Received: 16 May 2019 | Accepted: 19 July 2019

DOI: 10.1002/sctm.19-0148

TISSUE-SPECIFIC PROGENITOR AND STEM CELLS

STEM CELLS TRANSLATIONAL MEDICINE

Capillary-resident EphA7$^+$ pericytes are multipotent cells with anti-ischemic effects through capillary formation

Yuri Yoshida[1,2] | Maki Kabara[1] | Kohei Kano[1,3] | Kiwamu Horiuchi[1,3] | Taiki Hayasaka[1,3] | Yui Tomita[1,4] | Naofumi Takehara[3] | Akiho Minoshima[3] | Tatsuya Aonuma[3] | Keisuke Maruyama[3] | Naoki Nakagawa[3] | Nobuyoshi Azuma[2] | Naoyuki Hasebe[1,3] | Jun-ichi Kawabe[1]

[1]Department of Cardiovascular Regeneration and Innovation, Asahikawa Medical University, Asahikawa, Japan

[2]Department of Vascular Surgery, Asahikawa Medical University, Asahikawa, Japan

[3]Department of Medicine, Division of Cardiovascular, Respiratory and Neurology, Asahikawa Medical University, Asahikawa, Japan

[4]Department of Radiology, Asahikawa Medical University, Asahikawa, Japan

Correspondence
Jun-ichi Kawabe, MD, PhD, Department of Cardiovascular Regeneration and Innovation, Asahikawa Medical University, Asahikawa, 2-1-1 Midorigaoka-higashi, Asahikawa 078-8510, Japan.
E-mail: kawabeju@asahikawa-med.ac.jp

Funding information
OideCapiSEA, Inc; Asbio Pharmer Co. Ltd.; Daiichi Sankyo Co. Ltd.; Grant-in-Aid for Young Scientists, Grant/Award Numbers: 18K16379, 17K15980; Exploratory Research, Grant/Award Number: 17K19368; Grant-in-Aid for Scientific Research, Grant/Award Number: 17H04170

Abstract

The presence of pericytes (PCs) with multipotency and broad distribution along capillary suggests that microvasculature plays a role not only as a duct for blood fluid transport but also as a stem cell niche that contributes to tissue maintenance and regeneration. The lack of an appropriate marker for multipotent PCs still limits our understanding of their pathophysiological roles. We identified the novel marker EphA7 to detect multipotent PCs using microarray analysis of an immortalized PC library. PCs were isolated from microvessels of mouse subcutaneous adipose tissues, then EphA7$^+$ PCs called capillary stem cells (CapSCs) were separated from EphA7$^-$ control PCs (ctPCs) using fluorescence-activated cell sorting system. CapSCs had highly multipotency that enabled them to differentiate into mesenchymal and neuronal lineages compared with ctPCs. CapSCs also differentiated into endothelial cells and PCs to form capillary-like structures by themselves. Transplantation of CapSCs into ischemic tissues significantly improved blood flow recovery in hind limb ischemia mouse model due to vascular formation compared with that of ctPCs and adipose stromal cells. These data demonstrate that EphA7 identifies a subpopulation of multipotent PCs that have high angiogenesis and regenerative potency and are an attractive target for regenerative therapies.

KEYWORDS

angiogenesis, capillary, mesenchymal stem cells, neuronal stem cells, pericytes, peripheral ischemic diseases

1 | INTRODUCTION

Capillaries consist of endothelial cell (EC)-tubes covered with pericytes (PCs) as a minimal vascular unit of multicellular organisms that distribute blood fluids throughout the body and maintain cells through the exchange of nutrients, oxygen, and metabolities.[1] PCs play fundamental roles in the development and maturation of microvasculature. PCs wrap around the EC layers to provide scaffolding support and regulate EC functions including blood vessel integrity and permeability, and blood flow through direct cell-to-cell interaction or the release of paracrine mediators.[2] A number of recent studies suggest that PCs constitute multipotent cells such as mesenchymal stem cells

This is an open access article under the terms of the Creative Commons Attribution-NonCommercial-NoDerivs License, which permits use and distribution in any medium, provided the original work is properly cited, the use is non-commercial and no modifications or adaptations are made.
© 2019 The Authors. STEM CELLS TRANSLATIONAL MEDICINE published by Wiley Periodicals, Inc. on behalf of AlphaMed Press

(MSCs),[3,4] white adipocyte progenitors,[5-7] muscle stem cells,[3,8] and NSCs.[9-11] Therefore, the understanding that PCs contain multipotent cells and have broad distribution in association with capillaries provides an important insight into microvasculature capillaries and their role not only as a duct to transfer blood fluid but also as a stem cell reservoir that contributes to tissue maintenance and repair/regeneration upon injury in multicellular organisms.

Preclinical studies indicate the therapeutic potential of multipotent PCs in various disease models including limb ischemia, ischemic heart disease, muscular dystrophy, and retinal vasculopathy.[12] Transplantation of PCs in the clinical setting will require a scalable, well-defined multipotent PC cell source; however, PCs are a heterogenous population of mesenchymal cells that have several different developmental origins. There are limited phenotypes and markers that are unique to PCs, and highly dependent on the type of tissues and affected by the pathogenic state of the organ.[2] Previous studies have identified perivascular cells with MSC-like differentiation potential by lineage tracing approaches using platelet-derived growth factor receptor (PDGFR)β- and neuroglial 2 proteoglycan (NG2)-Cre mouse lines.[4,5,13] Multipotent PCs in culture are defined by the expression of certain molecular markers such as CD146 (melanoma cell adhesion molecule), ALP (alkaline phosphatase), and the absence of hematopoietic and endothelial markers (CD45 and CD31/CD34), as well as skeletal satellite cell markers, CD56.[3,8,14] However, none of these molecular markers are specific to multipotent PCs or distinguish them from other PC populations.

Studies on transgenic mouse lines expressing reporter genes have revealed that multipotent PCs can be defined by PPARγ-GFP and nestin-GFP transgenes.[5,15] Bribrair et al demonstrated that nestin$^+$ PCs may be a specific multipotent PC subtype with restricted lineage potential.[15] However, these molecules are located in an intracellular compartment and cannot be used as specific markers for the isolation of targeted living cells from human or animal organs. So far, there is no known molecular marker that specifically identifies or isolates living multipotent PCs among heterogenic PC populations. These challenges have hampered the characterization of multipotent PCs subtypes, research into their pathophysiological roles, and their clinical applications.

We recently established several immortalized PC cell lines from microvessels grown in remodeling tissue from injured arterial walls in temperature-sensitive SV40-T-antigen transgenic mice.[16] The feature of these clonal PCs is that they are identical in their genomic background and maintain their cellular phenotypes including multipotency during long-term subculture,[16] which is advantageous for the characteristic analysis of each population of PCs. In this study, we used microarray analysis of PC cell library that consisted of several clonal PC lines that possess different degrees of multipotency to identify EphA7 as PC multipotency-related candidate markers. Using an antibody against EphA7, we successfully isolate unique EphA7$^+$ PCs from peripheral tissues including subcutaneous adipose tissues. EphA7$^+$ PCs have high multipotency, differentiating mesenchymal and neuronal lineages, whereas EphA7$^-$ PCs have no or relatively lower multipotency. Thus, EphA7 is specific marker that distinguished multipotent PCs from other PC populations.

Significance statement

The present study characterizes the multipotency of pericyte populations isolated from mammalian capillaries using a novel genetic marker, EphA7. It is shown that this marker can be used to isolate living cells and that EphA7+ pericytes, termed capillary stem cells (CapSCs), have capillary formation by themselves and cross-germ layer plasticity to differentiate into mesenchymal and neuronal lineages, indicating its potential use in both disease models and regenerative therapies.

2 | MATERIALS AND METHODS

The detailed materials and methods are described in Appendix S1. Patent pending: PCT/JP2016/072259.

2.1 | Animals

All experiments involving animal studies were performed according to protocols approved by the Animal Care and Use Committee of Asahikawa Medical University. Animals were maintained in a temperature- and light-controlled facility and were fed normal chow. Male C57BL/6 and transgenic mice including actin-promoter-drived GFP (GFP) and NG2-promoter-drived DsRed (NG2-DsRed) mice aged 10-12 weeks were used for the experiments as described previously.[17] Only male mice were used in order to exclude any effects of female hormones such as estrogen in this study.

2.2 | Preparation of adipose stromal cells and microvessel-rich fractions from peripheral tissues

The subcutaneous adipose tissues were digested with collagenase buffer, then adipose stromal cells (ASCs) were prepared as described previously.[18] For preparation of microvessel-rich fractions, the collagenase-digested homogenate was filtered through 118-μm nylon mesh and sequentially filtered through a 100-μm cell strainer. The resultant filtrates were applied to a 40-μm cell strainer; the microvessel fragments on the strainer membrane were collected and used for isolation of microvessel-associated cells.

2.3 | Isolation of microvessel-associated cells

The microvessel fragments were incubated in complete medium at 37°C in 5% CO_2 incubator for 4-6 days. Among cells that grew out from microvessel fragments (Figure 2C,D), the NG2$^+$ cells (PCs) were separated with magnetic-activated cell sorting system (MACS) and/or fluorescence-activated cell sorting system (FACS) using anti-NG2 antibody (Miltenyi Biotec, Auburn, California) as previously described.[17] EphA7$^+$ cells and

EphA7^{-} cells (control PCs [ctPCs]) were isolated from prepared PCs by FACS using anti-EphA7 antibody (LSBio, LS-C3329513, Seattle, Waltham).

2.4 | In vitro cellular differentiation assay

Mesenchymal and neuronal cell differentiations were induced by incubation with appropriate differentiation media according to the manufacturer's manual (Mesenchymal Stem Cell Functional Identification Kit [SC-010] and Neural Lineage Functional Identification Kit [SC-028]; R&D Systems, Minneapolis, Minnesota, http://www.rndsystems.com).

2.5 | In vitro 3D-gel angiogenesis assay

The in vitro vascular formation assay was carried out as described previously.[16,17] Images of formed tubes in the gels were obtained with a phase-contrast and fluorescence microscope (BZ-X710; Keyence, Osaka, Japan).

2.6 | Hind limb ischemia mouse model and cell injection

The severe ischemic hind limb condition was induced as described in previous studies[17,19] with some modification. Briefly, mouse (C57BL/6, aged 16-24 weeks, male) was anesthetized, and the proximal common iliac artery and the distal deep femoral and superficial femoral arteries were ligated, and the intervening arteries were cut out. Then, capillary stem cells (CapSCs), ctPCs, ASCs or its vehicle, phosphate buffered solution (PBS) was intramuscularly transplanted into the ischemic hind limb. The blood flow in the hind limb at an indicated time was estimated using laser speckle contrast imaging (OMEGA ZONE; Omega Wave Co., Tokyo, Japan).

2.7 | Immunohistological analysis

Immunohistochemical and immunofluorescence analyses were conducted as described in previous reports.[16,17] Hematoxylin and eosin staining was carried out on paraffin sections using standard methods. In order to observe transplanted cells within skeletal muscle tissues in 3D view, we made fixed tissues transparence using CUBIC method.[20]

2.8 | Gene expression profiling and data analyses

Cells incubated in Matrigel for indicated times were isolated from Matrigel using Cell recovery solution (CORNING, Corning, New York), and used to further gene expression analyses. Gene expression profile was determined by reverse transcription-polymerase chain reaction (RT-PCR), quantitative PCR and flow cytometry analysis as described previously.[16] For gene expression microarrays, total RNA isolated from sorted CapSCs and ctPCs (three independent isolated cell samples) was applied to microarray analysis on a 3D-Gene Mouse Oligo Chip 24K (Toray Industries Inc., Tokyo, Japan) as described previously.[16]

2.9 | Statistical analysis

Results are presented as means ± SEM unless otherwise noted. Normally distributed data and homogeneity of variance of each data were

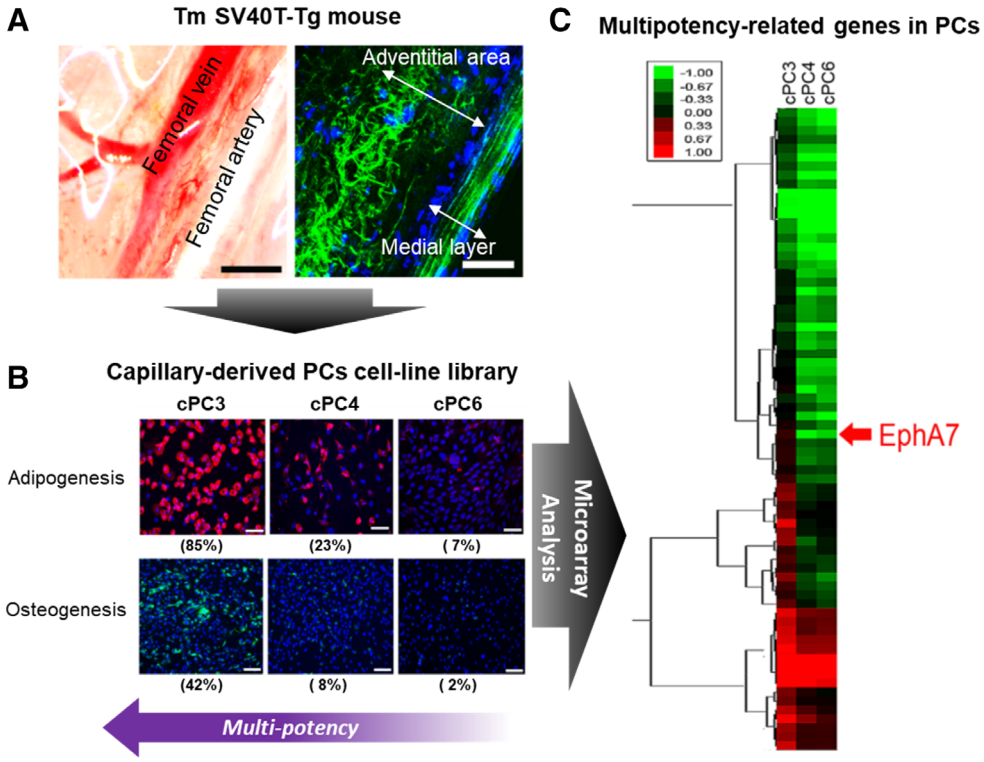

FIGURE 1 Screening of candidate genes associated with pericyte multipotency. A, Microvessels growing within remodeling tissues were prepared from adventitial microvessels of injured femoral arteries in temperature-sensitive SV40 T-antigen transgenic (Tm SV40 tg) mice, and immortalized capillary-derived PCs (cPCs) were established using these microvessels. B, Three clonal cPC cell lines were collected among 10 established cPCs that possess different degrees of multipotency. C, Representative parts from a dendrogram of a microarray cluster analysis illustrating the comparison among the three cPC lines. EphA7 was selected among the membrane-associated genes from the candidate genes. Scale bars = 500 μm (A) and 50 μm (B)

confirmed by *F* test and Bartlett test, respectively. Student's *t* test was used in two group comparisons. For comparisons of more than two groups, one-way analysis of variance (ANOVA) was used for normal distributions. Blood flow recovery in the ischemic hind limb was compared between the two groups by two-way repeated measurements ANOVA followed by Turkey-Kramer analyses. $P < .05$ was considered statistically significant.

3 | RESULTS

3.1 | EphA7 is a candidate gene associated with PC multipotency

In order to detect specific molecules that associate with multipotent PCs, we used a clonal PC cell library consisting of several PC lines with different degrees of multipotency. Previously, we established immortalized capillary-derived PCs (cPCs) from microvessels in the adventitial *vasa vasorum* of injured femoral arteries of temperature-sensitive SV40 T-antigen transgenic mice (Figure 1A).[16] All immortalized clonal cPCs lines (10 cell lines) equally possessed PC-specific characteristics including expression of PC-specific markers such as *PDGFRβ*, *NG2*, and *CD146* but not EC markers such as *vWF* and *PECAM* (*CD31*) (Figure S1 and Table S1). Some cPCs lines have multipotent potency and can differentiate to mesenchymal and neuronal cell lineages, as well as skeletal myogenesis and angiogenesis.[16]

We prepared the cPC library and selected three lines among the established cPC lines.[16] PC clone #3 (cPC3) possesses highly MSC-like multipotency and adipogenesis and osteogenesis differentiation efficiencies of 85% and 42%, respectively. cPC6 does not show any multipotency (7% and 2%), whereas cPC4 has moderate differentiation potency that is between that of cPC3 and cPC6 (23% and 8%) (Figure 1B). We performed microarray analysis and transcriptional comparison to define the genes related to the degree of multipotency

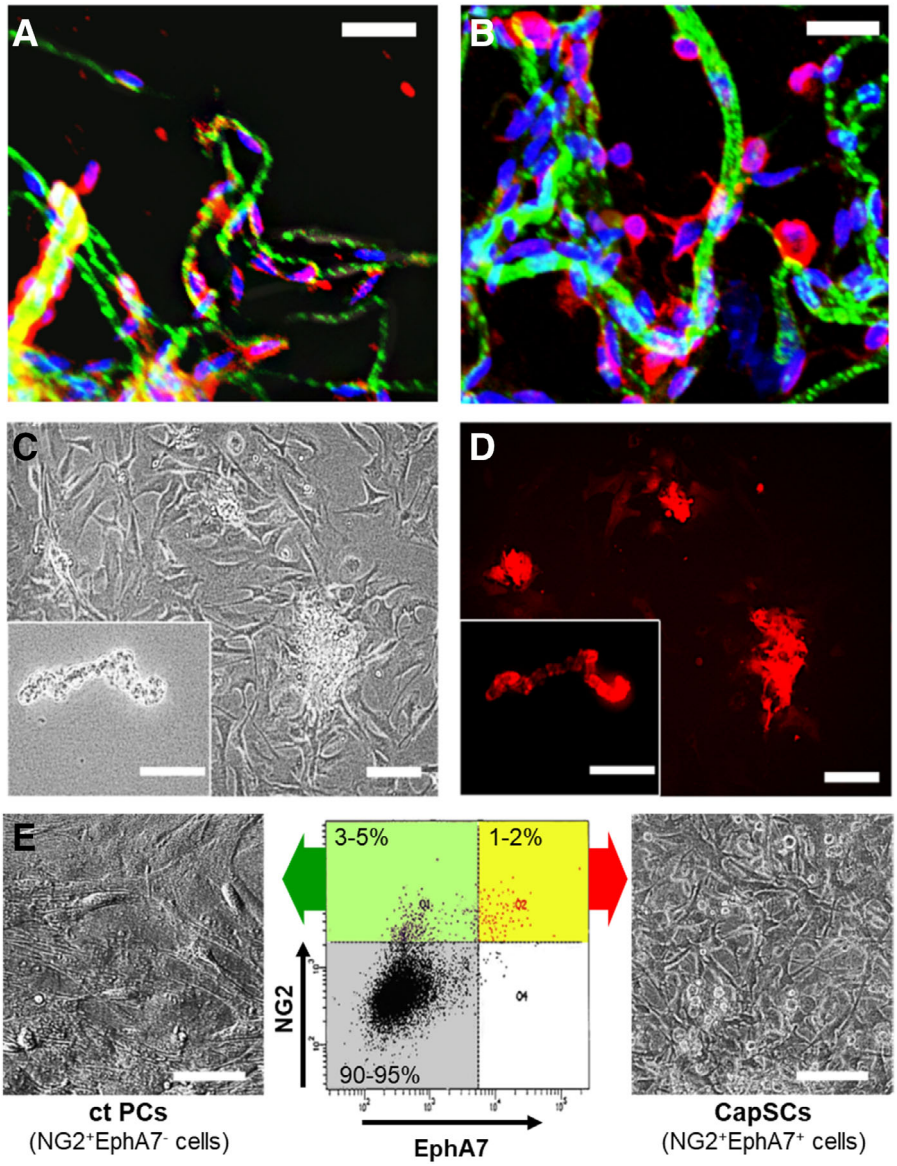

FIGURE 2 Isolation of EphA7$^+$ PCs from peripheral tissues. The vascular intraluminal endothelial layer was stained with FITC-lectin (green). Microvessels isolated from subcutaneous adipose tissues were immune-stained with anti-NG2 (red) (A), and anti-Epha7 antibodies (red) (B). Nuclei were counterstained with Hoechest 33 258 (blue). C and D, Microvessel fragments were collected from subcutaneous adipose tissues from NG2-DsRed mice. NG2$^+$ PCs (red) adhered to the isolated microvascular tubes (insert). After 6 days of incubation, NG2$^+$PCs grew out from microvessel fragments and made cell clusters. E, Adipose stromal cells were immuno-labeled with anti-NG2 and anti-EphA7 antibodies, and then NG2$^+$EphA7$^+$cells, that is, capillary stem cells (CapSCs) and NG2$^+$EphA7$^-$ cells, that is, control PCs (ctPCs) were isolated using fluorescence-activated cell sorting system. Scale bars = 100 μm (A, B, E), and 50 μm (C, D)

in these three cPC lines. Finally, among membrane-associated genes within the candidates, we selected *EphA7*, which was specifically expressed in cPC3, but not in cPC4 or cPC6 (Figure 1C and Table S1). Importantly, there was no apparent difference in the expression of PC-specific genes and well-known MSC-marker genes among the different cPC lines (Table S1).

3.2 | Isolation of EphA7$^+$ PCs from peripheral tissues

The PCs were detected as NG2$^+$ cells in microvessels of peripheral tissues such as subcutaneous adipose tissues and skeletal muscle.[1,17,21] Most of the NG2$^+$ cells had an elongated, flat-shaped morphology and adhered to the endothelium (Figure 2A). The EphA7$^+$ PCs were adjacent to the endothelium and highly branched in morphology; some protruded into the interstitial spaces (Figure 2B).

ASCs and microvessel-associated cells were prepared from collagenase-digested fraction of mouse subcutaneous adipose tissues. To enhance the yield of capillary-associated PC fraction, microvessels within collagenase-digested fraction were collected by a combination of enzyme digestion and membrane filtration as described in the Materials and Methods section. NG2$^+$ PCs grew out from small fragment of capillaries (ie, microvessel explants) were highly proliferative, and formed PC-made clusters (Figure 2C,D). NG2$^+$ PCs were isolated using a fluorescence-activated cell sorting system (FACS) or magnetic-activated cell sorting system (MACS), then EphA7$^+$ cells were isolated from NG2$^+$ PCs using FACS (Figure 2E and Figure S2). Among these stromal cell fractions, EphA7$^+$ cells were mostly in the NG2$^+$ cell fractions (Figure 2E). The ratio of EphA7$^+$ cells was approximately 5%-20% among crude PCs fractions. The yield efficiency of EphA7$^+$ PCs sorting was not different among these two purification strategies, that is, one step FACS (Figure 2E) and combination of MACS/FACS (Figure S2).

When sorted EphA7$^+$ PCs (*termed* capillary-derived stem cells, CapSCs) and EphA7$^-$ PCs (ie, ctPCs) were cultured, CapSCs were stellate-shaped with a highly branched morphology. In contrast, ctPCs are elongated and stellate but relatively flat-shaped cells (Figure 2E). Although proliferation of fleshly isolated CapSCs was relatively lower, the proliferation rate of CapSCs gradually increased within 2~3 subcultures and was higher than that of ctPCs. The CapSCs maintained a high proliferation rate up to 40 subcultures, resulting in a doubling time of 31.7 and 58.3 hours for CapSCs and ctPCs, respectively (Figure 3A). CapSCs, not ctPCs, formed sphere from a single cell under non-adherent culture condition (25.3%, 3.2% of isolated cells, respectively) (Figure 3B).

3.3 | Gene expression profile of EphA7$^+$ PCs, namely CapSCs

Microarray analysis demonstrated that both CapSCs and ctPCs highly and equally expressed PC-specific genes such as *PDGFRβ*,

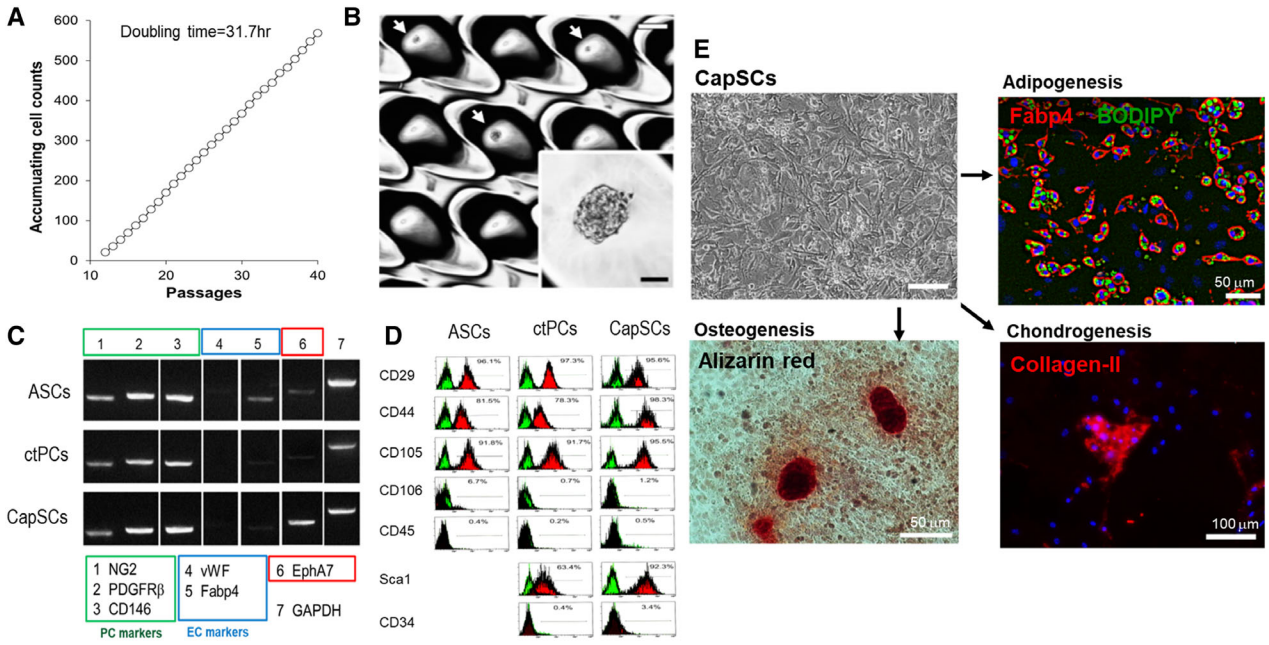

FIGURE 3 Cellular characteristics of isolated primary CapSCs. A, Accumulating cell numbers during subculture of CapSCs are shown. B, Isolated cells were incubated for 7-10 days in an ultra-low attachment dish and formed spheres were observed. C, The expression levels of PC- and EC-specific genes detected by RT-PCR. The cropped images were shown, and the full-length gel images were included in the Figure S4. D, Flow cytometry analysis. Specific antibody staining and isotype control IgG staining profiles are shown by red and green histograms, respectively. E, Representative images of CapSCs differentiation into mesenchymal lineage cells. Adipocytes were dual stained with Fabp4 and BODIPY, osteocytes were alizarin-red-stained mineral deposits, and chondrocytes were stained with collagenase II. Scale bars = 200 μm (B) and 50 μm (B, insert)

CD146, and *αSMA* (Table S2). PCs are precursors for smooth muscle cells (SMCs)[1,2] and CapSCs and ctPCs also highly expressed the SMC-marker genes *αSMA*, *calponin2*, and *tropomyosin1*. However, the expression level of these genes was higher in ctPCs than in CapSCs (Table S2). EC marker genes such as *CD31*, *Flt1*, and *vWF* were relatively weak or not detected in both CapSCs and ctPCs (Table S2). Interestingly, well-known MSC-marker genes such as *CD29*, *CD44*, and *CD105* were identically expressed in both CapSCs and ctPCs. Consistent with the results of the microarray analysis, the expression of PC-specific genes (eg, *NG2*, *PDGFRβ*, and *CD146*) was observed in CapSCs, ctPCs, and ASCs, and was identical between CapSCs and ctPCs (Figure 3C). The EC markers *vWF* and *Fabp4*, which is known as capillary-derived EC marker[22], were not detected in CapSCs and ctPCs (Figure 3C). Flow cytometry analysis revealed that CapSCs and ctPCs were both positive for the well-known MSC markers CD29, CD44, CD105, and Sca1, but negative for hematopoietic markers such as CD34 and CD45 (Figure 3D). These data confirmed that CapSCs and ctPCs were included in PC populations and also indicated that CapSCs and ctPCs were not contaminated with hematopoietic stem cells or endothelial progenitor cells (EPCs).

3.4 | CapSCs have cross-germ layer multipotency, differentiation to mesenchymal and neuronal cell lineages

After CapSCs were cultured in the appropriate differentiation media, they effectively differentiated to Fabp4$^+$ adipocytes containing Bodipy-stained lipid drops, osteoblasts with alizarin-red-stained mineral deposits, and collagen2-stained chondroblasts (Figures 3E and 4). In contrast, a few ctPCs differentiated into mesenchymal cells, adipocytes (~10%), and osteopontin-stained osteoblasts (~5%) (Figure 4).

Microarray analysis demonstrated that CapSCs had relatively high expression of the NSC marker, *nestin* (Table S2). Therefore, we tested if CapSCs could differentiate into neuronal lineages. When CapSCs were incubated in neural maintenance medium, the number of nestin-positive cells increased and nestin expression was further enhanced (Figure 5). The CapSCs that differentiated into neuronal lineage cells in neuronal differentiation medium stained for neuron-specific β3 tubulin and glial cells-specific markers S100β or GFAP (Figure 5 and Figure S3). Conversely, neurogenesis potency was rarely observed in ctPCs (Figure 5). These data indicate that CapSCs have multipotency, can differentiate into two germ layers, meso- and ectoblastic lineage cells (ie, mesenchymal and neuronal cells), and EphA7 can be used to isolate multipotent PCs from non-multipotent PCs.

ASCs, consisted of a heterogenous cell populations had the minimum criteria as MSCs,[23] expression of CD29, CD44, and CD105, and the lack of expression of CD45 (Figure 3D), differentiation capacity (adipogenesis and neurogenesis) (Figure 4 and Table S4). Although expression of MSC markers is mostly identical among ASC, ctPCs, and CapSCs, differentiation capacity of CapSCs was higher than that of ASCs and ctPCs.

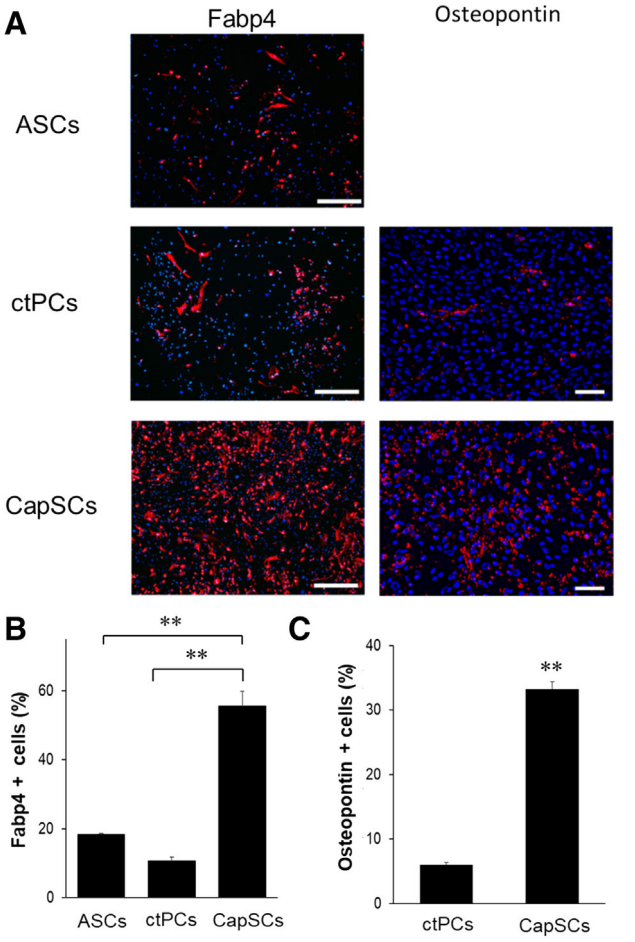

FIGURE 4 Adipogenic and osteogenic induction of primary CapSCs. A, Representative images of Fabp4- and osteopontin-stained adipocytes and osteoblasts respectively after differentiation induction in each tested cells. Nuclei were counterstained with Hoechest 33258 (blue). Scale bars = 500 μm. B and C, Fabp4$^+$ and osteopontin$^+$ cells were counted, and the ratio to total adherent cells were calculated. The results were presented as the means ± SEM. $^{**}P < .01$ compared with the ctPC (n = 4)

3.5 | CapSCs form capillary-like structure by themselves

It is well documented that ECs have tube-like formation potency on a gel-coated dish, but do not form in gel culture conditions.[24] Although PCs alone did not form tube-like structures in a gel, a mixture of PCs and ECs formed capillary-like structures, that is, EC-made tube surrounded with PCs as previously reported.[17,25,26] Similar to previous studies,[17,26] ctPCs alone formed small cell clusters with minimal branching (Figure 6A,C), whereas CapSCs formed tube-like networks with dense cells clusters and extensive branching in gel culture conditions in the presence of vascular endothelial growth factor (VEGF) and low-concentrated serum (Figure 6B,C). CapSCs tubes were relatively enlarged in their diameter compared with EC tubes and consisted of vWF-stained ECs surrounded by NG2-stained PCs (Figure 6D). CapSCs did not express any EC or its precursor markers under basal

culture conditions (Figure 3 and Table S2). However, when CapSCs were grown in a gel in the presence of VEGF, expression of EC marker genes such as *vWF* and *PECAM* was significantly induced (Figure 6E). These data indicate that some CapSCs differentiated into ECs and assembled with CapSCs and/or differentiated PCs to form capillary-like structure.

3.6 | Reproducibility of cellular functions of stocked CapSCs

CapSCs were independently prepared several times from mouse peripheral adipose tissues. The isolated cells were stocked at −80°C in parallel with their cellular analyses. After confirmation of cellular characteristics, stocked cells were used for further experiments. Among three stocked cell samples, CapSCs constantly demonstrated high adipogenesis and neurogenesis potency and angiogenesis compared with ctPCs and ASCs (Table S4). CapSCs were also successfully isolated from skeletal muscle tissues, and showed specific cellular functions similar to that of adipose-tissue derived CapSCs.

These data indicate that multipotent CapSCs can be prepared reproducibly from peripheral tissues including subcutaneous adipose and skeletal muscle tissues by the cell isolation procedure.

3.7 | Angiogenic effects of CapSCs in hind limb ischemia mouse model

We demonstrated that CapSCs have potent angiogenic effects and assemble capillary-like structure by themselves in vitro (Figure 6). Thus, to test the therapeutic effects of CapSCs on ischemic tissues, a mouse severe hind limb ischemia (HLI) was induced by deletion of left femoral vessels and femoral subcutaneous adipose tissues that contribute to the collateral route of ischemic area as performed previously.[17] Then, GFP-expressing cells, including CapSCs, ctPCs, ASCs, and PBS, were intramuscularly injected into the ischemic hind limb. Laser Doppler perfusion imaging showed that the blood perfusion in ischemic lower limb was decreased to 25% of right intact lower limb, and gradually recovered and plateaued at 21-28 days after HLI surgery (Figure 7A,B). The blood perfusion of CapSCs transplanted limbs, however, increased even at 28 days

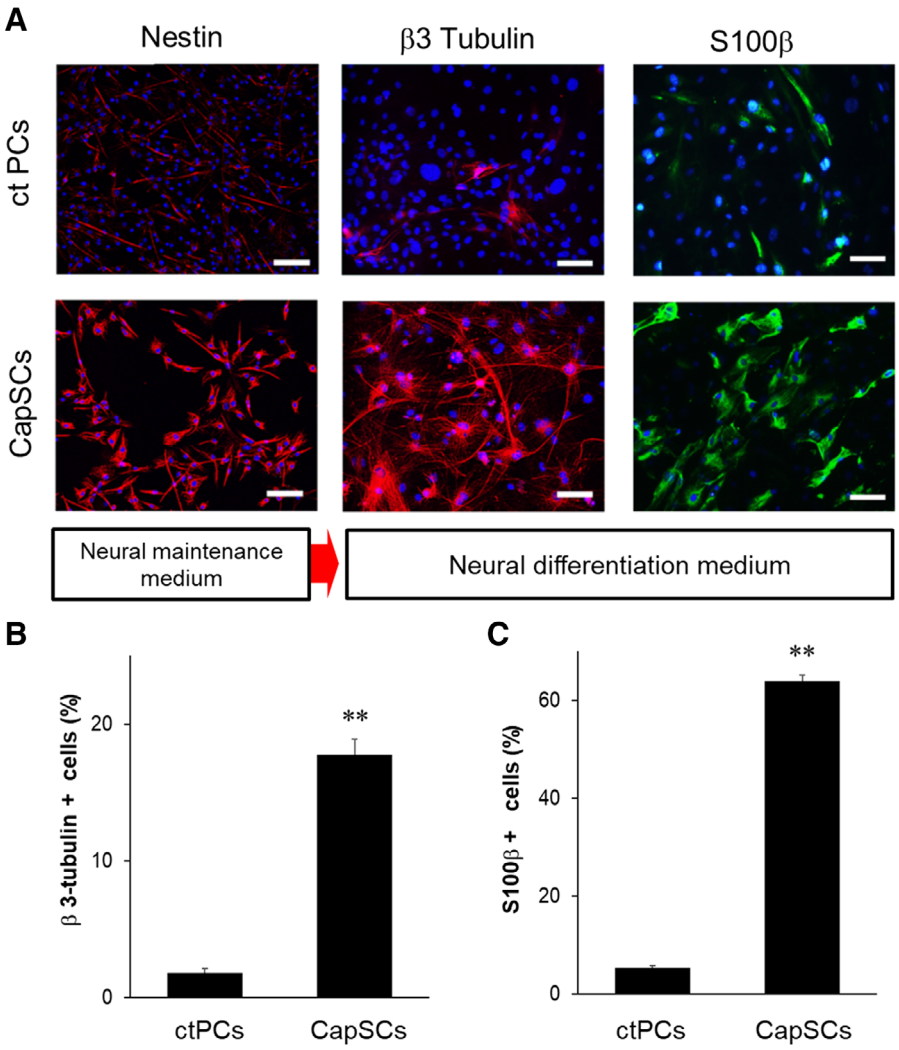

FIGURE 5 CapSCs can differentiate into neuronal lineages. A, Representative images of nestin-stained CapSCs and ctPCs after treatment with neural maintenance medium, and neuro-specific β3 tubulin- and S100β-stained neuronal cells and glial Schwann cells, respectively, after further treatment with differentiation medium. Nuclei were counterstained with Hoechest 33258 (blue). Scale bars = 100 μm. B and C, $\beta3tubulin^+$ and $S100\beta^+$ cells were counted and the ratio to total adherent cells was calculated. The results are presented as means ± SEM. $^{**}P < .01$ versus ctPC (n = 4)

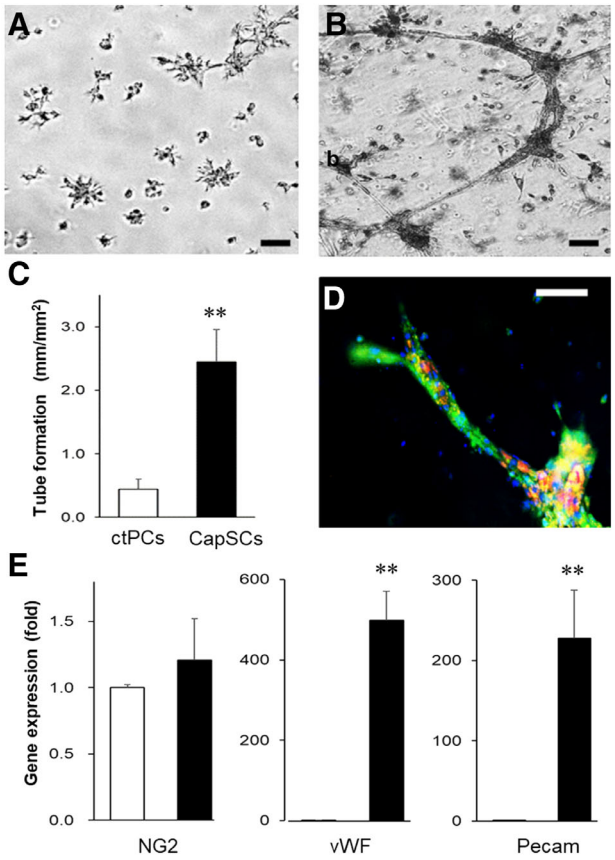

FIGURE 6 Endothelial differentiation and formation of capillary-like structure in CapSCs. Representative phase-contrast optical images of the tube formation potential in ctPCs (A) and CapSCs (B) that were cultured for 6 days in Matrigel. C, The tube length was significantly higher in CapSCs compared with ctPCs. D, Representative immunostaining image of CapSCs-made tubes using anti-vWF (red), and anti-NG2 (green) antibodies. E, Gene expression in CapSCs grown in gel (closed bar) or adherent culture conditions (control, open bar) was estimated by qRT-PCR. The results are presented as means ± SEM. $^{**}P < .01$ versus the control group (n = 4). Scale bars = 100 μm (A, B), and 50 μm (D)

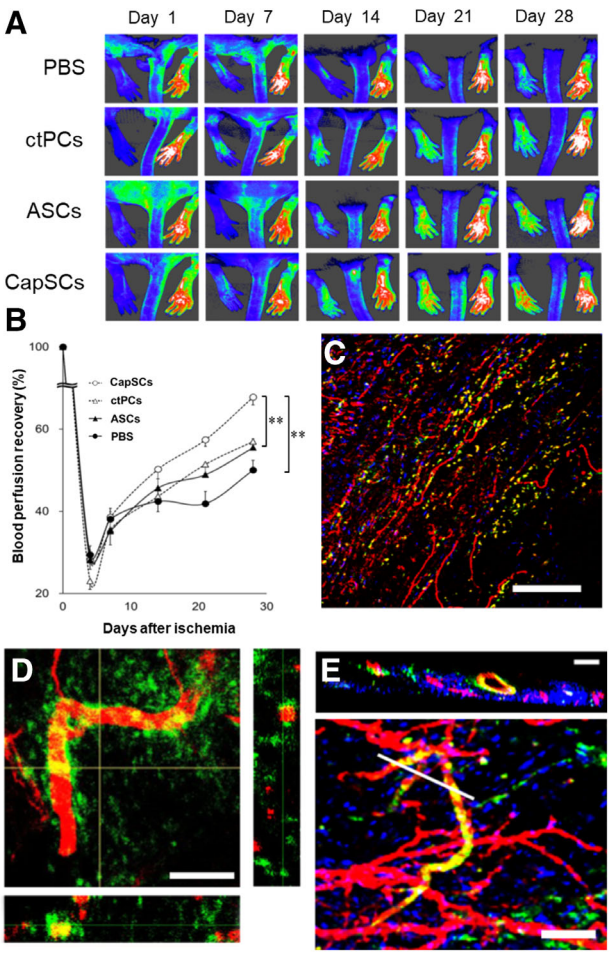

FIGURE 7 Recovery of blood flow and therapeutic effects in hind limb ischemia after CapSCs transplantation. A, Representative laser Doppler perfusion images of blood flow recovery in ischemic hind limbs. B, Quantitative analysis revealed improved blood perfusion in CapSCs compared with ctPCs, ASCs, and PBS-treated groups 4 weeks after cell injection. $^{**}P < .01$ (n = 10-12). C and D, Representative 3D fluorescent images of cleared ischemic skeletal muscles at 2 weeks, and (E) 4 weeks after GFP-CapSCs (green) transplantation. Endothelium was stained by rhodamine-lectin (red). Scale bars = 200 μm (B), 10 μm (D), and 100 μm (E)

after surgery and was significantly higher than that in limbs injected with ctPCs, ASCs, or PBS at 28 days (Figure 7A,B). Transplanted GFP-CapSCs were observed in ischemic area for up to 28 days after surgery (Figure 7C), some were adjacent to neovessels and incorporated into microvessels (Figure 7D,E).

4 | DISCUSSION

In the present study, we showed that distribution of EphA7$^+$ cells in the capillaries of peripheral tissues were perivascular. Multipotent PCs were selectively purified from capillary PCs of peripheral tissues including subcutaneous adipose tissues using the expression of EphA7 as a cell-specific marker. EphA7$^+$ PCs (CapSCs) were extracted from peripheral tissues and maintained their capability for differentiation across mesoderm and ectoderm germ layer for up to 20 passages. These finding indicate that EphA7 is a potential marker that defines some population of origin for both multipotent cells and perivascular cells in vitro and in vivo.

CapSCs are characterized as nestin$^+$ PCs (Table S2) and have multipotency as both MSCs and NSCs. Previous studies have demonstrated that PCs derived from brain and other peripheral tissues are neural cell precursors.[9,27,28] The coexpression of nestin and NG2 in PCs is well associated with neuronal lineage priming, similar to what is observed in mesenchymal lineages and potentially explains the cross-germ layer plasticity of PCs.[28] These data are consistent with the observation that PCs and MSCs originate from the neural crest, which consists of nestin-positive cells during embryogenesis.[29,30]

Expression pattern of two PDGFRs, namely PDGFRα and β, is somewhat specific for cell type. PDGF is released from ECs to act on

neighboring PDGFRβ$^+$ PCs to allow their proliferation and migration during blood vessel morphogenesis.[2,31] In contrast, PDGFRα is dominantly expressed in certain stem cells such as MSCs.[32] As shown in Table S2, expression level of PDGFRβ is not different between CapSCs and ctPCs. However, PDGFRα is dominantly expressed in CapSCs, suggesting that CapSCs are MSC-like cells among PDGFRβ$^+$ PCs.

Birbrair et al reported two PC subpopulations in the skeletal muscle using nestin-GFP transgenic mice, that identified nestin$^+$ type 2 PCs and nestin$^-$ type1 PCs.[33] Type 2 PCs have multipotency and regenerative abilities that contribute to angiogenesis and myogenesis in vitro and in vivo.[15,34] Recently, Maeda et al identified meflin as a marker for MSCs (immunoglobulin superfamily containing leucine-rich repeat, *Islr*).[35] Meflin$^+$ cells are found on stromal cells, perivascular cells including PCs in multiple organs. Meflin maintains the undifferentiated state of cultured MSCs and is downregulated upon their differentiation. Interestingly, expression of meflin in CapSCs was relatively higher than in ctPC (Table S2). A limitation in these studies is the absence of appropriate antibody for immunostaining and cell sorter analyses. In particular, nestin is located in intracellular compartments and cannot be used as a marker to isolate targeted living cells from normal tissues/organs.

A number of vascular progenitor cells including circulating EPCs delivered from bone marrow CD34$^+$ hematopoietic cells and tissue-resident EPCs.[36-38] Recently, Wakabayashi et al reported that bone marrow stromal antigen1 (bst1) CD157$^+$ vascular endothelial stem cells are present in peripheral blood vessels and have vascular regeneration potential.[39] Our gene expression profiling and flow cytometry analyses indicated that CapSCs did not include CD34$^+$ hematopoietic cells or CD157$^+$ EPCs (Figure 3, Table S2).

CapSCs have potent regenerative effects, including enhanced blood flow recovery following HLI. These effects would be mediated by multiple mechanisms. It is well documented that PCs play a key role during angiogenesis and are regulators of vascular stabilization and maturation through trophic effects and direct interaction with ECs.[1,2] The CapSCs are a subgroup of PCs that can act as original PCs and produce angiogenic growth factors. Interestingly, the trophic effect of CapSCs include the production of angiogenic factors such as VEGF, FGF, and angiopoietin was relatively higher compared with that in ctPCs (Table S3). In addition to their trophic effects, CapSCs could differentiate into vascular cells to form neovessels in vitro and in vivo. Our data suggest that CapSCs act as common progenitors, at least in peripheral skeletal muscle tissues that can differentiate into vascular cells depending on their microenvironment, although the mechanism of their differentiation regulation has not been elucidated.

The large Eph receptor families and their ephrin ligands transduce signals in a cell-cell interaction-dependent fashion and play critical roles in a variety of processes during embryonic development, adult pathophysiological actions such as angiogenesis, neurogenesis, and tumor growth.[40] Eph-ephrin signaling regulates the angiogenic remodeling of blood vessels and lymphatic vessels and plays roles in differentiation and assembly of ECs as well as PCs and VSMCs, and interacts with EphB4/ephrinB2 to establish arterial-venous EC specification.[40,41] Ephrin B2 is required for PC and EC assembly into cord like structures.[42] Some of Eph-ephrin are also regulators of stem and progenitor cell actions both during development and in adulthood.[43] Neural progenitor cells and neuroblasts in the subventricular zone express ephirnA2, whereas quiescent ependymal cells and stem cells express EphA7. EphA7 induces ephirnA2 reverse signaling, negatively regulating neural progenitor cell proliferation.[44] Recently, it was reported that EphA7 is prominently expressed during reprogramming of somatic cells to a pluripotent state, induces pluripotent stem cells (iPS), and plays a crucial role in reprogramming through inducing extracellular signal regulated kinase (ERK) activity reduction.[45] Although CapSCs expressed several kinds of Eph and ephrin family members including EphA1, 2, 3, 7, B2, 3, and 4; and ephrin A4, B1, and 2, EphA7 was mostly selectively expressed in CapSCs compared with ctPCs (Table S3). It is presently unclear whether EphA7 contributes to critical functions of CapSCs, like their multipotency and regenerative effects. CapSCs located at perivascular areas, may associate with other cells including ECs through EphA7-ephrin to regulate their functions. Alternatively, multiple Eph receptors and ephrins are often coexpressed in the same cells where they can be activated by the same ephrins and likely function in concert. Future studies will aim to understand how EphA7 signals in CapSCs and how CapSCs association with other cells could be essential for the role of EphA7 not only for isolation of CapSCs, but in their cellular function.

In conclusion, we have identified EphA7 as a novel marker for multipotent PCs, which we have termed CapSCs. These cells are found in microvessels of peripheral tissues and have unique characteristics that include the ability to form capillaries by themselves and cross-germ layer plasticity, that is, differentiation into mesenchymal and neuronal cells. CapSCs could retain their regenerative capability in peripheral ischemic mouse models. Thus, it is expected that CapSCs could act as fundamental multipotent cells that contribute to the angiogenesis/regeneration and maintenance of tissues in multicellular organs. These cells might be attractive targets for regenerative therapeutic approaches.

ACKNOWLEDGMENTS

The authors thank S. Yoshida (Asahikawa Medical University) and Y. Bando (Akita University) for helpful discussion. The authors thank S. Takahashi, K. Kanno, Y. Horikawa, and M. Kudo for laboratory assistance; and M. Kusakabe and A. Oda for assistance with FACS experiments. The authors also thank N. Ogawa for technology transfer support. This work was supported by Grant-in-Aid for Scientific Research (B) (17H04170 to J.K.), challenging Exploratory Research (17K19368 to J.K.), and Grant-in-Aid for Young Scientists (A) (17K15980 to M.K. and 18K16379 to Y.Y.) commissioned by the Ministry of Education, Culture, Sports, Science and Technology of Japan. The present study was supported in part by Daiichi Sankyo Co. Ltd., Asbio Pharmer Co. Ltd., and OideCapiSEA, Inc.

CONFLICT OF INTEREST

The authors declare no potential conflict of interest.

AUTHOR CONTRIBUTIONS

Y.Y. and M.K.: concept and design, collection and/or assembly of data, data analysis, and interpretation; K.K., K.H., T.H, Y.T., N.T., A.K., T.A., K.M., and N.N.: collection and or assembly of data, data analysis and interpretation; N.A. and N.H.: data analysis and interpretation; J.K.: conception and design, data interpretation, manuscript writing, final approval manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Jun-ichi Kawabe https://orcid.org/0000-0002-4837-6598

REFERENCES

1. Diaz-Flores L, Gutierrez R, Madrid JF, et al. Pericytes. Morphofunction, interactions and pathology in a quiescent and activated mesenchymal cell niche. *Histol Histopathol*. 2009;24:909-969.
2. Armulik A, Genove G, Betsholtz C. Pericytes: developmental, physiological, and pathological perspectives, problems, and promises. *Dev Cell*. 2011;21:193-215.
3. Crisan M, Yap S, Casteilla L, et al. A perivascular origin for mesenchymal stem cells in multiple human organs. *Cell Stem Cell*. 2008;3:301-313.
4. Feng J, Mantesso A, De Bari C, et al. Dual origin of mesenchymal stem cells contributing to organ growth and repair. *Proc Natl Acad Sci USA*. 2011;108:6503-6508.
5. Tang W, Zeve D, Suh JM, et al. White fat progenitor cells reside in the adipose vasculature. *Science*. 2008;322:583-586.
6. Uezumi A, Fukada S, Yamamoto N, et al. Mesenchymal progenitors distinct from satellite cells contribute to ectopic fat cell formation in skeletal muscle. *Nat Cell Biol*. 2010;12:143-152.
7. Olson LE, Soriano P. PDGFR beta signaling regulates mural cell plasticity and inhibits fat development. *Dev Cell*. 2011;20:815-826.
8. Dellavalle A, Sampaolesi M, Tonlorenzi R, et al. Pericytes of human skeletal muscle are myogenic precursors distinct from satellite cells. *Nat Cell Biol*. 2007;9:255-267.
9. Dore-Duffy P, Katychev A, Wang X, et al. CNS microvascular pericytes exhibit multipotential stem cell activity. *J Cereb Blood Flow Metab*. 2006;26:613-624.
10. Nakagomi T, Kubo S, Nakano-Doi A, et al. Brain vascular pericytes following ischemia have multipotential stem cell activity to differentiate into neural and vascular lineage cells. *Stem Cells*. 2015;33:1962-1974.
11. Yamamoto S, Muramatsu M, Azuma E, et al. A subset of cerebrovascular pericytes originates from mature macrophages in the very early phase of vascular development in CNS. *Sci Rep*. 2017;7:3855.
12. Cathery W, Faulkner A, Maselli D, et al. Concise review: the regenerative journey of pericytes toward clinical translation. *Stem Cells*. 2018;36:1295-1310.
13. Hosaka K, Yang Y, Seki T, et al. Pericyte-fibroblast transition promotes tumor growth and metastasis. *Proc Natl Acad Sci USA*. 2016;113:E5618-E5627.
14. Corselli M, Chen CW, Crisan M, et al. Perivascular ancestors of adult multipotent stem cells. *Arterioscler Thromb Vasc Biol*. 2010;30:1104-1109.
15. Birbrair A, Zhang T, Wang ZM, et al. Type-2 pericytes participate in normal and tumoral angiogenesis. *Am J Physiol Cell Physiol*. 2014;307:C25-C38.
16. Kabara M, Kawabe J, Matsuki M, et al. Immortalized multipotent pericytes derived from the vasa vasorum in the injured vasculature. A cellular tool for studies of vascular remodeling and regeneration. *Lab Invest*. 2014;94:1340-1354.
17. Minoshima A, Kabara M, Matsuki M, et al. Pericyte-specific ninjurin1 deletion attenuates vessel maturation and blood flow recovery in hind limb ischemia. *Arterioscler Thromb Vasc Biol*. 2018;38:2358-2370.
18. Bunnell BA, Flaat M, Gagliardi C, et al. Adipose-derived stem cells: isolation, expansion and differentiation. *Methods*. 2008;45:115-120.
19. Aburakawa Y, Kawabe J, Okada M, et al. Prostacyclin stimulated integrin-dependent angiogenic effects of endothelial progenitor cells and mediated potent circulation recovery in ischemic hind limb model. *Circ J*. 2013;77:1053-1062.
20. Susaki EA, Tainaka K, Perrin D, et al. Whole-brain imaging with single-cell resolution using chemical cocktails and computational analysis. *Cell*. 2014;157:726-739.
21. Crisan M, Corselli M, Chen CW, et al. Multilineage stem cells in the adult: a perivascular legacy? *Organogenesis*. 2011;7:101-104.
22. Iso T, Maeda K, Hanaoka H, et al. Capillary endothelial fatty acid binding proteins 4 and 5 play a critical role in fatty acid uptake in heart and skeletal muscle. *Arterioscler Thromb Vasc Biol*. 2013;33:2549-2557.
23. Dominici M, Le Blanc K, Mueller I, et al. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy*. 2006;8:315-317.
24. Staton CA, Reed MW, Brown NJ. A critical analysis of current in vitro and in vivo angiogenesis assays. *Int J Exp Pathol*. 2009;90:195-221.
25. Sacharidou A, Stratman AN, Davis GE. Molecular mechanisms controlling vascular lumen formation in three-dimensional extracellular matrices. *Cells Tissues Organs*. 2012;195:122-143.
26. Matsuki M, Kabara M, Saito Y, et al. Ninjurin1 is a novel factor to regulate angiogenesis through the function of pericytes. *Circ J*. 2015;79:1363-1371.
27. Montiel-Eulefi E, Nery AA, Rodrigues LC, et al. Neural differentiation of rat aorta pericyte cells. *Cytometry A*. 2012;81:65-71.
28. Birbrair A, Zhang T, Wang ZM, et al. Skeletal muscle neural progenitor cells exhibit properties of NG2-glia. *Exp Cell Res*. 2013;319:45-63.
29. Etchevers HC, Vincent C, Le Douarin NM, et al. The cephalic neural crest provides pericytes and smooth muscle cells to all blood vessels of the face and forebrain. *Development*. 2001;128:1059-1068.
30. Morikawa S, Mabuchi Y, Niibe K, et al. Development of mesenchymal stem cells partially originate from the neural crest. *Biochem Biophys Res Commun*. 2009;379:1114-1119.
31. Gaengel K, Genove G, Armulik A, et al. Endothelial-mural cell signaling in vascular development and angiogenesis. *Arterioscler Thromb Vasc Biol*. 2009;29:630-638.
32. Houlihan DD, Mabuchi Y, Morikawa S, et al. Isolation of mouse mesenchymal stem cells on the basis of expression of Sca-1 and PDGFR-alpha. *Nat Protoc*. 2012;7:2103-2111.
33. Birbrair A, Zhang T, Wang ZM, et al. Skeletal muscle pericyte subtypes differ in their differentiation potential. *Stem Cell Res*. 2013;10:67-84.
34. Birbrair A, Zhang T, Wang ZM, et al. Pericytes: multitasking cells in the regeneration of injured, diseased, and aged skeletal muscle. *Front Aging Neurosci*. 2014;6:245.
35. Maeda K, Enomoto A, Hara A, et al. Identification of meflin as a potential marker for mesenchymal stromal cells. *Sci Rep*. 2016;6:22288.
36. Zampetaki A, Kirton JP, Xu Q. Vascular repair by endothelial progenitor cells. *Cardiovasc Res*. 2008;78:413-421.
37. Torsney E, Xu Q. Resident vascular progenitor cells. *J Mol Cell Cardiol*. 2011;50:304-311.

38. Kawabe J, Hasebe N. Role of the vasa vasorum and vascular resident stem cells in atherosclerosis. *Biomed Res Int*. 2014;2014:1-8.
39. Wakabayashi T, Naito H, Suehiro JI, et al. CD157 marks tissue-resident endothelial stem cells with homeostatic and regenerative properties. *Cell Stem Cell*. 2018;22:384-397.e386.
40. Pasquale EB. Eph–ephrin bidirectional signaling in physiology and disease. *Cell*. 2008;133:38-52.
41. Hellstrom M, Phng LK, Hofmann JJ, et al. Dll4 signalling through Notch1 regulates formation of tip cells during angiogenesis. *Nature*. 2007;445:776-780.
42. Salvucci O, Maric D, Economopoulou M, et al. EphrinB reverse signaling contributes to endothelial and mural cell assembly into vascular structures. *Blood*. 2009;114:1707-1716.
43. Genander M, Frisen J. Ephrins and Eph receptors in stem cells and cancer. *Curr Opin Cell Biol*. 2010;22:611-616.
44. Holmberg J, Armulik A, Senti KA, et al. Ephrin-A2 reverse signaling negatively regulates neural progenitor proliferation and neurogenesis. *Genes Dev*. 2005;19:462-471.
45. Lee J, Nakajima-Koyama M, Sone M, et al. Secreted ephrin receptor A7 promotes somatic cell reprogramming by inducing ERK activity reduction. *Stem Cell Rep*. 2015;5:480-489.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Yoshida Y, Kabara M, Kano K, et al. Capillary-resident EphA7^{+} pericytes are multipotent cells with anti-ischemic effects through capillary formation. *STEM CELLS Transl Med*. 2020;9:120–130. https://doi.org/10.1002/sctm.19-0148