The specificity of NeuroTrace 500/525 in the human induced pluripotent stem cell-derived pericyte-like cells

Seo Young Kim

Department of Medical Science

The Graduate School, Yonsei University

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Directed by Professor Chul Hoon Kim

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Seo Young Kim

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This certifies that the Master's Thesis
of Seo Young Kim is approved.

Thesis Supervisor: Chul Hoon Kim

Thesis Committee Member #1: Ho Sung Jung

Thesis Committee Member #2: Heon Yung Gee

The Graduate School
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ABSTRACT

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Seo Young Kim

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Brain pericytes are located around the capillaries and are critical components of the neurovascular unit. Pericytes play essential roles in cerebral blood flow regulation, maintenance of the blood brain barrier and angiogenesis[1-3]. Pericyte dysfunction can cause the blood flow reduction and the blood brain barrier breakdown, which may ultimately lead to the neurological diseases such as stroke and Alzheimer's diseases[1].

Recently, several methods were suggested for differentiation of human pluripotent stem cells (hPSCs) into brain mural cells, specifically pericytes or vascular smooth muscle cells (SMCs)[4-9]. Unfortunately, identifying the pericytes from among such hPSC-derived mural cells has been challenging. This is due to the absence of pericyte-specific markers and to the loss of defining anatomical information inherent to culture

conditions. NeuroTrace 500/525, a fluorescent dye, is recently reported as a new method of labeling pericytes with high selectivity in the live mouse brain[10]. However, the utility of NeuroTrace 500/525 *in vitro* or in human cells has not been tested yet. In this study, the cellular specificity of NeuroTrace 500/525 uptake was investigated *in vitro* using the human induced pluripotent stem cell (hiPSC)-derived vascular cells. First, I found that NeuroTrace 500/525 specifically stains primary human brain pericytes, confirming its specificity *in vitro*. Second, I found that NeuroTrace 500/525 specifically labels hiPSC-derived pericyte-like cells, but neither endothelial cells nor SMCs derived from the same hiPSCs. Last, I found that neuroectoderm-derived SMCs, which have pericyte-like properties, also take up NeuroTrace 500/525. These data indicate NeuroTrace 500/525 is useful for identifying pericyte-like cells among hiPSC-derived brain vascular cells.

Key words: pericyte, brain, smooth muscle cell, endothelial cell, NeuroTrace 500/525, hiPSC

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I. INTRODUCTION

Brain pericytes are positioned around the capillaries and are important components of the neurovascular unit (NVU)[1-3]. They play an essential role in central nervous system (CNS) homeostasis including the blood brain barrier (BBB) formation and maintenance, vascular stability, and cerebral blood flow regulation[11-14]. Despite their importance, pericyte research has been hampered by prevalent confusion regarding the precise identification and definition of pericytes due to the lack of pericyte-specific marker molecules. The commonly known pericyte markers are platelet-derived growth factor receptor ß (PDGFRß), neuron-glial antigen 2 (NG2), desmin and CD13; however, these markers are also expressed by other cell types such as oligodendrocytes, glia and

vascular smooth muscle cells (SMCs)[15-18]. Moreover, pericytes and SMCs, collectively termed as mural cells, are very difficult to distinguish one from the other[3]. The primary distinction between pericytes and SMCs is dependent on the anatomical position in which pericytes are located on the small vessels, whereas SMCs are located on the large vessels[19, 20]. However, because there is no single marker gene available, it is challenging to study the roles of pericytes *in vivo* using genetic switches for pericyte-specific manipulation

Making human pericytes *in vitro* is promising because it will surely expand the opportunities for disease modeling and drug screening in pericytes. Recently, several protocols for differentiating hiPSCs into human vascular mural cells were developed[5-7], making it possible to produce human pericyte-like cells or SMCs in culture. Because there is no selective marker for pericytes, the identities of differentiated cells are sometimes ambiguous. Indeed, both hiPSC-derived human pericytes and SMCs show the expression of PDGFRß and NG2 proteins. Because anatomical information is lost under cell culture conditions, it is more challenging to discriminate pericytes from SMCs without cell-type specific markers.

Recently, NeuroTrace 500/525 was proved as an exclusive marker for pericytes in the mouse brain[10]. It was originally used to identify neurons in fixed brain tissues[21]. However, when NeuroTrace 500/525 is administered to the brains of *Pdgfrβ-Cre*;*tdTomato* transgenic mice where VMCs express tdTomato, the dye labels only tdTomato-positive cells that ensheathe capillaries (pericytes), but not ring-like cells lining pre-capillaries or arterioles (SMCs)[10]. Thus, I asked whether NeuroTrace 500/525 could help us discern pericyte-like cells differentiated from hiPSCs.

In this study, I found that NeuroTrace 500/525 selectively labels primary human brain pericytes. Moreover, by generating and comparing human brain microvascular

endothelial cells (BMECs), SMCs and pericyte-like cells from the same hiPSC line, I further validated the specificity of NeuroTrace 500/525 in hiPSC-derived pericyte-like cells. Moreover, I differentiated hiPSCs into SMCs using two different protocols that derive SMCs from neural crest stem cells (NCSCs) or neuroectoderm (NE), respectively. I discovered that NE-derived SMCs (NE-SMCs), which have pericyte-like characteristics, also take up NeuroTrace 500/525. In the present study, I demonstrate that NeuroTrace 500/525 is useful for pericyte identification *in vitro* especially in hiPSC-induced mural cell differentiation used in the field of NVU research.

II. MATERIALS AND METHODS

1. Primary cell culture

Human brain vascular pericytes and human brain vascular smooth muscle cells were purchased from ScienCell Research Laboratories (Carlsbad, CA, USA). They were plated onto poly-L-lysine-coated plates and maintained in pericyte medium containing 2% FBS and pericyte growth supplement or smooth muscle cell medium containing 2% FBS and smooth muscle cell growth supplement (ScienCell), respectively. This study used only pericytes and smooth muscle cells between passages 3 and 6. Human brain microvascular endothelial cells (BMECs) were purchased from Cell Systems (Kirkland, WA, USA), plated onto fibronectin-coated plates, and maintained in complete classic medium containing serum and CultureBoost™ (Cell Systems) between passages 3 and 5. Human umbilical vein endothelial cells (HUVECs) were purchased from Lonza (Basel, Switzerland) and cultured in EGM™-2 endothelial cell growth media.

2. Pericyte differentiation

Pericyte differentiation protocol was adopted from a recently published protocol[7]. Pericytes were differentiated from hiPSCs through NCSCs. For NCSC differentiation, ASE9209 (Applied StemCell, Milpitas, CA, USA) or IMR90-4 (WiCell Research Institute, Madison, WI, USA) cells were singularized using Accutase (STEMCELL Technologies, Vancouver, Canada) and seeded at 8.75×10^5 cells/well in Matrigel-coated 6-well plates with mTeSR1 (STEMCELL Technologies) and 10 μM Y27632

(Tocris, Minneapolis, MN, USA). After a 24 hr incubation, the medium was switched to E6 (Gibco, Grand Island, NY, USA) supplemented with 22.5 μg/ml heparin sodium salt (Sigma-Aldrich, St Louis, MO), 1 μM CHIR99021 (Tocris), 10 μM SB431542 (Tocris), 10 ng/ml bFGF (R&D Systems, Minneapolis, MN, USA), and 1 μM dorsomorphin (Sigma-Aldrich). The cells were then differentiated for 15 days by changing the medium (E6-CSFD) daily. During NCSC differentiation, when the cells reached 100% confluence, the differentiating neural crest cells were passaged at a 1:6 ratio. Closing in on day 15, the cells were passaged at a 1:2 or 1:3 ratio. On day 15, the cells (10^7 cells) were resuspended in 60 μl of buffer containing 0.5% bovine serum albumin (BSA) and 2 mM EDTA in DPBS (without $Ca2^+$/$Mg2^+$). Then, 20 μl of NCSC magnetic microbeads (conjugated to $p75^{NTR}$ antibodies) and 20 μl of FcR blocking reagent (Miltenyi Biotec, Bergisch Gladbach, Germany) were added. After a 15 min-incubation at 4℃, cells labeled with anti-$p75^{NTR}$ NCSC microbeads were sorted through MS columns using a MiniMACS magnetic separator (Miltenyi Biotec) according to the manufacturer's instructions. After MACS, the sorted $p75^{NTR}$+ NCSCs were replated onto 6-well plates at 2×10^5 cells/well in E6-CSFD with 10 μM Y27632. After a 24-hr incubation, the medium was switched to E6 medium supplemented with 10% FBS. The cells were further differentiated into pericytes for 9 days with daily changes of the E6-FBS medium.

3. BMEC differentiation

Our BMEC differentiation protocol was adopted from a previously published protocol[22]. First, hiPSCs were seeded at 2×10^5 cells/well onto Matrigel-coated 6-well

plates in mTeSR1 with 10 μM Y27632. The cells were maintained in mTeSR1 for 3 days. When the cells reached a density of $3 \times 10^5 \sim 5 \times 10^5$ cells/well, the medium was switched to unconditioned medium (UM) containing DMEM/Ham's F12 (Gibco) with 20% Knockout Serum Replacement (Gibco), 1× MEM nonessential amino acids (Gibco), 1 mM L-glutamine (Gibco), and 0.1 mM β-mercaptoethanol (Sigma-Aldrich) for 6 days. On day 6, the medium was switched from UM to endothelial cell (EC) medium supplemented with 10 μM retinoic acid (Sigma-Aldrich). EC medium is composed of human endothelial serum-free medium (hESFM, Gibco), 20 ng/ml bFGF, and 1% platelet-poor plasma-derived human serum (Sigma-Aldrich). On day 8, the cells were dissociated with Accutase and seeded onto collagen/fibronectin-coated plates. The plates were coated with a 4:1:5 ratio of collagen, fibronectin, and water and incubated for at least 1 hr at 37℃. The cells were then cultured in EC medium for 3 days. NeuroTrace 500/525 staining was performed on the live cells. Cells were treated with NeuroTrace 500/525 (Invitrogen, Carlsbad, CA, USA) diluted 1: 2,000 in essential 6 (E6) medium (Gibco, Grand Island, NY, USA) for 20 min at 37℃. Then cells were incubated for 10 min with DAPI (Thermo Fisher Scientific, Rutherford, NJ, USA) diluted 1: 1,000 in PBS. Cells were washed three times with PBS and mounted with mounting solution (Invitrogen, Carlsbad, CA, USA). Images were taken by using confocal laser-scanning microscope (LSM 710, Carl Zeiss, Jena, Germany).

4. NCSC-derived SMC differentiation

Our NCSC-SMC differentiation protocol was adopted from a previously published protocol[5]. hiPSCs were seeded at 8.75×10^5 cells/well in Matrigel-coated 6 well plates with mTeSR1 and 10 μM Y27632. After 24 hrs, the medium was switched to E6-CSFD.

NCSCs were differentiated for 14 days by changing E6-CSFD daily. At day 15, NCSCs were sorted through MACS, and sorted $p75^{NTR}$ + NCSCs were replated onto 6 well plates with seeding density of 2×10^5 cells/well in E6-CSFD with 10 μM Y27632. After 24 hrs, the medium was switched to E6-PT. E6-PT is E6 medium (Gibco) supplemented with 10 ng/ml PDGF-BB (Peprotech, Rocky Hill, New Jersey, USA) and 2 ng/ml TGF-β1 (Peprotech). Cells were differentiated for 12 days by changing E6-PT every day.

5. Neuroectoderm-derived SMC differentiation

Our NE-SMC differentiation protocol was adopted from a previously published protocol[4]. First, hiPSCs were passaged onto Matrigel-coated 6-well plates at 10–20 clusters/well in mTeSR1. After a 24-hr incubation, the hiPSCs were cultured in E6 medium supplemented with 10 μM SB431542 and 12 ng/ml bFGF for 5 days. Then, the cells were subcultured onto new Matrigel-coated plates at a density of $2 \times 10^5 \sim 3 \times 10^5$ cells/well. On day 6, the medium was switched to E6-PT, and the cells were further differentiated into NE-SMCs in E6-PT for 12 days.

6. Immunocytochemistry

Cells were fixed for 15 min at room temperature with 4% (v/v) paraformaldehyde (BioSolution, Gyeonggi-do, Korea). Cells were washed three times in PBS without $Ca2^+/Mg2^+$ Cells were blocked for 1 hr in blocking buffer at room temperature and incubated overnight at 4℃ with primary antibodies diluted in primary antibody staining buffer. Antibodies and staining conditions are listed in table 1. The next day, cells were washed three times with PBS and incubated with secondary antibodies diluted 1: 1,000

in primary antibody staining buffer. Cells were incubated for 1 hr in the dark at room temperature on a rocking platform. Then cells were incubated for 10 min with DAPI (Thermo Fisher Scientific, Rutherford, NJ, USA) diluted 1: 1,000 in PBS. Cells were washed three times with PBS and mounted with mounting solution (Invitrogen, Carlsbad, CA, USA). Images were taken by using a confocal laser-scanning microscope (LSM 710, Carl Zeiss, Jena, Germany) and fluorescence microscopy (BX-40, Olympus, Japan).

Table 1. List of primary antibodies and antibody staining conditions

Antibodies	Source	Identifier	Staining solution	Dilution
p75-NGFR	Advanced Targeting System	AB-N07	1% BSA	1:500
NG2	Millipore	MAB2029	5% goat serum + 0.4% TX-100	1:100
PDGFRβ	CST	3169	5% goat serum + 0.4% TX-100	1:100
PECAM	Abcam	ab32457	5% goat serum + 0.4% TX-100	1:200
Occludin	Thermo	71-1500	5% goat serum + 0.4% TX-100	1:300
SM22	Abcam	ab14106	3% BSA + 0.1% TX-100	1:2,000
αSMA	Abcam	ab7817	5% NFDM + 0.4% TX-100	1:200

* NFDM – Non-fat dry milk

7. RNA extraction and real time PCR (qRT-PCR)

Cells were collected in TRIzol Reagent (Thermo). cDNA was synthesized from RNA samples by PrimeScript™ 1st strand cDNA Synthesis Kit (TAKARA, Shiga,

Japan) following the manufacturer's instructions. The gene expression levels of pericytes and smooth muscle cells were tested by quantitative real time PCR (qRT-PCR) using SYBR Green Master Mix (Applied Biosystems, Waltham, MA, USA) on a StepOne Real-Time PCR system (Applied Biosystems). The primers used in this study are listed in Table 2

Table 2. List of primers

Gene	Forward (5'-3')	Reverse (5'-3')
NGFR	GTGGGACAGAGTCTGGGTGT	AAGGAGGGGAGGTGATAGGA
PDGFRβ	GCTCACCATCATCTCCCTTATC	CTCACAGACTCAATCACCTTCC
CSPG4	IDT DNA Hs.PT.58.39417158 Predesigned Probe	
ACTA2	TGTTCCAGCCATCCTTCATC	GCAATGCCAGGGTACATAGT
TAGLN	TCTTTGAAGGCAAAGACATGG	TTATGCTCCTGCGCTTTCTT
CNN1	GTCCACCCTCCTGGCTTT	AAACTTGTTGGTGCCCATCT

8. *In vitro* endothelial cord formation assay and quantification

Eight-well glass chamber slides were coated with 200 μl of Matrigel/well and incubated for 1 hr at 37°C to solidify the Matrigel. HUVECs were plated at 4.4×10^4 cells/well in 500 μl of EGM2 medium alone or with 2.2×10^4 hiPSC-derived pericyte-like cells, NCSC-SMCs, or NE-SMCs. The cells were incubated for 24 hrs at 37°C, and bright-field images were taken before fixation. Then, the cords were fixed for 15 min at room temperature with 4% PFA and stained according to the immunocytochemistry methods described above. The cords were mounted onto glass slips and imaged using a Zeiss confocal microscope. In a defined area of interest, Angiogenesis Analyzer

(ImageJ software) represents vascular cord structures as vectorial objects and provides a quantitative evaluation of their junctions, branches, segments. Angiogenesis Analyzer calculates average segment length as the sum of the lengths of all segments (binary line linked with two junctions) divided by the total number of the segments.

9. Statistical analysis

All data analysis was conducted with GraphPad Prism version 7 (GraphPad Software, Inc., La Jolla, CA, USA). *P*-values were calculated via an unpaired Student's *t* test or one-way analysis of variance (ANOVA) followed by multiple comparisons with Bonferroni *post hoc* test. All data are presented as means ± standard error of the mean (SEM). The significance level was set at $p < 0.05$.

III. RESULTS

1. NeuroTrace 500/525 selectively labels primary human brain pericytes

A previous study reported that NeuroTrace 500/525 selectively labels pericytes in the live mouse brain *in vivo*. To know whether NeuroTrace 500/525 can specifically label human brain pericytes *in vitro*, I first performed NeuroTrace 500/525 staining in primary human brain pericytes, SMCs, BMECs and HUVECs. I tried NeuroTrace 500/525 staining of different dilution ratios from 1:5,000 to 1:500 and different time conditions of 5 min, 15 min and 30 min. A dilution ratio of 1:500 was too strong that primary human brain pericytes as well as SMCs, BMECs and HUVECs were all labeled with NeuroTrace 500/525. At the dilution of 1:5,000 of NeuroTrace 500/525, primary human brain pericytes had a weak fluorescence signal. Moreover, there were no significant differences in dye intensity with increased incubation times of up to 1 hr.

Therefore, I tested the dilution ratio of 1: 2,000 and incubation time of 20 min as the best staining condition in this experiment. I found that primary human brain SMCs, BMECs and HUVECs are not labeled with NeuroTrace 500/525 (Fig. 1). In contrast, primary human brain pericytes revealed bright green fluorescent signals (Fig. 1). These results indicate that NeuroTrace 500/525 is selective for human brain pericytes *in vitro*.

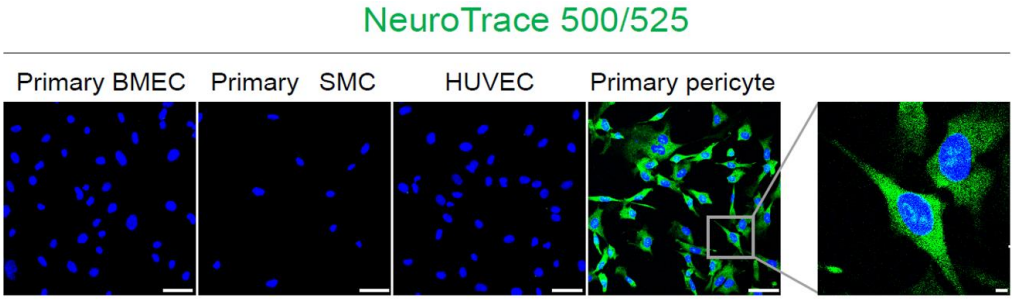

Figure 1. NeuroTrace 500/525 labels primary human brain pericytes *in vitro*. NeuroTrace 500/525 staining was performed in primary human BMECs, SMCs, HUVECs and pericytes. Cells were incubated with NeuroTrace 500/525 diluted 1:2,000 in E6 medium for 20 min at 37℃. Then, E6 medium with dye was removed from cells for subsequent fixation. Images were taken on a confocal microscope. Cellular uptake of dye is shown as green fluorescent signals. Scale bars, 50 μm (5 μm in magnified area).

1. Generation of hiPSC-derived pericyte-like cells

Because of the limited availability of human cells, hiPSC-derived pericyte model will be a valuable tool for pericyte research; however, identification of pericytes in differentiation studies is unclear due to the lack of specific markers. After establishing that NeuroTrace 500/525 exclusively labels human brain pericytes *in vitro*, I sought to test whether NeuroTrace 500/525 identifies hiPSC-derived pericyte-like cells. I differentiated the hiPSCs into pericyte-like cells via NCSCs (Fig. 2). Initially, hiPSCs showed highly compact colony morphology and as they were differentiated into pericyte-like cells, they adopted mesenchymal morphology gradually (Fig. 2).

PDGFRβ and NG2 are the commonly used mural cell markers. I used these two markers to verify that the differentiated pericytes have the expression of mural cell markers. Immunocytochemistry (ICC) was performed to examine the protein expressions of NCSC marker and mural cell markers. $p75^{NTR}$ was highly expressed at day 16 (NCSC stage) and the protein expression of $p75^{NTR}$ decreased rapidly at day 20 and day 25 (pericyte-like cell stage) (Fig. 3). In contrast, the mural cell markers, PDGFRβ and NG2 were nearly negative at day 16, and they were highly expressed at day 20 and day 25 (Fig. 3).

Moreover, the mRNA levels of hiPSC-derived NCSCs and mural cell markers were assessed by qRT-PCR. Compared to the mRNA levels at day 0 (hiPSC stage) in which the expressions of NCSC and mural markers were absent, the mRNA expression of *NGFR* ($p75^{NTR}$) was high at day 16 and decreased at day 25 during pericyte differentiation (Fig. 4). The mRNA levels of *PDGFRβ* and *CSPG4* (NG2) were nearly negative at day 0 and day 16, but they were noticeably upregulated at day 25 (Fig. 4). The mRNA results were consistent with the protein results. Overall, these results

demonstrate that hiPSCs are differentiated into pericyte-like cells properly.

Angiogenesis is one of main functions of pericytes[1]. An *in vitro* endothelial cell cord formation assay closely mimics the vessel formation *in vivo*[23]. I performed this assay to evaluate the capacity of differentiated pericytes to surround endothelial cells and to maintain vascular stability. For this experiment, I seeded HUVECs alone or HUVECs and hiPSC-derived pericyte-like cells together into thick Matrigel-coated 8 well glass plate. After 24 hrs, the vascular cords were formed (Fig. 5). In HUVEC monoculture, the endothelial cells formed many small branching cords whereas in co-cultures with hiPSC-derived pericyte-like cells, pericyte-like cells self-assembled with endothelial cells, forming longer cords. To observe whether pericyte-like cells surround endothelial cords, I performed ICC and observed that pericyte-like cells (NG2, red) are located around endothelial cords (CD31, green) (Fig. 5). The vascular cord networks showed that pericyte-like cells promote the formation of longer vascular cords. These results indicate that hiPSC-derived pericyte-like cells *in vitro* functionally recapitulate pericytes *in vivo*. In combination with marker expressions, the functional assay reaffirmed that the hiPSC-derived pericyte model is well developed for validating the selectivity of NeuroTrace 500/525.

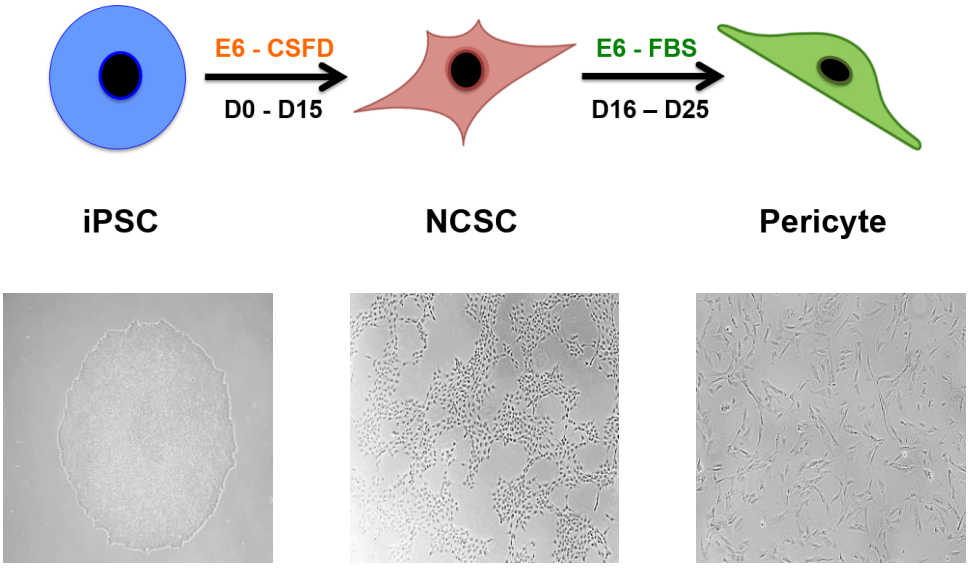

Figure 2. Schematic representation of hiPSC-derived pericyte-like cell differentiation. Pericyte-like cells were differentiated from hiPSC via NCSCs. hiPSCs were differentiated into NCSCs for 14 days in E6-CSFD and then NCSCs were differentiated into pericyte-like cells for 9 days in E6-10% FBS. E6-CSFD: Essential 6 medium containing CHIR 99021, SB431542, FGF2 and dorsomorphin. E6-FBS: Essential 6 medium with 10% FBS.

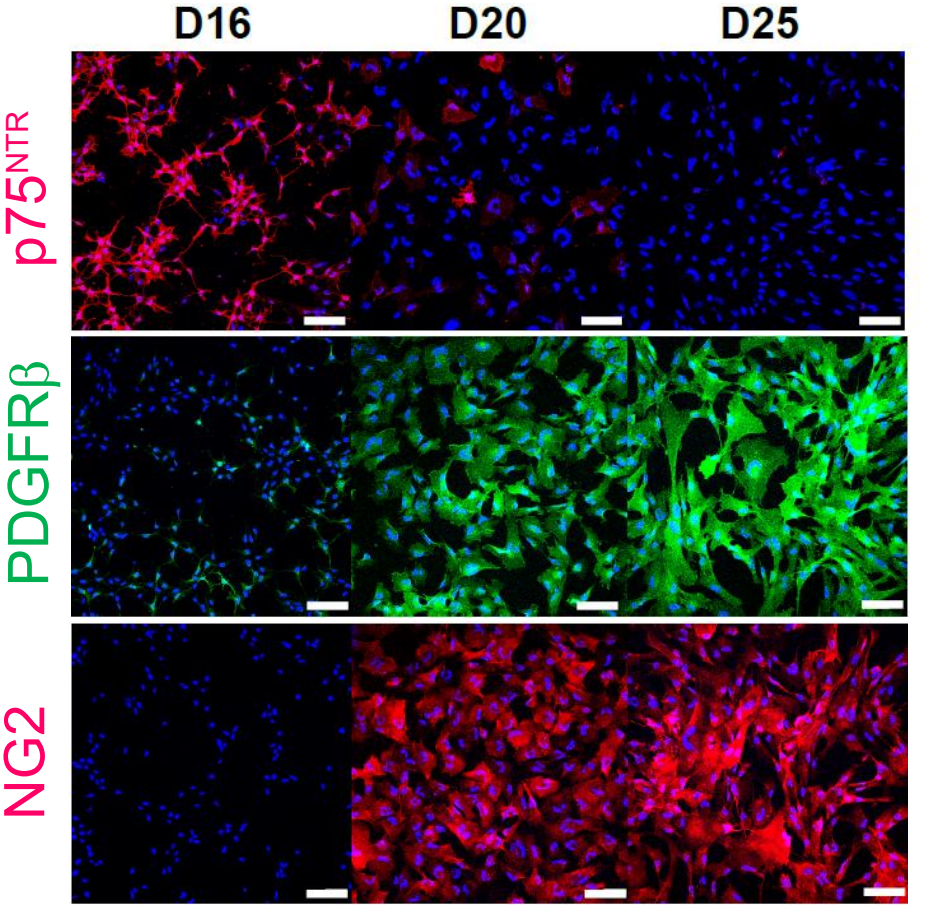

Figure 3. Changes in protein expressions of molecular markers for NCSCs and pericytes during pericyte-like cell differentiation from hiPSCs. ICC was used to analyze the protein expressions of neural crest-specific marker ($p75^{NTR}$) and mural cell markers (PDGFRβ and NG2) at day 16 (NCSC stage), day 20 and day 25 (pericyte-like cell stage). Scale bars, 100 μm.

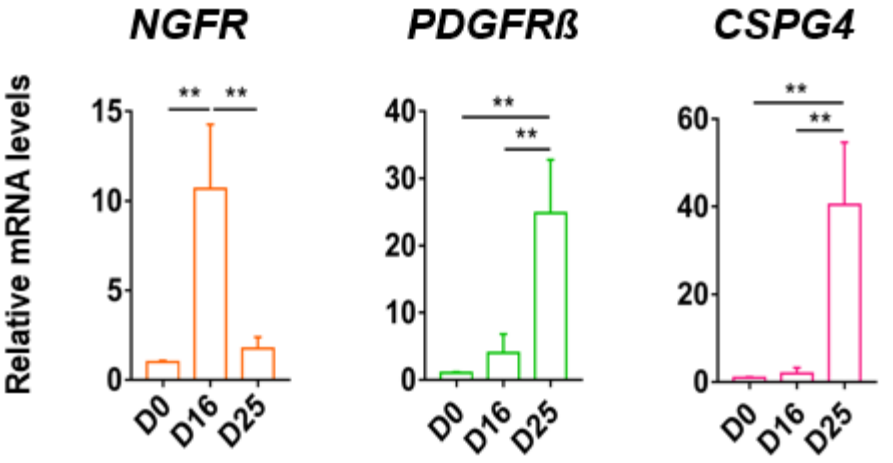

Figure 4. Changes in mRNA levels of molecular markers for NCSCs and pericytes during pericyte-like cell differentiation from hiPSCs. qRT-PCR analysis showed the mRNA expressions of neural crest marker (*NGFR*) and mural cell markers (*PDGFRβ* and *CSPG4*) at day 0 (hiPSC stage), day 16 (NCSC stage), and day 25 (pericyte-like cell stage). mRNA expression level of each gene was normalized to *GAPDH* as a reference gene. *P*-value was calculated by one-way ANOVA, followed by Bonferroni *post hoc* tests for multiple comparisons. *NGFR:* F (2,6) = 19.36, p =0.0024, *PDGFRβ*: F (2,6) = 21.10, p =0.0019, *CSPG4*: F (2,6) = 22.20, p =0.0017. Results are presented as means ± SEM of three independent experiments. ** $p < 0.01$.

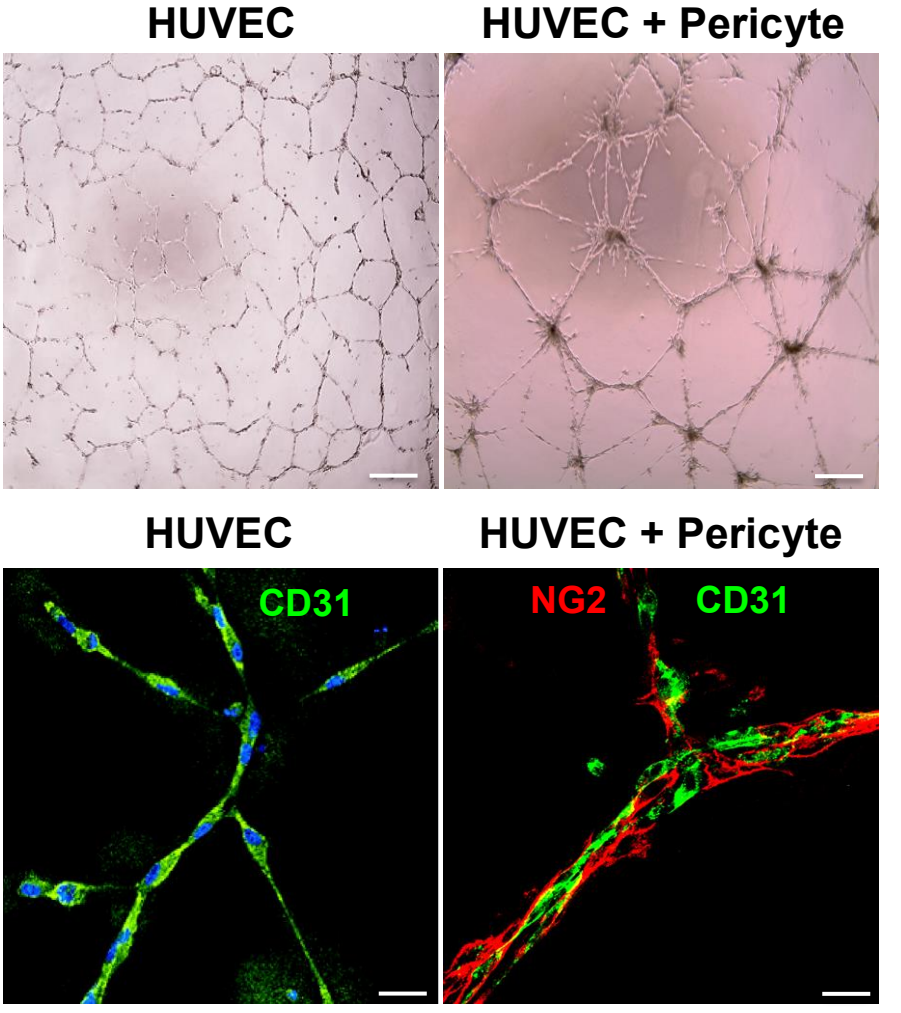

Figure 5. ***In vitro*** **endothelial cord formation assay using HUVECs and hiPSC-derived pericyte-like cells.** Representative bright field images of HUVECs alone or co-cultures with hiPSC-derived pericyte-like cells (upper panels). Scale bars, 500 μm. Immunostaining of HUVECs alone or co-cultures with hiPSC-derived pericyte-like cells (lower panels). HUVECs were stained with CD31, green, and pericyte-like cells were stained with NG2, red. Scale bars, 50 μm.

2. NeuroTrace 500/525 selectively labels hiPSC-derived pericyte-like cells

To see whether NeuroTrace 500/525 is also selective for pericyte-like cells derived from hiPSCs, NeuroTrace 500/525 staining was performed. I co-differentiated the same hiPSCs line into endothelial cells and pericyte-like cells via NCSCs, avoiding genetic variations that occur when using different hiPSC lines. hiPSC-derived NCSCs, pericyte-like cells and BMECs showed the expression of $p75^{NTR}$, NG2 and ZO-1, a tight junction protein, respectively, and these cells did not express other types of cell markers (Fig. 6). These data indicate that each cell type is distinctly differentiated. Differentiated cells were incubated with NeuroTrace 500/525 1: 2,000 diluted in E6 medium, incubated at 37℃ for 20 min and then fixed with 4% PFA for 15 min. We found that hiPSC-derived pericyte-like cells are stained bright green while hiPSC-derived NCSCs and BMECs are not stained (Fig. 6). Overall, these results indicate that NeuroTrace 500/525 is selective for hiPSC-derived pericyte-like cells.

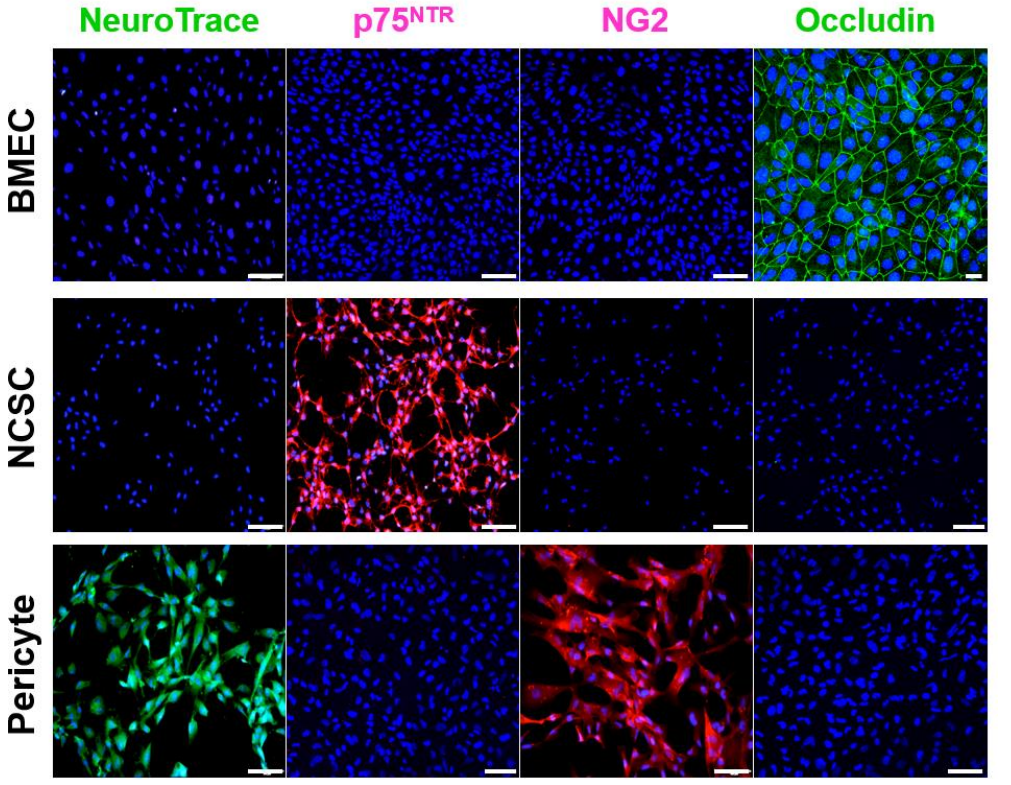

Figure 6. NeuroTrace 500/525 selectively labels hiPSC-derived pericyte-like cells. NeuroTrace 500/525 staining of hiPSC-derived NCSCs, pericyte-like cells and BMECs. hiPSC-derived NCSCs, pericyte-like cells, and BMECs were incubated with NeuroTrace 500/525 for 20 min (first column). hiPSC-derived NCSCs, pericyte-like cells, and BMECs were stained with antibodies against cell type-specific markers such as $p75^{NTR}$ (magenta), NG2 (red), and Occludin (green). Scale bars, 100 μm.

3. NeuroTrace 500/525 distinguishes hiPSC-derived pericyte-like cells from hiPSC-derived SMCs

It has been challenging to distinguish between pericytes and SMCs *in vitro* where the anatomical context is lacking. To observe whether NeuroTrace 500/525 can discriminate pericytes from SMCs *in vitro*, we differentiated pericyte-like cells and SMCs from the same hiPSCs. Brain pericytes and SMCs have a common origin of neural crest[24, 25]. Therefore, we adopted methods to induce pericyte-like cells and SMCs via NCSCs[5, 7] (Fig. 7A). First, ICC was performed and we found that both pericyte-like cells and SMCs express mural cell markers including PDGFRβ and NG2 (Fig. 7B), suggesting these two proteins cannot distinguish brain pericytes. Interestingly, only SMCs expressed αSMA (Fig. 7B) which is consistent with previous studies demonstrating that brain capillary pericytes generally do not express αSMA[26-28]. We also found that the qRT-PCR results are consistent with ICC results (Fig. 8). It is difficult to distinguish these two mural cells by marker genes. Incubation of pericyte-like cells and SMCs with NeuroTrace 500/525 demonstrated that only pericyte-like cells show selective dye uptake (Fig. 9). These results indicate that NeuroTrace 500/525 can distinguish between hiPSC-derived pericyte-like cells and hiPSC-derived SMCs.

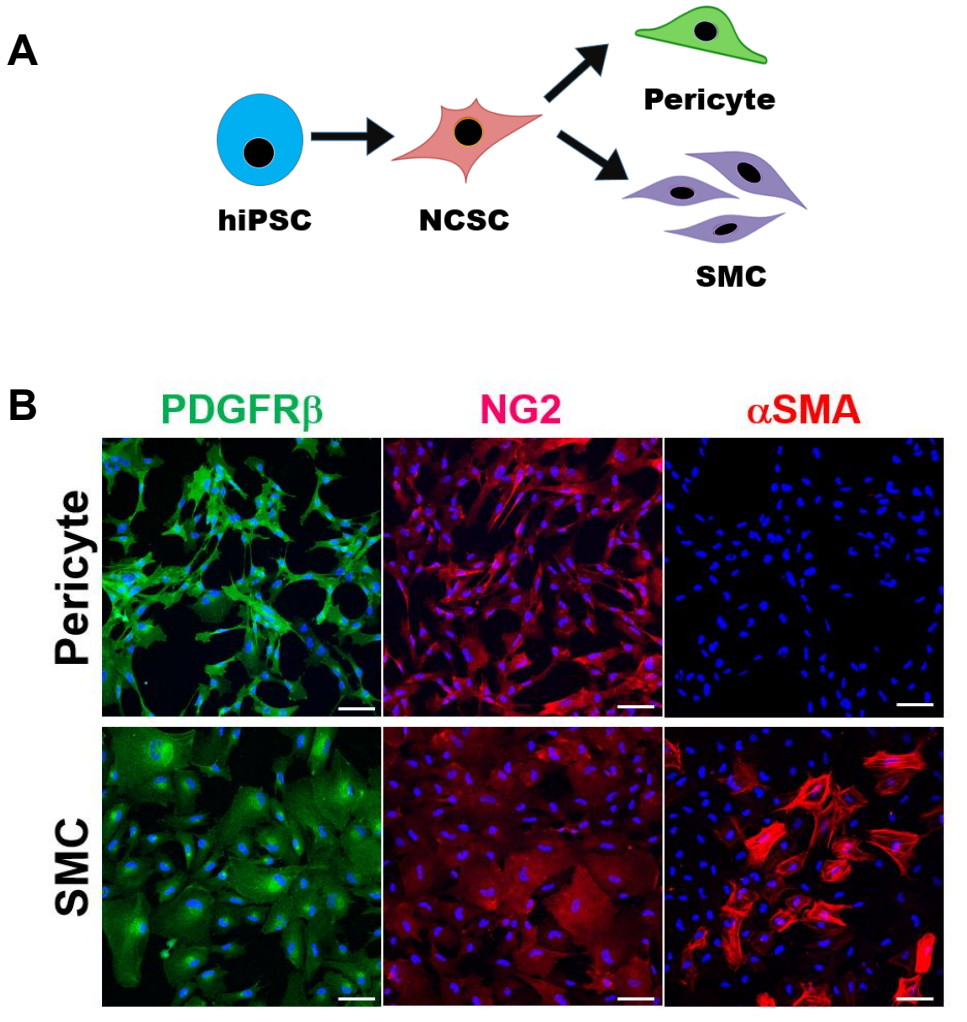

Figure 7. hiPSC-derived pericyte-like cell and SMC differentiation (A) Schematic illustration of hiPSC-derived pericyte-like cell and SMC differentiation. hiPSCs were differentiated into NCSCs for 2 weeks. After MACS of NCSCs, sorted $p75^{NTR}$+ NCSCs were differentiated into both pericytes and SMCs by adding FBS for 9 days and adding SMC inducers including PDGF-BB and TGFβ for 12 days, respectively. (B) Immunostaining of mural cell markers in both cell types. Differentiated pericyte-like cells (D25) and SMCs (D28) were stained with mural cell markers including PDGFRβ

(green), NG2 (magenta) and αSMA (red). Images are representatives of n = 3 independent experiments. Scale bars, 100 μm.

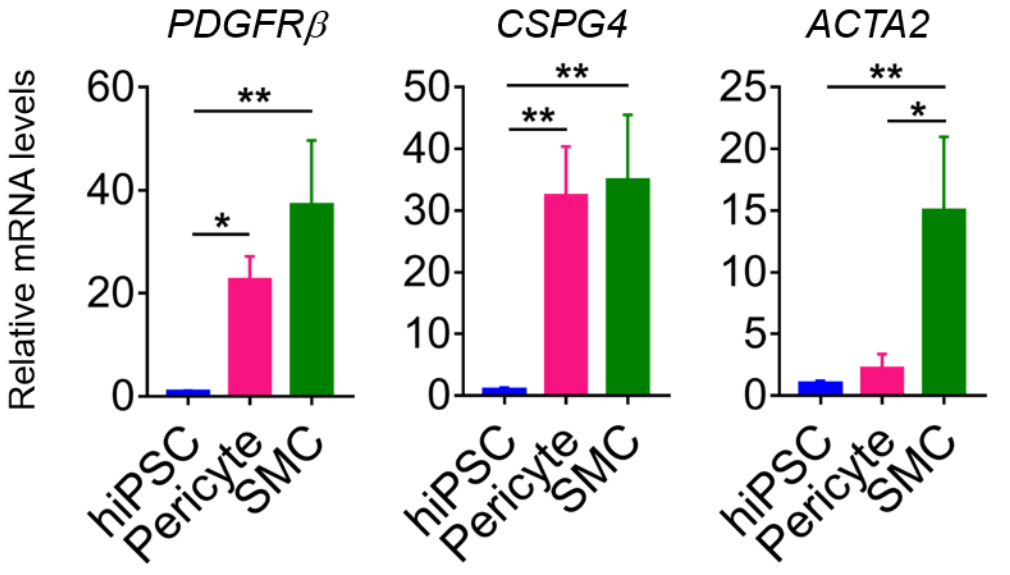

Figure 8. mRNA levels of mural cell markers in hiPSC-derived pericyte-like cells and SMCs. qRT-PCR analysis showed the mRNA expression of mural cell markers (*PDGFRβ, CSPG4* and *ACTA2*) in hiPSC, hiPSC-derived pericyte-like cell and SMCs. mRNA expression level of each gene was normalized to *GAPDH* as a reference gene. *P*-value was calculated by one-way ANOVA, followed by Bonferroni *post hoc* tests for multiple comparisons. *PDGFRβ*: F (2,6) = 17.30, p =0.0032, *CSPG4*: F (2,6) = 18.69, p =0.0026, *ACTA2*: F (2,6) = 14.89, p =0.0047. Results are presented as means ± SEM of three independent experiments. * $p < 0.05$, ** $p < 0.01$.

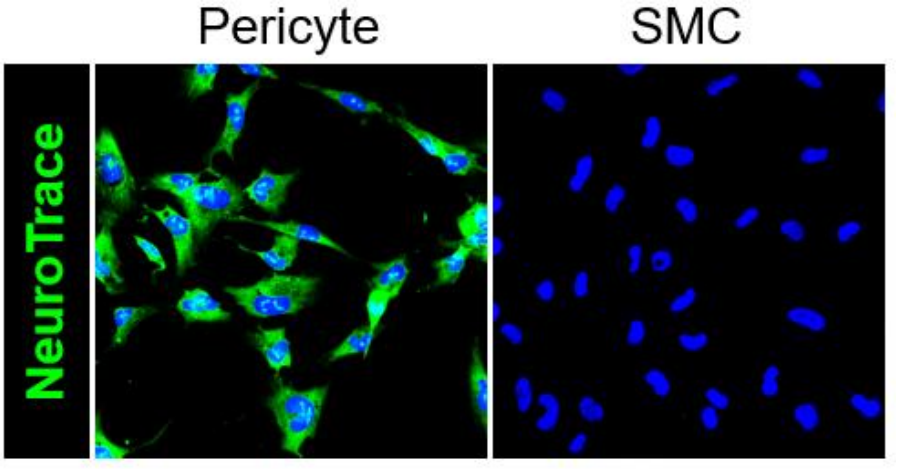

Figure 9. NeuroTrace 500/525 differentiates between hiPSC-derived pericyte-like cells and SMCs. Confocal microscope images of NeuroTrace 500/525 labeling in hiPSC-derived pericyte-like cells and SMCs. Cells were incubated with NeuroTrace 500/525 1:2000 diluted in E6 for 20 min at 37℃. Then, E6 medium with dye was removed from cells for subsequent fixation. Incubation of hiPSC-derived pericyte-like cells and SMCs with NeuroTrace 500/525 reveals the selective dye uptake in pericyte-like cells. Images are representatives of n = 3 independent experiments. Scale bars, 50 μm

4. NeuroTrace 500/525 labels neuroectoderm-derived SMCs that possess properties of pericytes

There exist two reported methods of generating cerebral SMCs[4, 5] (Fig. 10A). The main difference between two approaches is that the first one induces SMCs directly from neuroectodermal cells (NE-SMCs)[4] and the second one induces SMCs via NCSCs (NCSC-SMCs)[5]. The molecular signature of NCSC-SMCs is reportedly more like that of human brain SMCs than NE-SMCs. Ironically, a previous study demonstrated that NE-SMCs have pericyte-like characteristics[9]. I therefore wondered whether NeuroTrace 500/525 can discriminate NCSC-SMCs and NE-SMCs. I split and differentiated the same hiPSC line into NCSC-SMCs and NE-SMCs [4, 5]. ICC revealed that both NCSC-SMCs and NE-SMCs express mural cell markers of PDGFRβ, NG2, SM22 and αSMA (Fig. 10B). There were no significant differences in morphology and marker expression between NCSC-SMCs and NE-SMCs. In contrast, I found that NeuroTrace 500/525 labels only NE-SMCs (Fig. 11).

NE-SMCs reportedly have enough pericyte-like properties to support capillary structures[9]. To determine whether NE-SMCs have functional properties of pericytes. I performed *in vitro* endothelial cord formation assay by co-culturing HUVECs with NE-SMCs or NCSC-SMCs. I discovered that NE-SMCs contribute to forming longer capillary cords whereas NCSC-SMCs pull cords apart (Fig. 12A, B), suggesting that these two SMCs are certainly distinct. Taken together, these data suggest that NE-SMCs indeed have key pericyte attributes accounting for NeuroTrace 500/525 uptake, and NeuroTrace 500/525 is useful in discerning cells with pericyte-like characteristics.

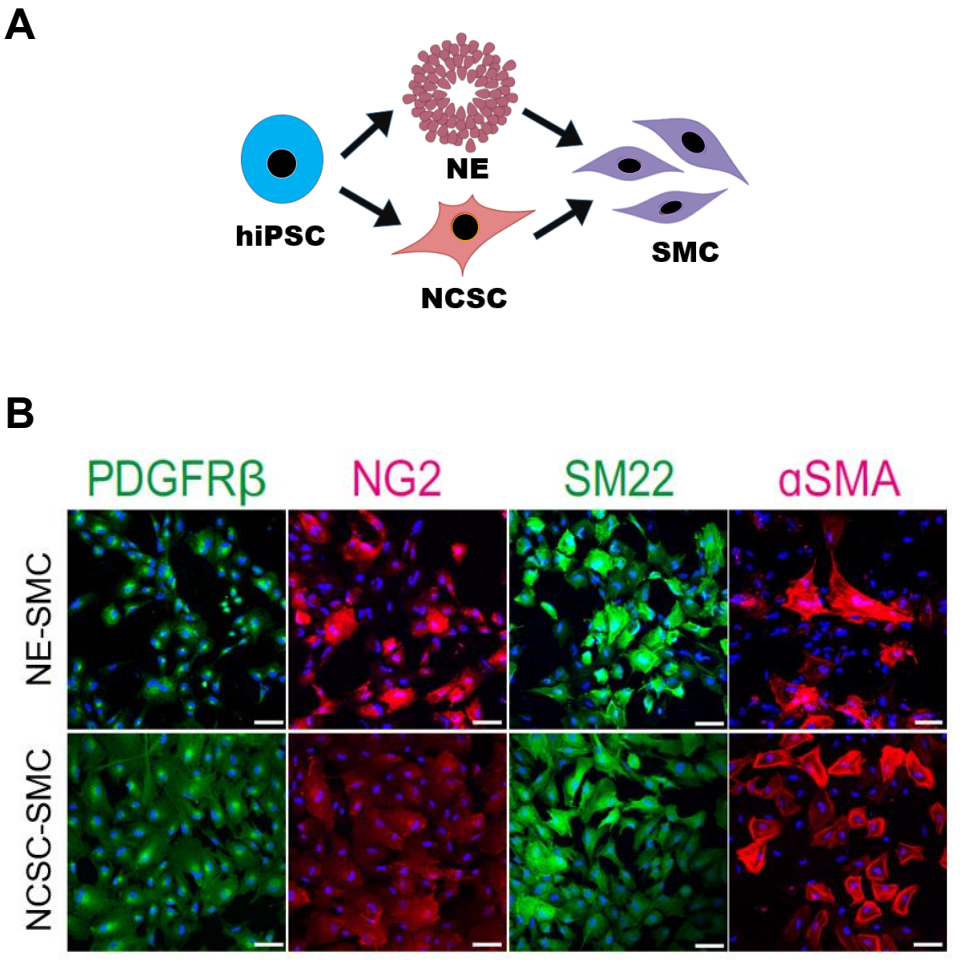

Figure 10. Neuroectoderm-derived SMC (NE-SMC) and NCSC-derived SMC (NCSC-SMC) differentiation from hiPSCs. (A) Schematic diagram of NE-SMC and NCSC-SMC differentiation. hiPSCs were differentiated into NE-SMCs for 18 days and NCSC-SMCs for 28 days. Then, neuroectodermal cells and NCSCs were commonly differentiated into SMCs by treating SMC inducers including PDGF-BB and TGFβ. (B) Immunostaining of mural cell markers in both cell types. NE-SMCs (D18) and NCSC-SMCs (D28) were stained with mural cell markers including PDGFRβ (green), NG2

(magenta), SM22 (green) and αSMA (red). Images are representatives of n = 3 independent experiments. Scale bars, 100 μm.

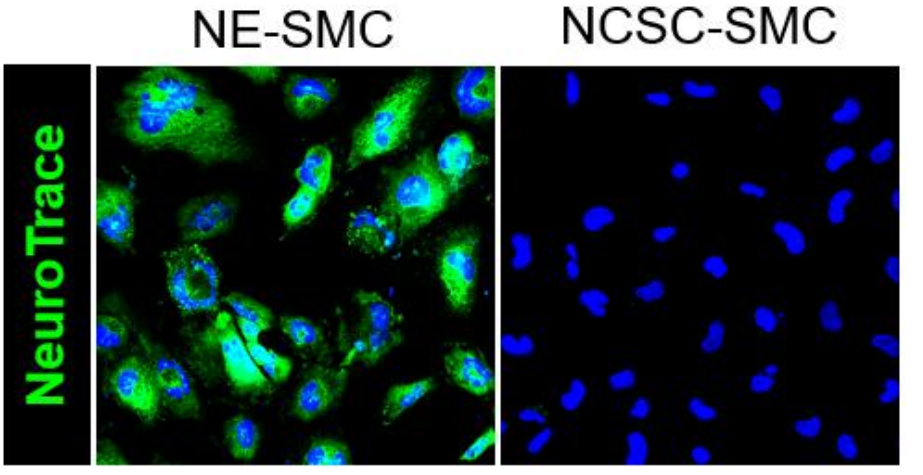

Figure 11. NeuroTrace 500/525 labels neuroectoderm-derived SMCs. Confocal microscope images of NeuroTrace 500/525 labeling in NE-SMCs and NCSC-SMCs. Cells were incubated with NeuroTrace 500/525 1:2000 diluted in E6 for 20 min at 37℃. Then, E6 medium with dye was removed from cells for subsequent fixation. NeuroTrace 500/525 specifically labels NE-SMCs. Images are representatives of $n = 3$ independent experiments. Scale bars, 50 μm

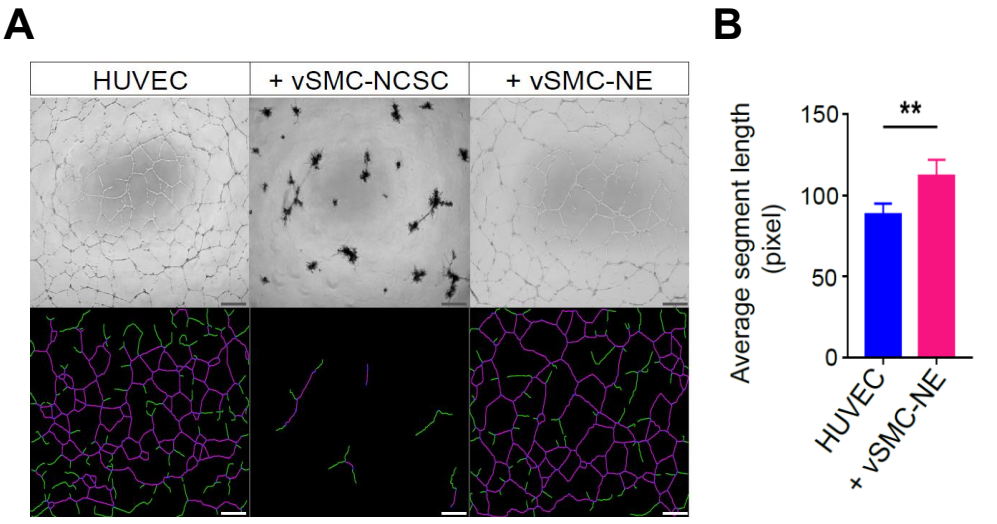

Figure 12. *In vitro* endothelial cord formation assay using HUVECs, NE-SMCs or NCSC-SMCs. (A) Representative bright field images of vascular cords (upper panels) and maps of cord networks analyzed by angiogenesis analyzer in ImageJ software, showing segments (magenta) and branches (green) (lower panels). Scale bars, 500 μm. (B) Quantification of average segment length of vascular cord networks formed by HUVECs alone or co-cultures with NE-SMCs. *P*-value was calculated by unpaired Student's *t* test. $^{**}p < 0.01$ ($n = 5$ for each group).

IV. DISCUSSION

Currently, pericyte identification *in vivo* as well as *in vitro* largely depends on transgenic promoter-dependent expression of reporter genes[2, 28, 29]. Transgenic mouse models including *Cspg4-Cre and Pdgfrb-Cre* mice are commonly used, but they have disadvantages of labeling all mural cells[28, 29]. Recently, Nikolakopoulou et al. generated pericyte-specific *Cre* lines using double promoters of *Cspg4* and *Pdgfrb* to specifically target pericytes[30]. Although the double promoters help to reduce targeting non-pericyte cell types, these promoters are still active in SMCs[30]. In case of *in vitro* studies, the use of NG2 and PDGFRβ is neither a reliable way to identify pericytes, so *in vitro*-generated pericytes are sometimes broadly referred to as pericyte-like cells or mural cells[6-8]. Our data also clearly showed the problems of pericyte identification using those markers. In our data, both hiPSC-derived pericytes and SMCs express NG2 and PDGFRβ limiting the utility of their use in distinguishing between hiPSC-derived pericytes and SMCs. The presence of αSMA in hiPSC-derived SMCs and the absence of αSMA in hiPSC-derived pericyte-like cells seem to discriminate the latter cells from the former cells; however, being αSMA-negative is inadequate to identify pericytes. Hence, a selective marker that specifically targets pericytes is necessary. In contrast to αSMA, NeuroTrace 500/525 exclusively labels pericytes without labeling SMCs. Taken together, NeuroTrace 500/525 is a selective marker which helps the precise identification of brain pericytes both *in vitro* and *in vivo*.

Cheung et al. first generated NE-SMCs, defining them as SMCs from mural cell marker expressions and contractile ability[4]. Recently, Kelleher et al. differentiated hiPSCs into SMCs by adopting a Cheung's protocol[4, 9]. Ironically, they reported that

NE-SMCs have pericyte-like attributes of supporting capillary structures, suggesting that NE-SMCs are also pericyte-like to some extent[9]. Thus, we wondered whether NeuroTrace 500/525 can also label the cells that reportedly have pericyte-like traits. We sought to compare pericyte-like NE-SMCs with the NCSC-SMCs that closely resemble primary human brain SMCs[5]. According to the previous study, NCSC-SMCs share the closest molecular signatures and functional characteristics with primary human brain SMCs than NE-SMCs[5]. We therefore generated cerebral SMCs using two protocols that derive SMCs from neural crest stem cells (NCSCs) or neuroectoderm (NE), respectively[4, 5]. Both NE-SMCs and NCSC-SMCs are differentiated from a common SMC differentiation protocol by adding SMC inducers such as PDGF-BB and TGFβ1[4, 5]. However, there is a clear distinction of NeuroTrace 500/525 uptake between NE-SMCs and NCSC-SMCs, suggesting that these cells are indeed different. We do not exactly know how these cell types are different, but we can at least conclude that NeuroTrace 500/525 discriminates cells with pericyte-like characteristics. Several studies demonstrated that pericytes have plastic properties in which pericytes can be differentiated into SMCs and vice versa[31-33]. In our data, NE-SMCs are not as potent as NCSC-derived pericyte-like cells in supporting endothelial cord formation, so they may represent a transition state between SMCs and mature pericytes.

Brain pericytes highly express genes that are related with transmembrane transporter activity, suggesting that brain pericytes are heavily involved in molecular transport at the BBB[27]. Brain pericytes have many ATP-binding cassette (ABC) and solute carrier (SLC) transporters[26,27]. In the recent study, *Atp13a5* is reported as a new CNS pericyte marker[34]. The *Atp13a5* genetic marker and the transgenic mouse model targeting *Atp13a5* allele demonstrated the high specificity to pericytes without labeling SMCs and peripheral pericytes[34]. In RNA-seq data, *Atp13a5* is abundant in mouse brain

microvessels; however, the expression of *ATP13A5* is low in human brain microvessels. *Atp13a5* can be a pericyte marker in mice, but whether it can be a pericyte marker in humans is questionable. In contrast to *ATP13A5*, we have shown that NeuroTrace 500/525, which was first reported in *in vivo* mouse studies, is also selective for human brain pericytes[10]. To our knowledge, NeuroTrace 500/525 is the only way to selectively label pericytes among vascular cells in *vitro* and *in vivo* in both humans and rodents, suggesting a universal mechanism across species. When pinpointing its transporting molecules, we can discover the endogenous molecular markers for pericytes and it will help to develop pericyte-specific antibodies and *Cre* recombinase system that will greatly expedite pericyte research.

V. CONCLUSION

This study demonstrates that the selectivity of NeuroTrace 500/525 for pericytes is also recapitulated *in vitro* as well as in human cells, suggesting that the transporting molecules responsible for NeuroTrace 500/525 are universally present in both humans and rodents. In this study, NeuroTrace 500/525 selectively labels hiPSC-derived pericytes and differentiates from other hiPSC-derived vascular cells including endothelial cells and SMCs. The use of NeuroTrace 500/525 will help not only better identify pericytes by discriminating pericytes from other vascular cells, but also facilitate the effort to identify the novel putative marker of pericytes.

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ABSTRACT (IN KOREAN)

역분화 줄기세포로부터 분화된 사람 혈관주위세포 모델에서 NeuroTrace에 의한 혈관주위세포 특이성 확인

< 지도교수 김철훈 >

연세대학교 대학원 의과학과

김 서 영

뇌의 혈관주위세포는 모세혈관 바깥을 둘러싸고 있는 가늘고 긴 세포로 혈액뇌장벽을 조절하는 데 있어 중요한 역할을 한다. 혈관주위세포의 대표적인 기능으로는 혈액뇌장벽 융합막 강화와 투과성 및 뇌 혈류조절이 보고되어 있다. 혈관주위세포의 기능장애는 혈액뇌장벽 붕괴와 혈류장애를 일으킴으로써 뇌졸중, 혈관성 치매와 같은 뇌혈관 질환을 유발하기도 한다.

혈관주위세포의 역할과 기능 연구를 하기 위해서는 혈관주위세포를 확인할 수 있는 단백질을 특정하는 것이 중요하다. 하지만 혈관주위세포에 특이성이 있을 것으로 여겨졌던 단백질들은 희소돌기신경세포와 평

활근세포 등 다른 세포들과 공유하고 있어 혈관주위세포만을 구별해낼 수 있는 특정한 마커가 없다. 따라서 조직학적 위치 정보가 없는 *in vitro* 에서는 혈관주위세포를 정의하는데 더 큰 어려움이 있다. 본 연구는 최근에 보고된 생쥐 뇌에서 모세혈관의 혈관주위세포를 특정하게 염색하는 물질인 NeuroTrace 500/525 를 사람 역분화줄기세포에서 유도된 혈관주위세포 모델에 적용함으로써 *in vitro* 모델과 사람혈관주위세포에서도 특이성을 검증해보고자 하였다. Neurovascular unit을 구성하는 혈관주위세포, 내피세포, 평활근세포를 사람 역분화줄기세포에서 분화시켜서 NeuroTrace 500/525 처리를 하였을 때 혈관주위세포에서만 단독적으로 염색이 된 것을 확인하였다. 특히 구분하기 어렵다는 평활근세포로부터 혈관주위세포를 구별해낼 수 있었다. 이를 기반으로 NeuroTrace 500/525의 혈관주위세포 특이적 염색은 *in vitro* 혈관주위세포 구별방법으로 유용하게 쓰일 것으로 예상되며 향후 혈관주위세포 관련 내인성 단백마커 발굴과 기능 연구에 도움이 될 것으로 기대한다.

핵심되는 말 : 혈관주위세포, 뇌, 혈액뇌장벽, 사람 역분화줄기세포, neurotrace 500/525, 평활근세포