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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. (*Circ J* 2015; **79:** 1363–1371)

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 (EC) tubes to form microvessels, are implicated in the regulation of vascular morphogenesis and functions.[1,2] Abnormal pericyte function or loss of pericytes leads to increased vascular permeability, immature vessel formation, and further loss of microvessels.[3–5] The role of pericytes is mediated through specific trophic effects and the direct interaction with ECs.[1,2] 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,[6,7] the mechanisms that regulate their actions in microvascular physiology have been largely underinvestigated.

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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.[8,9] 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.[8] Recently, it has been reported that Ninj1 affects non-neuronal cells and has a role in neuronal and non-neuronal patho-physiological conditions. Ninj1 is responsible for the progression of multiple sclerosis, an autoimmune inflammatory central nervous system disease.[10] Ninj1 participates in the invasion of myelin-reactive T cells across the blood-brain barrier to mediate neuronal inflammation in multiple sclerosis.[11] 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.[12] Recently, Yin et al reported that attenuation of Ninj1 function via neutralizing anti-Ninj1 antibodies, successfully restored erectile function,

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.[14] 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 48–72 h 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 AnaeroPack system (Mitsubishi Gas Chemical Company, Inc; Tokyo, Japan), which absorbs O_2 in the jar and generates an environment of 6–12% O_2. Hypoxia was measured using an anaerobic indicator (Becton, Dickinson and Company, Sparks, MD, USA). After predefined 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 10 ng/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 Matirgel 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 1×10^4 cells/well in 100 μl medium (EBM2 with 2% FBS and 10 ng/ml VEGF). The assay was performed in a CO_2 incubator, with plates incubated at 37°C for 24 h. Images were obtained by phase contrast microscopy and the length of the tube-like structure in each well was measured at 40× magnification.

Hind Limb Ischmia 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 (HLI) 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 (200 mg/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 300 μl phosphate-buffered saline (PBS) containing rhodamine-labeled G. simplicifolia lectin I (100 μg/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 (Bioss, Woburn, MA, USA) and an Alexa488-conjugated secondary antibody (Invitrogen). Nuclei were counterstained with Hoechst 33258 (Lonza, Basel, Switzerland).
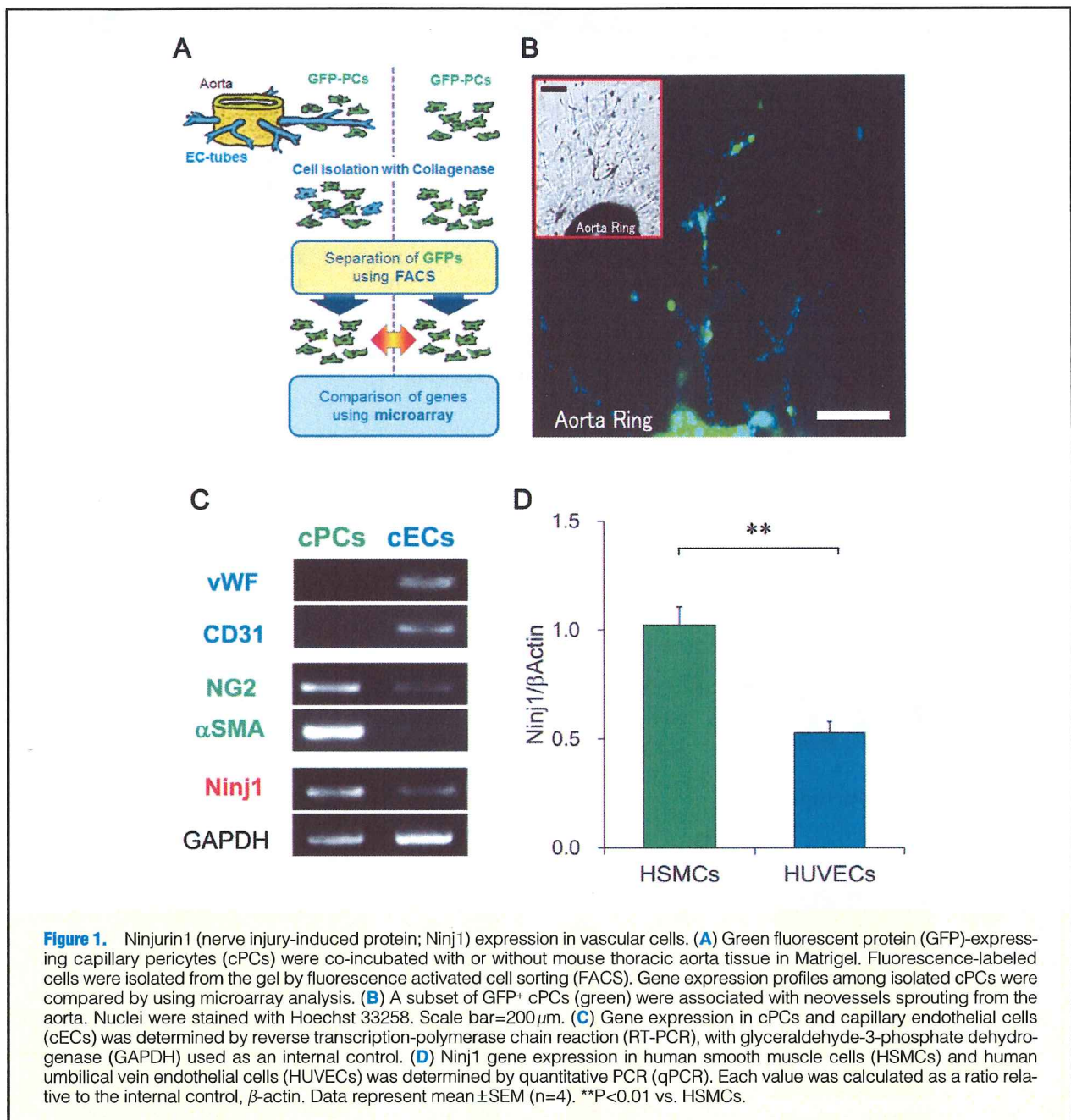
Gene Microarray Analysis
GFP-expressing cPCs ($5\times10^3/20\,\mu$l gel) were cultured in growth factor-reduced Matrigel with or without aortic rings, as described above. After 7 days of incubation, all cells within the gel were isolated using Liberase DL (Roche; Indianapolis, IN, USA). Living cPCs were separated by FACS by means of 7-amino-actinomycinD-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 10 μmol/L sense and anti-sense primers. The primers of mouse Ninj1, VEGF were: sense, 5'-ACTGAGGAGTATGAGCTCA-3', anti-sense, 5'-TCCATTACAGGCTTCTGGA-3', and sense, 5'-ATGAACTTTCTGCTCTCTTGGGTGC-3', anti-sense, 5'-TCACCGCCTTGGCTTGTCACATCTG-3', respectively. For quantitative PCR analysis, total RNA was reverse-transcribed using a Transcriptor High Fidelity cDNA Synthesis

Figure 1. Ninjurin1 (nerve injury-induced protein; Ninj1) expression in vascular cells. (**A**) Green fluorescent protein (GFP)-expressing capillary pericytes (cPCs) were co-incubated with or without mouse thoracic aorta tissue in Matrigel. Fluorescence-labeled cells were isolated from the gel by fluorescence activated cell sorting (FACS). Gene expression profiles among isolated cPCs were compared by using microarray analysis. (**B**) A subset of GFP+ cPCs (green) were associated with neovessels sprouting from the aorta. Nuclei were stained with Hoechst 33258. Scale bar=200 μm. (**C**) Gene expression in cPCs and capillary endothelial cells (cECs) was determined by reverse transcription-polymerase chain reaction (RT-PCR), with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) used as an internal control. (**D**) Ninj1 gene expression in human smooth muscle cells (HSMCs) and human umbilical vein endothelial cells (HUVECs) was determined by quantitative PCR (qPCR). Each value was calculated as a ratio relative to the internal control, β-actin. Data represent mean±SEM (n=4). **P<0.01 vs. HSMCs.

Kit (Roche; Basel, Switzerland), and cDNA was quantified by real-time PCR on a 7300 PCR system (Applied Biosystems; Foster City, CA, USA). Real-time quantitative PCR was carried out using the TaqMan Gene Expression Master Mix (Applied Biosystems), according to the manufacturer's instructions. The relative mRNA expression level was calculated by using the comparative threshold cycle (Ct) method, with mouse β-actin used as an internal control. The mouse angiogenesis RT^2 Profiler PCR Array (QIAGEN) was used to obtain angiogenesis-related gene expression profiles of cPCs. Ct values were used to calculate folds changes in mRNA abundance, with the levels in the control group set to 1.

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

Ninj1 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-

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

Symbol	Angiogenic factors	Neovessels		
	Gene	Without	With	(Fold)
VEGFα	Vascular endothelial growth factor α	83	398	4.8
Ang1	Angiopoietin 1	106	224	2.1
FGF7	Fibroblast growth factor 7	1,083	3,558	3.3
HGF	Heparin-binding EGF-like growth factor	52	269	5.2
IGF1	Insulin-like growth factor 1	37	498	13.3
TGFβ1	Transforming growth factor β1	75	210	2.8
MMP3	Matrix metallopeptidase 3	23	2,509	109.8
MMP12	Matrix metallopeptidase 12	10	1,775	177.5
Ninj1	Ninjurin1	131	555	4.2

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.
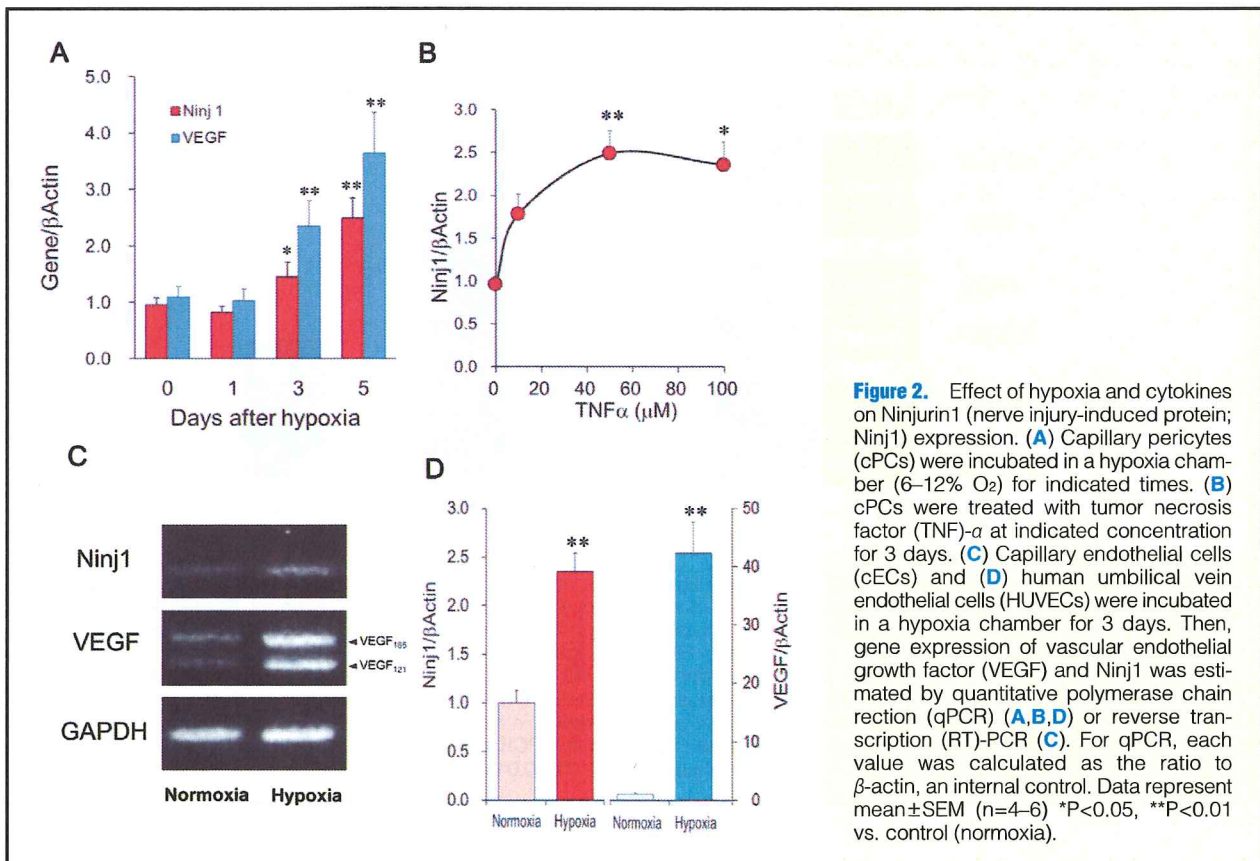
Figure 2. Effect of hypoxia and cytokines on Ninjurin1 (nerve injury-induced protein; Ninj1) expression. (**A**) Capillary pericytes (cPCs) were incubated in a hypoxia chamber (6–12% O2) for indicated times. (**B**) cPCs were treated with tumor necrosis factor (TNF)-α at indicated concentration for 3 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 rection (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).

esis assay. Consistent with an earlier study,[15] 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,[17] 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**).

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
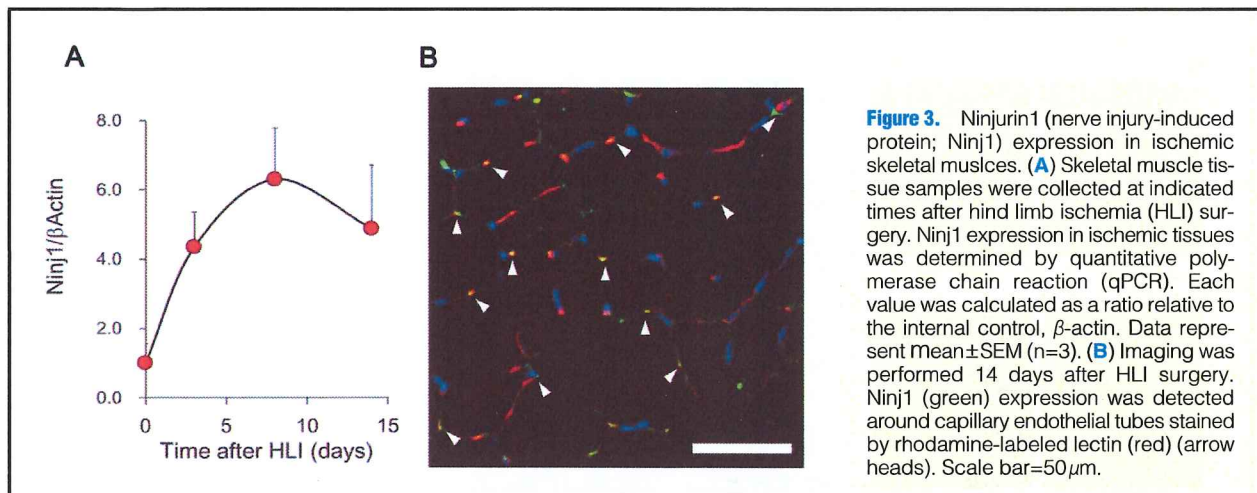
Figure 3. Ninjurin1 (nerve injury-induced protein; Ninj1) expression in ischemic skeletal muslces. (**A**) Skeletal muscle tissue samples were collected at indicated times after hind limb ischemia (HLI) surgery. Ninj1 expression in ischemic tissues was determined by quantitative polymerase chain reaction (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=50μm.
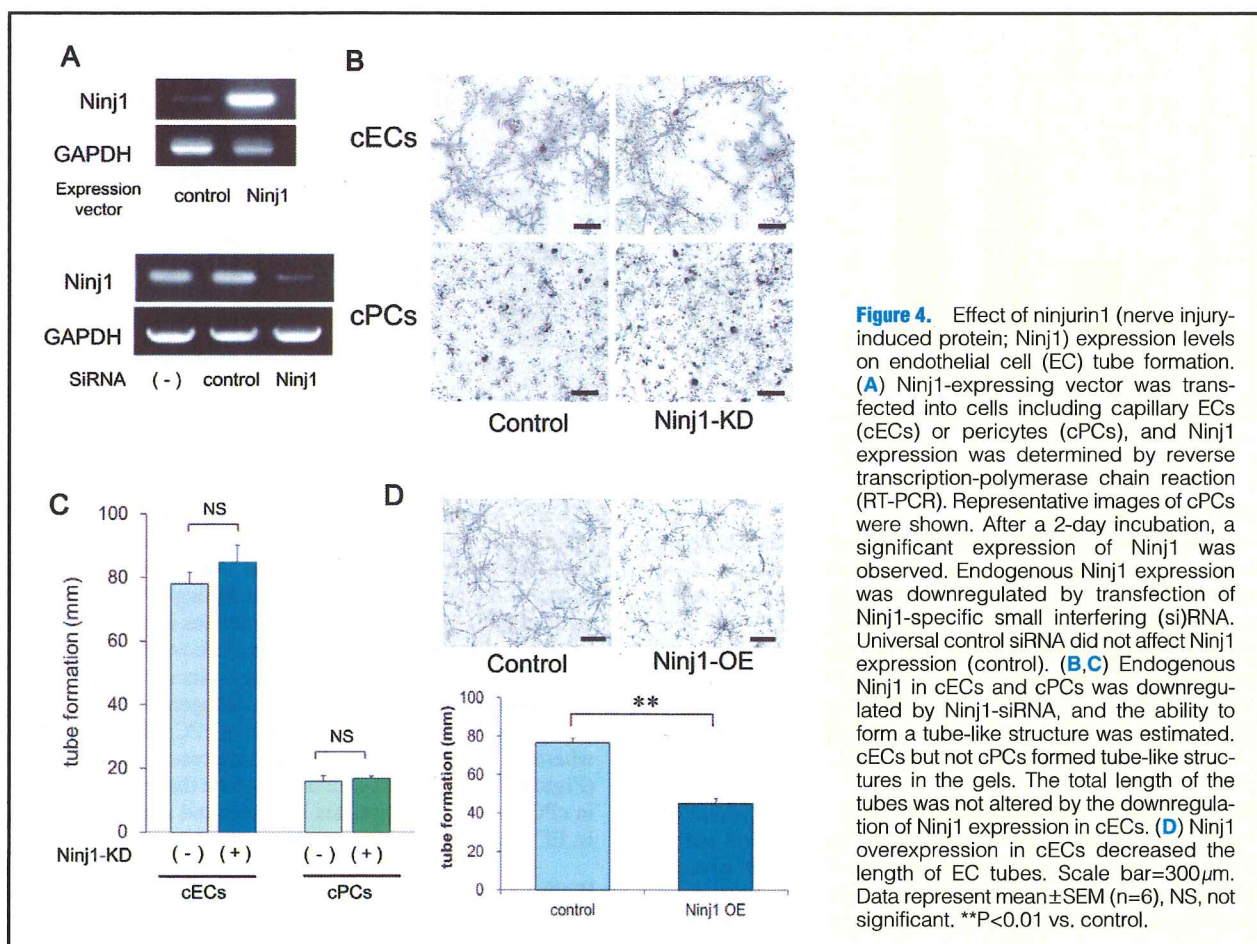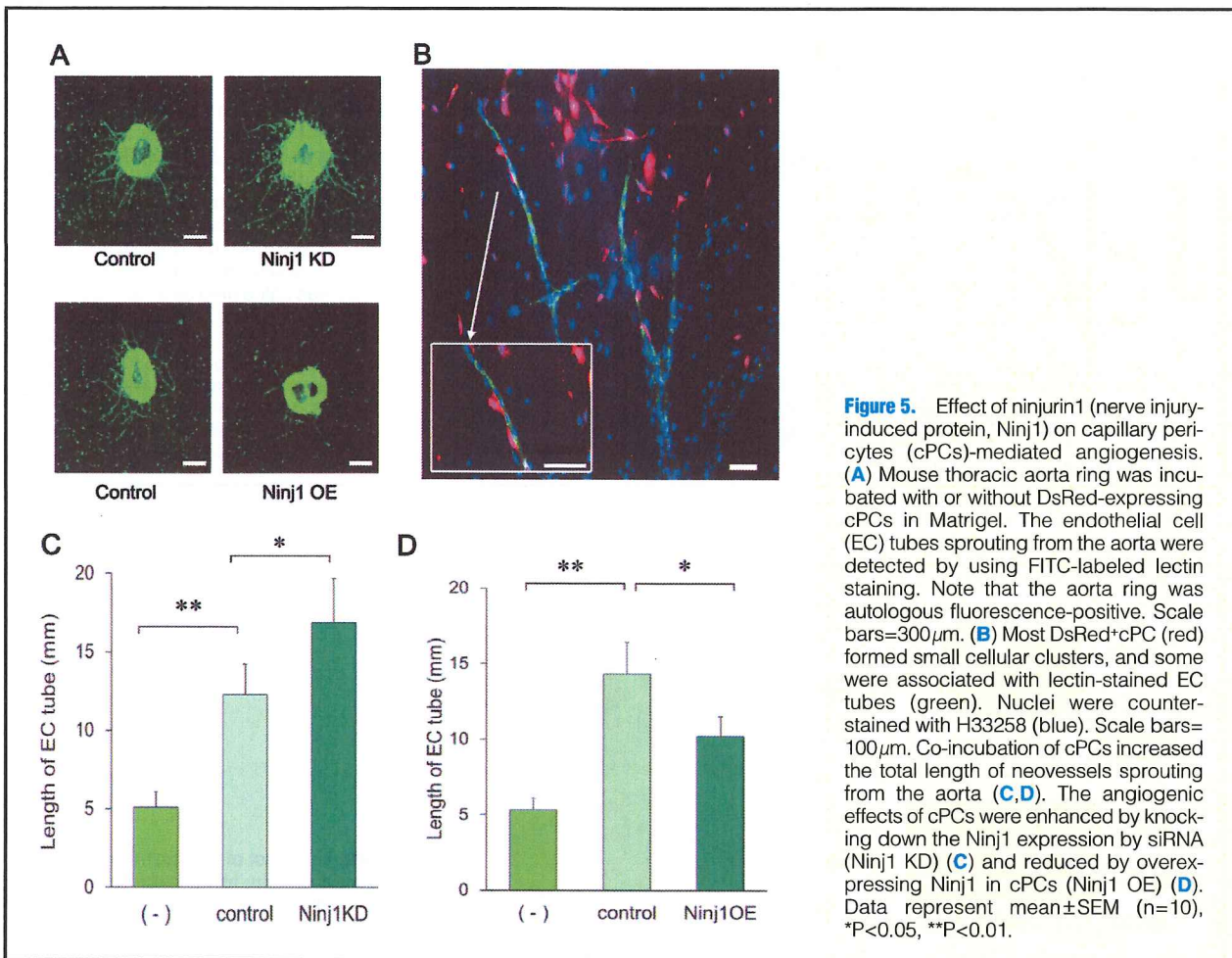

Figure 4. Effect of ninjurin1 (nerve injury-induced protein; Ninj1) expression levels on endothelial cell (EC) tube formation. (**A**) Ninj1-expressing vector was transfected into cells including capillary ECs (cECs) or pericytes (cPCs), and Ninj1 expression was determined by reverse transcription-polymerase chain reaction (RT-PCR). Representative images of cPCs were shown. After a 2-day incubation, a significant expression of Ninj1 was observed. Endogenous Ninj1 expression was downregulated by transfection of Ninj1-specific small interfering (si)RNA. Universal control siRNA did not affect Ninj1 expression (control). (**B,C**) Endogenous Ninj1 in cECs and cPCs was downregulated by Ninj1-siRNA, and the ability to form a tube-like structure was estimated. cECs but not cPCs formed tube-like structures in the gels. The total length of the tubes was not altered by the downregulation of Ninj1 expression in cECs. (**D**) Ninj1 overexpression in cECs decreased the length of EC tubes. Scale bar=300μm. Data represent mean±SEM (n=6), NS, not significant. **P<0.01 vs. control.

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.[17] 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.[18,19] 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

Figure 5. Effect of ninjurin1 (nerve injury-induced protein, Ninj1) on capillary pericytes (cPCs)-mediated angiogenesis. (**A**) Mouse thoracic aorta ring was incubated with or without DsRed-expressing cPCs in Matrigel. The endothelial cell (EC) tubes sprouting from the aorta were detected by using FITC-labeled lectin staining. Note that the aorta ring was autologous fluorescence-positive. Scale bars=300 μm. (**B**) Most DsRed+cPC (red) formed small cellular clusters, and some were associated with lectin-stained EC tubes (green). Nuclei were counterstained with H33258 (blue). Scale bars= 100 μm. Co-incubation of cPCs increased the total length of neovessels sprouting from the aorta (**C,D**). The angiogenic effects of cPCs were enhanced by knocking down the Ninj1 expression by siRNA (Ninj1 KD) (**C**) and reduced by overexpressing Ninj1 in cPCs (Ninj1 OE) (**D**). Data represent mean±SEM (n=10), *P<0.05, **P<0.01.

HUVECs (**Figures 2C,D**).

The HLI mouse model was used to determine whether Ninj1 expression is upregulated in ischemic tissues. Ninj1 expression in skeletal muscle tissues was increased and reached at peak 7 days after HLI (**Figure 3A**), as determined by real-time PCR. An immunocytochemical analysis found that Ninj was predominantly expressed in capillary cells in ischemic skeletal muscle tissue (**Figure 3B**). These observations suggest that Ninj1 expressed in vascular cells — including PCs and ECs — contributes to angiogenesis.

Ninj1 Inhibits EC Tube Formation by Acting on cECs and cPCs
Gain- and loss-of-function experiments were carried out to clarify the role of Ninj1 in angiogenesis. Ninj1 was overexpressed in cells by transfecting a Ninj1-expressing vector and was knocked down using Ninj1-targeted siRNA (**Figure 4A**). As previously reported,[14] cECs can form EC tubes in 3D gel (**Figure 4B**). Inhibiting Ninj1 in cECs did not affect EC tube formation (**Figures 4B,C**); however, overexpressing Ninj1 significantly reduced the length of tube-like structures (**Figure 4D**).

cPCs cultured alone formed small cell clusters but did not form tubes in the gel (**Figure 4B**), and this tendency was unaltered by knocking down Ninj1expression in cPCs (**Figure 4C**). To observe the angiogenic effect of cPCs, DsRed-expressing cells were co-incubated with mouse thoracic aorta tissue in a 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 whicn 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

| Symbol	Angiogenic factors	Change (fold) by...	
	Gene	Ninj1 overexpression	Ninj1 knockdown
Angpt1	Angiopoietin 1	−1.48	3.81
Angpt2	Angiopoietin 2	3.41	1.44
CTGF	Connective tissue growth factor	−7.55	1.57
EGF	Epidermal growth factor	−1.65	2.30
IGF1	Insulin-like growth factor 1	−14.28	6.56
VEGFα	Vascular endothelial growth factor A	−1.50	1.32
VEGFβ	Vascular endothelial growth factor B	−1.43	1.23
TGFβ1	Transforming growth factor, β 1	1.07	−1.61
MMP9	Matrix metallopeptidase 9	−2.32	1.21

cPCs were transfected with: (1) Ninj1-expressing or control vectors; or (2) Ninj1-specific siRNA or control small interfering (si)RNA. After a 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 idenftified 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,[17,22] 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 anigiogenic 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 anigiogenesis 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
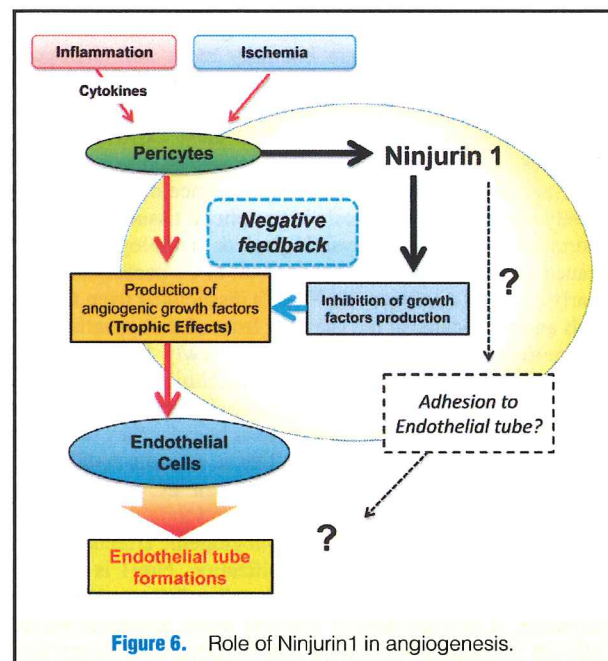
Figure 6. Role of Ninjurin1 in angiogenesis.

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 myofibroblats in fibrosis demonstrated that resident microvascular PCs were a source of myofibroblast progenitors.[2,33,34] 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.[13] 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.[35] cPCs dissociated from the vessels and are located at the growing front of the sprouting ECs, promoting EC survival and guiding newly formed vessels through their trophic effects.[1,35] 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.[2,7,36] For stabilization of neovessels, the proliferation and migration of ECs are attenuated. Thus, the anti-angiogenic effects of Ninj1 would contribute to vascular stabilization. Ninj1 is an adhesion molecule that is essential for cell-cell interactions;[8] for instance, it is expressed in myeloid cells, including macrophages and monocytes, and mediates adhesion between these and ECs.[37] 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.

Acknowledgments

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Supplementary Files

Supplementary File 1

Figure S1. Co-incubation of capillary pericytes (cPCs) with capillary endothelial cells (cECs) to form capillary-like structures.