Original Article

Umbilical artery tissue contains p75 neurotrophin receptor-positive pericyte-like cells that possess neurosphere formation capacity and neurogenic differentiation potential

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ABSTRACT

Introduction: The p75 neurotrophin receptor (p75NTR) is known as an efficient marker for the prospective isolation of mesenchymal stem cells (MSCs) and neural crest-derived stem cells (NCSCs). To date, there is quite limited information concerning p75NTR-expressing cells in umbilical cord (UC), although UC is known as a rich source of MSCs. We show for the first time the localization, phenotype, and functional properties of p75NTR$^+$ cells in UC.
Methods: Human UC tissue sections were subjected to immunohistochemistry for MSC markers including p75NTR. Enzymatically isolated umbilical artery (UA) cells containing p75NTR$^+$ cells were assessed for immunophenotype, clonogenic capacity, and differentiation potential. To identify the presence of neural crest-derived cells in the UA, P0-Cre/Floxed-EGFP reporter mouse embryos were used, and immunohistochemical analysis of UC tissue was performed.
Results: Immunohistochemical analysis revealed that p75NTR$^+$ cells were specifically localized to the subendothelial area of the UA and umbilical vein. The p75NTR$^+$ cells co-expressed PDGFRβ, CD90, CD146, and NG2, phenotypic markers of MSCs and pericytes. Isolated UA cells possessed the potential to form neurospheres that further differentiated into neuronal and glial cell lineages. Genetic lineage tracing analysis showed that EGFP$^+$ neural crest-derived cells were detected in the subendothelial area of UA with p75NTR immunoreactivity.
Conclusions: These results show that UA tissue harbors p75NTR$^+$ pericyte-like cells in the subendothelial area that have the capacity to form neurospheres and the potential for neurogenic differentiation. The lineage tracing data suggests the p75NTR$^+$ cells are putatively derived from the neural crest.

1. Introduction

Mesenchymal stem cells (MSCs) are multipotent stromal cells originally identified in bone marrow. These cells can be isolated and expanded due to their property of plastic adherence and differentiate into osteoblasts, adipocytes, and chondrocytes under the proper culture conditions. The differentiation potential of MSCs is not only restricted to mesodermal lineage but also ectoderm lineages such as neurons and glial cells. Of note, MSCs partially originate from the neuroepithelium and neural crest and therefore can differentiate into neural lineage cells [1–3].

Umbilical cord (UC) is a birth-associated tissue that connects the developing embryo with the placenta. The UC contains two umbilical arteries (UAs), an umbilical vein (UV), surrounding connective tissue called Wharton's jelly (WJ), and outer amnion-derived epithelium. It is well documented that UC is a rich source of MSCs [4,5]. Because UC cells can be isolated by a non-invasive procedure, there are no ethical problems with obtaining biomaterial, and their lack of tumorigenicity makes the cells an attractive cell source for cell-based therapy [6]. Although several studies showed that UC-derived cultured MSCs formed neurospheres and differentiated into neurogenic cells under appropriate induced conditions [7,8], it is still unclear whether UC tissue contains neural crest-like progenitor cells *in situ*.

The p75 neurotrophin receptor (p75NTR, the low affinity nerve growth factor receptor and CD271) is a member of the tumor necrosis factor superfamily of transmembrane glycoprotein receptors, which play important roles in regulating axon growth and neuron/oligodendrocyte survival during nervous system development and repair [9–11]. In human bone marrow, p75NTR is an efficient MSC cell surface marker that is not expressed in hematopoietic lineage cells [12,13]. Moreover, p75NTR is known as a good marker for the prospective isolation of mammalian neural crest-derived stem cells (NCSCs) [14]. Nevertheless, there is quite limited information concerning p75NTR-expressing cells in UC tissue.

In this study, we examined the localization, phenotype, and functional properties of p75NTR$^+$ cells in the UC. As a result, we showed the existence of p75NTR$^+$ cells that were localized in the subendothelial area of umbilical vessels in human UC tissue. We also identified neural crest-derived cells in the identical area of UA in the P0-Cre reporter mouse.

2. Materials and methods

2.1. Isolation of human UCs

Human UCs (n = 18) were obtained from newborns of 36—39 weeks gestation delivered from healthy pregnant women planning to undergo cesarean sections at Nihon University Itabashi Hospital, Tokyo, Japan and Yokosuka City Hospital, Yokosuka, Japan. Informed consent was obtained from the pregnant women prior to collecting their UC tissues. All experiments using human UCs were approved by the Ethics Committee of Nihon University Itabashi Hospital and Yokosuka City Hospital and were conducted in accordance with the principles of the Declaration of Helsinki. Collected UC tissues were immediately stored in phosphate-buffered saline (PBS) at room temperature and were used for experiments within 24 h of collection.

2.2. Animals

P0-Cre/Floxed-enhanced green fluorescent protein (EGFP) double transgenic mice were created as described previously [3]. In brief, transgenic mice expressing the Cre enzyme induced by the myelin protein zero (P0) promoter [15] were crossed with the CAG-CAT-EGFP transgenic line [16]. In P0-Cre/Floxed-EGFP double transgenic mice, neural crest-derived cells were identified by evaluating the expression of EGFP after P0-Cre-mediated DNA recombination. To eliminate pigmentation in the embryonic tissue of the double transgenic lines, the male P0-Cre/Floxed-EGFP double transgenic mice were crossed with female Crlj:CD1 (ICR) mice (Japan Charles River, Tokyo, Japan). The mid-day of identifying a vaginal plug was considered as E0.5. At E15.5, the P0-Cre/Floxed EGFP mice embryos were identified by GFP fluorescence in their heads under ultraviolet light exposure and used for experiments. Embryos negative for GFP were used as a negative control. The embryos with UCs and placentas were removed, washed with PBS, and placed into 1 mL PBS containing 2 mM EDTA. The UCs were cut at a position close to the embryos, and fetal cord blood was allowed to flow freely into the PBS. Then, the UC tissue fragments were cut from placenta, fixed with 4% paraformaldehyde (Muto Pure Chemicals, Tokyo, Japan), and used for immunohistochemistry. All experimental animal procedures described in this study were approved by the Ethics Committee for Animal Experiments of Nihon University School of Medicine and were in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health.

2.3. UA cells isolation

Human UCs cut into lengths of 1—1.5 cm were washed in sterile PBS several times to remove red blood cells. Two UAs were longitudinally dissected from the UC and were mechanically separated by forceps. UA samples were minced into small pieces and incubated with a mixed enzyme solution containing 0.04% collagenase type I (Sigma-Aldrich, St. Louis, MO), 0.04% collagenase type II (Sigma-Aldrich), and 1.6 ng/mL dispase (Roche Diagnostics, Mannheim, Germany) at 37 °C for 1 h. Digested cells were filtrated through a 250-μm filter and washed with Dulbecco's modified Eagle medium (DMEM, Life Technologies, Carlsbad, CA) containing 2% fetal bovine serum (FBS, SAFC Biosciences, St. Louis, MO). The cells were suspended in culture medium and used for experiments.

2.4. Immunofluorescence

Human and mouse UC samples were fixed with 4% paraformaldehyde at 4 °C for 24 h, embedded in Tissue-Tek OCT compound (Sakura Finetek, Tokyo, Japan), snap-frozen in liquid nitrogen, and cut into 10-μm-thick sections. In some experiments, UC samples were fixed with 10% buffered formalin (Wako, Osaka, Japan) and embedded in paraffin. Paraffin-embedded sample sections (4 μm thick) were deparaffinized and rehydrated. The sections were activated by Target Retrieval Solution (DakoCytomation, Glostrup, Denmark) and then incubated in 10% goat serum (Vector Laboratories, Burlingame, CA) for 20 min to block endogenous antigens. For human UC tissue, samples were incubated overnight at 4 °C with primary antibodies to anti-human p75NTR (1:100; Sigma-Aldrich), anti-human CD34 (1:100; Leica Microsystems, Wetzlar, Germany), anti-human CD90 (1:200; AbD Serotec, Oxford, UK), anti-human CD105 (1:100; Proteintech Group, Rosemont, IL), anti-human CD146 (1:100; Miltenyi Biotec, Bergisch Gladbach, Germany), anti-human platelet-derived growth factor receptor β (PDGFRβ, 1:100, Epitomics, Burlingame, CA), anti-human von Willebrand factor (vWF, 1:5000; DakoCytomation), anti-human neuron-glial antigen 2 (NG2) (1:100; Millipore, Temecula, CA), and anti-human α-smooth muscle actin (ASMA, 1:200; DakoCytomation). For mouse UC tissue, samples were incubated with anti-mouse p75NTR (1:5000; Millipore), anti-mouse CD31 (1:100; BD Biosciences, San Jose, CA), and anti-GFP (1:1000; Medical & Biological Laboratories, Nagoya, Japan). The slides were washed with
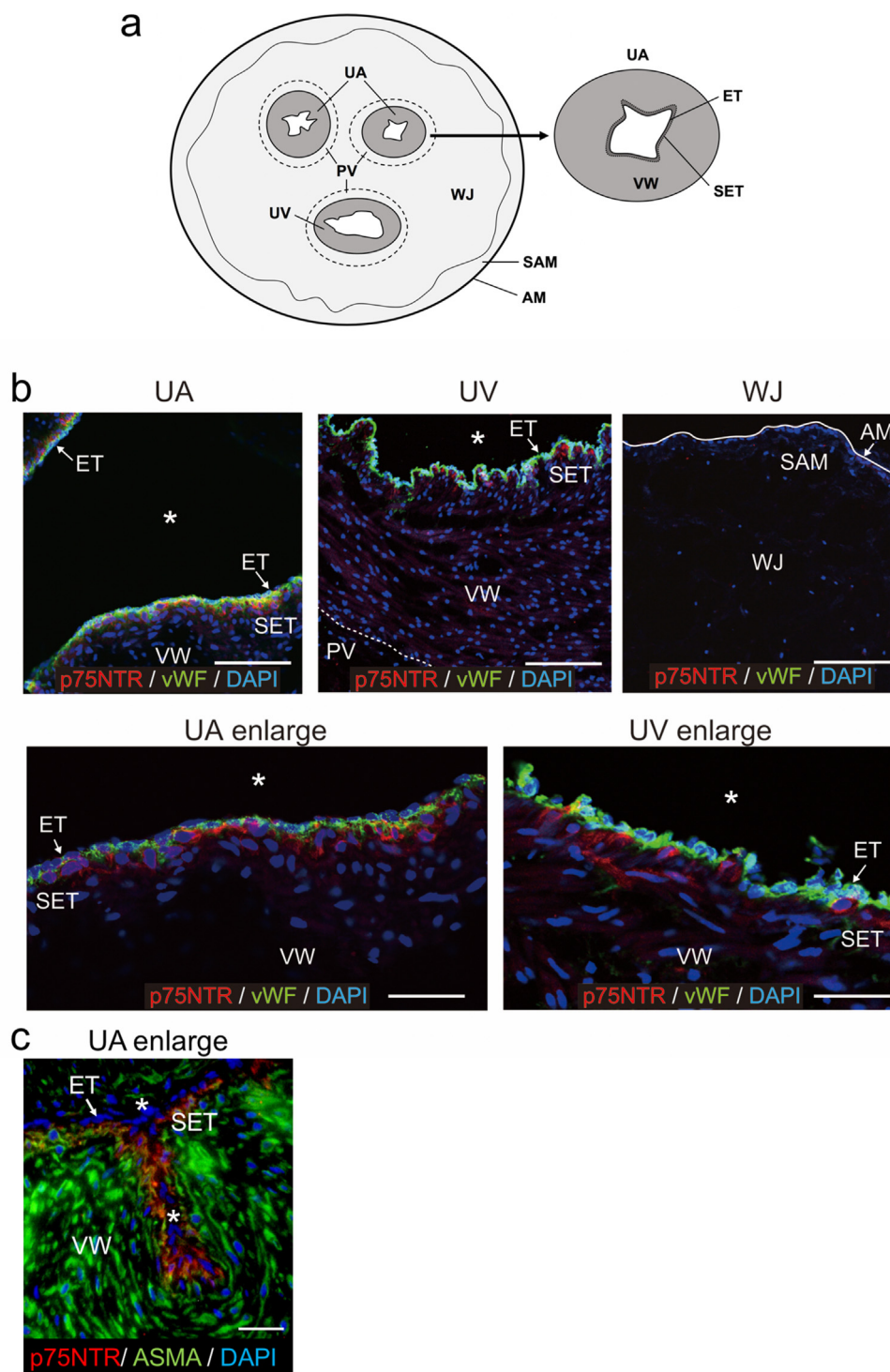
Fig. 1. Localization of p75NTR⁺ cells in human umbilical cords. (a) Schematic illustration of human umbilical cord compartments. (b, c) Photomicrograph of umbilical cord samples stained for p75NTR (red). (b) Vascular endothelial cells and nuclei were stained for von Willebrand factor (vWF, green), and DAPI (blue), respectively. (c) Vessel wall smooth muscle cells and nuclei were stained for α-smooth muscle actin (ASMA, green), and DAPI (blue), respectively. Asterisks indicate the vascular lumen. Dotted line indicates the border of the vascular wall (VW) and perivascular area (PV) of Wharton's jelly (WJ). Scale bar: 100 μm in UA, UV, and WJ, 20 μm in UA enlarge and UV enlarge. Abbreviations: UA, umbilical artery; UV, umbilical vein; ET, endothelium; SET, subendothelium; SAM, subamnion; AM, amnion.

PBS and incubated for 1 h at room temperature with species-specific secondary antibodies of Alexa Fluor 488 or Alexa Fluor 594 (1:500; Invitrogen, Carlsbad, CA). Nuclear counterstaining was performed with 5 μg/mL 4',6-diamino-2-phenylindole (DAPI, Invitrogen). For enzymatically digested human UA cells, a 100-μL aliquot of 1×10^4/mL concentrated cell suspension in DMEM with 2% FBS was applied to single-well slides using a Cytospin3 (Thermo Fischer Scientific, Waltham, MA). The samples were fixed with 4% paraformaldehyde and stained with primary antibodies and secondary antibodies as described above. The sections were mounted

in Fluoromount-G (Southern Biotech, Birmingham, AL) and visualized under a Fluoview FV10i fluorescence confocal microscope (Olympus, Tokyo, Japan).

2.5. Cell culture

The generation of neurospheres from UA cells was performed as described previously [17] with slight modification. Briefly, UA cells of 1×10^4 cells/100 μL were plated into low-attachment plastic 96-well plates (Thermo Fisher Scientific) in neural stem cell medium composed of DMEM/F12 (Invitrogen), B-27 supplement without vitamin A (Invitrogen), 20 ng/mL epidermal growth factor (EGF, R&D Systems, Minneapolis, MN), 10 ng/mL fibroblast growth factor-2 (FGF-2, R&D Systems), and 10 ng/mL recombinant mouse leukemia inhibitory factor (Millipore) at 37 °C in 5% CO$_2$. Half the amount of medium was renewed twice a week for 7–10 days to generate spheres. The spheres were picked out and applied to single-well slides using Cytospin3 then fixed with 4% paraformaldehyde at 4 °C for 30 min. The samples were stained with primary antibodies of rabbit anti-human nestin (1:100; Sigma-Aldrich) and mouse anti-human p75NTR (1:100; Sigma-Aldrich). Secondary antibodies used were goat anti-rabbit IgG conjugated with Alexa Fluor 594 and goat anti-mouse IgG conjugated with Alexa Fluor 488 (Invitrogen). After staining nuclei with DAPI, the samples were examined with a Fluoview FV10i fluorescence confocal microscope.

A 5-ethynyl-2′-deoxyuridine (EdU) assay was performed using Click-iT EdU imaging kits (Invitrogen) according to the manufacturer's instruction. Briefly, 10 μM of EdU (Invitrogen) was added into the neurosphere culture 24 h before fixation, fixed with 4% paraformaldehyde for 15 min, then incubated with 0.5% Triton X-100 (Sigma-Aldrich) in Tris-buffered saline (TBS) at room temperature for 20 min. Then, the cells were incubated with Click-iT EdU reaction cocktail (Invitrogen) at room temperature for 30 min, and nuclear counterstaining was performed with 5 μg/mL Hoechst33342 (Sigma-Aldrich). Images of the stained cells were captured under the Fluoview FV10i fluorescence confocal microscope.

For plastic adherence culture to detect MSCs, UA cells (3×10^5 cells/well) were plated on 35-mm^2 plastic culture dish in DMEM supplemented with 10% FBS and were incubated at 37 °C in 5% CO$_2$. The cells were then fixed with 4% paraformaldehyde at 4 °C for 30 min and incubated overnight at 4 °C with primary antibodies of mouse anti-human p75NTR (1:100) and rabbit anti-human CD90 (1:100). After three washes with PBS, samples were incubated for 1 h at room temperature with the appropriate secondary antibody of goat anti-mouse IgG conjugated with Alexa Fluor 488 and goat anti-rabbit IgG conjugated with Alexa Fluor 594. Nuclear counterstaining was performed with DAPI. To evaluate colony-forming unit fibroblast (CFU-F), the UA cells were plated at a density of 100 cells/35-mm^2 dish in NH CFU-F medium (Miltenyi Biotec) for 14 days. Cell clusters of 50 or more cells were considered a colony, and the number of colonies was counted in quadruplicate dishes.

2.6. Neural cell differentiation

For neurogenic cell differentiation, the neurospheres were plated into low-attachment plastic 96-well dishes incubated at 37 °C for 24 h with alpha-modified minimum essential medium (α-MEM, Invitrogen) containing 1 mM β-mercaptoethanol (βME). The media were removed, washed with PBS (pH 7.4), and replaced with new media consisting of α-MEM, 10% FBS, and 35 ng/mL all-trans-retinoic acid (RA, Sigma-Aldrich) for 3

days. Neurospheres were washed with PBS and transferred to fibronectin-coated 8-well chamber slides (BD Biosciences) containing α-MEM, 10% FBS, 5 μM forskolin (FSK, Sigma-Aldrich), 20 ng/mL EGF, 10 ng/mL FGF-2, and 10 ng/mL human brain-derived neurotrophic factor (BDNF, Sigma-Aldrich) for 7 days. For glial cell differentiation, the neurospheres were plated into low-attachment plastic 96-well dishes incubated at 37 °C for 24 h with α-MEM containing 1 mM βME. The media were then removed, washed with PBS (pH 7.4), and replaced with new media of α-MEM containing 1 mM βME and 35 ng/mL RA for 3 days. Neurospheres were washed with PBS and transferred to fibronectin-coated 8-well chamber slides containing α-MEM, 10% FBS, 5 μM FSK, 10 ng/mL FGF-2, 5 ng/mL PDGF-BB (R&D Systems), and 200 ng/mL neuregulin 1-β1/heregulin 1β1 EGF domain (R&D Systems) for 7 days. Samples were then fixed with 4% paraformaldehyde at 4 °C for 30 min and permeabilized with 0.5% Triton X-100 in TBS at room temperature for 30 min followed by an incubation in blocking buffer (10% goat serum, 1% bovine serum albumin in TBS) at room temperature for 1 h. Samples were incubated overnight at 4 °C with the following primary antibodies: mouse anti-microtubule-associated protein 2 (MAP2, clone HM-2, 1:100; Sigma-Aldrich), mouse anti-neurofilament 200 (NF200, 1:50; Sigma-Aldrich), mouse anti-β III tubulin (Clone TU-20, 1:100; Abcam, Cambridge, UK), mouse anti-oligodendrocyte marker O4 (clone 04, 1:50, R&D Systems), and mouse anti-glial fibrillary acidic protein (GFAP, clone G-A-5, 1:200, Sigma-Aldrich). After three washes with PBS, samples were incubated for 1 h at room temperature with the appropriate secondary antibody of Alexa Fluor 594 or Alexa Flour 488 goat anti-mouse IgG (Invitrogen). Nuclear counterstaining was performed with DAPI. The samples were examined using a Fluoview FV10i fluorescence confocal microscope.

3. Results

3.1. Human umbilical vessels contain p75NTR$^+$ cells in the subendothelial area

We first examined whether p75NTR-expressing cells are present in the UC tissue. A schematic illustration of the compartments of UC tissue is shown in Fig. 1a. Immunohistochemical analysis revealed that p75NTR immunoreactivity was specifically observed in the subendothelial area of the UA and UV (Fig. 1b, see SET in UA, UV). We found that the p75NTR was expressed in stromal cells adjacent to vWF$^+$ endothelial cells but not in endothelial cells or vascular wall cells mainly consisting of ASMA$^+$ smooth muscle cells (Fig. 1b, UA, UV enlarge, and Fig. 1c). The number of p75NTR$^+$ cells in UV was relatively smaller than that in UA. The p75NTR$^+$ cells were not detected in other areas such as WJ including perivascular region, subamnion, and amnion (Fig. 1b, see PV, WJ, SAM, and AM). These findings indicate that p75NTR$^+$ cells were specifically localized in the subendothelial area of the umbilical vessels.

Because UC tissue is known to contain several different types of MSCs, we next determined *in situ* characterization of MSC markers expression in the UC tissue. Our data showed that expression of the MSC marker CD90 was observed throughout the vessel walls including the subendothelial area in both UA and UV (Fig. 2a, see VW in UA, UV, Enlarge). The expression in vessel wall smooth muscle cells were confirmed by immunostaining for ASMA (Fig. 2a, UA-VW). The expression was also detected in the perivascular region of WJ, but not in other areas of WJ and amnion (Fig. 2a, see PV in UA, UV, Enlarge). The other MSC marker CD105 was widely expressed in the UA and UV including endothelium, subendothelial area, and vascular wall (Fig. 2b, UA, UV, Enlarge, UA-VW). The

expression was also detected in WJ including the perivascular region and amnion (Fig. 2b, WJ). The pericyte marker CD146 was strongly expressed in the subendothelial area and vessel wall in both UA and UV and weakly expressed in the perivascular region of WJ (Fig. 2c). Expression of the other pericyte marker PDGFRβ was markedly observed in the subendothelial area of UA and UV, and in WJ including the perivascular region and subamnion (Fig. 2d). Similar results were obtained from five independent UC samples. The expression profile of these markers in each region is summarized in Table 1. These results indicated that MSC and pericyte marker-expressing cells are relatively rich in the subendothelial area of umbilical vessels and the perivascular region of WJ.

3.2. UA p75NTR$^+$ cells express MSC/pericyte marker proteins

We next examined the immunophenotype of p75NTR$^+$ cells localized in the subendothelial area of UA. Immunohistochemical analysis revealed that p75NTR$^+$ cells co-expressed certain MSC markers such as CD90 and pericyte markers CD146, PDGFRβ, and NG2 (Fig. 3a—d). Although the expression of these 4 markers was also observed in p75NTR$^-$ cells except for endothelial cells in UA, the fluorescence intensity of PDGFRβ and CD146 in the p75NTR$^+$ cells was relatively higher than that in the p75NTR$^-$ cells (Fig. 3a, c, arrows).

To confirm the expression of these markers in the p75NTR$^+$ cells, UA tissue fragments were enzymatically digested, and isolated cells were analyzed by immunocytochemistry. Two types of p75NTR$^+$ cells were detected: p75NTR strongly positive cells with round-shaped morphology (Fig. 4a, arrows) and p75NTR weakly positive cells with spindle-shaped morphology (Fig. 4a, arrowheads). PDGFRβ, CD90, CD146, and NG2 were expressed in both types of p75NTR$^+$ cells (Fig. 4a—d). However, the p75NTR$^+$ cells did not express endothelial markers vWF and CD34 (Fig. 4e). Taken together, these data suggested that UA p75NTR$^+$ cells have a phenotypic feature of MSCs and pericytes.
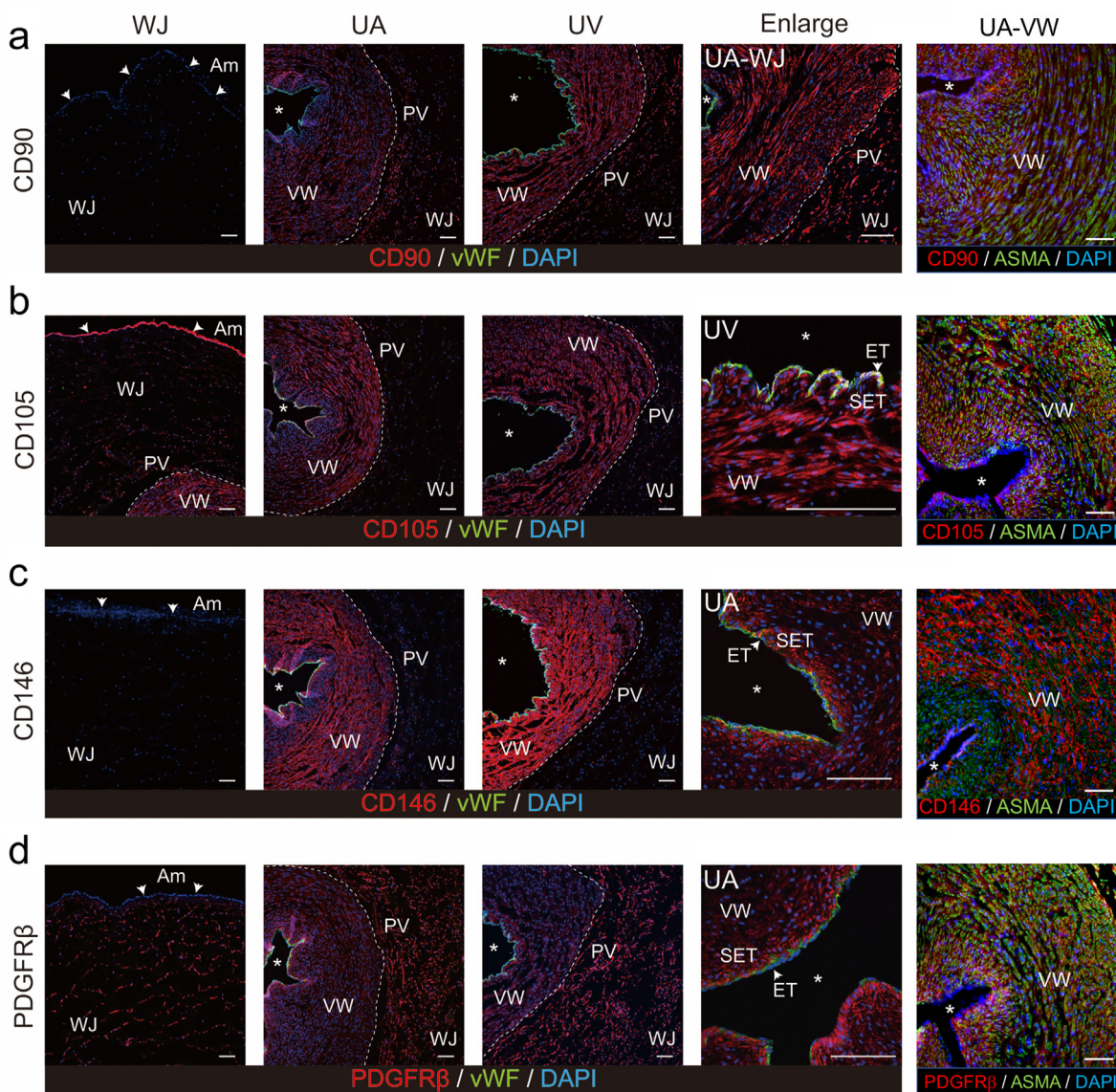

Fig. 2. Localization of mesenchymal stromal cell markers in umbilical cords. Umbilical cord samples were stained for CD90 (a), CD105 (b), CD146 (c), and PDGFRβ (d) (red). Vascular endothelial cells, vessel wall smooth muscle cells and nuclei were stained for von Willebrand factor (vWF, green), α-smooth muscle actin (ASMA, green), and DAPI (blue), respectively. Asterisks indicate the vascular lumen. Dotted line indicates the border of the vascular wall (VW) and perivascular area (PV) of Wharton's jelly (WJ). Other abbreviations as in Fig. 1. Scale bar: 100 μm.

Table 1
The expression of MSC markers and their localization in umbilical cord tissue by immunohistochemistry.

	UA			UV			WJ		Am
	ET	SET	VW	ET	SET	VW	PV	WJ	
p75NTR	ND	+	ND	ND	±	ND	ND	ND	ND
CD90	ND	+	++	ND	+	++	+	ND	ND
CD105	+	+	+	+	+	+	++	+	+
CD146	ND	++	++	ND	++	++	+	ND	ND
PDGFRβ	ND	++	+	ND	++	+	+++	+++	ND

UA: umbilical artery, UV: umbilical vein, WJ: Wharton's jelly, ET: endothelium, SET: subendothelium, VW: vessel wall, PV: perivascular region, Am: amnion, ND: not detected.

3.3. Isolated UA cells have neurosphere-forming capacity and neuronal differentiation potential

We next examined the clonogenic potential of the p75NTR+ cells using two different culture conditions (MSC culture condition and neurosphere culture condition). We had tried to perform these experiments using isolated p75NTR+ cells from the enzymatically digested UA cell fraction by flow sorting. However, we failed to expand them, probably due to the lack of paracrine factors from the other types of cells. Therefore, we performed these experiments using the bulk of the enzymatically digested UA cells containing p75NTR+ cells. In the MSC culture condition, we confirmed that the UA cells were highly adhesive to plastic and showed proliferative ability with fibroblast-like morphology (Fig. 5a). CFU-F colonies were observed when the UA cells were cultured at the low initial cell density of 100 cells/35-mm^2 dish (Fig. 5b). The frequency of CFU-F was 3.25 ± 1.3%. Immunocytochemical analysis revealed that the cultured MSCs strongly expressed CD90 but not p75NTR (Fig. 5c).

In the neurosphere culture condition, we observed that the UA cells formed floating colonies that were morphologically similar to neurospheres by 24 h after plating. Most of the spheres grew larger in size during the culture and reached a diameter of 100 μm until 7–8 days after plating (Fig. 5d). When the spheres were mechanically dissociated into single-cell suspensions and reseeded in the neurosphere culture medium, secondary neurospheres could be obtained although the sphere size became smaller. The EdU incorporation assay revealed that EdU-positive cells were detected in nuclei of the spheres (Fig. 5e), indicating the presence of dividing cells. Immunocytochemical analysis revealed that most of the cells in the spheres expressed p75NTR (Fig. 5f) and the neuronal
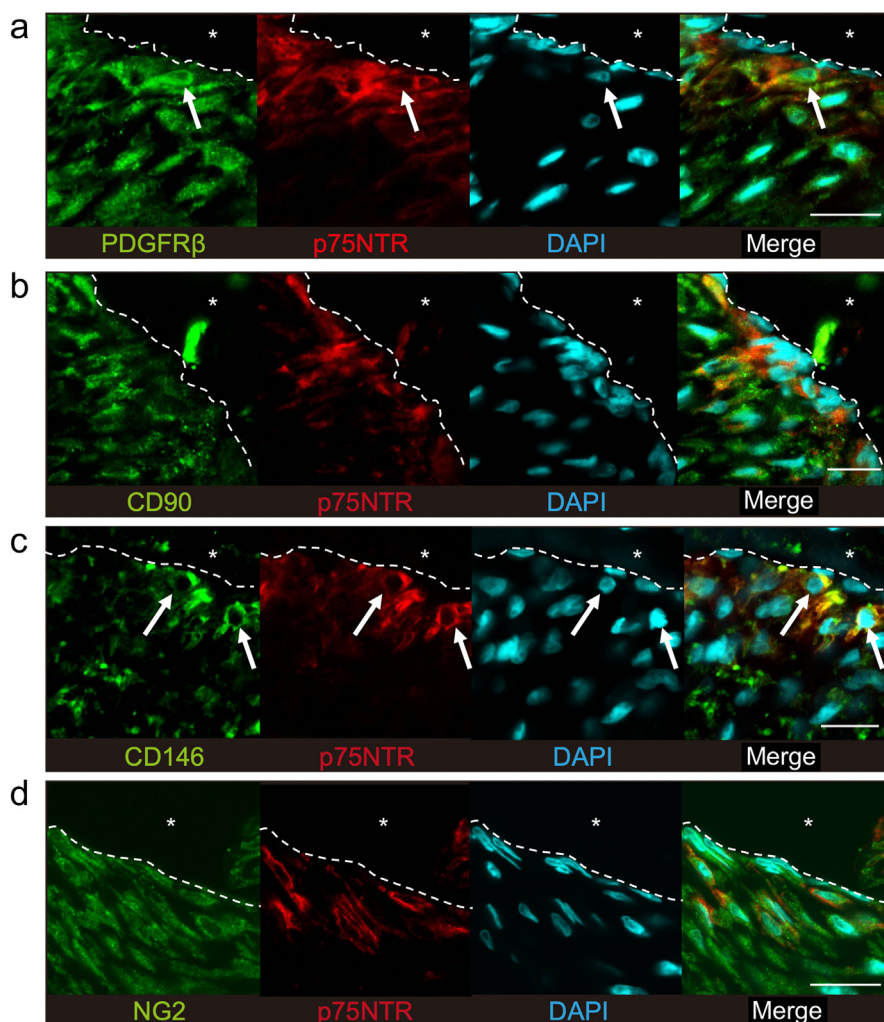

Fig. 3. Immunohistochemical analysis of subendothelium in umbilical artery. Umbilical cord samples were co-stained for p75NTR with PDGFRβ (a), CD90 (b), CD146 (c), and NG2 (d). Nuclei were stained for DAPI. Arrows indicate p75NTR+ cells co-expressing PDGFRβ and CD146 at high intensity. Asterisks indicate the vascular lumen. Scale bar: 20 μm.
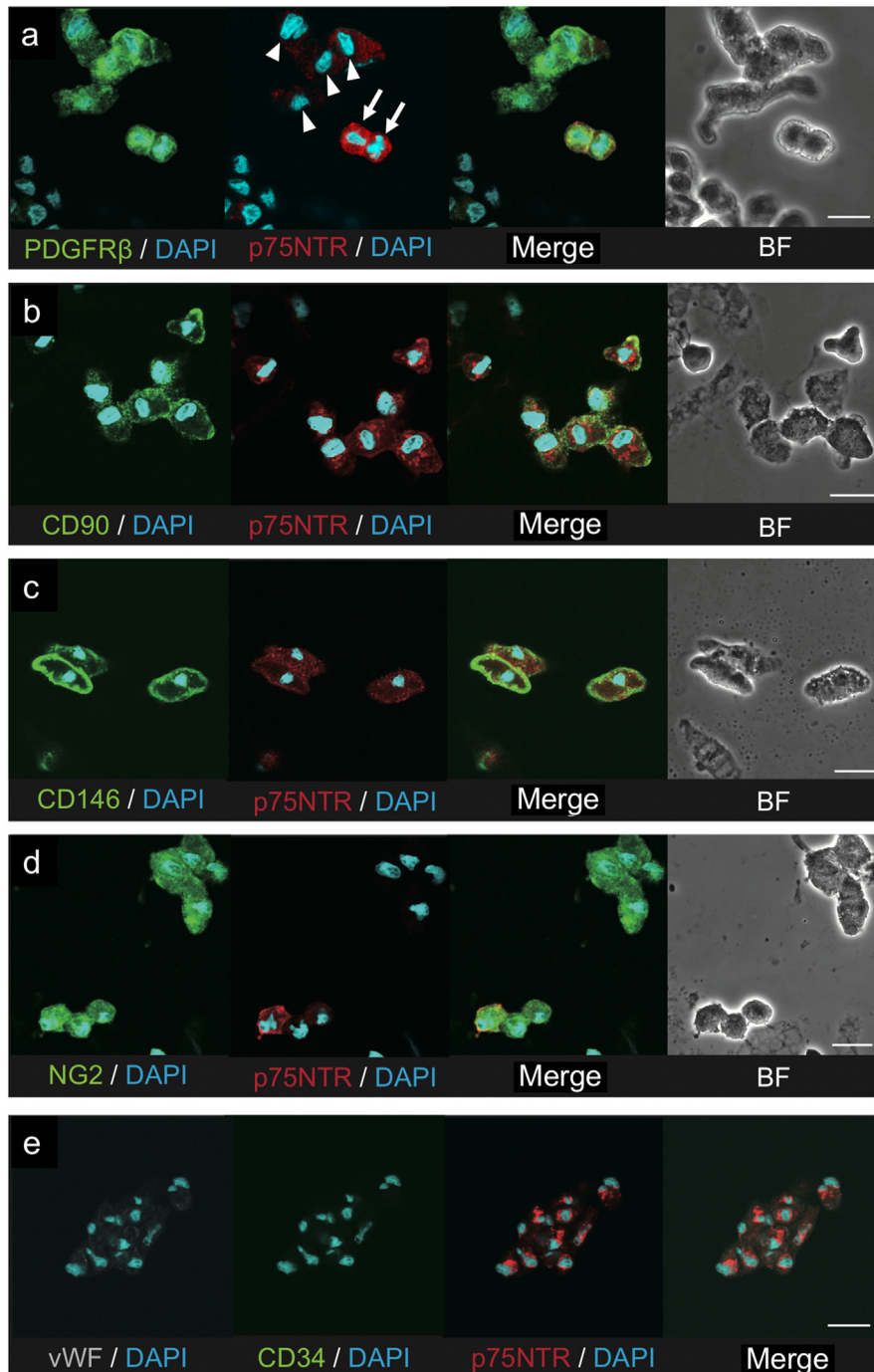
Fig. 4. Immunocytochemical analysis of umbilical artery-derived cells. Umbilical arteries were mechanically pulled out from umbilical cords and then enzymatically digested to isolate cells. Cytospin preparation of isolated cells was co-stained for p75NTR with PDGFRβ (a), CD90 (b), CD146 (c), NG2 (d), vWF and CD34 (e). Nuclei were stained for DAPI. Arrows and arrowheads show cells strongly positive and weakly positive for p75NTR, respectively. Scale bar: 20 μm.

progenitor cell marker nestin (Fig. 5g). These data suggested that the UA cells contain a progenitor/stem cell population that could form neurospheres with self-renewal capacity.

Next, we evaluated the neurogenic differentiation potential of UA cell-derived spheres. In the neurogenic differentiation culture, the sphere cells attached and converted to a neuron-like morphology with long thin processes. The neuronal differentiation was confirmed by positive immunostaining for NF200, βIII-tubulin, and MAP2, which are markers for neuronal cells (Fig. 6a). In the glial cell differentiation culture, the sphere cells attached and converted to a spindle-shaped morphology that expressed GFAP and O4, which are markers for glial cells (Fig. 6b). These findings indicated that UA cell-derived spheres possess the ability to differentiate into neuronal and glial cell lineages.
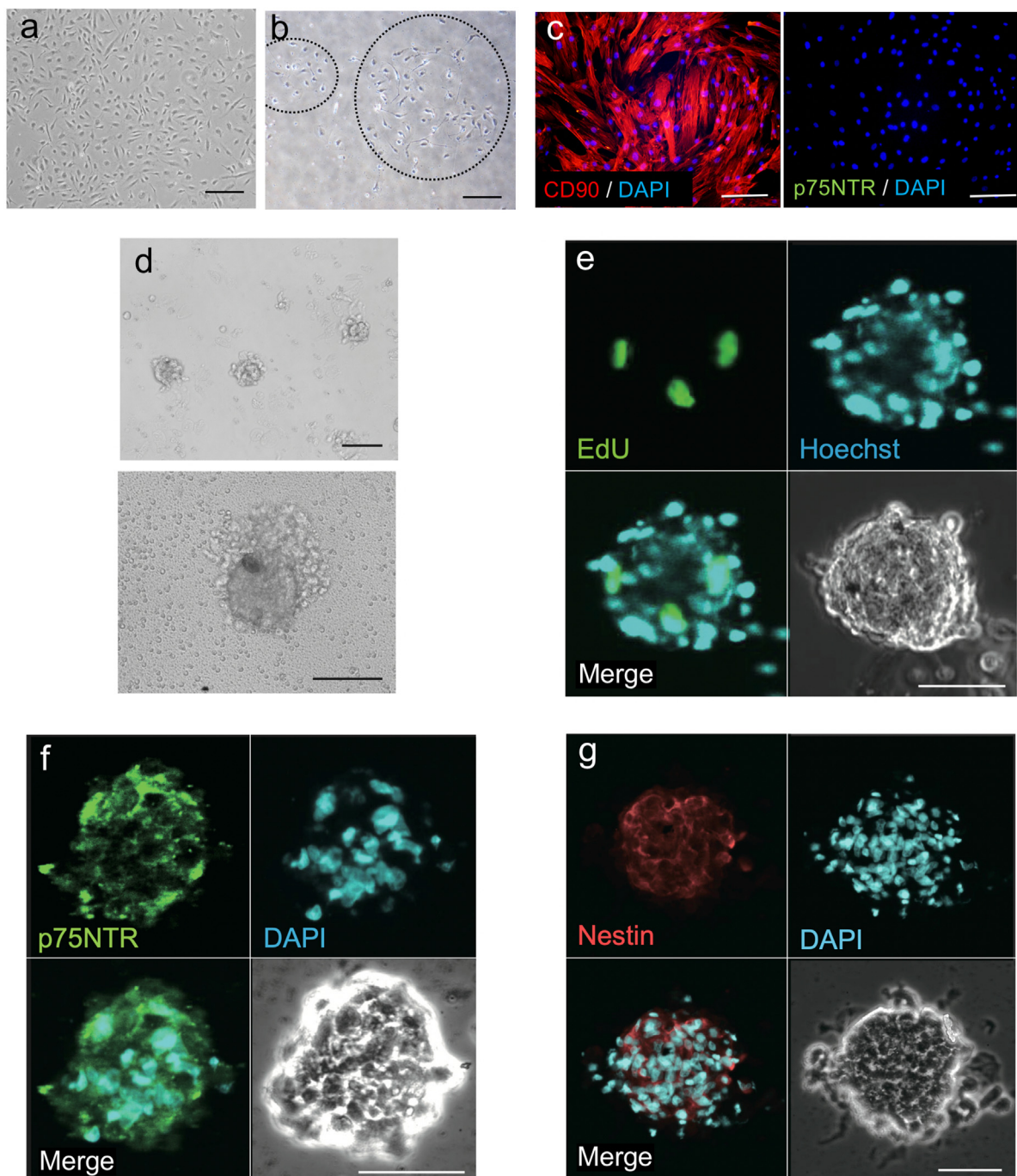
Fig. 5. Clonogenic potential of umbilical artery-derived cells. Cells enzymatically isolated from umbilical arteries were cultured in a mesenchymal stem cell (MSC) culture condition or in a neurosphere culture condition. (a) A representative image of cells cultured in the MSC culture condition at day 7. Scale bar: 100 μm. (b) Morphology of CFU-F colonies at day 14 of culture. Scale bar: 100 μm. (c) Immunostaining of cultured MSCs for CD90 and p75NTR. Nuclei were stained for DAPI. Scale bar: 50 μm. (d) Representative images of cell aggregates at day 7 (upper panel) and day 8 (lower panel) of neurosphere culture. Scale bar: 100 μm. (e) EdU incorporation in the cellular aggregates was visualized by Alexa Fluor 488 Azide. Nuclei were stained for Hoechst33342. Scale bar: 50 μm. (f, g) The cellular aggregates were stained for p75NTR (f) and nestin (g). Nuclei were stained for DAPI. Scale bar: 50 μm.

3.4. Identification of neural crest-derived cells in mouse UC tissue

To explore the hypothesis that UA p75NTR$^+$ cells are derived from the neural crest, we used P0-Cre/Floxed-EGFP mice whose neural crest-derived cells can be genetically labeled and performed immunohistochemical analysis of their extraembryonic tissue. We found that GFP-immunoreactive cells, which suggested neural

crest-derived cells, were observed in the subendothelial area of UA (Fig. 7a and b). The p75NTR$^+$ cells were also detected in the sub-endothelial area of UA but not in the other regions including in endothelial cells (Fig. 7c and d). The number of p75NTR$^+$ cells was much smaller than that of GFP$^+$ cells, although both types of cells localized in a similar position in serial sections. Double staining for GFP and p75NTR revealed that a part of GFP$^+$ cells co-expressed
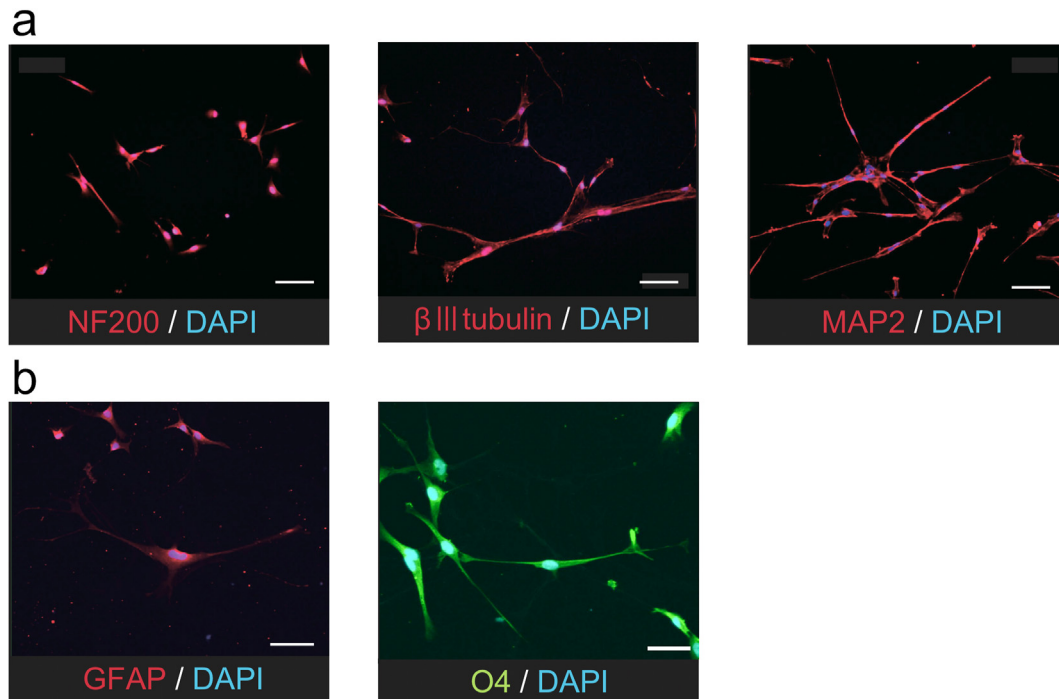
Fig. 6. Neurogenic differentiation of umbilical artery-derived cells. Cells enzymatically isolated from umbilical arteries were plated into low-attachment plastic 96-well plates and cultured in neural stem cell medium. After 7 days, formed spheres were picked up and cultured in neuronal or glial differentiation medium for 11 days. (a) Representative photomicrographs of cells stained for NF200, βIII tubulin, and MAP2 after neuronal induction. Nuclei were stained for DAPI. (b) Representative photomicrographs of cells stained for GFAP and O4 after glial induction. Scale bar: 100 μm.

p75NTR (Fig. 7e). These data suggested that neural crest-derived cells that express p75NTR are present in the UA subendothelial area in mice.

4. Discussion

In this study, we showed for the first time, to our knowledge, that human UA tissue contains p75NTR$^+$ cells that have the capacity for self-renewal and the potential for neurogenic differentiation. Our immunohistology data showed that p75NTR$^+$ cells are specifically localized in the subendothelial area of umbilical vessels and are mostly in close contact with endothelial cells. The p75NTR$^+$ cells co-expressed CD90, CD105, CD146, PDGFRβ, and NG2 but did not express the endothelial markers von Willebrand factor and CD34. The immunophenotype and the localization data strongly suggested that the P75NTR$^+$ cells are pericyte-like perivascular cells as defined previously [18]. The p75NTR$^+$ cells were not detected in other areas of UC tissue. These results support previous studies showing that p75NTR expression was absent or low *in situ* in fresh fragments of WJ [19,20]. Interestingly, our data showed that there were at least two types of p75NTR$^+$ cells with different expression levels and different morphologies. That both types of p75NTR$^+$ cells expressed PDGFRβ, CD90, CD146, and NG2, suggests that the cells have a phenotypic feature of pericytes. Thus, it is supposed that UA tissue contains several different types of pericyte-like cells that express p75NTR. To support this supposition, the presence of at least three different subsets of pericytes have been shown in human UA tissue [18]. In diverse human tissues, perivascular cells, especially pericytes, give rise to multipotent MSCs [21]. Previous studies found that umbilical vessel-derived cultured MSCs showed high potential for proliferation and multilineage differentiation [22–24]. Our data confirmed that the isolated UA cells could proliferate on plastic dishes and exhibit CFU-F-forming ability. Interestingly, our data showed that UA-derived

MSCs did not express p75NTR. This result is in agreement with a previous report [22]. Because it is possible that a p75NTR$^-$ cell fraction contributed CFU-Fs, further studies are needed to clarify whether the p75NTR$^+$ cells possess clonogenic and multi-lineage differentiation potential as MSCs.

In the neurosphere culture condition, we found that the UA cells form neurospheres that can further differentiate into neuronal and glial cell lineages. Most of the neurosphere-forming cells expressed p75NTR, suggesting that the neurospheres originated from subendothelial p75NTR$^+$ cells. To our knowledge, this is the first study to report that neurospheres can be generated from freshly isolated UA cell fraction, although previous studies showed that UC-derived cultured MSCs exhibit neurosphere formation capacity [8,25,26]. Our data showed that the neurospheres could be generated from the bulk of UA cells but not from freshly isolated p75NTR$^+$ cells. These findings suggested that UA cells other than p75NTR$^+$ cells support the development of neurospheres.

Genetic lineage tracing analysis using the P0-Cre reporter line showed that GFP$^+$ neural crest-derived cells are present in the subendothelial area of UA. Moreover, a part of the subendothelial GFP$^+$ cells also expressed p75NTR. These mouse data suggested that human UA p75NTR$^+$ cells also originate from the neural crest. Interestingly, neural crest-derived cells were reported to appear in fetal blood circulation before reaching bone marrow in the reporter mouse [3]. We also confirmed that the neural crest-derived cells in the mouse fetal blood expressed p75NTR [27]. These findings raise the possibility that the neural crest-derived cells in UA had migrated from the fetal blood circulation because their time of appearance (E12.5–E14.5) corresponds to the timing of UC development in mouse embryo [28].

It is expected that NCSCs generated from clinically accessible sources will be applied to cell-based therapy for neurodegenerative diseases. In addition, recent studies found that NCSCs can be
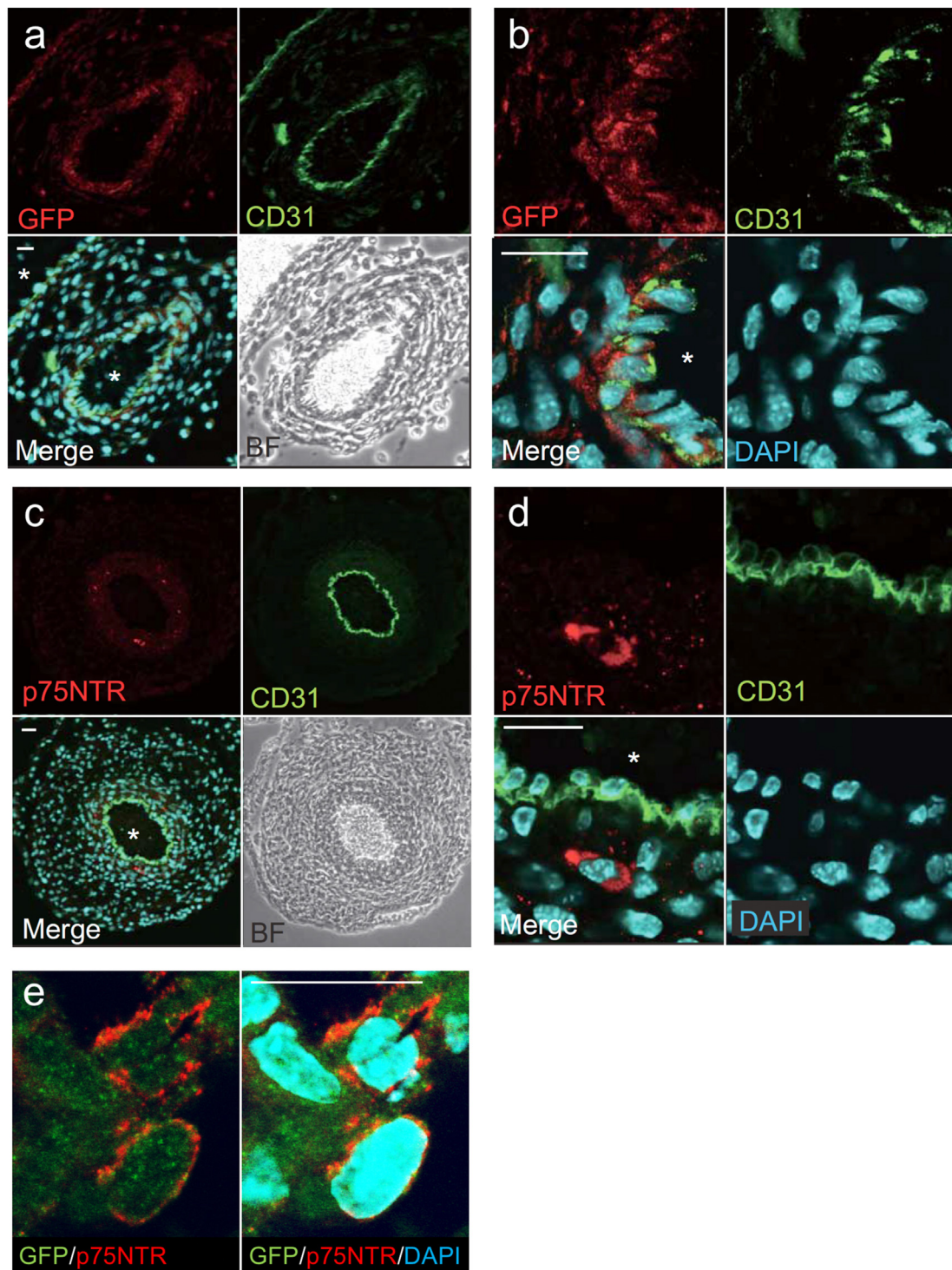
Fig. 7. Immunohistochemical analysis of umbilical cords in P0-Cre/Floxed-EGFP mice. Umbilical cords were isolated and sectioned from P0-Cre/Floxed-EGFP mice at E15.5. Samples were immunostained for GFP, p75NTR, and CD31. Nuclei were stained for DAPI. (a) Representative photomicrographs of umbilical arteries stained for GFP and CD31. (b) Higher magnification views of (a). (c) Representative photomicrographs of umbilical arteries stained for p75NTR and CD31. (d) Higher magnification views of (c). (e) Representative photomicrographs of subendothelial area stained for GFP and p75NTR. Asterisks indicate the lumen of umbilical artery. Scale bar: 20 μm.

generated from human-induced pluripotent stem cells [29]. However, such NCSCs tend to form teratomas even after the exclusion of pluripotent markers [30]. The UA p75NTR$^+$ cells may be suitable for neuroregenerative strategies because they lack tumorigenicity and can be isolated from UC tissue that is usually discarded as medical waste.

There are several limitations in this study. First, we failed in the long-term culture of UA p75NTR$^+$ cells. Although we could generate secondary neurospheres, we failed to expand them further. To obtain large quantities of cells for clinical applications, further studies are needed to explore efficient methods for the expansion of neurosphere-forming cells. Second, we did

not examine the functional properties and therapeutic activities of the UA p75NTR$^+$ cells *in vivo*. Additional experiments are needed to examine whether the UA p75NTR$^+$ cells possess migration ability in the body and therapeutic potential using proper animal models. Nevertheless, the present results are encouraging for the potential future use of p75NTR$^+$ cells as a cellular therapy.

5. Conclusion

We showed that p75NTR$^+$ cells were specifically present in the subendothelial area of UA and UV. These cells possess a phenotypic feature of MSCs and pericytes. Although the association of the p75NTR$^+$ cells with neurosphere formation has yet to be confirmed, isolated UA cells showed the ability to form neurospheres followed by differentiation into nerve cells and glial cells. Moreover, neural crest-derived cells were identified in the corresponding area of UA in the P0-Cre reporter mouse. These data provide new insight into the unique stem/progenitor cell population in UC that may be clinically applicable for cell-based therapy.

Declaration of competing interest

The authors declare no conflicts of interests in relation to this article.

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