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Brain Vascular Pericytes Following Ischemia Have Multipotential Stem Cell Activity to Differentiate Into Neural and Vascular Lineage Cells

TAKAYUKI NAKAGOMI,[a] SHUJI KUBO,[b] AKIKO NAKANO–DOI,[a] RIKA SAKUMA,[a] SHAN LU,[a,c] AYA NARITA,[a] MAIKO KAWAHARA,[a,d] AKIHIKO TAGUCHI,[e] TOMOHIRO MATSUYAMA[a]

Key Words. Blood-brain barrier • Ischemia • Pericyte • Stem cell plasticity • Reprogramming • Neurovascularization

[a]Institute for Advanced Medical Sciences and [b]Department of Genetics, Hyogo College of Medicine, Nishinomiya, Hyogo, Japan; [c]Department of Neurology of Hangzhou First People's Hospital, Hangzhou, People's Republic of China; [d]Graduate School of Science and Technology, Kwansei Gakuin University, Sanda, Hyogo, Japan; [e]Department of Regenerative Medicine Research, Institute of Biomedical Research and Innovation, Kobe, Hyogo, Japan

Correspondence: Takayuki Nakagomi, Ph.D., M.D., Institute for Advanced Medical Sciences, Hyogo College of Medicine, 1-1 Mukogawacho, Nishinomiya, Hyogo 663–8501, Japan. Telephone: 81–798-45–6821; Fax: 81–798-45–6823; e-mail: nakagomi@hyo-med.ac.jp

Received August 1, 2014; accepted for publication January 27, 2015; first published online in STEM CELLS *EXPRESS* February 19, 2015.

1066-5099/2014/$30.00/0

http://dx.doi.org/10.1002/stem.1977

ABSTRACT

Brain vascular pericytes (PCs) are a key component of the blood-brain barrier (BBB)/neurovascular unit, along with neural and endothelial cells. Besides their crucial role in maintaining the BBB, increasing evidence shows that PCs have multipotential stem cell activity. However, their multipotency has not been considered in the pathological brain, such as after an ischemic stroke. Here, we examined whether brain vascular PCs following ischemia (iPCs) have multipotential stem cell activity and differentiate into neural and vascular lineage cells to reconstruct the BBB/neurovascular unit. Using PCs extracted from ischemic regions (iPCs) from mouse brains and human brain PCs cultured under oxygen/glucose deprivation, we show that PCs developed stemness presumably through reprogramming. The iPCs revealed a complex phenotype of angioblasts, in addition to their original mesenchymal properties, and multidifferentiated into cells from both a neural and vascular lineage. These data indicate that under ischemic/hypoxic conditions, PCs can acquire multipotential stem cell activity and can differentiate into major components of the BBB/neurovascular unit. Thus, these findings support the novel concept that iPCs can contribute to both neurogenesis and vasculogenesis at the site of brain injuries. STEM CELLS *2015;33:1962–1974*

INTRODUCTION

Brain vascular pericytes (PCs) are a perivascular element constituting the blood-brain barrier (BBB)/neurovascular unit, along with neural and vascular cells including endothelial cells (ECs). Besides their crucial role in maintaining the BBB [1], increasing evidence shows that PCs can transform into multipotent stem cells and differentiate into various cells including neural and vascular cells [2–6]. Thus, it would be ideal if the BBB/neurovascular unit could be reconstructed by such endogenous stem cells. However, the mechanism through which PCs develop stemness is unknown.

A recent study using gene transduction showed that reprogramming is required for PCs to differentiate into non-PC lineage cells, such as neural cells [7]. In addition, hypoxia can increase stemness [8] via reprogramming [9], suggesting that PCs within the ischemic/hypoxic regions undergo reprogramming in vivo. Indeed, we demonstrated that ischemia-induced neural stem cells (iNSCs), which are PC derivatives, developed only within ischemic areas and expressed various stem cell and undifferentiated cell markers [10, 11]. These findings lead us to hypothesize that brain PCs develop stemness through reprogramming in response to ischemia/hypoxia and that such ischemia-induced stem cells in the central nervous system (CNS) exhibit multipotency.

In this study, using PCs extracted from ischemic regions (iPCs) from mouse brains and human brain PCs (hPCs) cultured under oxygen/glucose deprivation (hPC-OGD), we show that iPCs develop stemness presumably via cellular reprogramming and acquire multipotential stem cell activity to produce major components of the BBB/neurovascular unit, including neural and vascular cells. Because brain injuries such as a stroke disrupt the BBB/neurovascular unit [12], which forms in the CNS as a minimal functional unit [1], iPCs can be a targeted to repair the injured CNS.

MATERIALS AND METHODS

Induction of Focal Cerebral Ischemia

All procedures were performed under the auspices of the Animal Care Committee of Hyogo College of Medicine. Six-week-old male CB-17/Icr-+/+Jcl mice (CB-17 mice; Clea Japan, Inc.,

Tokyo, Japan, http://www.clea-japan.com) were subjected to cerebral ischemia as described previously [10, 11, 13, 14]. Permanent focal cerebral ischemia was produced by ligation and interruption of the distal portion of the left middle cerebral artery (MCA) [10, 11, 13, 14]. In brief, the left MCA was isolated, electrocauterized, and disconnected just distal to its crossing of the olfactory tract (the distal M1 portion) under halothane inhalation.

iPC Isolation

iPCs were extracted from poststroke CB-17 mice as described previously [10, 11, 13, 14]. In brief, mice were deeply anesthetized with sodium pentobarbital (50 mg/kg) on poststroke day 3. The postischemic areas containing leptomeninges that have abundant iPCs [11, 15, 16] were carefully removed under a microscope (Carton, Pathum Thani, Thailand, http://www.carton-opt.co.jp). The removed tissues were then mechanically dissociated by passage through 18-, 23-, and 27-gauge needles to prepare a single-cell suspension. The resulting cell suspensions were incubated with adherent cultures in Dulbecco's modified Eagle's medium (DMEM)/F-12 medium (Invitrogen, Carlsbed, CA, http://www.lifetechnologies.com) containing fibroblast growth factor-basic (bFGF; 20 ng/ml; Peprotech, Rocky Hill, NJ, http://www.peprotech.com), epidermal growth factor (EGF; 20 ng/ml; Peprotech), 1% N2 supplement (Invitrogen), and 2% fetal bovine serum (FBS). On day 7 after incubation, iPCs were treated with trypsin and used for experiments. In another sets of experiments, iPCs were subjected to magnetic cell sorting (MACS) as described previously [11]. Then, MACS-sorted PDGFRβ^+ iPCs (β^+iPCs) were used in some experiments.

Cell Culture

On day 7 after incubation, adhered iPCs were treated with trypsin and reincubated with floating cultures in neural-conditioned medium (NCM) (DMEM/F-12, EGF, FGF-2, and N2) [10, 11, 13, 14] or endothelial-conditioned medium (ECM) (DMEM, FGF-2, 10% FBS, and N2) [17, 18]. After incubation, cell clusters had formed (NCM-treated iPCs [iPC-NCM] or ECM-treated iPCs [iPC-ECM]) and were subjected to immunohistochemistry or reverse transcriptase-polymerase chain reaction (RT-PCR) analysis. To induce neural differentiation, iPC-NCM was incubated on poly(L-lysine)-coated glass coverslips for 7 days in neurobasal medium (Invitrogen) with B-27 supplement (Invitrogen) and all-trans retinoic acid (0.2 μM; Sigma, St. Louis, http://www.sigmaaldrich.com) [14]. To analyze tube formation, iPC-ECM was incubated on Matrigel-treated plates (BD Matrigel, Bedford, MA, http://www.bdbiosciences.com). To promote pericytic differentiation, iPC-NCM was incubated in DMEM with 10% FBS [18]. Some iPCs were incubated under NCM including recombinant mouse leukemia inhibitory factor (LIF) (0.025 μg/ml; Millipore, Temecula, CA, http://www.merckmillipore.com). Embryonic stem cell (ESC) lines derived from murine 129/SVEV (Millipore) or human brain ECs (hECs; ACBRI-376, Cell Systems, Kirkland, WA, http://www.cell-systems.com) were purchased and the extracted m-RNA was used as a control in the RT-PCR analysis. Commercially available mouse brain ECs (mECs; CRL-2299, ATCC, Manassas, VA, http://www.atcc.org) were obtained and were maintained in EC growth medium (Cell Systems). A detailed explanation is provided in Materials and Methods of Supporting Information.

OGD Treatment

Primary human brain PCs (hPCs; ACBRI-499, Cell Systems) were incubated under OGD to mimic ischemic/hypoxic condition. In brief, hPCs (2.5×10^4 cells/well) were plated on 12-well dishes (Iwaki, Tokyo, Japan, http://www.atg.ushop.jp) in PC growth medium (Cell Systems). One day later, the medium was removed and replaced with glucose-free DMEM and FBS (2%). hPCs were then incubated under hypoxia for 3 days (1% O_2) using a hypoxia-inducing system (Bionix, SUGIYAMA-GEN, Tokyo, Japan, http://www.sugiyama-gen.co.jp). In some experiments, recombinant human LIF (0.025 μg/ml; Millipore) or bFGF (20 ng/ml; Peprotech) was added to the cultures.

Immunohistochemistry

Coronal brain sections were prepared and they were subjected to immunohistochemistry as described previously [10, 11, 13, 14]. The iPC-NCM was fixed in paraformaldehyde (4%), cut on a cryostat, and subjected to immunohistochemistry. Samples were stained with antibodies to nestin (Millipore), CD31 (Santa Cruz Biotechnology, Santa Cruz, CA, http://www.scbt.com), PDGFRβ (Santa Cruz), NG2 (Millipore), Tuj1 (Stem Cell Technologies, Vancouver, Canada, http://www.stemcell.com), glial fibrillary acidic protein (GFAP) (Millipore), MAP2 (Millipore), O4 (Millipore), α-smooth muscle actin (αSMA) (Millipore; LifeSpan Biosciences, Seattle, WA, https://www.lsbio.com), CDH5 (Santa Cruz), Sox2 (Millipore), c-myc (Santa Cruz), or Klf4 (Santa Cruz). Primary antibodies were visualized using Alexa Fluor 488- or 555-conjugated secondary antibodies (Molecular Probes). Nuclei were stained with 4′,6-diamino-2-phenylindole (Kirkegaard & Perry Laboratories, Gaithersburg, MD, http://www.kpl.com). Images of sections were captured using a confocal laser microscope (LSM510; Carl Zeiss, Jena, Germany, http://www.zeiss.com). The semiquantitative analysis for positive cells was performed as described previously [13, 14].

RT-PCR

Total RNA was extracted using the RNeasy Micro Kit (Qiagen, Hilden, Germany, http://www.qiagen.com); cDNA was amplified according to the manufacture's protocols as described [10, 11]. RNA levels were normalized to β-actin using Image J analysis software downloaded from NIH Image as described previously [14]. Primer sequences are listed in Supporting Information Tables S1 and S2.

Sox2-Green Fluorescent Protein Lentiviral Transduction in iPCs

iPCs were transduced with a Sox2-green fluorescent protein (GFP)-expressing lentivirus as described previously [13, 14, 19]. In brief, the lentivirus vector plasmid pLVSK-c-Sox2-internal ribosome entry site (IRES)-enhanced green fluorescent protein (EGFP) was generated by cloning a mouse Sox2 IRES fragment of pMXs-Sox2-IP (Addgene, #15919, Cambridge, MA, https://www.addgene.org) into pLVSKc-EGFP (sinSKcmv-EGFP) [19, 20] between a cytomegalovirus promoter and an EGFP. A lentivirus vector pseudotyped with vesicular stomatitis virus-G was produced by transient cotransfection of 293T cells as described previously [19, 20]. The titers for the vector were determined by EGFP expression using fluorescence-activated cell sorting (FACS) (FACS Calibur, BD Pharmingen), and expressed in terms of transducing units per milliliter. Next, iPCs were incubated in six-well

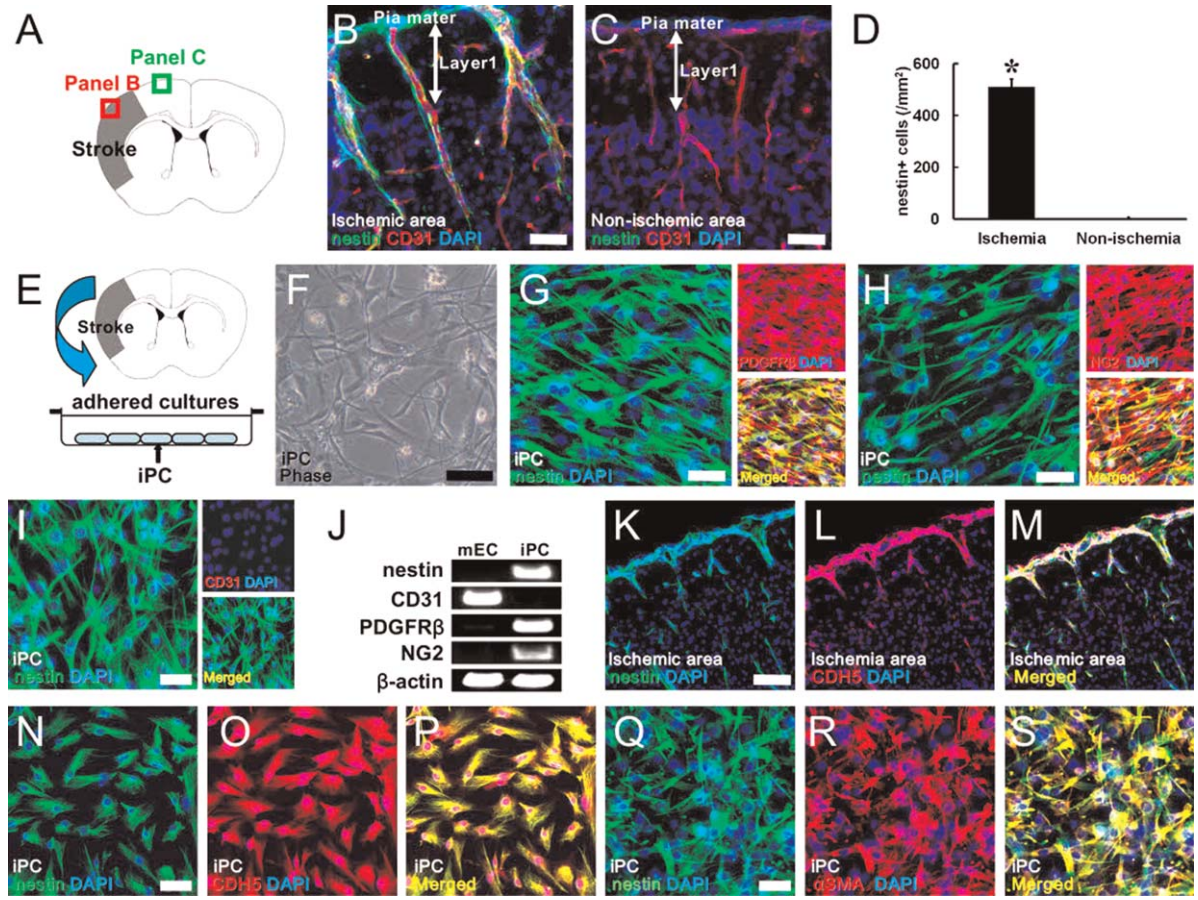

Figure 1. Isolation and characterization of iPCs. Brain sections demonstrating that nestin$^+$ iPC developed nearby CD31$^+$ ECs from the poststroke pia mater to the cortex **(A, B)**, but were rarely present in the nonischemic ipsilateral regions (A, **C**) (nestin [B, C, green], CD31 [B, C, red], DAPI [B, C, blue]). The number of nestin$^+$ cells increased significantly in ischemic areas compared with nonischemic areas **(D)**. iPCs were isolated from ischemic areas **(E)** and incubated with adhered cultures **(F)**. Immunohistochemistry showed that nestin$^+$ iPCs expressed pericytic markers such as PDGFRβ (nestin [**G**: green], PDGFRβ [G: red], DAPI [G: blue]) and NG2 (nestin [**H**: green], NG2 [H: red], DAPI [H: blue]) but lacked CD31 (nestin [**I**: green], CD31 [I: red], DAPI [I: blue]). PCR analysis also revealed that iPCs possessed nestin, PDGFRβ, and NG2 but not CD31 **(J)**. Immunohistochemistry showed that nestin$^+$ iPCs expressed CDH5 in vivo **(K–M)** and in vitro **(N–P)** (nestin [K, M, N, P: green], CDH5 [L, M, O, P: red], DAPI [K–P: blue]). Nestin$^+$ iPCs expressed αSMA (nestin [**Q**, **S**: green], αSMA [**R**, S: red], DAPI [Q–S: blue]). *, $p < .05$ versus nonischemic areas ($n = 5$; D). Scale bars = 50 μm (B, C, G, H, I, N, Q) and 100 μm (F, K). Abbreviations: DAPI, 4′,6-diamino-2-phenylindole; iPC, pericyte following ischemia; mEC, mouse endothelial cell.

plates and transduced 72 hours later with a Sox2-GFP-expressing lentivirus as described previously [13, 14]. The virus was removed and the medium was changed at 96 hours; nontransduced controls were treated equivalently (without the virus). Finally, the cells were harvested and subjected to RT-PCR.

Statistical Analysis

The data are presented as the mean ± SD. Statistical comparisons among groups were performed using one-way analysis of variance. Where indicated, the statistical significance of differences was calculated using Student's *t* test; a *p*-value less than .05 was considered statistically significant.

Results

Isolation and Characterization of iPC

Using a murine model of cortical infarction, we previously found that iNSCs develop in ischemic areas [10, 13, 14] and originate partially from iPCs derived from the poststroke leptomeninges and cortex [11]. Because iPCs highly express nestin as well as pericytic markers such as PDGFRβ and NG2 [11, 14], we used immunohistochemistry to determine the location of nestin$^+$ cells on day 3 after stroke. Consistent with our previous findings [11, 14], nestin$^+$ iPCs were induced near CD31$^+$ ECs in the poststroke leptomeninges (pia mater) and cortex (Fig. 1A, 1B, 1D) but were rarely observed in the ipsilateral nonischemic areas (Fig. 1A, 1C, 1D).

To characterize iPCs, we isolated them from ischemic areas as described previously [11]. Because brain PCs are neural crest derivatives [21] and have mesenchymal phenotypes [22], we initially incubated iPCs as "mesenchymal" monolayer-adhered cultures in a medium including bFGF and EGF (Fig. 1E). On day 7 after initial incubation, adhered cells (Fig. 1F) were subjected to immunohistochemistry. These cells largely expressed nestin (more than 95%) and contained PDGFRβ^+ (Fig. 1G) and NG2$^+$ cells (Fig. 1H), showing that iPCs selectively proliferated under these conditions.

Although nestin$^+$ iPCs expressed the pericytic markers, it is known that nestin is expressed in vascular lineage cells

including ECs [23, 24]. To rule out potential contamination of ECs in iPCs, immunohistochemistry and RT-PCR were performed using the adhered cells. Immunohistochemistry showed that CD31$^+$ ECs were not observed in iPCs (Fig. 1I). The RT-PCR analysis also showed that iPCs possessed genes for nestin, PDGFRβ, and NG2 but not for CD31, which was expressed in mECs that were used as a positive control (Fig. 1J, Supporting Information Fig. S1). Thus, we next investigated whether iPCs express CDH5, which is widely expressed in vascular lineage cells, including vascular stem cells (VSCs) [25], hematopoietic stem cells (HSCs), and ECs [26]. We found that nestin$^+$ iPCs expressed CDH5 in vivo (Fig. 1K–1M) and in vitro (Fig. 1N–1P). Furthermore, CDH5 was expressed in PDGFRβ^+ iPCs in ischemic areas (Supporting Information Fig. S2). These findings show that iPCs are not ECs, but they do share vasculogenic traits.

Certain PCs are the likely source of VSCs, which are defined as vascular resident stem cells that can differentiate into multiple lineages [27]. Because VSCs express CDH5 and the pericytic/mesenchymal marker αSMA [25], we investigated whether iPCs express αSMA. Immunohistochemistry showed that nestin$^+$ iPCs expressed αSMA (Fig. 1Q–1S). These results suggest that iPCs have traits similar to those of VSCs.

iPCs Have the Potential to Generate Vascular Lineage Cells

Because VSCs can generate ECs as well as αSMA$^+$ cells [28], we further examined whether iPCs have angiogenic potential. We first examined various angiogenesis-related genes in adhered iPCs. We found that iPCs possessed CDH5, endoglin (CD105), thrombomodulin (TM), VCAM1, and CD34 (Fig. 2A, 2B) but lacked markers for endothelial progenitor cells (EPCs; Flk1) and ECs (CD31) (Fig. 2A, 2B). Thus, to investigate the ability of iPCs to differentiate into ECs, they were then incubated as floating cultures in ECM that promotes EC differentiation (Fig. 2C). The iPC clusters (iPC-ECM; Fig. 2D) expressed both Flk1 and CD31 (Fig. 2A, 2B). Immunohistochemistry also showed that iPC-ECM differentiated into CD31$^+$ mature ECs (Fig. 2E). These results suggest that iPCs generate ECs via Flk1-expressing EPCs after incubation in ECM. Next iPC-ECM was placed on a Matrigel-coated dish to examine whether they form tube-like structures. We found that iPC-ECM formed tube-like structures in a network (Fig. 2F).

To confirm whether angiogenic iPCs were derived from PCs, PDGFRβ^+ cells expressing hallmarks of PC were selectively isolated from adhered iPCs by MACS (Fig. 2G) as described previously [11]. All sorted cells expressed PDGFRβ (Fig. 2H), and β^+iPCs expressed nestin (Fig. 2I, 2J). Because PCs have mesenchymal phenotypes [22], we further investigated whether iPCs possess mesenchymal markers. We found that iPCs expressed various mesenchymal markers, including αSMA, Snail, and Slug (Fig. 2K).

It has been reported that ECs also transform into cells with mesenchymal phenotypes [29]. However, mECs did not show mesenchymal markers, except for a weak expression of Snail (Fig. 2K), or iPC-like phenotypes (Supporting Information Fig. S3), suggesting that iPCs are not derived from pre-existing ECs.

In contrast, β^+iPCs expressed CDH5 (Fig. 2L), and similar to the traits of iPCs, they expressed several angiogenic genes such as CDH5, CD105, TM, VCAM1, and CD34 but not Flk1 and CD31; all these genes were expressed in the control mECs (Fig. 2M, 2N). After incubating in ECM, β^+iPCs formed cell clusters (β^+iPC-ECM; Fig. 2O) and also formed tube-like structures (Fig. 2P). These results show that PDGFRβ^+ PCs likely gained angioblastic potential presumably in a mesenchymal-angioblastic transition (MAT)-like manner after ischemia/hypoxia. Together, these data show that iPCs have the same traits as mesenchymoangioblast, which have a complex phenotype of both the mesenchyme and angioblast [30].

iPCs Have Activity to Produce Neural Lineage Cells

Although our data show that iPCs have vasculogenic potential, we recently showed that iPCs can produce iNSCs, which differentiate into neural cells, including neurons [11]. To confirm this capacity of iPCs, adhered iPCs were incubated as floating cultures in a NCM that promoted the formation of neurosphere-like cell clusters (Fig. 3A) [10, 11, 13, 14]. On day 3 after incubation, iPC-NCM formed cell clusters (Fig. 3B). Immunohistochemistry with continuous sections indicated that they contained nestin$^+$ cells, which coexpressed CDH5 (Fig. 3C) and pericytic markers PDGFRβ (Fig. 3D), NG2 (Fig. 3E), and αSMA (Fig. 3F). iPC-NCM differentiated into cells coexpressing CDH5 and αSMA (Fig. 3G). However, they had not yet differentiated into Tuj1$^+$ immature neurons (Fig. 3H). These results suggest that iPC-NCM in the early period of incubation still maintain the traits toward vasculogenesis rather than neurogenesis. Thus, floating iPCs were incubated in NCM for a longer period. We found that iPCs-NCM obtained after 3 weeks of incubation differentiated into Tuj1$^+$ neurons (Fig. 3H) as well as CDH5$^+$ cells (Fig. 3I–3K, Supporting Information Fig. S4). In addition, the presence of Tuj1$^+$ cells coexpressing αSMA (Fig. 3L–3N) suggests that such immature neurons are partially produced by iPCs, which is consistent with a previous report showing that nestin$^+$ PCs (which presumably contain an iPC population) directly produce Tuj1$^+$ immature neurons [6]. To confirm this, β^+iPCs were sorted by MACS and then incubated in NCM (β^+iPCs-NCM) (Fig. 3O). Similar to our previous study [11], β^+iPCs-NCM obtained after 4 weeks of incubation differentiated into Tuj1$^+$ (Fig. 3P) and MAP2$^+$ neurons (Fig. 3Q). In contrast, mECs did not show such activities (Supporting Information Fig. S5). These results show that iPCs have the potential to differentiate into neural lineage cells.

iPCs Gain the Potential to Generate iNSCs

The mechanism through which iPCs produce iNSCs remains unclear. To clarify this mechanism, we investigated iPC-NCM traits for more than 6 weeks. Although PDGFRβ was highly expressed in adhered iPCs, its expression was gradually downregulated in iPC-NCM (Fig. 4A–4C), suggesting that iPCs decreased their pericytic traits. In addition, we found that the iPC marker nestin was also downregulated in iPC-NCM (Fig. 4A, 4C). Next, we examined whether they expressed the persistent neural stem cell (NSC) marker Sox2 [31] because we had previously demonstrated that iPC-derived iNSCs possessed this marker [10, 11, 14, 32]. Although Sox2 was weakly observed in iPCs, it was increased in iPC-NCM (Fig. 4A, 4C). Immunohistochemistry also revealed that Sox2 expression was increased in spheres obtained after 4 weeks of incubation compared with that observed only after 1 week of incubation (Supporting Information Fig. S6). Sox2 is expressed not only in NSCs but also in pluripotent stem (iPS) cells [33]. Recent studies, including ours, demonstrated that NSC/iNSC express c-myc, Klf4, and Sox2 of the four Yamanaka factors [10, 11, 34]. Another study showed

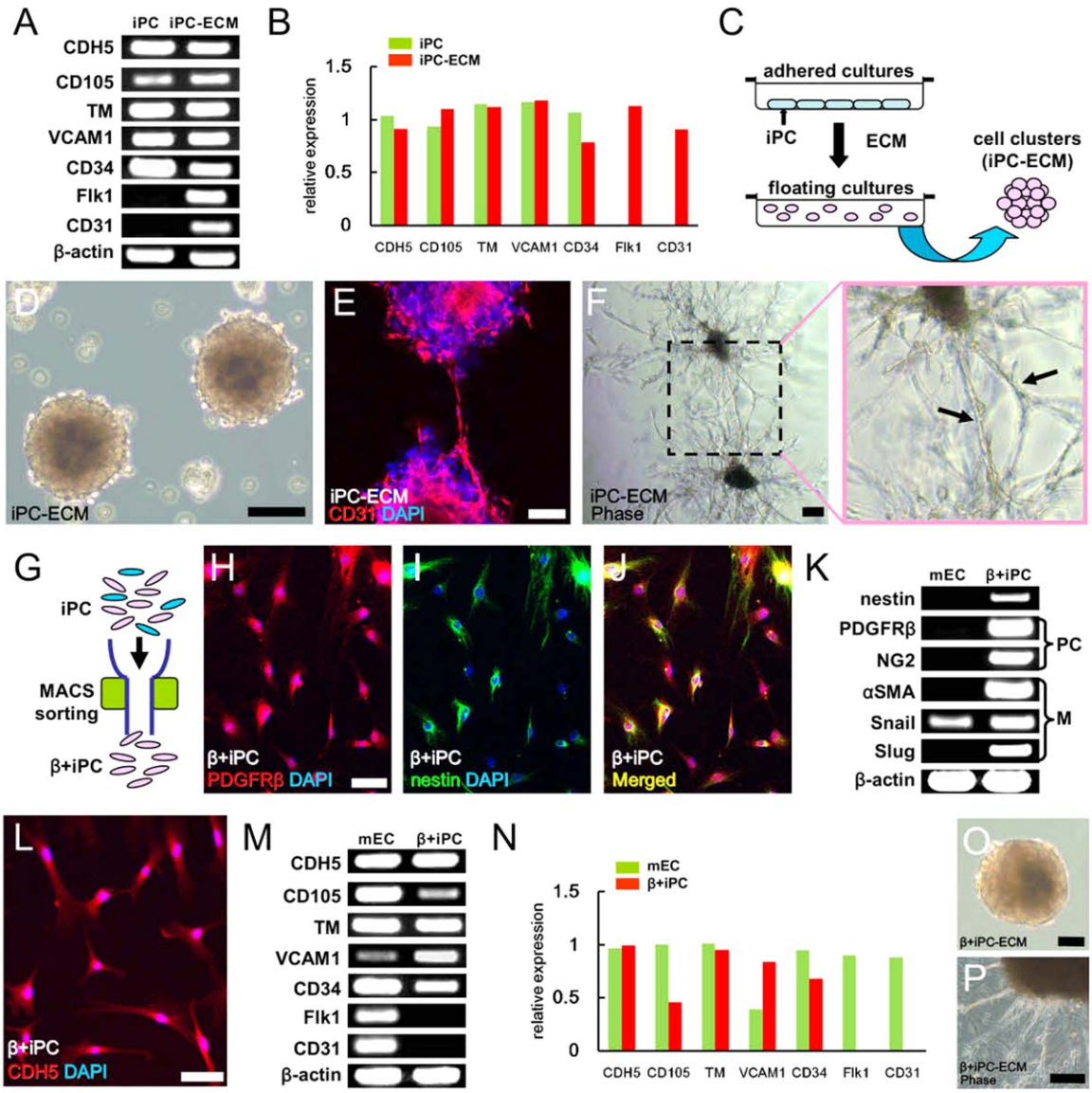

Figure 2. Vasculogenic potential of iPCs. iPCs possessed vasculogenic genes, including CDH5, CD105, TM, VCAM1, and CD34 **(A, B)**. Following incubation in ECM **(C)**, iPC-ECM formed cell clusters **(D)** and expressed Flk1 and CD31 (A, B). iPC-ECM differentiated into $CD31^+$ mature ECs (CD31 [**E**: red], DAPI [E: blue]) that formed tube-like structures (**F**: arrow). $PDGFR\beta^+$ iPCs (β^+ iPCs) were isolated by MACS **(G)**. The sorted β^+ iPCs expressed nestin (PDGFRβ [**H**, **J**: red], nestin [**I**, J: green], DAPI [H–J]). β^+ iPCs expressed nestin and markers for PCs and the mesenchyme, including PDGFRβ, NG2, αSMA, Snail and Slug, whereas, except Snail, ECs did not express these genes **(K)**. Immunohistochemistry revealed that most β^+iPCs expressed CDH5 (CDH5 [**L**: red], DAPI [L: blue]). β^+iPCs expressed angiogenic genes, except for Flk1 and CD31, whereas all the angiogenic genes were expressed in the control mECs **(M, N)**. After incubating in ECM, β^+iPCs formed cell clusters (β^+iPCs-ECM; **O**) that formed tube-like structures **(P)**. Scale bars = 100 μm (D, E, F, H, L, P) and 50 μm (O). Abbreviations: β^+iPC, $PDGFR\beta^+$ iPC; DAPI, 4′,6-diamino-2-phenylindole; iPC, pericyte following ischemia; iPC-ECM, iPC incubated in endothelial-conditioned medium; M, mesenchyme; PC, pericyte.

that somatic cells such as mesenchymal fibroblasts could be reprogrammed into NSCs by transduction of these three factors [35]. Thus, we examined whether iPCs possessed these markers. We found that c-myc and Klf4 were present in both iPCs and iPC-NCM (Fig. 4A, 4C). However, similar to the phenotypes of NSCs/iNSCs [10, 11, 34], iPC/iPC-NCM lacked Oct3/4 of the four Yamanaka factors, which was highly expressed in the ESCs used as a positive control (Supporting Information Fig. S7). These results indicate that iPC-NCM develops characteristics similar to those of NSCs/iNSCs during incubation in NCM for a long time.

We further investigated the expression of neural markers in iPCs and iPC-NCM. Although the neuronal marker MAP2 was observed at low level in iPCs, its expression was increased in iPC-NCM (Fig. 4B, 4C). Glial markers of astrocytes [GFAP and aquaporin4 (APQ4)] and oligodendrocytes (Olig2) were observed in iPC-NCM at later time points (Fig. 4B, 4C). Immunohistochemistry showed that iPC-NCM differentiated into $Tuj1^+$ (Fig. 4D) and $MAP2^+$ neurons (Fig. 4E) and $GFAP^+$ astrocytes (Fig. 4D) and $O4^+$ oligodendrocytes (Fig. 4F), which is consistent with the traits of iNSCs [10, 11, 13, 14].

Sox2 is a key factor for directing cells to the neuronal lineage [7, 36–39] as well as acquiring stemness by somatic cells [33]. Thus, we next investigated the role of Sox2 in neurogenesis. We conducted the transduction of the Sox2 gene in iPCs using a lentivirus vector

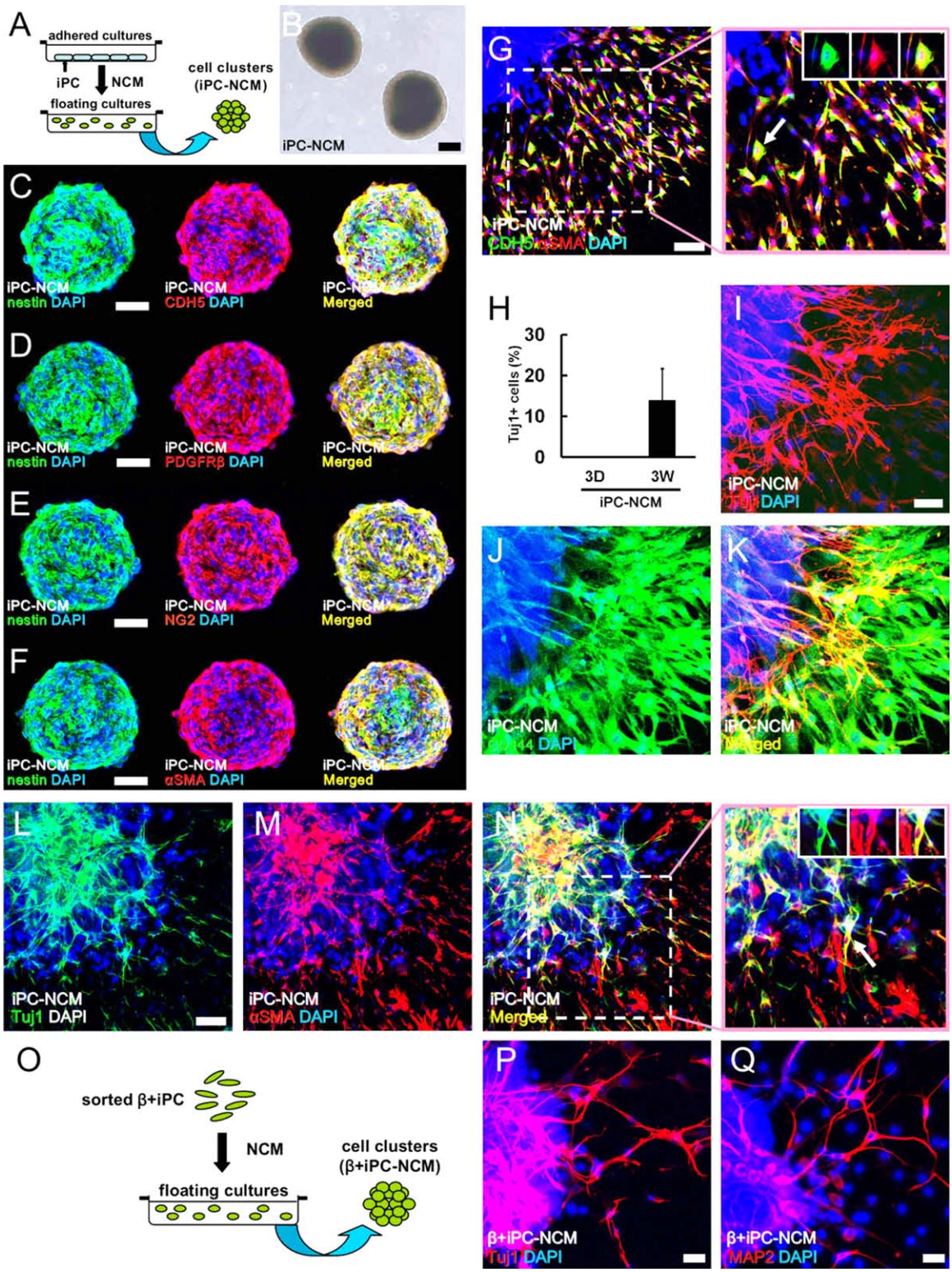

Figure 3. iPCs have the potential to generate neural lineage cells. iPCs in adhered cultures were incubated in NCM under floating cultures **(A)**. On day 3 after incubation, iPC-NCM formed cell clusters **(B)**. Immunohistochemistry at that time showed that nestin$^+$ iPCs expressed CDH5 (nestin [**C**: green], CDH5 [C: red], DAPI [C: blue]) and pericytic markers, including PDGFRβ (nestin [**D**: green], PDGFRβ [D: red], DAPI [D: blue]), NG2 (nestin [**E**: green], NG2 [E: red], DAPI [E: blue]), and αSMA (nestin [**F**: green], αSMA [F: red], DAPI [F: blue]). iPC-NCM obtained 3 days after incubation largely differentiated into vascular stem cell-like cells coexpressing CDH5 and αSMA (CDH5 [**G**: green], αSMA [G: red], DAPI [G: blue]). iPC-NCM obtained 3 days after incubation rarely differentiated into Tuj1$^+$ cells, whereas iPC-NCM obtained 3 weeks after incubation differentiated into Tuj1$^+$ cells **(H)** as well as CDH5$^+$ (Tuj1 [**I**, **K**: red], CDH5 [**J**, K: green], DAPI [I–K: blue]), or αSMA cells (Tuj1 [**L**, **N**: green], αSMA [**M**, N: red], DAPI [L–N: blue]). The sorted β^+ iPCs were incubated in NCM **(O)**. β^+ iPCs formed cell clusters differentiated into neuronal cells expressing Tuj1 (Tuj1 [**P**: red], DAPI [P: blue]) and MAP2 (MAP2 [**Q**: red], DAPI [Q: blue]). Scale bars = 100 μm (B, G, L), 50 μm (C, D, E, F, I), and 20 μm (P, Q). Abbreviations: DAPI, 4′,6-diamino-2-phenylindole; iPC, pericyte following ischemia; iPC-NCM, iPC incubated in neural-conditioned medium.

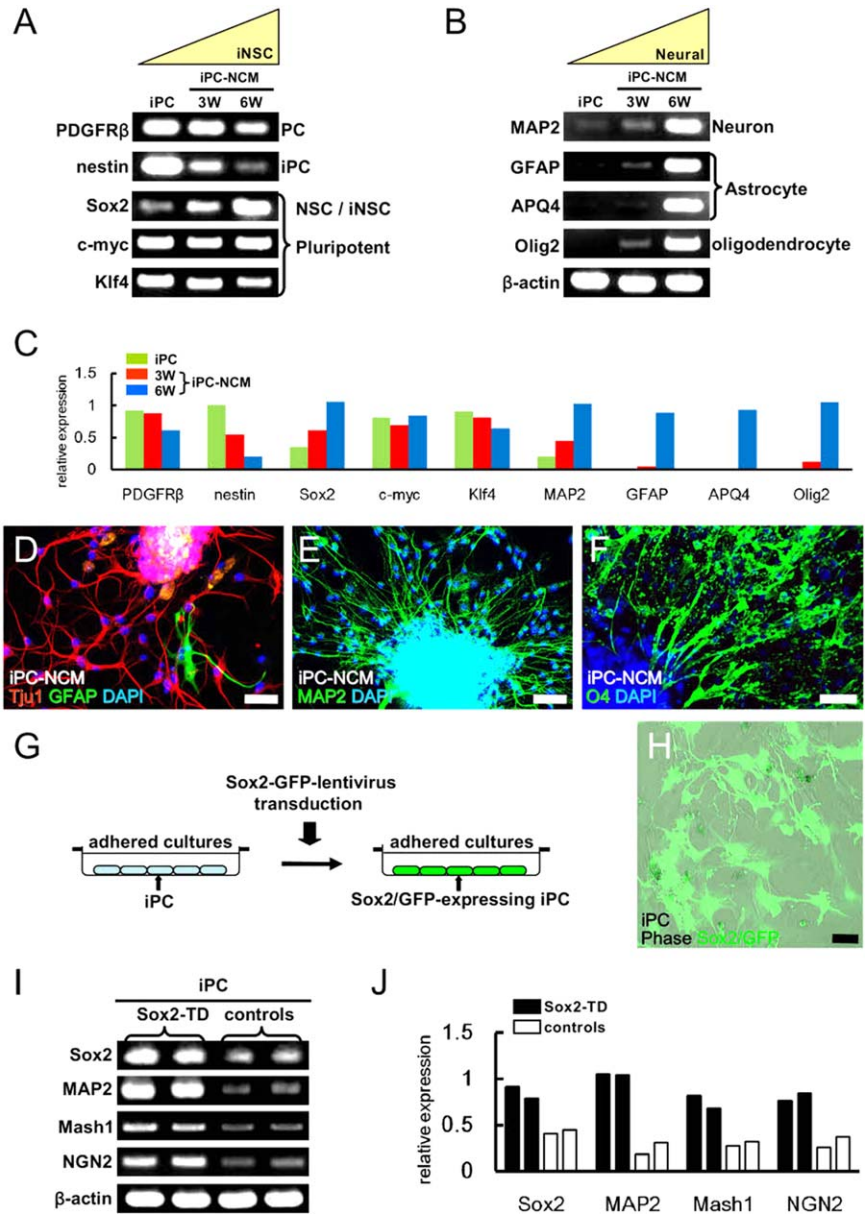

Figure 4. iPCs acquire the potential to generate iNSCs. Gene expression for PC/iPC, NSC/iNSC, pluripotent, or neural cells was analyzed in iPCs/iPC-NCM **(A–C)**. PC (PDGFRβ) and iPC markers (nestin) were gradually decreased in iPC-NCM (A, C), whereas NSC/iNSC (Sox2) and neural markers (MAP2, GFAP, APQ4, and Olig2) were increased (A–C). After differentiation, iPC-NCM expressed neuronal (Tuj-1 [**D**: red], MAP2 [**E**: green]), astrocyte (GFAP [D: green]), and oligodendrocyte (O4 [**F**: green]) markers (DAPI [D–F: blue]). Sox2 transduction (Sox2-TD) was performed in iPCs via a lentivirus vector carrying GFP **(G)**. Sox2-transducted iPCs **(H)** increased the expressions of MAP2, Mash1, and NGN2 **(I, J)**. Scale bars = 100 μm (F) and 50 μm (D, E, H). Abbreviations: DAPI, 4′,6-diamino-2-phenylindole; GFP, green fluorescent protein; iNSC, ischemia-induced neural stem cell; iPC, pericyte following ischemia; iPC-NCM, iPC incubated in neural-conditioned medium; NSC, neural stem cell.

carrying GFP (Fig. 4G). Sox2-transducted iPCs (Fig. 4H) increased the expression of the MAP2 gene and neurogenic transcription factors, such as Mash1 and Neurog2 (NGN2) compared with the controls (Fig. 4I, 4J). In contrast, small interfering RNA knockdown of Sox2 resulted in the inhibition of neurogenic transcription factors (Supporting Information Fig. S8). These results show that Sox2 plays an important role in the acquisition of neuronal traits in iPCs.

iPCs Give Rise to Neural Lineage Cells in a Mesenchymal-Epithelial Transition-Like Manner

Brain PCs are partially derived from the neural crest [21], in which some mesenchymal markers (e.g., Snail and Slug) are observed. Thus, we investigated whether iPCs possess neural crest genes. Consistent with our previous study [11], iPCs expressed neural crest markers such as Sox9 and twist (Fig. 5A, 5B). This shows that iPCs have traits of the mesenchymal lineage, including the neural crest. However, these markers were downregulated (Fig. 5A, 5B). Because the mesenchymal neural crest can acquire "neuroepithelial" phenotypes when they express the Sox2 gene through mesenchymal-epithelial transition (MET) [39], we investigated whether iPCs change their mesenchymal or epithelial markers during incubation. Similar to the results for Sox9 and twist genes, mesenchymal or epithelial-mesenchymal transition markers such as αSMA,

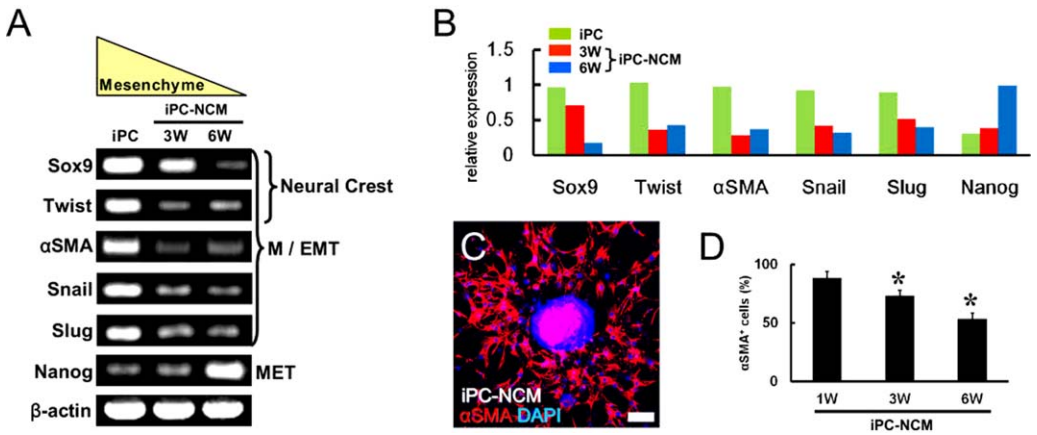

Figure 5. iPCs produce neural lineage cells in a MET-like manner. iPCs expressed neural crest/mesenchymal/EMT markers, including Sox9, Twist, αSMA, Snail, and Slug **(A, B)**. However, these genes were downregulated (A, B) and the production of αSMA^+ cells (αSMA [**C**: red], DAPI [C: blue]) decreased gradually in iPC-NCM **(D)**. *, $p < .05$ versus iPC-NCM (1 week [W]) group ($n = 5$) (D). Scale bars = 100 μm (C). Abbreviations: DAPI, 4′,6-diamino-2-phenylindole; EMT, epithelial-mesenchymal transition; iPC, pericyte following ischemia; iPC-NCM, iPC incubated in neural-conditioned medium; M, mesenchyme; MET, mesenchymal-epithelial transition.

Snail, and Slug were downregulated (Fig. 5A, 5B) as iPCs gained neural traits (Fig. 4A–4C). Immunohistochemistry also showed iPCs gradually reduced their activity to generate αSMA^+ cells (Fig. 5C, 5D) as they obtained neural traits (Fig. 4A–4C). These results suggest that iPCs undergo MET during incubation by "epithelial" floating cultures in NCM.

However, unexpectedly, we could not obtain evidence for the reported MET, which showed that epithelial markers such as E-cadherin and Crb3 emerged or increased (Supporting Information Fig. S9). Therefore, we examined the expression of Nanog in iPC because a pluripotent marker, Nanog, is turned on when mesenchymal fibroblasts undergo MET in iPS cell reprogramming [40]. Nanog was upregulated as mesenchymal markers decreased (Fig. 5A, 5B). These results suggest that iPCs acquire neural traits in a MET-like manner.

Expression of Reprogramming Factors in PCs in Ischemic Areas

iPCs isolated from ischemic areas expressed various stem cell markers with multidifferentiation potentials including neurovascular cells. However, we could not identify these cells in the nonischemic areas, as described previously [10, 11, 13, 14]. This suggests that PCs within ischemic areas undergo reprogramming, thereby developing stemness. To examine this result, the essential factors for reprogramming, such as c-myc, Klf4, and Sox2, were examined in poststroke brains because these markers were expressed in iPCs (Fig. 4A, 4C). Although c-myc (Fig. 6A, 6B, 6D), Klf4 (Fig. 6A, 6B, 6H), and Sox2 (Fig. 6A, 6B, 6L) were rarely observed in the nonischemic areas, they were frequently observed in ischemic areas (Fig. 6A, 6B, 6E–6G, 6I–6K, 6M–6O). Such reprogramming factors were localized in the nucleus and/or cytoplasm, as described previously [41–44]. Some of them (especially $Sox2^+$ cells) were observed in iPCs around $CD31^+$ ECs within ischemic regions (Fig. 6C, 6E, 6I, 6M) and coexpressed an iPC marker nestin (Fig. 6P–6R). These results suggest that ischemia activates reprogramming factors in PCs in ischemic areas.

PCs Acquire Multidifferentiation Potential into Neurovascular Cells Via Ischemia-Induced Reprogramming

To further investigate the mechanism through which iPCs developed multipotency to differentiate into neurovascular cells, brain-derived hPCs were subjected to OGD for 3 days, which can mimic ischemia/hypoxia in vivo. hPCs under normal/normoxia conditions (hPC-N) showed that they have the markers of PCs/mesenchyme, including PDGFRβ, NG2, αSMA, Snail, and Slug (Fig. 7A). C-myc was stably expressed in cultured hPC-N, and it was not increased in hPC-OGD (Fig. 7B). Sox2 was rarely observed in hPC-N, but it could be detected in hPC-OGD (Fig. 7B). Klf4, which can promote reprogramming into the vascular lineage [45] in part by binding to and activating the CDH5 promoter [46], increased rapidly in hPC-OGD (Fig. 7B), with dramatic changes in cell morphology (hPC-N [Fig. 7C], hPC-OGD [Fig. 7D, Supporting Information Video S1]). The expression of vascular endothelial growth factor (VEGF), a strong stimulator for EC differentiation, also significantly increased in hPC-OGD (Fig. 7E). Thus, we further examined whether hPC-OGD might gain vascular traits following incubation in ECM (Fig. 7F). Although vascular markers such as CDH5, Flk1, and CD31 were expressed in hECs and used as positive controls, hPC-N did not possess these markers (Fig. 7G); however, hPC-OGD/ECM (Fig. 7F) expressed these genes (Fig. 7G). In addition, they formed tube-like structures (data not shown) and differentiated into $CDH5^+$ (Fig. 7H) and $CD31^+$ cells (Fig. 7I). As expected, some $CDH5^+$ cells overlapped with mesenchymal αSMA cells (data not shown), similar to the traits of iPCs. These results suggest that hPCs can be reprogrammed from mesenchymal into angiogenic lineage cells in a MAT-like manner.

We showed that reprogramming factors (especially Sox2) were observed in iPCs near ECs (Fig. 6C). This suggests that endothelial factors also contribute to the acquisition of stemness in PCs under ischemia/hypoxia conditions. Because stimulated ECs produce LIF [47], which activates the Sox2 gene [39], we investigated Sox2 expression in hPC-OGD

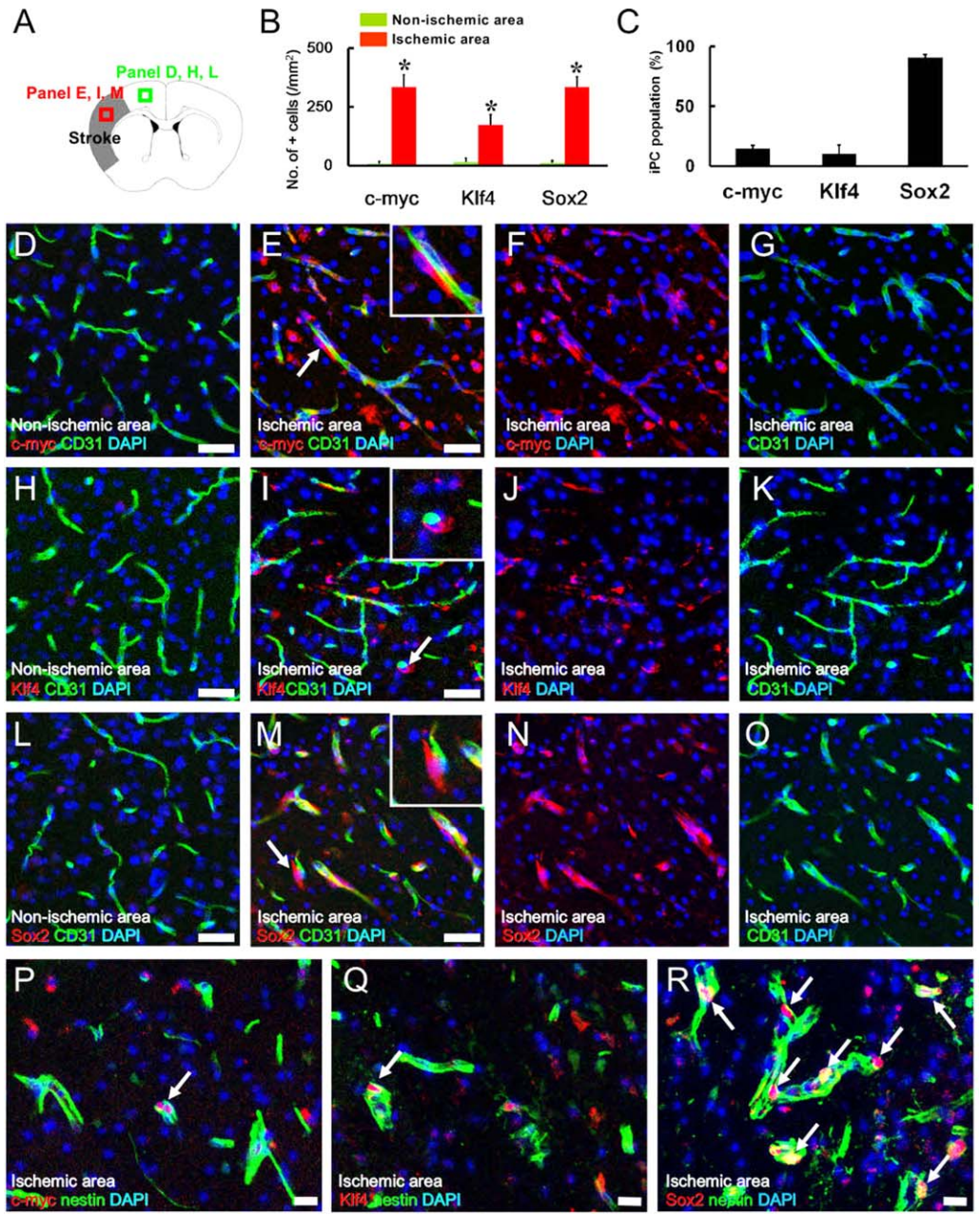

Figure 6. Ischemia activates reprogramming factors in PCs in ischemic areas. Immunohistochemical analysis **(A–O)** showed that the reprogramming factors such as c-myc, Klf4, and Sox2 were rarely present in nonischemic areas, but they were significantly increased in ischemic areas (c-myc [D, E, F: red], Klf4 [H, I, J: red], Sox2 [L, M, N: red], CD31 [D, E, G, H, I, K, L, M, O: green], DAPI [D–O: blue]). Note that some of them (especially $Sox2^+$ cells) were observed in iPCs around $CD31^+$ ECs (E, I, M: arrows) and they expressed nestin (**P–R**: arrows). *, $p < .05$ versus nonischemic areas ($n = 5$) (B). A population of iPCs is shown within ischemic areas (C). Scale bars = 50 μm (D, E, H, I, L, M) and 20 μm (P, Q, R). Abbreviations: DAPI, 4′,6-diamino-2-phenylindole; iPC, pericyte following ischemia.

treated with LIF (hPC-OGD/LIF) or without LIF (hPC-OGD). Immunohistochemistry showed that $Sox2^+$ cells increased significantly in hPC-OGD/LIF compared with hPC-OGD (Fig. 7J). Moreover, because ECs secrete bFGF in response to ischemia/hypoxia [48], we further examined the role of bFGF in Sox2 expression. Similar to the immunohistochemistry results, Sox2 expression increased in LIF-treated hPCs (L^+/F^-) compared with LIF-lacking hPCs (L^-/F^-). Such Sox2 expression was further increased by adding bFGF (L^+/F^+; Fig. 7K). Thus, it is likely that both the OGD stimulus and environmental factors are involved in the acquisition of stemness in PCs.

In addition, we found that the immature neuronal gene Tuj1 increased in parallel with the Sox2 gene (Fig. 7K), which is similar to the findings that Sox2 promoted iPC acquisition of neuronal traits (Fig. 4G–4J). Thus, hPC-OGD-derived cell clusters treated with LIF + NCM (hPC-OGD/NCM+LIF) were analyzed (Fig. 7L). The control hPC-N-derived cell clusters did not possess neuronal genes, such as Tuj1 and MAP2 (Fig. 7M); however, hPC-OGD/NCM+LIF expressed these genes (Fig. 7M), and they differentiated into neuron-like cells (Fig. 7N). Finally, to confirm whether mesenchymal hPCs generate immature neurons, hPCs were incubated for 5 days under conditions promoting

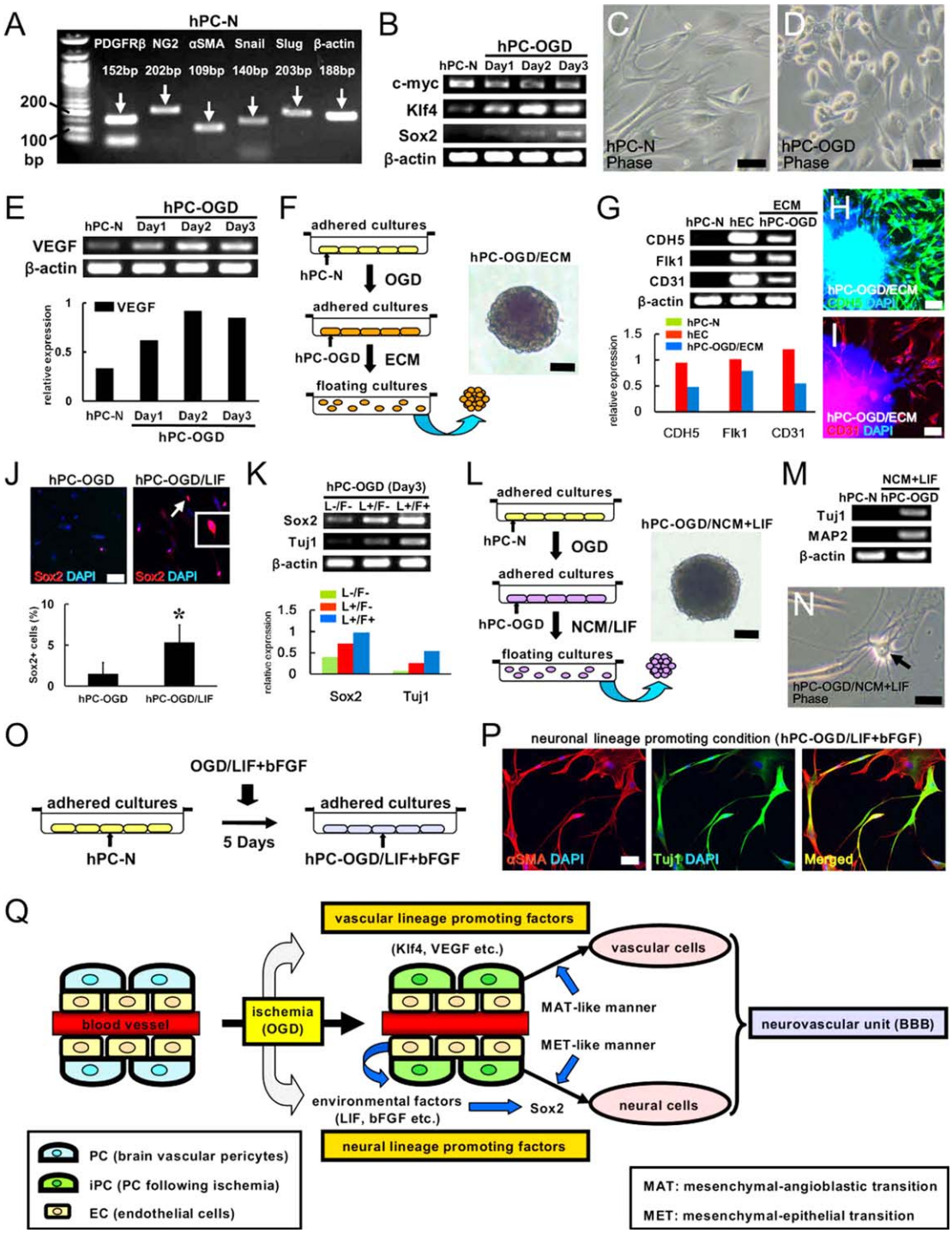

Figure 7. PCs develop multidifferentiation potential toward neurovascular cells via ischemia-induced reprogramming. hPC-N displayed genes related to PCs and the mesenchyme **(A)**. Reprogramming factors (c-myc, Klf4, Sox2) were examined in hPC-OGD using RT-PCR **(B)**. Compared with hPC-N **(C)**, the cell morphology was dramatically changed in hPC-OGD **(D)**. hPC-OGD revealed upregulated expression of VEGF **(E)**. hPC-OGD/ECM **(F)** displayed increased expressions for CDH5, Flk1, and CD31 compared with hPC-N **(G)**. hPC-OGD/ECM differentiated into $CDH5^+$ (CDH5 [**H**: green], DAPI [H: blue]) and $CD31^+$ cells (CD31 [**I**: red], DAPI [I: blue]). Immunohistochemistry showed that hPC-OGD/LIF increased Sox2 expression compared with hPC-OGD (Sox2 [**J**: red], DAPI [J: blue]). PCR analysis showed that Sox2 expression increased in hPC-OGD/LIF (L^+/F^-) compared with hPC-OGD (L^-/F^-). Sox2 expression was further increased by adding bFGF (L^+/F^+) **(K)**. The immature neuronal gene Tuj1 increased in parallel with Sox2 expression (K). hPC-OGD/NCM+LIF formed cell clusters **(L)** that expressed neuronal genes **(M)**, and it differentiated into neuron-like cells **(N)**. hPC-N were incubated under conditions that promoted neuronal lineage differentiation **(O)**. hPC-OGD/LIF+bFGF showed that αSMA^+ cells began expressing Tuj1 (αSMA [**P**: red], Tuj1 [P: green], DAPI [P: blue]). Schematic representation summarizing PC fates under ischemic condition **(Q)**. Scale bars = 50 μm (C, D, H, I, N) and 100 μm (F, J, L, P). Abbreviations: DAPI, 4′,6-diamino-2-phenylindole; hEC, human brain endothelial cell; hPC-N, human brain pericyte cultured under normal/normoxia condition; hPC-OGD, hPC cultured under oxygen/glucose deprivation.

neuronal differentiation (Fig. 7O). Immunohistochemistry for αSMA and Tuj1 showed that mesenchymal αSMA$^+$ PCs began to express Tuj1 (Fig. 7P). These findings suggest that hPCs can be reprogrammed from mesenchymal into neural lineage cells in a MET-like manner. The proposed fates of PCs under ischemic condition are summarized in Figure 7Q.

Discussion

This study demonstrates for the first time that brain PCs develop stemness following ischemia/hypoxia. Furthermore, these iPCs express nestin at high levels both in vivo and in vitro. Whether nestin is expressed specifically in neural (e.g., NSC) [49] or in vascular lineage cells such as ECs [23, 24] and vascular PCs [4, 6] has been debated for several years. However, these findings lead us to the conclusion that nestin is expressed in iPCs, which can generate major component of the BBB/neurovascular unit including neural and vascular cells.

In the CNS, neurogenesis and angiogenesis interactions are coordinated during development [50] and in pathological conditions, such as ischemic strokes [13, 51]. Although NSCs reside near ECs in conventional neurogenic regions, such as subventricular zone (SVZ) [52], our previous study demonstrated that iNSCs are also located near ECs within areas affected by stroke [14]. This suggests that both new neurons and ECs can be produced at the same site in poststroke brains. In support of this notion, this study demonstrated that iPCs extracted from the poststroke mouse brains could differentiate into neurons or ECs. This leads to the novel concept that brain PCs can be targeted after a CNS injury to promote both neurogenesis and angiogenesis.

Why do iPCs have the potential to differentiate into both a neural and vascular lineage? In this study, we showed that nestin$^+$ iPCs in part coexpress PDGFRβ and/or NG2, and that they can differentiate into both lineages. Similar to our findings, recent studies by Birbrair et al. showed that nestin$^+$/NG2$^+$ type 2 PCs have the potential to produce both neural [6] and vascular cells [5]. In addition, previous studies demonstrated that certain NSCs differentiate into not only neural but also into vascular lineage cells [17, 18, 53–55], although their precise traits are unclear. However, this study demonstrates that CDH5$^+$ iPCs can form neurosphere-like cell clusters, some of which generate cells of both lineages. Although CDH5 has been considered to be a specific marker for vascular lineage cells, such as ECs, HSCs [26], and VSCs [25], extravascular expression of CDH5 was observed in neural lineage cells [56] and specifically in glioblastoma stem-like cells [53, 57] that differentiate into neural lineage cells or vascular lineage cells, including ECs and vascular PCs [53, 54, 58]. Thus, iPCs and these glial progenitors might have similar traits. However, iPCs have no tumorigenesis and initially lack glial markers such as GFAP, APQ4, and Olig2, suggesting that iPCs are not glial progenitors. Alternatively, they express various PCs and neural crest markers along with CDH5, similar to ESC-derived neural crest cells [39]. These findings support the hypothesis that iPCs are derived from PCs of the neural crest [11]. Because the neural crest has a stem cell potential [59] that can differentiate into neural [60, 61] and vascular lineage cells [60, 62], it would not be surprising if iPCs produced every components of the neurovascular unit.

Ischemia/hypoxia promotes angiogenesis in a VEGF-dependent manner [63]. We found that PCs increased VEGF expression under OGD, suggesting that PCs act on ECs to promote their proliferation in vivo. However, this study showed that proliferating or stimulated ECs did not express pericytic markers. In contrast, iPCs displayed a mesenchymoangioblast nature [30]. These findings may explain why certain PCs exhibit vasculogenic traits expressing CDH5 [64], CD105 [2], and CD34 [65]. Furthermore, immature PCs share angioblastic phenotypes during the early stage of vasculogenesis [66, 67]. Thus, ischemia/hypoxia may convert somatic PCs into mesenchymoangioblasts, which are typically observed during the developmental stage. However, the precise source, lineage, subtypes, and traits of iPCs warrant further investigation.

Ischemia in vivo is a very complex event, and that OGD treatment in vitro cannot completely mimic ischemia in vivo. Indeed, this study showed that Sox2 expression was not so dramatic as compared with Klf4 expression, although ischemic stroke induced the increased expression of Sox2 in vivo. In addition, we found that environmental factors other than OGD can increase Sox2 expression in hPCs. We have not yet determined as to which factors/signaling pathways specifically induced either the vascular and/or the neural lineage cells in iPCs; however, these results showed that iPCs establish a neuronal phenotype via Sox2 transduction. These results are consistent with those of previous studies showing that Sox2 induced neuronal cells from various cells including PCs [7], fibroblasts [36], and hematopoietic cells [38]. In addition, Sox2 directed axial stem cells [37] and neural crest cells [39] to a neural lineage. In this study, we found that LIF and/or bFGF activated the Sox2 gene, thereby promoting PC differentiation to neuronal cells. Thus, these factors may serve as the prostemness and proneurogenic factors for PCs during ischemia-induced cell reprogramming in vivo. Because stimulated ECs secrete such factors (LIF and bFGF) [47, 48], EC-mediated neurogenesis via iPC/iNSC [13, 14] may be attributed, at least in part, to these factors.

Using a highly reproducible mouse model of stroke, we could easily isolate iPCs from ischemic areas, whereas we could not obtain normal PCs from nonischemic areas in mice, as described previously [10, 11, 13, 14]. Although PCs in nonischemic areas rarely express Yamanaka factors in vivo, commercially available hPCs already expressed c-myc. However, it is possible that such "naïve" hPCs develop stemness during in vitro treatment (repeated passages, specific chemical treatment, etc.). Alternatively, these findings may be because of the difference in species (mice or human) or ages (younger or older). However, this study showed that ischemic stroke or OGD stimulation promoted stemness of PCs with increased expressions of reprogramming factors. This strongly suggests that iPCs developed stemness via reprogramming. Although iPCs developed the multipotency, they lacked Oct3/4 of the four Yamanaka factors. This shows that iPC traits differ from those of pluripotent stem cells, such as ESCs and iPS cells [33]. This suggests that even under strong stimulus conditions that promote reprogramming, such as ischemia/hypoxia, adult somatic cells do not easily acquire phenotypes that are similar to those of pluripotent stem cells. However, iPCs expressed some pluripotent markers such as c-myc, Klf4, Sox2, and Nanog. Thus, the potential for iPC pluripotency should be carefully investigated in future studies.

Conclusions

In conclusion, we demonstrated that iPCs develop multipotency to differentiate into neurovascular cells. Because they are major components of the BBB/neurovascular unit, iPCs are a promising source for repairing the BBB/neurovascular unit after a CNS injury. Further experiments are required to reconstitute this unit in vivo because multiple factors (e.g., inflammatory cells, niche cells, cytokines, and growth factors) influence the fate of iPCs/iNSCs [13, 14, 32].

Acknowledgments

This work was partially supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology (24650173; to T. Nakagomi) and Grant-in-Aid for researchers, Hyogo College of Medicine (2013; to T. Nakagomi). We would like to thank Y. Kimoto, H. Kato, T. Soma, Y. Fujimori, H. Yagi, R. Eguchi, Y. Kawamura, N. Nakagomi, Y. Okinaka, and Y. Tatsumi for technical assistance and helpful discussions.

Author Contributions

T.N.: conception and design, financial support, collection and assembly of data, data analysis and interpretation, manuscript writing, and final approval of manuscript; S.K.: provision of study material and final approval of manuscript; A.N.-D., R.S., S.L., A.N., and M.K.: collection and assembly of data and data analysis and interpretation; A.T.: data analysis and interpretation and final approval of manuscript; T.M.: conception and design, data analysis and interpretation, and final approval of manuscript.

Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

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