causes ischemia in diseases such as peripheral arterial diseases, myocardial infarction, and stroke; whereas excessive vascular growth also promotes diseases including diabetic retinopathy. Pericytes, the mural cells that wrap around endothelial cell (ECs) tubes to form microvessels, are implicated in the regulation of vascular morphogenesis and functions. Abnormal pericyte function or loss of pericytes leads to increased vascular permeability, immature vessel formation, and further loss of microvessels. The role of pericytes is mediated through specific trophic effects and the direct interaction with ECs. While several signaling pathways to mediate the function of pericytes such as platelet-derived growth factor (PDGF)/PDGF receptor-β, angiopoietin (Ang)1/Tie2, and transforming growth factor (TGF)-β, have been documented, the mechanisms that regulate their actions in microvascular physiology have been largely underinvestigated.

Nerve injury-induced protein 1, Ninjurin1 (Ninj1), was originally discovered as a cell-surface protein expressed in neuronal and Schwann cells after nerve injury, and it has been shown to promote neurite outgrowth from dorsal root ganglion neurons. Ninj1 is not restricted to neuronal cells and has been shown to be expressed in many kinds of tissues and cells, predominantly in those of epithelial origin. Recently, it has been reported that Ninj1 affects non-neuronal cells and has a role in neuronal and non-neuronal pathophysiological conditions. Ninj1 is responsible for the progression of multiple sclerosis, an autoimmune inflammatory central nervous system disease. Ninj1 participates in the invasion of myelin-reactive T cells across the blood-brain barrier to mediate neuronal inflammation in multiple sclerosis. Ninj1 expressed in macrophages contributes to the regression of hyaloid blood vessels, a transient vascular system involved in the maturation of the lens during the embryonic period. Recently, Yin et al reported that attenuation of Ninj1 function via neutralizing anti-Ninj1 antibodies, successfully restored erectile function,

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which was associated with enhanced penile angiogenesis in diabetic mice.13 Thus, it is suggested that Ninj1 is implicated in the regulation of vascular formation in pathological conditions. However, the mechanism by which Ninj1 affects the vascular formation is currently unclear.

We recently established immortalized vascular cell lines — namely, of capillary ECs (cECs) and pericytes (cPCs) — from microvessels of peripheral tissues.14 When cPCs are co-incubated with neovessel EC-tube in a 3-dimensional (3D) gel, cPCs elongate the EC-tube to form a capillary-like structure (Figure S1). Our in vitro angiogenesis system using these cell lines can serve as a useful tool to examine the mechanisms of angiogenesis.14 After cPCs were incubated with or without neovessels in the gel, a comprehensive microarray analysis of genes expressed in cPCs was performed. Ninj1 was selected from among candidate genes whose expression was altered in cPCs after co-incubation with neovessels.

**Methods**

**Cell Preparation**

Immortalized cells derived from capillaries, namely cECs and cPCs, were prepared as described previously.15 For some experiments, cells were infected with a retrovirus harboring the green fluorescent protein (GFP)- or Red fluorescent protein (DsRed)-genes to label living cells. Then, fluorescence-expressing cells were sorted using fluorescence activated cell sorting (FACS) flow cytometry (FACS Aria II, BD Biosciences; Rockville, MD, USA). Human umbilical vein ECs (HUVECs) and human smooth muscle cells (HSMCs) were purchased from Kurabo (Osaka, Japan).

For gene knockdown experiments, small interfering (si)RNAs against mouse Ninj1 and a universal negative control siRNA were obtained from Nippon Gene Material Co, Ltd (Toyama, Japan). For Ninj1 overexpression, the full-length mouse Ninj1 gene sequence was purchased from GenScript (Piscataway, NJ, USA) and cloned into the pcDNA3 expression vector (Invitrogen, Carlsbad, CA, USA). Cells were analyzed for gene expression 48–72h after transfection and used for in vitro angiogenesis assays.

**Hypoxia Treatment**

Cells were cultured in anaerobic jars without medium changes under continuous hypoxic conditions using the AnaeroPac system (Mitsubishi Gas Chemical Company, Inc; Tokyo, Japan), which absorbs O2 in the jar and generates an environment of 6–12% O2. Hypoxia was measured using an anaerobic indicator (Becton, Dickinson and Company, Sparks, MD, USA). After predetermined incubation times, cells were collected for experiments.

**Aortic Ring Angiogenesis Assay**

Aortic ring cultures were established as previously described, with minor modifications.15 Briefly, aorta rings were embedded in growth factor-reduced Matrigel (BD Biosciences; Bedford, MA, USA) and incubated in endothelial basal medium-2 (EBM2) (PromoCell, Heidelberg, Germany) containing 2% fetal bovine serum (FBS) and 10ng/ml vascular endothelial growth factor (VEGF). EC tubes sprouting from aortic rings were identified by staining with fluorescein isothiocyanate-labeled Griffonia simplicifolia lectin I (Vector Laboratories, Burlingame, CA, USA). A quantitative analysis of the number of sprouting microvessels per aortic ring was carried out using angiogenesis image analyzer software (Kurabo, Osaka, Japan), and total microvessel lengths were also determined.

**EC Tube Formation Angiogenesis Assay**

The EC tube formation assay was performed as previously described.14 A 40-μl volume of growth factor-reduced Matrigel was added to each well of a 96-well plate at room temperature. The gel was solidified at 37°C for at least 30 min and then seeded with 1x106 cells/well in 100μl medium (EBM2 with 2% FBS and 10ng/ml VEGF). The assay was performed in a CO2 incubator, with plates incubated at 37°C for 24h. Images were obtained by phase contrast microscopy and the length of the tube-like structure in each well was measured at 40x magnification.

**Hind Limb Ischemia Surgery and Immunocytochemistry**

Male C57BL/6 mice 12 weeks of age were used for experiments. All animal protocols were approved by the Animal Care and Use Committee of Asahikawa Medical University. Unilateral hindlimb ischemia (HIL) models were established by ligation and excision of the femoral artery/vein as previously described.16 At predetermined time points, mice were sacrificed by overdose with a pentobarbital-based euthanasia solution (200mg/kg by intraperitoneal injection). Gastrocnemius muscle samples were collected and stored at −80°C for quantitative polymerase chain reaction (qPCR) analysis. The histological assessment of ischemic limb tissue was performed 14 days after hindlimb surgery. Functional vessels were stained by injecting 300μl phosphate-buffered saline (PBS) containing rhodamine-labeled G. simplicifolia lectin (100μg/ml, Vector Laboratories) via the tail vein. After 5 min, mice were sacrificed and perfused through the heart with PBS followed by 4% paraformaldehyde in PBS, and the gastrocnemius muscle was dissected and embedded in tissue-Tek OCT medium. Ninj1 expression in 10-μm cross-sections was detected by immunocytochemistry using an anti-Ninj1 antibody (Bios, Woburn, MA, USA) and an Alexa488-conjugated secondary antibody (Invitrogen). Nuclei were counterstained with Hoechst 33258 (Lonza, Basel, Switzerland).

**Gene Microarray Analysis**

GFP-expressing cPCs (5×103/20μl gel) were cultured in growth factor-reduced Matrigel with or without aortic rings, as described above. After 7 days of incubation, all cells within the gel were isolated using Liberase DL (Roche; Indianapolis, IN, USA). Living cPCs were separated by FACS by means of 7-aminocyclooctynid-D-negative and fluorescent (GFP)-positive cells. The living cells (~92–95%) were applied to microarray analysis using the 3D Gene Mouse Oligo Chip 24K (Toray Industries Inc, Tokyo, Japan). Intensity values greater than 2 standard deviations above the background signal were considered valid. The signal corresponding to each gene was normalized by the global normalization method (Cy3/Cy5 ratio median=1).

**Reverse Transcription (RT)-PCR and qPCR**

RNA was prepared with a RNeasy kit (QIAGEN, Venlo, Netherlands). Reverse transcription-PCR was performed using a superscript one-step RT-PCR kit (Invitrogen) in the presence of 10μmol/L sense and anti-sense primers. The primers of mouse Ninj1, VEGF were: sense, 5'-ACTGAGGAATGAGCCTCA-3', anti-sense, 5'-TCCATTACGGCCTTGGGA-3', and sense, 5'-ATGAATCTTTCGTCCTTGGTG-3', anti-sense, 5'-TCACCCGGCTGGGTGTGGTTC-3', respectively. For quantitative PCR analysis, total RNA was reverse-transcribed using a Transcriptor High Fidelity cDNA Synthesis
Statistical Analysis

Results are presented as mean±SEM unless otherwise noted. Differences between two measurements were evaluated with the unpaired Student’s t-test, and multiple comparisons were carried out by analysis of variance followed by Fisher’s test. P values <0.05 were considered statistically significant.

Results

Ninjurin1 Expression is Enhanced During Angiogenesis in cPCs

In order to identify factors expressed in cPCs that regulate angiogenesis, cPCs were co-incubated with mouse thoracic aorta tissue in a 3D gel using the ex vivo aortic ring angiogen-
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(IGF)1, and matrix metalloproteinase was upregulated in cPCs after incubation with neovessels (Table 1). In addition to these factors, Ninj1 was selected from among candidate genes whose expression was altered by co-incubation with neovessels (Table 1).

Hypoxia and Inflammatory Cytokines Stimulate the Expression of Ninj1 in Vascular Cells

Ninj1 was expressed in both cECs (vWF+ CD31+ cells) and cPCs (NG2+ αSMA+ cells), but was dominantly expressed in cPCs compared to cECs (Figure 1C). Ninj1 expression was stimulated by hypoxia and inflammatory cytokines (Figure 2). Consistent with an earlier study, after a 7-day incubation in 3D gel with medium containing VEGF, EC tubes sprouted from aortic tissue. The cPCs formed capillary-like structures in association with these tubes, while those incubated alone in the 3D gel formed only small cell clusters (Figures 1A, B). Cells were separated from the gel using a collagenase solution, and fluorescence-labeled cPCs were isolated by FACS followed by a microarray analysis to obtain gene expression profiles (Figure 1A). As previously reported, the expression of angiogenesis-related genes such as VEGF, hepatocyte growth factor (HGF), insulin-like growth factor (IGF)1, and matrix metalloproteinase was upregulated in cPCs after incubation with neovessels (Table 1). In addition to these factors, Ninj1 was selected from among candidate genes whose expression was altered by co-incubation with neovessels (Table 1).

**Table 1. Gene Expression in cPCs Incubated in the Presence or Absence of Neovessels**

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Angiogenic factors</th>
<th>Neovessels</th>
</tr>
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<tbody>
<tr>
<td>VEGFα</td>
<td>Vascular endothelial growth factor α</td>
<td>Without: 83, With: 398, (Fold): 4.8</td>
</tr>
<tr>
<td>Ang1</td>
<td>Angiopoietin 1</td>
<td>Without: 106, With: 224, (Fold): 2.1</td>
</tr>
<tr>
<td>FGF7</td>
<td>Fibroblast growth factor 7</td>
<td>Without: 1,083, With: 3,558, (Fold): 3.3</td>
</tr>
<tr>
<td>HGF</td>
<td>Heparin-binding EGF-like growth factor</td>
<td>Without: 52, With: 269, (Fold): 5.2</td>
</tr>
<tr>
<td>IGF1</td>
<td>Insulin-like growth factor 1</td>
<td>Without: 37, With: 498, (Fold): 13.3</td>
</tr>
<tr>
<td>TGFβ1</td>
<td>Transforming growth factor β1</td>
<td>Without: 75, With: 210, (Fold): 2.8</td>
</tr>
<tr>
<td>MMP3</td>
<td>Matrix metallopeptidase 3</td>
<td>Without: 23, With: 2,509, (Fold): 109.8</td>
</tr>
<tr>
<td>MMP12</td>
<td>Matrix metallopeptidase 12</td>
<td>Without: 10, With: 1,775, (Fold): 177.5</td>
</tr>
</tbody>
</table>

Gene expression analysis on cPCs, which were incubated with or without neovessels, was performed using the 3D-Gene mouse Oligo Chip (Toray). Fold-change differences in gene expression between cPCs incubated with and without neovessels are expressed as Log2 (ratio). cPCs, capillary pericytes.

**Figure 2.** Effect of hypoxia and cytokines on Ninjurin1 (nerve injury-induced protein; Ninj1) expression. (A) Capillary pericytes (cPCs) were incubated in a hypoxia chamber (6–12% O₂) for indicated times. (B) cPCs were treated with tumor necrosis factor (TNF)-α at indicated concentration for 3 days. (C) Capillary endothelial cells (cECs) and (D) human umbilical vein endothelial cells (HUVECs) were incubated in a hypoxia chamber for 3 days. Then, gene expression of vascular endothelial growth factor (VEGF) and Ninj1 was estimated by quantitative polymerase chain reaction (qPCR) (A,B,D) or reverse transcription (RT)-PCR (C). For qPCR, each value was calculated as the ratio to β-actin, an internal control. Data represent mean±SEM (n=4–6) *P<0.05, **P<0.01 vs. control (normoxia).
Ninjurin1 Regulates Angiogenesis

Ninjurin1 (nerve injury-induced protein; Ninj1) expression in ischemic skeletal muscles. (A) Skeletal muscle tissue samples were collected at indicated times after hind limb ischemia (HLI) surgery. Ninj1 expression in ischemic tissues was determined by quantitative polymerase chain reaction (qPCR). Each value was calculated as a ratio relative to the internal control, β-actin. Data represent mean±SEM (n=3). (B) Imaging was performed 14 days after HLI surgery. Ninj1 (green) expression was detected around capillary endothelial tubes stained by rhodamine-labeled lectin (red) (arrow heads). Scale bar=50 μm.

We found that TNF-α increased expression of Ninj1 in cPCs (Figure 2B). Although Ninj1 expression in ECs was relatively weak under normoxic conditions (Figures 1C,D), Ninj1 and VEGF expression was enhanced by hypoxia in both cECs and also detected in both HSMCs and HUVECs, and was higher in the former (Figure 1D). Hypoxia is a major trigger of angiogenesis that induces the secretion of VEGF. Both VEGF and Ninj1 expression was significantly enhanced in cPCs cultured under hypoxic conditions (Figure 2A). Pro-inflammatory cytokines including tumor necrosis factor (TNF)-α released from immune and other cell types, act directly or indirectly on vascular cells to mediate angiogenesis. We found that TNF-α increased expression of Ninj1 in cPCs (Figure 2B). Although Ninj1 expression in ECs was relatively weak under normoxic conditions (Figures 1C,D), Ninj1 and VEGF expression was enhanced by hypoxia in both cECs and...
3D gel. As previously reported, 16 neovessels — which are mainly composed of lectin-positive EC tubes — sprouted from the adventitia of aortic rings after 7 days incubation (Figures 5A, B). EC tubes extended among cPC cell clusters; some cPCs formed capillary-like structures in association with EC tubes (Figure 5B). The growth of neovessels from aortic tissue was significantly increased by co-incubation with cPCs (Figures 5A, C, D). Knocking down endogenous Ninj1 expression enhanced the angiogenic effect of cPCs (Figures 5A, C), whereas overexpressing Ninj1 suppressed these effects (Figures 5A, D). These data demonstrate that Ninj1 expressed in cPCs negatively regulates PC-mediated angiogenesis; that is, EC tube formation.

Ninj1 Negatively Regulates the Production of Angiogenic Factors in cPCs

The angiogenic function of cPCs is mediated through their trophic effects and through direct interaction with ECs. 2, 20, 21 To investigate the mechanism by which Ninj1 regulates the angiogenic effects of cPCs, the expression of angiogenesis-related factors in these cells was assessed. As shown in Table 2, siRNA-mediated reduction of Ninj1 increased the expression of a number of angiogenic factors, including Ang1, VEGF, IGF, and connective tissue growth factor (CTGF). When Ninj1 was overexpressed in cPCs, the production of these factors was attenuated.
Table 2. Role of Ninj1 on the Production of Angiogenic Factors by cPCs

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Angiogenic factors</th>
<th>Ninj1 overexpression</th>
<th>Ninj1 knockdown</th>
</tr>
</thead>
<tbody>
<tr>
<td>Angpt1</td>
<td>Angiopoietin 1</td>
<td>−1.48</td>
<td>3.81</td>
</tr>
<tr>
<td>Angpt2</td>
<td>Angiopoietin 2</td>
<td>3.41</td>
<td>1.44</td>
</tr>
<tr>
<td>CTGF</td>
<td>Connective tissue growth factor</td>
<td>−7.55</td>
<td>1.57</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
<td>−1.65</td>
<td>2.30</td>
</tr>
<tr>
<td>IGF1</td>
<td>Insulin-like growth factor 1</td>
<td>−14.28</td>
<td>6.56</td>
</tr>
<tr>
<td>VEGFα</td>
<td>Vascular endothelial growth factor A</td>
<td>−1.50</td>
<td>1.32</td>
</tr>
<tr>
<td>VEGFβ</td>
<td>Vascular endothelial growth factor B</td>
<td>−1.43</td>
<td>1.23</td>
</tr>
<tr>
<td>TGFβ1</td>
<td>Transforming growth factor, β 1</td>
<td>1.07</td>
<td>−1.61</td>
</tr>
<tr>
<td>MMP9</td>
<td>Matrix metalloproteinase 9</td>
<td>−2.32</td>
<td>1.21</td>
</tr>
</tbody>
</table>

cPCs were transfected with: (1) Ninj1-expressing or control vectors; or (2) Ninj1-specific siRNA or control small interfering (si)RNA. After 2-day incubation, gene expression of angiogenesis-related genes was determined by quantitative PCR using RT² Profiler PCR Array (QIAGEN). Changes in mRNA levels after: (1) Ninj1 overexpression; or (2) Ninj1 downregulation were calculated. Genes that showed an inverse change between these 2 experiments are indicated. Ninj1, Ninjurin1; PCR, polymerase chain reaction. Other abbreviation as in Table 1.

Discussion

In the present study, we identified Ninj1 as a potential negative regulator of angiogenesis through the action of cPCs. PCs have a potent effect on angiogenesis, in part through trophic effects. Consistent with previous reports,23,24 the expression of angiogenesis-related genes — including VEGF, Ang1, and HGF — in cPCs was increased during neovessel formation and under hypoxic conditions, and the expression of Ninj1 was also enhanced along with the production of angiogenic factors. Moreover, Ninj1 inhibited the production of angiogenic factors by cPCs and mitigated their angiogenic effects. Collectively, these results suggest that Ninj1 expressed in cPCs negatively regulates angiogenesis by suppressing the trophic effects of cPCs (Figure 6).

The expression of Ninj1 in cPCs was enhanced when these cells were co-incubated with growing neovessels under normoxic conditions. Although the underlying mechanism is not well understood, it may be that the direct and/or indirect action of neovessels on cPCs induces Ninj1 expression. It is well-documented that pro- and anti-angiogenic factors, including inflammatory cytokines and microRNAs, are produced by vascular as well as immune cells and mediate angiogenesis.19,23,24 Thus, factors released from neovessel ECs may indirectly affect the gene expression profile of cPCs. Indeed, we found that TNF-α-induced Ninj1 expression in cPCs. Because some cPCs were associated with sprouting EC tubes, we speculate that direct association of cECs with cPCs affects genomic parameters in cPCs.6 Investigating the possibility of cell-cell interaction would require developing a method of isolating cPCs that are associated with neovessels.

Among the angiogenic factors changing by the expression of Ninj1, the reciprocal change of Ang1 and 2 is particularly interesting (Table 2). It is well-documented that Ang1 is essential for the promotion of angiogenesis.25,26 Ang1 expression was decreased, and then expression of Ang2, an endogenous inhibitor of Ang1,27 was upregulated in Ninj1-overexpressed PCs. These changes were reversed when the expression of endogenous Ninj1 was reduced (Table 2). Expressions of IGF1 and CTGF in cPCs were drastically altered by the expression of Ninj1. IGF1 promotes migration and tube formation in ECs,28 and stabilizes neovessels generated from ECs through extracellular signal-regulated kinase activation.29 CTGF plays a central role in diseases in which tissue remodeling and fibrosis occur.30 CTGF interacts with various molecules, including VEGF, IGF1, and TGF-β, to mediate angiogenesis and extracellular matrix deposition and activate myofibroblasts.31,32 Recent studies using genetic fate mapping to address the origin of myofibroblasts in fibrosis demonstrated that resident microvascular PCs were a source of myofibroblast progenitors.23,33,34 Therefore, Ninj1 also may contribute to tissue remodeling and the pathogenesis of fibrosis, in addition to angiogenesis.

Recently, Yin et al reported that expression of Ninj1 was increased in penile tissue in streptozotocin-induced diabetic mice, and attenuation of Ninj1 function by injection of neutralizing anti-Ninj1 antibody successfully restored erectile function through enhanced penile angiogenesis.13 This group examined the role of Ninj1 on EC tube formation in vitro using mouse cavernous ECs (MCECs). High glucose enhanced the expression of Ninj1, and siRNA-mediated Ninj1-downreg-
ulation was only able to rescue EC-tube formation in the high-glucose-damaged MCECs. Indeed, we demonstrated that downregulation of Ninj1 in cECs did not affect EC-tube formation under normal glucose conditions. Thus, anti-angiogenic effects of Ninj1 would be explained by the effects on ECs only in the hyperglycemic, but not normal conditions. Furthermore, it is not clear if Ninj1 affects angiogenesis only in specific tissue types such as penile tissue, and if Ninj1 affects angiogenesis only under special pathological conditions such as diabetes mellitus. In this study, we demonstrated that Ninj1 expression was enhanced in vascular cells (ie, cPCs and cECs) under hypoxic conditions and in the microvasculature of ischemic skeletal muscle tissue. Therefore, Ninj1 may also contribute to the angiogenesis in the ischemic diseases in addition to diabetic mellitus.

Inhibiting Ninj1 expression in cECs had no effect on their ability to form tube-like structures; however, Ninj1 overexpression decreased EC tube formation. This discrepancy between the loss- and gain-of-function phenotypes could be due, in part, to a low expression level of endogenous Ninj1 in cECs under normal conditions. Given that hypoxia markedly enhanced Ninj1 expression, Ninj1 might not affect cEC tube formation under normoxia but may exert anti-angiogenic effects under conditions of high Ninj1 expression such as in hypoxia. However, these results require cautious interpretation because Ninj1 was overexpressed in these experiments at a super-physiological level.

The current study provides new evidence that Ninj1 negatively regulates angiogenesis in peripheral tissues; that is, the aorta adventitia. The physiological or pathological significance of this finding remains unclear. cPCs participate in the early phase of angiogenesis. cPCs dissociated from the vessels and are located in the growing front of the sprouting ECs, promoting EC survival and guiding newly formed vessels through their trophic effects. Thus, Ninj1 may inhibit EC tube sprouting in early phases of angiogenesis. cPCs have another important role in angiogenesis because they contribute to the later stage of angiogenesis; that is, vascular stability and maturation by recruiting PCs to nascent EC-tubes. For stabilization of neovessels, the proliferation and migration of ECs are attenuated. Thus, anti-angiogenic effects of Ninj1 would contribute to vascular stabilization. Ninj1 is an adhesion molecule that is essential for cell-cell interactions; for instance, it is expressed in myeloid cells, including macrophages and monocytes, and mediates adhesion between these and ECs. Therefore, Ninj1 may contribute to vascular stability and maturation by mediating the interaction between PC and EC tubes (Figure 6).

The formation of EC tubes, a somewhat type of immature vessel, can be assessed by most in vitro angiogenesis assays, including the aortic ring angiogenesis assay. When cPCs were incubated with nascent vessels, cPCs associated with EC tubes to form capillary-like structures. However, this is rarely observed in our in vitro angiogenesis system, making it difficult to quantitatively evaluate EC-PC interactions and vascular maturation. Additional studies are required to clarify the role of Ninj1 in capillary cells under physiological and pathological conditions using in vivo methods that allow the observation of EC-PC interactions, such as in transgenic mice in which Ninj1 is specifically overexpressed or inhibited in PCs.

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Ninjurin1 Regulates Angiogenesis


**Supplementary Files**

**Figure S1.** Co-incubation of capillary pericytes (cPCs) with capillary endothelial cells (cECs) to form capillary-like structures. Please find supplementary file(s); http://dx.doi.org/10.1253/circj.CJ-14-1376