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ORIGINAL ARTICLE

# Long-term expression of periostin during the chronic stage of ischemic stroke in mice

Munehisa Shimamura[1], Yoshiaki Taniyama[2], Hironori Nakagami[1], Naruto Katsuragi[3], Kouji Wakayama[4], Hiroshi Koriyama[1], Hitomi Kurinami[1], Akiko Tenma[1], Hideki Tomioka[1] and Ryuichi Morishita[2]

**Periostin is an extracellular matrix glycoprotein and has various cellular effects. Previously, we demonstrated the neuroprotective effects of periostin during the acute stage of cerebral ischemia. However, its expression during the chronic stage remains unknown. Herein, we examined the expression of full-length periostin (periostin 1; *Pn1*) and its splicing variant lacking exon 17 (periostin 2; *Pn2*) during the 28 days following transient middle cerebral artery occlusion in mice. Real-time reverse transcription-PCR showed that the expression of *Pn2* was dramatically upregulated between days 3 and 28, and the highest expression was observed on day 7. The expression of *Pn1* was also increased, but delayed compared with *Pn2*. Immunohistochemistry showed that periostin was weakly expressed in reactive astrocytes in the peri-infarct region and in microglia/macrophages in infarct regions, on days 3 and 7. Periostin was also expressed around CD31-positive cells in both the peri-infarct and the sub-ventricular zone (SVZ) on days 3 and 7. SOX-2 positive cells, which are neural stem cells, also expressed periostin on day 7. The highest periostin immunoreactivity that occurred co-localized with collagen I and fibronectin in the peri-infarct region between days 7 and 28. Thus, the expression pattern of periostin mRNA was dependent on the splicing variant, and it continued to be expressed up to 28 days after cerebral ischemia. As periostin was expressed in various cells, such as reactive astrocytes/microglia, fibroblasts and neuronal progenitor cells, periostin might be associated with pathophysiology in post-ischemic inflammation and neurogenesis.**
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**Keywords:** extracellular matrix; ischemia; middle cerebral artery occlusion; periostin

## INTRODUCTION

Periostin is a 93-kDa secreted extracellular matrix (ECM) *N*-glycoprotein and has variable sequences in the C-terminal region. In humans, exons 17, 18, 19 and 21 are alternatively spliced, whereas exons 17, 20 and 21 are alternatively spliced in mice.[1] Recently, periostin was reported to be a critical marker of progression/reversal of hypertensive nephropathy[2] and another study showed that angiotensin II increased periostin expression in a rat model of continuous angiotensin II infusion and in cultured adult rat cardiac fibroblasts.[3] Ischemic stroke is also one of major complications in hypertension[4] and we previously showed the expression in reactive astrocytes and periendothelium in the acute phase of ischemic stroke, where periostin 2 (Pn2, splicing variant lacking exon 17) but not periostin 1 (Pn1, full-length periostin) worked as neuroprotective.[5] However, the expression of periostin has not been clarified in the chronic stage of ischemic stroke.

Recent studies showed that periostin has important roles in (1) the migration and proliferation of fibroblasts,[6] which express ECM molecules such as laminin and fibronectins,[7] (2) the expression of matrix metalloproteinases (MMPs) in macrophages,[8] and (3) T regulatory cell differentiation induced by transforming growth factor (TGF)-β1.[9] From the viewpoint, we hypothesized that periostin would be expressed in the chronic stage of ischemic stroke, as the fibroblasts invading the lesion site with secreting ECMs[10] and T regulatory cells[11] as well as MMPs[12] in the chronic phase have been reported to have important roles in the recovery of injured brain. In addition, periostin was reported to be dependent on the integrin-Akt pathway[13] in cell proliferation and the integrin-FAK pathway in cell migration.[14] Because integrin receptors are involved in migration[15,16] and proliferation[16] of neuronal progenitor cells in the SVZ, we also hypothesized that periostin might be expressed in the SVZ.

To clarify these hypothesis, we examined the expression of periostin mRNA and protein in mice during the 28 days after transient middle cerebral artery occlusion (tMCAo). Although mice have several alternative splice variants of the periostin gene in exons 17, 20 and 21, we focused on Pn1 (full-length periostin) and Pn2 (splicing

[1]Division of Vascular Medicine and Epigenetics, Department of Child Development, United Graduate School of Child Development, Osaka University, Kanazawa University and Hamamatsu University School of Medicine, Osaka, Japan; [2]Department of Clinical Gene Therapy, Graduate School of Medicine, Osaka University, Osaka, Japan; [3]Biomedical Research Laboratories, Asubio Pharma Co, Ltd, Hyogo, Japan and [4]Department of Advanced Clinical Science and Therapeutics, Graduate School of Medicine, University of Tokyo, Tokyo, Japan
Correspondence: Professor R Morishita, Department of Clinical Gene Therapy, Osaka University, Graduate School of Medicine, 2-2 Yamada-oka, Osaka 565-0871, Japan.
E-mail: morishit@cgt.med.osaka-u.ac.jp
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variant lacking exon 17) in the present study, because the functions of Pn1 and Pn2 are relatively well known from our previous study.[5]

## METHODS

### Surgical procedure

All procedures were approved by the Institutional Animal Care and Use Committee of Osaka University. Experiments were performed in 6- to 8-week-old male C57BL/6 mice (CLEA Japan, Tokyo, Japan).

### tMCAo

Mice were anesthetized with 1.4% isoflurane. Cerebral blood flow was measured using a laser Doppler flowmeter (Unique Acquisition software; Unique Medical, Tokyo, Japan). A 6.0 monofilament surgical suture was advanced into the internal carotid artery to obstruct the origin of the medical cerebral artery (MCA). The filament was left in place for 90 min and then removed. Only animals that exhibited more than an 82% reduction in cerebral blood flow (CBF) during MCA occlusion and in which CBF recovered by 50% after 5 min of reperfusion were included in the study. Mouse rectal

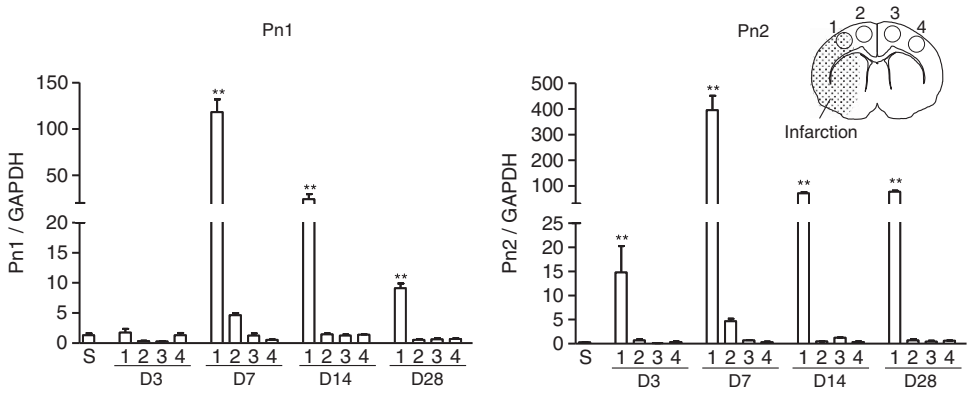


**Figure 1** Expression of periostin after transient MCA occlusion in the brain. Real-time reverse transcription-PCR results from sham-operated mice (S), the infarct and peri-infarct regions (1), the intact region in the ischemic hemisphere (2) and the contralateral intact regions corresponding to the ischemic hemisphere (3, 4). $n=4$. $^{**}P<0.01$ *vs.* sham-operated mice. Pn1 and Pn2, periostin 1 and periostin 2.

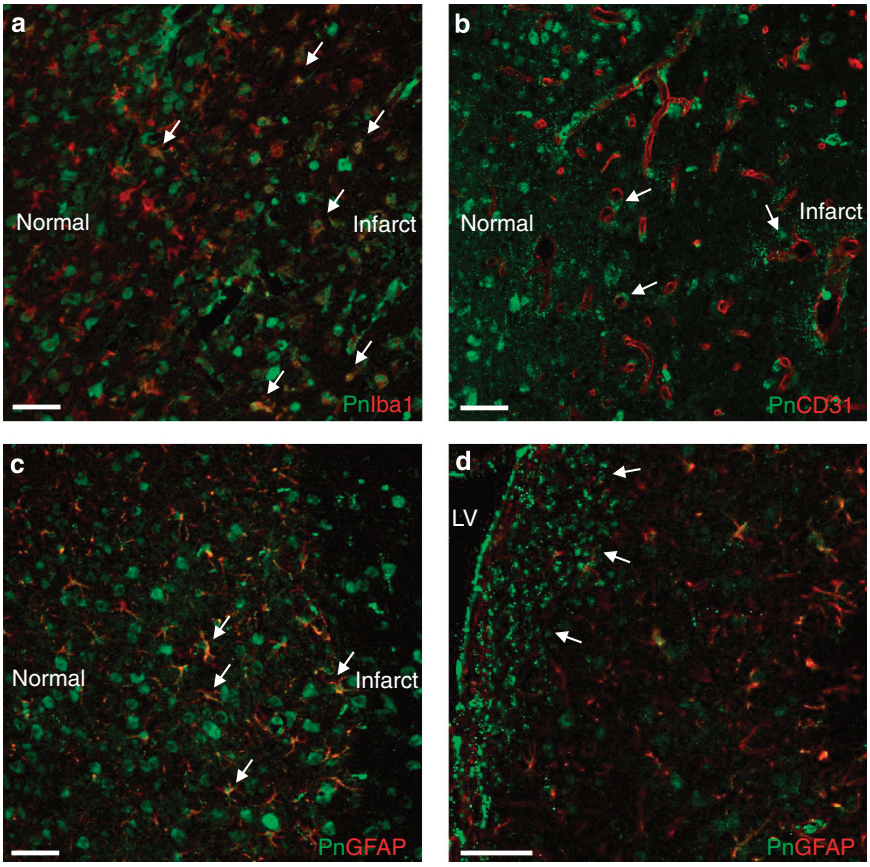


**Figure 2** Expression of periostin (Pn) on day 3 after transient middle cerebral artery occlusion. Pn was expressed in Iba1-positive cells (**a**, arrows), around CD31-positive cells (**b**, arrows) and GFAP-positive cells (**c**, arrows) in the infarct (**a**, **b**) and peri-infarct region (**c**). Pn was also diffusely expressed in the sub-ventricular zone (**d**, arrows). Bar = 100 μm.

temperature was kept at 37.0 ± 0.5 °C during surgery and in the recovery period, until the animals regained consciousness.

### Primer pairs for real-time reverse transcription-PCR

The cerebral cortex was collected using a punch (FST No.18035-80). RNA from the brain punch was extracted using the QIAGEN RNeasy Lipid TissueMini Kit (Qiagen, Germantown, MD, USA), according to the manufacturer's instructions. Each quantitative PCR analysis was performed using an ABI Prism 7700 Sequence Detection System (Applied Biosystems, Applera Co., Norwalk, CT, USA) with SYBR green staining of DNA double strands. The following primer pairs were used: Pn1 (sense: 5′-ATAACCAAAGTCGTGGAACC-3′, antisense: 5′-TGTCTCCCTGAAGCAGTCTT-3′, 415 bp), Pn2 (sense: 5′-CCATGACTGTC TATAGACCTG-3′, antisense: 5′-TGTCTCCCTGAAGCAGTCTT-3′, 360 bp), mouse glyceraldehyde-3-phosphate dehydrogenase (sense: 5′-GGGTGGAGCCA AAAGGGTC-3′, anti-sense: 5′-GGAGTTGCTGTTGAAGTCGCA-3′, 534 bp).[2] Each mRNA value was normalized to the expression of glyceraldehyde-3-phosphate dehydrogenase.

### Immunohistochemical staining

Mice were perfused with 4% paraformaldehyde (PFA), and the brain was cut into 12-μm thick sections. Sections were incubated in an anti-periostin antibody (1:25; goat polyclonal; Santa Cruz Biotechnology, Dallas, TX, USA, #SC-49480). This antibody was previously used to examine the expression of periostin in skin wounds.[17] As a negative control, normal control IgG (Santa Cruz Biotechnology, #SC-2028) was used instead of the anti-periostin antibody. For double immunostaining, sections were fixed again and blocked. The sections were incubated with anti-MAP2 (1:1000; mouse monoclonal; Sigma-Aldrich, St Louis, MO, USA), glial fibrillary acidic protein (GFAP) (1:1000; mouse monoclonal; Sigma-Aldrich), Iba-1 (1:1000; rabbit polyclonal; Wako, Tokyo, Japan), CD31 (1:100; rat monoclonal; Becton Dickinson, San Jose, CA, USA), SOX-2 (1:500; rabbit polyclonal; Merck Millipore, Billerica, MA, USA), Fibronectin (1:200; rabbit polyclonal; Abcam, Cambridge, MA, USA), collagen I (1:500; rabbit polyclonal; Novotec, Lyon, France) or anti-αSMA antibody (1:1000, Sigma-Aldrich).

### Statistical analysis

All values are expressed as the mean ± s.e.m. Multiple comparisons were performed using an analysis of variance followed by Dunnett's Multiple Comparison Test.

## RESULTS

### Expression of mRNA of Pn1 and Pn2 after tMCAo

Elevated expression of *Pn2* mRNA was observed at an earlier time point (Day 3) in the infarct regions compared with *Pn1* mRNA (Figure 1). The expression of Pn2 mRNA was already increased by day 3 and continued to increase until day 7. Pn2 expression level on days 14 and 28 was lower than that on day 7, but still at a high level. By contrast, Pn1 mRNA was increased on day 7 in the infarct regions and gradually decreased between days 14 and 28.

### Immunohistochemical analysis of expression of Pn protein after tMCAo

We previously showed that periostin was expressed in neurons in the normal brain and in reactive astrocytes around endothelial cells in the peri-infarct region, 24 h after ischemia. In this study, on day 3,

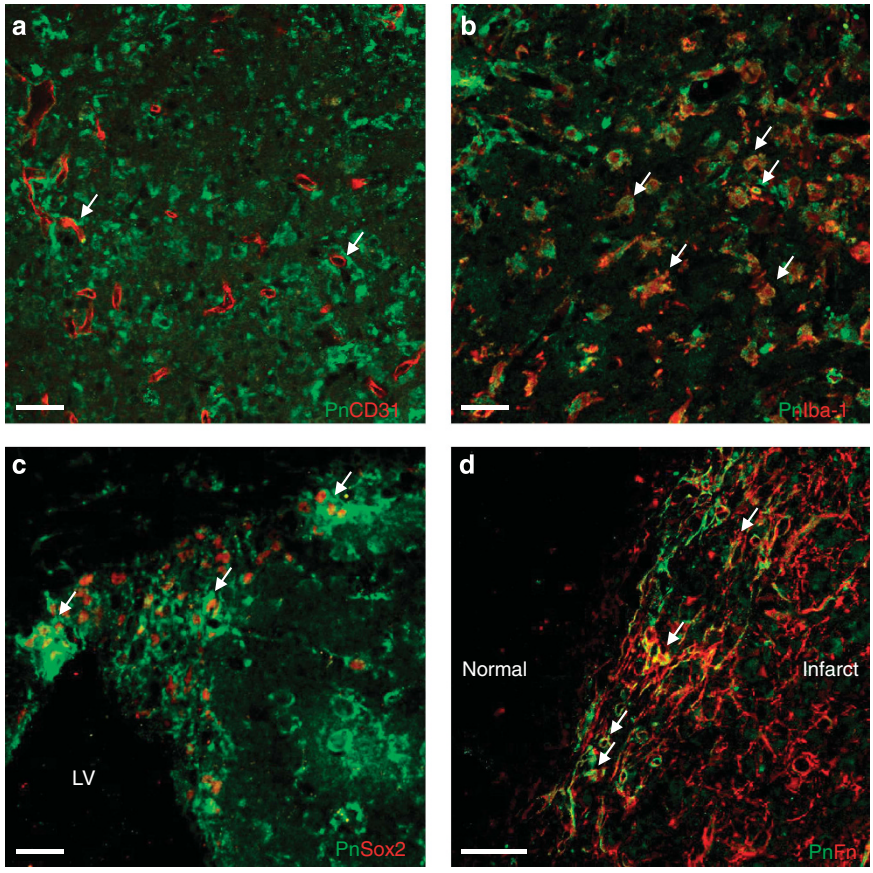


**Figure 3** Expression of periostin (Pn) on day 7. Pn was expressed around CD31-positive cells (**a**, arrows) and in Iba-1-positive microglia/macrophages (**b**, arrows) in the infarct region. SOX2-postitive cells also expressed Pn in the sub-ventricular zone (**c**, arrows). High expression of Pn was co-localized with fibronectin (Fn) (**d**), arrows). LV, lateral ventricle. Bar = 100 μm (**a**–**c**) and 50 μm (**d**).

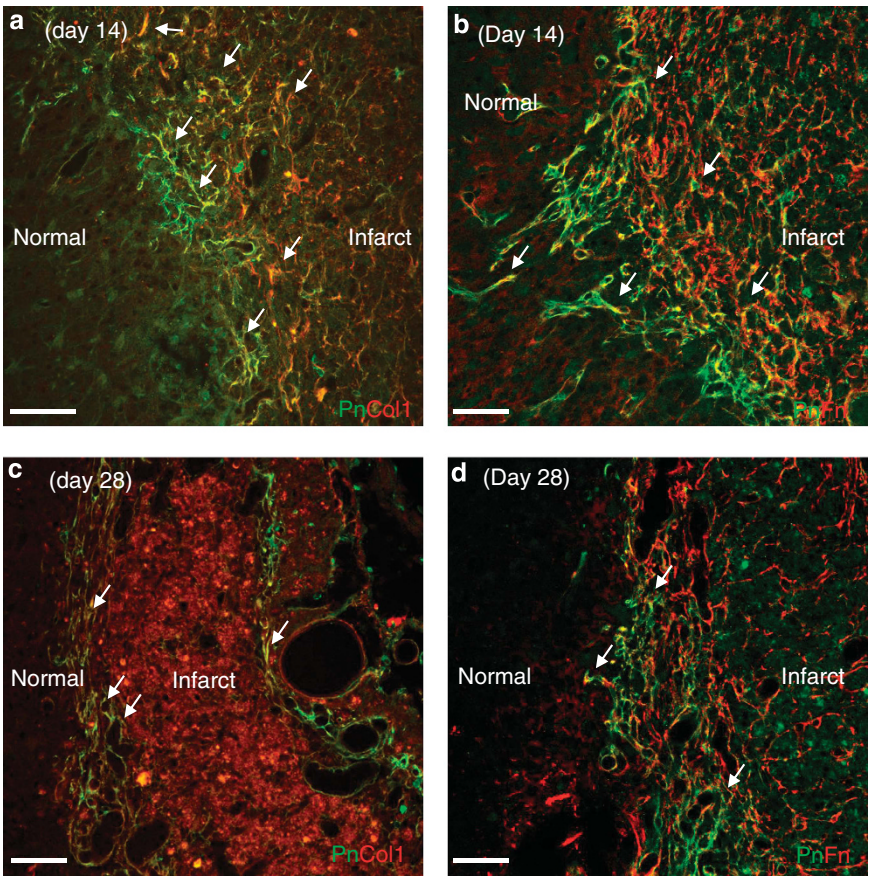


**Figure 4** Expression of periostin (Pn) on days 14 and 28. Pn was strongly expressed and co-localized with collagen I (**a** and **c**, arrows) and fibronectin (**b** and **d**, arrows) in the infarct region. The infarct core showed weak expression of Pn. Col 1, collagen 1; Fn, fibronectin. Bar = 200 μm.

periostin protein could be detected in Iba-1-positive microglia (Figure 2a) and around CD31-positive cells (Figure 2b) in the peri-infarct and infarct regions and in GFAP-positive reactive astrocytes in the peri-infarct regions (Figure 2c). Notably, scattered expression of periostin was also observed in the SVZ in the ischemic hemisphere (Figure 2d).

On day 7, periostin protein was still detected in astrocytes (Supplementary Figures 1A–C), microglia and around CD31-positive cells in the peri-infarct and infarct regions (Figures 3a and b). Periostin was also expressed in SOX2-positive cells, which is a marker for neural progenitor cells, in the SVZ (Figure 3c). Notably, much higher expression was observed in fibronectin- (Figure 3d) and collagen I-positive cells (Supplementary Figures 1D–F) in the peri-infarct regions, but not in the infarct core. Periostin was also co-localized with fibronectin (Supplementary Figures 1G–I) but not with CD-31 or αSMA (data not shown) in pial arteries in ischemic injured brain.

On days 14 and 28, periostin was strongly expressed in the peri-infarct region, but was weakly expressed in the infarct core, where it was co-localized with collagen I (Figures 4a and c) and fibronectin (Figures 4b and d). The expression level in the peri-infarct and infarct region was much higher than that in neurons in normal region (Supplementary Figure 2). The expression of periostin protein was observed in astrocytes on day 14, but not on day 28 (Supplementary Figures 3A, C). Expression of periostin was also observed in microglia in the infarct region (Supplementary Figures 3B, D), but not in the SVZ (data not shown). The temporal and spatial profile of periostin expression are summarized in Table 1. Thus, periostin was continued to be expressed in various cells in the chronic stage of ischemic brain.

**Table 1 Temporal and spatial profile of the expression of periostin in the ischemic brain**

| *Day* | *Peri-infarct region* | *Core* | *SVZ* |
|---|---|---|---|
| 3 | Astrocyte + | Microglia/macrophage + | + |
| | Microglia/macrophage + | Periendothelium + | |
| | Periendothelium + | | |
| 7 | Fibroblast + + | Microglia/macrophage + | + |
| | Astrocyte + | Periendothelium + | |
| | Microglia/macrophage + | | |
| | Periendothelium + | | |
| 14 | Fibroblast + + + | Fibroblast + | – |
| 28 | Fibroblast + + + | Fibroblast + | – |

Abbreviation: SVZ, subventricular zone.
The expression of periostin was observed in various regions and cells in ischemic brain and SVZ.

## DISCUSSION

Our previous study demonstrated that Pn1 and Pn2 mRNA were expressed in normal cortical tissue, and the expression level of Pn2 mRNA, but not Pn1, was increased in the infarct region 24 h after tMCAo.[5] Compared with the expression level of Pn2 mRNA/glyceraldehyde-3-phosphate dehydrogenase (8.6 ± 3.3 in the infarct regions and 0.29 ± 0.05 in sham) at 24 h,[5] the expression of Pn2 mRNA between days 3 and 28 was much higher. Considering that the

increased expression of Pn1 mRNA and the co-localization of periostin with type I collagen and fibronectin were first observed starting on day 7, Pn1 might be mainly produced in fibroblasts, and Pn2 might be expressed in reactive astrocytes, microglia, fibroblasts and around the endothelium, although further studies are necessary to develop antibodies that allow the discrimination of the expression of Pn1 and Pn2.

Although the role of periostin was not fully clarified in the present study, we speculate that periostin might have various roles in the chronic stages of ischemic brain injury because of their expression in various cells. One possibility is that periostin might promote the protection and regeneration of the surviving neuronal network in the peri-infarct regions, because Pn2 has exhibited neuroprotection and neurite outgrowth in primary cultured adult neurons.[5] Another possibility is that secreted periostin might act on the activated macrophages, because recent reports showed that periostin enhanced adhesion of macrophages to hyaloid vessels[18] and increased the expression of MMP-2, MMP-9 and MMP-13 in bone marrow-derived macrophages.[8] Considering that MMPs in the subacute stage of ischemia (24–72 h) have deleterious effects following injury of the blood–brain barrier, edema and hemorrhage,[19] secreted Pn2 from activated astrocytes and microglia/macrophages in the subacute stage may be associated with the regulation of the blood–brain barrier. In contrast, Pn1 and Pn2 expression during the chronic stage may be related to recovery after stroke through modulation of MMPs, because the delayed phase of MMPs (7–14 days) in the late stages of stroke has been shown to be required for improving recovery and enhancing long-term functional outcomes.[12] An important finding of the present study is the high expression of periostin in fibroblasts during the chronic stage. There is a possibility that some amount of periostin might be leaked from the serum with fibronectin because of the breakdown of blood–brain barrier occurring. However, the majority of the periostin was likely produced in the brain because the highest expression of periostin did not occur during the time period when the blood–brain barrier damage is most severe, which has been reported as 48 h after tMCAo.[20] As fibronectin and type I collagen are expressed in fibroblasts and periostin is strongly expressed in cardiac fibroblasts[21] and dermal fibroblasts,[6] periostin could have originated in fibroblasts in the brain during days 7–28.

Although the role of fibroblasts in the chronic stage has not been elucidated in the ischemic brain, studies of traumatic brain injury showed that fibroblasts began to invade the lesion site 3 days after injury and proliferated with secretion of ECMs, such as type IV collagen, fibronectin and laminin.[10] Because the latter two ECMs have been shown to enhance neurite outgrowth,[22] periostin might work with these ECMs to enhance neurite outgrowth. Considering that the proliferation of fibroblasts[10,23,24] and the production of ECMs including periostin[25] are enhanced by TGF-β1, TGF-β1 activated after ischemic stroke might be one of cytokines to stimulate the expression of periostin. The secreted periostin might act on fibroblasts in an autocrine manner and might promote the migration and proliferation of fibroblasts.[6] Because periostin was also shown to be involved in the augmentation of TGF-β-induced T regulatory cells differentiation,[9] periostin might be also involved with the protective and adaptive immune response during the delayed stage.[26]

Interestingly, immunohistochemistry showed the expression of periostin on days 3 and 7, which coincides with the proliferation of neural progenitors in the ischemic SVZ regions.[27] Because previous studies showed that integrin receptor β1,[28] which have been suggested as the receptors of periostin,[13] were related to the migration and proliferation of neural stem cells or neural progenitor cells,[15,16] periostin might have a role in endogenous neurogenesis after cerebral ischemia.

In conclusion, the present study demonstrated that periostin was expressed in various cells throughout the subacute to chronic stages in the ischemic brain. Because periostin has various roles in the functions of neurons, fibroblasts and immune cells, periostin might be one of the key molecules involved in regeneration and the post-ischemic immune system. In addition, the expression of periostin in the SVZ suggests a role in neurogenesis. Further studies, including examining other splice variants, are necessary to clarify the function of periostin during the chronic stage of ischemic stroke.

## CONFLICT OF INTEREST

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Supplementary Information accompanies the paper on Hypertension Research website (http://www.nature.com/hr)