Research Article

Nestin Protein Is Phosphorylated in Adult Neural Stem/Progenitor Cells and Not Endothelial Progenitor Cells

Jun Namiki,1 Sayuri Suzuki,1 Takeshi Masuda,2 Yasushi Ishihama,2,3 and Hideyuki Okano4

1 Department of Emergency and Critical Care Medicine, Keio University School of Medicine, Shinanomachi 35, Shinjuku, Tokyo 160-8582, Japan
2 Institute for Advanced Biosciences, Keio University, 403-1 Daiba-ji, Tsuruoka, Yamagata 997-0017, Japan
3 Graduate School of Pharmaceutical Sciences, Kyoto University, Kyoto 606-8501, Japan
4 Department of Physiology, Keio University School of Medicine, Shinanomachi 35, Shinjuku, Tokyo 160-8582, Japan

Correspondence should be addressed to Jun Namiki, namikijun@a8.keio.jp

Received 1 July 2012; Accepted 3 August 2012

Academic Editor: Oscar Gonzalez-Perez

Copyright © 2012 Jun Namiki et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

An intermediate filament protein, Nestin, is known as a neural stem/progenitor cell marker. It was shown to be required for the survival and self-renewal of neural stem cells according to the phenotypes of Nestin knockout mice. Nestin expression has also been reported in vascular endothelial cells, and we recently reported Nestin expression in proliferating endothelial progenitor cells, but not in mature endothelial cells. Using quantitative phosphoproteome analysis, we studied differences in phosphorylation levels between CNS Nestin in adult neural stem cells and vascular Nestin in adult bone-marrow-derived endothelial progenitor cells. We detected 495 phosphopeptides in the cell lysates of adult CNS stem/progenitor cells and identified 11 significant phosphorylated amino acid residues in the Nestin protein. In contrast, endothelial progenitor cells showed no significant phosphorylation of Nestin. We also measured neoplastic endothelial cells of the mouse brain and identified 13 phosphorylated amino acid residues in the Nestin protein. Among the 11 phosphorylated amino acids of adult CNS Nestin, five (S565, S570, S819, S883, and S886) were CNS Nestin-specific phosphorylation sites. Detection of the CNS-specific phosphorylation sites in Nestin, for example, by a phospho-specific Nestin antibody, may allow the expression of CNS Nestin to be distinguished from vascular Nestin.

1. Introduction

Nestin is a class VI intermediate filament protein expressed in undifferentiated central nervous system (CNS) cells during development. The protein is known as a neural stem/progenitor cell marker and required for the survival and self-renewal of neural stem cells (NSCs) [1]. Nestin expression is downregulated when CNS stem/progenitor cells differentiate into neurons or glial cells [2, 3], and the expression is kept in adult CNS stem/progenitor cells that reside in the forebrain neurogenic regions [4, 5]. Nestin expression has also been reported in vascular endothelial cells (ECs) from a variety of adult human non-CNS tissues [6, 7]. We recently reported Nestin expression in proliferating endothelial progenitor cells (EPCs), but not in mature ECs [8]. We utilized E/nestin: EGFP transgenic mice using its second intronic enhancer element to study neural-specific nestin gene expression [9, 10] and demonstrated that vascular nestin expression is not activated by the CNS-specific nestin enhancer of the nestin gene [8]. This finding indicated that the Nestin expressed in EPCs is cytochemically similar to the protein expressed in CNS stem/progenitor cells, but the regulatory mechanism of gene expression is different.

The reversible phosphorylation of proteins results in a conformational change that alters their function. Many proteins, including cellular receptors, enzymes, and intracellular signaling molecules, are activated/deactivated by phosphorylation/dephosphorylation. Thus, reversible phosphorylation plays a significant role in the regulation of cellular processes. Nestin protein in the cytoplasm of CNS stem/progenitor cells is thought to play a role in distributing Vimentin from copolymerized intermediate filaments to daughter cells during cell division [11]. Elevated phosphorylation of Nestin...
has been observed to accompany the mitotic reorganization
of Nestin in an immortalized CNS precursor rat cell line
[12]. Concerning the developing mouse brain, more than
500 phosphorylation sites on proteins, including Nestin, have
been identified by phosphoproteomic analysis [13]. How-
ever, Nestin phosphorylation has not been investigated in
adult NSCs. Using quantitative phosphoproteome analysis,
we demonstrate in the present study that different phos-
phorylation levels are found among CNS Nestin in adult NSCs
and vascular Nestin in adult bone-marrow-derived EPCs.

2. Materials and Methods

2.1. Animals. Adult (8 to 10 weeks old) wild-type C57BL/6J
mice were purchased from SLC (Shizuoka, Japan). All
animal-related procedures were approved by the Laboratory
Animal Care and Use Committee of Keio University and
conducted in accordance with the guidelines of the National
Institutes of Health, USA.

2.2. Primary Neural Stem/Progenitor Cell Culture. Neuro-
spheres were generated from adult mouse forebrain as
described previously [8, 10, 14, 15]. Briefly, the striata
from adult mice were dissected, incubated with trypsin
solution for 15 min at 37°C, triturated, and then trypsin
inhibitor solution added. Dissociated cells (5000 cells/mL)
were seeded in neurosphere culture medium composed
of DMEM-F12 (1:1), glucose (0.6%), glutamine (2 mM),
sodium bicarbonate (13.4 mM), HEPES (5 mM), insulin
(25 μg/mL), transferrin (100 μg/mL), progesterone (20 nM),
sodium selenate (30 nM), and putrescine (60 nM) supple-
mented with recombinant human epidermal growth factor
(EGF, 20 ng/mL) and recombinant human basic fibroblast
growth factor (bFGF, 20 ng/mL). Cells were cultured for 7
days in vitro (DIV) and formed floating cell clusters of neural
stem/progenitor cells (neurospheres). Primary neurospheres
were collected and mechanically triturated. Dissociated cells
were counted and stored at −20°C.

2.3. EPC Culture. EPCs were cultured from mononuclear
cells (MNCs) under previously reported culture conditions
[8]. The femurs and tibias of adult mice were crushed,
suspended in αMEM (#11900, Gibco Invitrogen, Carlsbad,
CA) supplemented with 10% fetal bovine serum (FBS)
and 1% penicillin G (10,000 units/mL) streptomycin sul-
phate (10,000 μg/mL) (PS), and filtered through a 70-μm
filter (Cell Strainer #352350, Falcon, Bedford, MA). MNCs
were isolated from bone marrow cells by Ficoll density-
gradient centrifugation (Ficoll-Paque Plus, 1.077 g/mL, GE
Healthcare, Uppsala, Sweden). Cells (1 × 10^6 cell/mL) were
plated on fibronectin-coated 6-well plates (#140675, Nunc,
Roskilde, Denmark) in endothelial basal medium supple-
mented with 5% FBS, vascular endothelial growth factor
(VEGF), bFGF, recombinant analog of insulin-like growth
factor-1 (R²-IGF-1), EGF, hydrocortisone, ascorbic acid, and
gentamicin/amphotericin-B (EGM-2-MV Bullet Kit CC-3202,
Lonza, Walkersville, MD). The medium was changed
after 24 hours to remove nonadherent cells and renewed
every week. At 21 DIV, cells were lifted by incubation with
0.25% trypsin and 1 mM EDTA and then stored at −20°C.

Although a unique EPC marker has not been identified,
EPCs are characterized as cells with a high proliferative
potential that display typical endothelial characteristics and
differentiate into ECs in vitro [16]. EPCs obtained under the
above culture conditions were positive for the proliferation
marker Ki67, positive for EC lineage marker CD31 and vas-
cular endothelium cadherin or the uptake of 1,1′ dioctadecyl-
3,3′,3′-tetramethylindolo-carbocyanine perchlorate Ac-LDL
(Dii–Ac-LDL), negative for the mature EC marker von
Willebrand factor (vWF), and capable of differentiating into
mature ECs [8].

2.4. EC Line. To compare phosphorylation between CNS
Nestin and vascular endothelial Nestin in proliferative cells
further, we prepared neoplastic ECs. Cells from a mouse
brain endothelioma cell line (bEnd.3 cells CRL-2299, ATCC,
Manassas, VA) were characterized as proliferative endothelial
cells expressing vascular Nestin similar to EPCs and positive
for mature EC marker vWF [8].

The EC line was cultured according to the manufac-
turer’s instructions. Briefly, cells were maintained in DMEM
(#12699, Gibco Invitrogen) supplemented with 10% FBS and
1% PS. The medium was renewed every 3 to 4 days. Cells
were harvested by incubation with 0.25% trypsin and 1 mM
EDTA and stored at −20°C.

2.5. Quantitative Phosphoproteome Analysis. Cells were pro-
cessed for phosphoproteome analysis based on mass spec-
trometry (MS) coupled with miniaturized on-line liquid
chromatography (LC). Proteins were extracted from cells
(100,000 cells from adult neurospheres; 1,000,000 cells
from EPCs and the neoplastic ECs) using 12 mM sodium
deoxycylolate and 12 mM sodium lauyrol sarcosinate, and
digested with Lys-C and trypsin [17]. Phosphopeptides were
enriched by aliphatic hydroxy acid-modified metal oxide
chromatography with titania [18] and analyzed by nanoLC-
MS/MS using an LTQ-Orbitrap instrument (Thermo Fisher
Scientific, Bremen, Germany). Peptides and proteins were
identified using Mascot version 2.3 (Matrix Science, Lon-
don) with the SwissProt database. Label-free quantitation
was performed based on the peak areas of extracted ion
chromatograms for identified phosphopeptides using Mass
Navigator (Mitsui Knowledge Industry, Tokyo, Japan).

3. Results

3.1. Protein Phosphorylation of CNS Stem/Progenitor Cells,
EPCs, and Neoplastic ECs. CNS stem/progenitor cells were
obtained from striata of the lateral wall of the lateral
ventricles in the adult mouse brain and grown as neuro-
spheres in vitro. Phosphoproteome analysis detected 495
phosphopeptides in the cell lysates (Table 1). Approximately
90% of the peptides detected in neurosphere cells were phos-
phorylated. A similar percentage of phosphopeptides was
measured in neoplastic ECs. However, approximately 60%
of peptides in nonneoplastic proliferative endothelial cells,
Table 1: Protein phosphorylation of adult CNS stem/progenitor cells, EPCs, and neoplastic ECs.

|                | pPeptides (pPeptides/total peptides) | pSites (pSite/total pSites) | Multi-pPeptides (single or multi-pSite peptides/total pPeptides) |
|----------------|---------------------------------------|----------------------------|---------------------------------------------------------------|
| Neurospheres   | 495 (91.2%)                           | 443 (84.2%)                | 401 (81.0%)                                                    |
| EPCs           | 250 ± 5 (59.7%)                       | 194 ± 8 (87.4%)            | 228 ± 6 (91.0%)                                                |
| Neoplastic ECs | 980 (97.8%)                           | 896 (88.1%)                | 675 (68.9%)                                                    |

pPeptides, phosphopeptides; pSites, phosphorylation sites; multi-pPeptides, multi-phosphorylated peptides; 1p, single-phosphorylated site; 2p, two phosphorylated sites. Data are mean ± standard deviation.

Neurospheres: Serine 495 (91.2%), Threonine 71 (13.5%), Tyrosine 12 (2.3%); EPCs: Serine 194 ± 8 (90.0%), Threonine 8 ± 0 (3.6%), Tyrosine 19 ± 0 (7.4%); Neoplastic ECs: Serine 896 (88.1%), Threonine 103 (10.1%), Tyrosine 18 (1.8%).

EPCs, were phosphorylated, indicating that intracellular and cell membrane proteins were activated overall in neurosphere cells compared to EPCs. Generally, phosphorylation occurs most commonly on serine, followed by threonine. More than 80% of phosphorylated sites were serine residues in our samples, and the ratio of phosphorylated amino acids was not different between neurosphere cells, EPCs, and neoplastic ECs (Table 1). We tabulated phosphorylated proteins and their phosphorylated amino acid residues in Figure 1 in Supplementary Material (available online at doi:10.1155/2012/430138).

3.2. Nestin Phosphorylation of CNS Stem/Progenitor Cells, EPCs, and Neoplastic ECs. Quantitative phosphoproteome analysis identified 10 phosphopeptides and 11 significant phosphorylated amino acid residues (a peak area >1.E+04) in the Nestin protein from adult neurosphere cells (Figure 1). In contrast, EPCs derived from adult mouse bone marrow showed no significant phosphorylation of Nestin (n = 2). In neoplastic ECs, 13 significant phosphorylated amino acid residues were identified. Thus, the finding that Nestin is not phosphorylated in EPCs is not likely due to the tissue specificity of ECs. All phosphorylated amino acid residues found in neurosphere cells and neoplastic ECs were serine. Although phosphothreonine was reported in samples of Nestin from an immortalized rat cell line [12] and human HeLa cells (cervical carcinoma) [19, 20], we did not detect the phosphorylation of threonine residues in the Nestin proteins from our samples. Five of the phosphorylated amino acid residues (S565, S570, S575, S570, and S570) were detected in neurosphere cells only, eight (S575, S668, S813, S816, S1216, S1562, S1860, and S1861) were detected in EPCs, and four (S575, S570, S570, and S570) were detected in neoplastic ECs.
in neoplastic ECs only, and six (S169, S728, S731, S1010, S1565, and S1837) were detected in both neurosphere cells and neoplastic ECs (Figure 1).

In CNS stem/progenitor cells, Nestin protein preferentially forms heterodimers and heterotetramers with a variety of intermediate filament proteins, particularly type III Vimentin and type IV α-Internexin [21, 22]. Phosphoproteome analysis detected phosphorylation of Vimentin but not α-Internexin from the samples of adult neurospheres, EPCs, and neoplastic ECs (Figure 1 in Supplementary Material).

4. Discussion

Recent investigations of nestin-knockout mice have reported that Nestin deficiency results in embryonic lethality with the neuroepithelium of the developing neural tube exhibiting low numbers of NSCs and high levels of apoptosis [1]. The downregulation of nestin in the embryonic cerebral cortex using small interference RNAs against nestin mRNA results in G1 cell-cycle arrest and a severe reduction in the generation of neurons [23]. However, no data have been reported on the in vivo function of adult Nestin. Transient transfection of nestin-non-expressing cells with an expression vector carrying rat nestin cDNA has been shown to promote the disassembly of phosphorylated Vimentin intermediate filaments in the cytoplasm during mitosis [11]. Our phosphoproteome analysis detected phosphorylated Vimentin in the adult neurosphere sample. Thus, Nestin in adult NSCs is likely to mediate the distribution of Vimentin protein to daughter cells during self-renewal and neurogenesis.

In a rat neuronal progenitor cell line, the mitotic reorganization of Nestin was accompanied by the elevated phosphorylation of Nestin, and T316 was identified as a Nestin phosphorylation site [12]. Phosphorylated threonine was not detected in Nestin from adult CNS stem/progenitor cells, EPCs, or neoplastic ECs in the present study. However, we identified 11 significant phosphorylation sites at serine residues in Nestin protein from adult mouse CNS stem/progenitor cells using quantitative phosphoproteome analysis. The difference in phosphorylated amino acids and the number of phosphorylated sites may be due to technological advances in phosphoproteome analysis and/or the difference in cell sources. Phosphorylated serine residues have been reported in Nestin protein from the brain of mouse embryos [13] and mouse skin melanoma [24] and have been assumed by similar data in human HeLa cells [19, 20]. Among the 11 phosphorylated serine residues we identified in Nestin protein, only two (S565 and S1010) were reported previously; the other nine (S169, S570, S728, S731, S819, S883, S886, S1565, and S1837) were newly identified in the present study.

Nestin protein is expressed not only in NSCs, but also in tissue stem/progenitor cells beyond the germ layers, including mesenchymal stem cells [25], vascular endothelium [6–8], muscle [26–28], testes [29], and teeth [30]. Nestin is also abundant in progenitor cells derived from embryonic stem cells that have the potential to develop into neuroectodermal, endodermal, and mesodermal lineages [31]. We recently reported that Nestin is expressed in proliferating ECs and may be useful as a marker of neovascularization [8]. Nestin expression has been reported in the angiogenic endothelium of cancers [32, 33]. Independent cell-type-specific elements of the nestin gene are identified in transgenic mice; the first intron directs reporter gene expression to the mesodermal somite, and the second intron contains enhancer that functions in NSCs [28]. Although the regulatory mechanisms underlying nestin gene expression in proliferative vascular cells are different from those in NSCs, the protein expression of vascular Nestin is cytochemically similar to CNS Nestin [8]. The phosphorylation of Nestin protein from adult neurospheres can allow it to be distinguished from Nestin in EPCs. Although Nestin phosphorylation was also observed in neoplastic ECs, our phosphoproteome analysis identified CNS-specific phosphorylation sites, suggesting that a phospho-specific Nestin antibody may distinguish between the expression of CNS and vascular Nestin proteins.

5. Conclusions

Quantitative phosphoproteome analysis identified phosphorylated serine residues in Nestin from adult mouse CNS stem/progenitor cells. Phosphorylation was not observed in Nestin from EPCs. Detection of the CNS-specific phosphorylation sites in Nestin, for example, by a phospho-specific Nestin antibody, may allow the expression of CNS Nestin to be distinguished from vascular Nestin.

Acknowledgment

This study was supported by Grants-in-Aid for Scientific Research from the Japanese Ministry of Education, Culture, Science, Sports, and Technology to J.N.

References

[1] D. Park, A. P. Xiang, F. F. Mao et al., "Nestin is required for the proper self-renewal of neural stem cells," Stem Cells, vol. 28, no. 12, pp. 2162–2171, 2010.
[2] J. Dahlstrand, M. Lardelli, and U. Lendahl, "Nestin mRNA expression correlates with the central nervous system progenitor cell state in many, but not all, regions of developing central nervous system," Developmental Brain Research, vol. 84, no. 1, pp. 109–129, 1995.
[3] K. Frederikson and R. D. G. McKay, "Proliferation and differentiation of rat neuroepithelial precursor cells in vivo," Journal of Neuroscience, vol. 8, no. 4, pp. 1144–1151, 1988.
[4] C. M. Morshed, B. A. Reynolds, C. G. Craig et al., "Neural stem cells in the adult mammalian forebrain: a relatively quiescent subpopulation of subependymal cells," Neuron, vol. 13, no. 5, pp. 1071–1082, 1994.
[5] B. A. Reynolds and S. Weiss, "Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system," Science, vol. 255, no. 5052, pp. 1707–1710, 1992.
[6] T. Klein, Z. Ling, H. Heimberg, O. D. Madsen, R. S. Heller, and P. Serup, "Nestin is expressed in vascular endothelial cells
in the adult human pancreas,” *Journal of Histochemistry and Cytochemistry*, vol. 51, no. 6, pp. 697–706, 2003.

[7] J. Mokry, D. Cizkova, S. Filip et al., “Nestin expression by newly formed human blood vessels,” *Stem Cells and Development*, vol. 13, no. 6, pp. 658–664, 2004.

[8] S. Suzuki, J. Namiki, S. Shibata, Y. Mastuzaki, and H. Okano, “The neural stem/progenitor cell marker nestin is expressed in proliferative endothelial cells, but not in mature vasculature,” *Journal of Histochemistry and Cytochemistry*, vol. 58, no. 8, pp. 721–730, 2010.

[9] C. B. Johansson, C. Lothian, M. Molin, H. Okano, and S. Suzuki, J. Namiki, S. Shibata, Y. Mastuzaki, and H. Okano, “Nestin expression in vimentin intermediate filaments during mitosis,” *Molecular Biology of the Cell*, vol. 14, no. 4, pp. 1468–1478, 2003.

[10] J. V. Olsen, B. Blagoev, F. Gnad et al., “Global, stem cell-specific phosphorylation dynamics in signaling networks,” *Molecular and Cellular Proteomics*, vol. 6, no. 6, pp. 1103–1109, 2007.

[11] C. Eliasson, C. Sahlgren, C. H. Berthold et al., “Intermediate filament protein partnership in astrocytes,” *The Journal of Biological Chemistry*, vol. 274, no. 34, pp. 23996–24006, 1999.

[12] J. E. Eriksson, “The intermediate filament protein nestin mediates filament nestin during skeletal muscle development,” *Journal of Cell Science*, vol. 106, part 4, pp. 1291–1300, 1993.

[13] C. M. Sahlgren, A. Mikhailov, J. Hellman et al., “Mitotic reorganization of the intermediate filament protein nestin involves phosphorylation by cdc2 kinase,” *The Journal of Biological Chemistry*, vol. 276, no. 19, pp. 16456–16463, 2001.

[14] A. Kawaguchi, T. Miya, K. Sawamoto et al., “Nestin-GFP transgenic mice: visualization of the self-renewal and multipotency of CNS stem cells,” *Molecular and Cellular Neuroscience*, vol. 17, no. 2, pp. 259–273, 2001.

[15] Y. H. Chou, S. Khoun, H. Herrmann, and R. D. Goldman, “Nestin promotes the phosphorylation-dependent disassembly of vimentin intermediate filaments during mitosis,” *Molecular Biology of the Cell*, vol. 14, no. 4, pp. 1468–1478, 2003.

[16] F. Timmermans, J. Plum, M. C. Yöder, D. A. Ingram, B. Vandekerckhove, and J. Case, “Endothelial progenitor cells: identity defined?” *Journal of Cellular and Molecular Medicine*, vol. 13, no. 1, pp. 87–102, 2009.

[17] T. Masuda, C. Eliasson, S. Sahlgren, C. H. Berthold et al., “Intermediate filament protein partnership in astrocytes,” *The Journal of Biological Chemistry*, vol. 274, no. 34, pp. 23996–24006, 1999.

[18] M. Aihara, K. I. Sugawara, S. Torii et al., “Angiogenic endothelium-specific nestin expression is enhanced by the first intron of the nestin gene,” *Molecular and Cellular Life Sciences*, vol. 61, no. 19-20, pp. 2510–2522, 2004.

[19] A. M. Kachinsky, J. A. Dominov, and J. B. Miller, “Intermediate filament protein partnership in astrocytes,” *The Journal of Histochemistry and Cytochemistry*, vol. 43, no. 8, pp. 843–847, 1995.

[20] K. Froydman, L. J. Pelliniemi, U. Lendahl, I. Virtanen, and J. E. Eriksson, “The intermediate filament protein nestin occurs transiently in differentiating testis of rat and mouse,” *Differentiation*, vol. 61, no. 4, pp. 243–249, 1997.

[21] K. Terling, A. Rass, T. A. Mitsiadis, K. Fried, U. Lendahl, and J. Wroblewski, “Expression of the intermediate filament nestin during rodent tooth development,” *International Journal of Developmental Biology*, vol. 39, no. 6, pp. 947–956, 1995.

[22] C. Wiese, A. Rolletschek, G. Kania et al., “Nestin expression—a property of multi-lineage progenitor cells?” *Cellular and Molecular Life Sciences*, vol. 61, no. 19-20, pp. 2510–2522, 2004.

[23] C. Eliasson, C. Sahlgren, C. H. Berthold et al., “Intermediate filament protein partnership in astrocytes,” *The Journal of Biological Chemistry*, vol. 274, no. 34, pp. 23996–24006, 1999.

[24] J. E. Eriksson, “The intermediate filament protein nestin mediates filament nestin during skeletal muscle development,” *Journal of Cell Science*, vol. 106, part 4, pp. 1291–1300, 1993.

[25] S. Mezendi, T. V. Michurina, F. Ferraro et al., “Mesenchymal and haematopoietic stem cells form a unique bone marrow niche,” *Nature*, vol. 466, no. 7308, pp. 829–834, 2010.

[26] A. M. Kachinsky, J. A. Dominov, and J. B. Miller, “Intermediate filament protein partnership in astrocytes,” *The Journal of Histochemistry and Cytochemistry*, vol. 43, no. 8, pp. 843–847, 1995.

[27] T. Sejersen and U. Lendahl, “Transient expression of the intermediate filament nestin during skeletal muscle development,” *Journal of Cell Science*, vol. 106, part 4, pp. 1291–1300, 1993.

[28] L. Zimmerman, B. Parr, U. Lendahl et al., “Independent regulatory elements in the nestin gene direct transgene expression to neural stem cells or muscle precursors,” *Neuron*, vol. 12, no. 1, pp. 11–24, 1994.

[29] K. Froydman, L. J. Pelliniemi, U. Lendahl, I. Virtanen, and J. E. Eriksson, “The intermediate filament protein nestin occurs transiently in differentiating testis of rat and mouse,” *Differentiation*, vol. 61, no. 4, pp. 243–249, 1997.

[30] C. Wiese, A. Rolletschek, G. Kania et al., “Nestin expression—a property of multi-lineage progenitor cells?” *Cellular and Molecular Life Sciences*, vol. 61, no. 19-20, pp. 2510–2522, 2004.

[31] M. Aihara, K. I. Sugawara, S. Torii et al., “Angiogenic endothelium-specific nestin expression is enhanced by the first intron of the nestin gene,” *Molecular and Cellular Proteomics*, vol. 6, no. 6, pp. 1103–1109, 2007.

[32] K. Froydman, L. J. Pelliniemi, U. Lendahl, I. Virtanen, and J. E. Eriksson, “The intermediate filament protein nestin occurs transiently in differentiating testis of rat and mouse,” *Differentiation*, vol. 61, no. 4, pp. 243–249, 1997.

[33] C. Eliasson, C. Sahlgren, C. H. Berthold et al., “Intermediate filament protein partnership in astrocytes,” *The Journal of Biological Chemistry*, vol. 274, no. 34, pp. 23996–24006, 1999.

[34] M. Aihara, K. I. Sugawara, S. Torii et al., “Angiogenic endothelium-specific nestin expression is enhanced by the first intron of the nestin gene,” *Molecular and Cellular Proteomics*, vol. 6, no. 6, pp. 1103–1109, 2007.

[35] N. Teranishi, Z. Naito, T. Ishiwata et al., “Identification of neovascularity using nestin in colorectal cancer,” *International Journal of Oncology*, vol. 30, no. 3, pp. 593–603, 2007.