

MICRO REPORT

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NeuroTrace 500/525 identifies human induced pluripotent stem cell-derived brain pericyte-like cells

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Abstract

In the CNS, pericytes are important for maintaining the blood–brain barrier (BBB) and for controlling blood flow. Recently, several methods were suggested for the differentiation of human pluripotent stem cells (hPSCs) into brain mural cells, specifically pericytes or vascular smooth muscle cells (vSMCs). Unfortunately, identifying the pericytes from among such hPSC-derived mural cells has been challenging. This is due both to the lack of pericyte-specific markers and to the loss of defining anatomical information inherent to culture conditions. We therefore asked whether NeuroTrace 500/525, a newly developed dye that shows cell-specific uptake into pericytes in the mouse brain, can help identify human induced pluripotent stem cell (hiPSC)-derived brain pericyte-like cells. First, we found that NeuroTrace 500/525 specifically stains primary cultured human brain pericytes, confirming its specificity in vitro. Second, we found that NeuroTrace 500/525 specifically labels hiPSC-derived pericyte-like cells, but not endothelial cells or vSMCs derived from the same hiPSCs. Last, we found that neuroectoderm-derived vSMCs, which have pericyte-like features, also take up NeuroTrace 500/525. These data indicate NeuroTrace 500/525 is useful for identifying pericyte-like cells among hiPSC-derived brain mural cells.

Keywords: Pericytes, hiPSCs, NeuroTrace 500/525

Pericytes are vascular mural cells (VMCs) that surround endothelial cells. They support vessel stability, blood flow regulation, angiogenesis, and inflammation [1]. The blood vessel coverage by pericytes is highest in the CNS [2]. Brain pericytes ensheath the capillary endothelium and contribute to BBB integrity by working with specialized endothelial cells, astrocytic end-feet, and neurons in structures called neurovascular units [1]. It has been difficult to study pericytes because of a lack of pericyte-specific marker molecules. Neural/glial antigen 2 (NG2) and platelet-derived growth factor receptor β (PDGFR β) are often used as pericyte markers, but both

are also expressed in vSMCs, another VMC subtype [3]. Recently, NeuroTrace 500/525 was reported as an exclusive marker for pericytes in the mouse brain [4]. It was originally used to identify neurons in fixed brain tissues, but when administered to the brains of *Pdgfr β -Cre; tdTomato* transgenic mice, the VMCs of which express tdTomato, NeuroTrace 500/525 labeled only tdTomato-positive cells ensheathing capillaries (pericytes). It did not label the ring-like cells that line pre-capillaries or arterioles (vSMCs) [4]. This pericyte-specific uptake of NeuroTrace 500/525 suggests the presence of molecular transport mechanisms exclusive to pericytes. Although these results are promising, the utility of NeuroTrace 500/525 has not yet been tested in human cells, which may have different transport mechanisms, or in in vitro culture systems that lack many of the non-autologous effects of in vivo systems.

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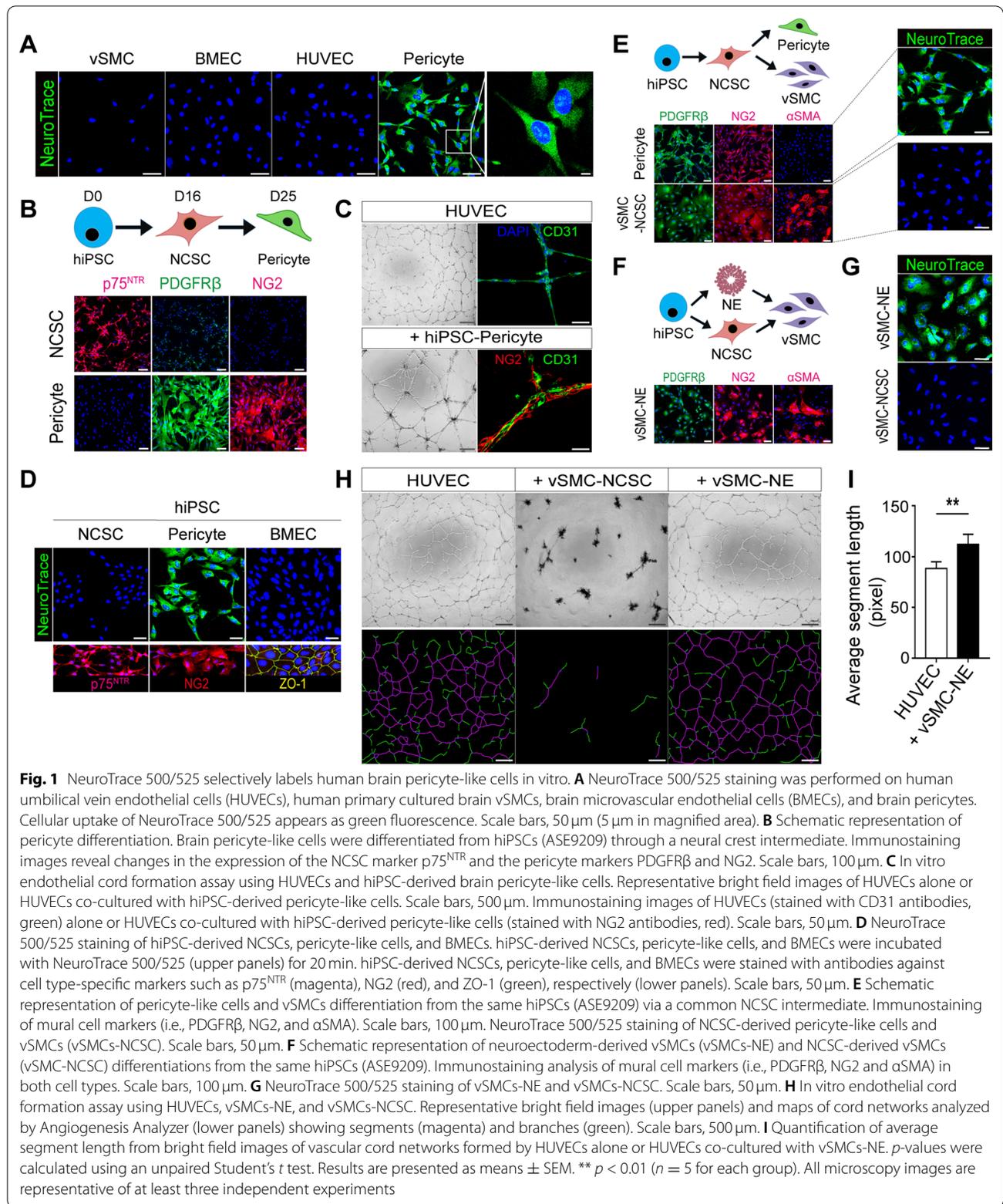
There are various protocols for differentiating hiPSCs into cell types of interest. Recently, differentiation protocols to produce human VMCs were developed, making it possible to produce human pericyte-like cells or vSMCs in culture [5–8]. Making human pericytes in vitro will surely improve the relevance of disease modeling and drug screening work related to pericytes. The problem is that both hiPSC-derived pericyte-like and vSMC cells express NG2 and PDGFR β without any anatomical information that would otherwise, in live mice, help discriminate these two mural cell types. Thus, we asked whether NeuroTrace 500/525 could help us discern pericyte-like cells differentiated from hiPSCs.

To determine whether NeuroTrace 500/525 specifically labels human pericytes in vitro, we added the dye to various types of primary cultured cells. We found green fluorescence only in human primary cultured brain pericytes, indicating that NeuroTrace 500/525 exclusively labels human pericytes in culture conditions (Fig. 1A). Next, we co-differentiated the same hiPSC line into endothelial cells and pericyte-like cells via neural crest stem cells (NCSCs), which are the embryonic precursor to forebrain mural cells [9] (Fig. 1B, D; see Additional file 1 for the detailed methods). We observed that as the NCSCs progressed to pericyte-like cells, their expression of the NCSC marker p75^{NTR} fell, while their expression of NG2 and PDGFR β increased (Fig. 1B, Additional file 2: Fig. S1). Among DAPI-positive cells, $95.9 \pm 1.2\%$ were NG2-positive and $97.1 \pm 0.7\%$ were PDGFR β -positive. We also found pericyte-like cells can self-assemble with endothelial cells in an endothelial cord forming assay in vitro and promote the formation of longer vascular cords (Fig. 1C). These data suggest hiPSC-derived pericyte-like cells possess the key attributes of brain pericytes. After adding NeuroTrace 500/525 to hiPSC-derived NCSCs, endothelial and pericyte-like cells, we found that the dye specifically stains pericyte-like cells (Fig. 1D and $97.2 \pm 1.2\%$ of DAPI-positive cells were NeuroTrace-positive).

Next, we differentiated the same hiPSC line into pericyte-like cells and vSMCs to determine whether NeuroTrace 500/525 can discriminate these two VMC subtypes. We noted that both cell types express NG2 and PDGFR β , ruling out these markers for the discrimination of pericytes from vSMCs (Fig. 1E; Additional file 2: Fig. S2). Interestingly, we found only vSMCs express α SMA, suggesting that the pericyte-like cells we produced may recapitulate capillary pericytes [10] (Fig. 1E). When we added NeuroTrace 500/525, we found that these capillary pericyte-like cells exhibit clear and selective uptake ($97.7 \pm 2.1\%$ of DAPI-positive cells were NeuroTrace-positive), whereas vSMCs did not exhibit any dye uptake (Fig. 1E).

Next, we focused on two protocols designed to differentiate hiPSCs into vSMCs. The main difference between the two approaches is that the first one induces vSMCs directly from neuroectodermal cells (vSMCs-NE) [7], while the second one induces vSMCs via NCSCs (vSMCs-NCSC) [8]. vSMCs-NE reportedly have enough pericyte-like properties to support capillary structures [11]. Furthermore, the molecular signature of vSMCs-NCSC is more like that of human brain vSMCs [8]. We therefore wondered whether NeuroTrace 500/525 can discriminate vSMCs-NCSC and vSMCs-NE. We split and differentiated the same hiPSC line into vSMCs-NCSC and vSMCs-NE [7, 8] (Fig. 1F). Immunostaining revealed that vSMCs-NE express NG2, PDGFR β and α SMA (Fig. 1F). We were surprised to find that NeuroTrace 500/525 also labels vSMCs-NE (Fig. 1G and $96.4 \pm 0.5\%$ of DAPI-positive cells were NeuroTrace-positive). To determine whether vSMCs-NE have pericyte-like attributes, we performed a Matrigel-based endothelial cord forming assay by co-culturing HUVECs with vSMCs-NCSC or vSMCs-NE. We found vSMCs-NE co-cultured with HUVECs contribute to forming longer capillary cords; the vSMCs-NCSC seem to pull the growing cords apart due to their contractile properties (Fig. 1H, I). Because vSMCs-NE are not as potent as NCSC-derived pericyte-like cells in supporting endothelial cord formation, they may represent a transition state between vSMCs and mature pericytes. We also confirmed the specificity of NeuroTrace 500/525 in cells derived from a different hiPSC line [12] (Additional file 2: Fig. S3).

These data suggest NeuroTrace 500/525 is a useful marker for the discrimination of hiPSC-derived pericyte-like cells. To our knowledge, NeuroTrace 500/525 is the only way to selectively label pericytes among vascular cells in vitro and in vivo in both human and rodents, suggesting a universal mechanism across species. NeuroTrace 500/525 enters neither neurons nor glia in mouse brains [4]. Considering this selectivity, it is unlikely that NeuroTrace 500/525 stains neural cells of human origin. Hence, it is plausible that the transport system for NeuroTrace 500/525 comprises molecules expressed in pericytes of both humans and rodents. Thus, although NeuroTrace 500/525 has proven to be a useful marker of pericytes, its utility will likely be extended when the molecules that transport it into pericytes are identified. Their discovery will pave the way to the development of pericyte-specific antibodies and even a Cre recombinase line that will greatly expedite pericyte research.



Abbreviations

hiPSCs: Human induced pluripotent stem cells; vSMCs: Vascular smooth muscle cells; NCSCs: Neural crest stem cells; NE: Neuroectoderm.

Supplementary Information

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Additional file 1. Materials and methods.

Additional file 2. Figure S1–S3.

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Authors' contributions

SYK and CHK conceptualized and designed the research. SYK, JR and JC conducted the experiments and analysed the data. SYK and CHK prepared the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

All data and materials are available upon request.

Declarations**Ethics approval and consent to participate**

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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