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Neural differentiation of human embryonic stem cells as an *in vitro* tool for the study of the expression patterns of the neuronal cytoskeleton during neurogenesis

Chao Liu [a,b,c,*], Yongwang Zhong [b], Andria Apostolou [b], Shengyun Fang [b,d,*]

[a] Department of Histology and Embryology, Anhui Medical University, Hefei, Anhui 230032, China
[b] Center for Biomedical Engineering and Technology (BioMET), University of Maryland, Baltimore, MD 21201, USA
[c] Institute of Stem Cell and Tissue Engineering, Anhui Medical University, Hefei, Anhui 230032, China
[d] Department of Physiology, University of Maryland, Baltimore, MD 21201, USA

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ABSTRACT

The neural differentiation of human embryonic stem cells (ESCs) is a potential tool for elucidating the key mechanisms involved in human neurogenesis. Nestin and β (beta)-III-tubulin, which are cytoskeleton proteins, are marker proteins of neural stem cells (NSCs) and neurons, respectively. However, the expression patterns of nestin and β-III-tubulin in neural derivatives from human ESCs remain unclear. In this study, we found that neural progenitor cells (NPCs) derived from H9 cells express high levels of nestin and musashi-1. In contrast, β-III-tubulin was weakly expressed in a few NPCs. Moreover, in these cells, nestin formed filament networks, whereas β-III-tubulin was distributed randomly as small particles. As the differentiation proceeded, the nestin filament networks and the β-III-tubulin particles were found in both the cell soma and the cellular processes. Moreover, the colocalization of nestin and β-III-tubulin was found mainly in the cell processes and neurite-like structures and not in the cell soma. These results may aid our understanding of the expression patterns of nestin and β-III-tubulin during the neural differentiation of H9 cells.
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1. Introduction

Neural stem cells (NSCs) and neurons derived from human embryonic stem cells (ESCs) are potential cell sources that can be used to acquire a better understanding of the key mechanisms involved in neural development [1,2]. The complex processes of neural development, such as neurogenesis, neuronal migration, axon pathfinding, and synapse formation, are regulated by intrinsic and extrinsic pathways that ultimately affect the cytoarchitectural network [3]. An elaborate multi-component filament cytoarchitecture in the eukaryotic cell includes three distinct and interconnected filament systems: intermediate filaments (IFs) made from fibrous proteins, microtubules made from α/β-tubulin heterodimers, and microfilaments of actin [4].

Nestin, which is a cytoskeletal IF protein, plays a complex role in regulating the assembly and disassembly of IFs and other structural proteins during cell remodeling [5]. This protein is expressed by neural progenitor cells (NPCs) during the development of the central nervous system (CNS) and peripheral nervous system (PNS) [6,7] and has long been used as an exclusive marker for CNS progenitors [3,8]. Nestin is also involved in the cytoskeleton remodeling required for neural plasticity [9]. Its deficiency results in embryonic lethality through the development of neural tubes with low numbers of NSCs [10]. β-III-tubulin, which is another cytoskeletal protein that is also known as neuron-specific class III β-tubulin, is a popular specific identifier for neurons [11]. This protein is also regulated during PNS and CNS neural development [12]. Mutations in the human β-III-tubulin-encoding gene TUBB3 affect microtubule dynamics in neurons to yield a diverse set of disease symptoms [13]. A recent study demonstrated the colocalization of nestin and β-III-tubulin in mature neurons, which suggests that both proteins may function in cytoskeleton remodeling [9]. To elucidate the role of the coexistence of nestin and β-III-tubulin in neurons, it is probably necessary to analyze a large amount of neurons that express both nestin and β-III-tubulin. Different protocols used for the generation of nestin-positive and β-III-tubulin-positive neurons from human ESCs have been reported [14–16], and these provide potential cell sources for the *in vitro* dissection of the expression and distribution of the neuronal cytoskeleton. However, the expression patterns of nestin and

Abbreviations: ESC, embryonic stem cell; NSC, neural stem cell; NPC, neural progenitor cell; IF, intermediate filament; CNS, central nervous system; PNS, peripheral nervous system; RA, retinoic acid; IFA, immunofluorescence assay.

* Corresponding authors. Address: Department of Histology and Embryology, 81 Meishan Road, Anhui Medical University, Hefei, Anhui 230032, China. Fax: +86 551 65161135 (C. Liu).
E-mail addresses: chaol1974@ahmu.edu.cn (C. Liu), sfang@umaryland.edu (S. Fang).

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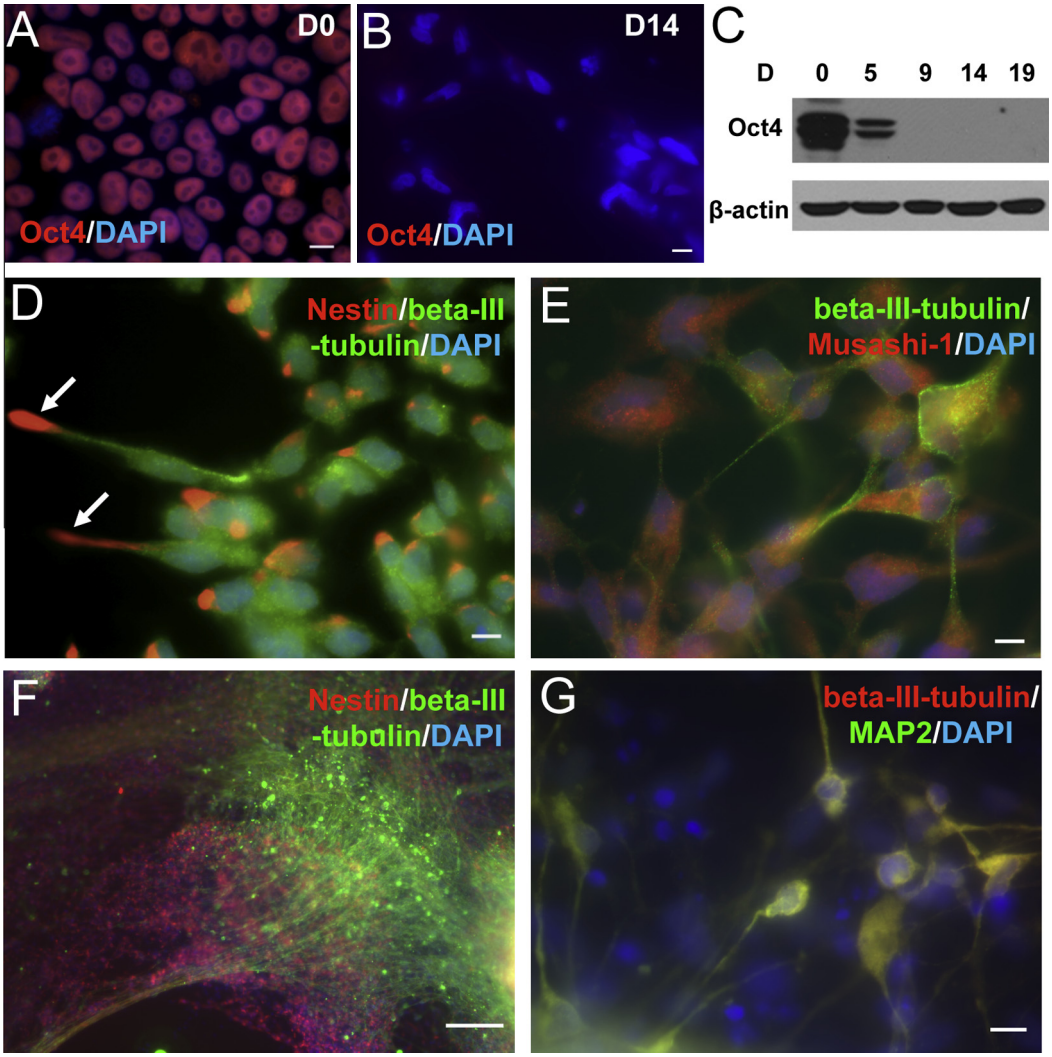

Fig. 1. Neural differentiation of H9 cells. (A–C) Robust Oct4 expression in undifferentiated H9 cells (A, red) and absence of Oct4 in differentiated H9 derived cells (B). Bar = 20 μM. The presence of the indicated proteins in the cell lysates was analyzed through an IB assay (C). (D–E) Neural progenitors are characterized by the expression of nestin (D and F, red) and β-III-tubulin (D and E, green) at D14 of the differentiation. Bar = 10 μM. (F) The expression of nestin and β-III-tubulin (F, green) in the neurites of neurons was shown at D42 of the differentiation. Bar = 100 μM. (G) The coexpression of β-III-tubulin (red) and MAP2 (Fig. 2G, green) was shown through an IFA. Bar = 10 μM. The nuclei were stained with DAPI. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

β-III-tubulin during human ESC neural differentiation are not well understood.

In this manuscript, we report the induction of NPCs and neurons from the human ESC cell line H9 and the expression pattern of nestin and β-III-tubulin in the derived NPCs and neurons. Nestin was abundant and formed a filament network in these NPCs and neurons. In contrast, the expression of β-III-tubulin was low and distributed randomly in the NPCs. As the differentiation proceeded, β-III-tubulin was found mainly in the cellular processes of the derived neurons. Moreover, the colocalization of nestin and β-III-tubulin was found mainly in the cellular processes with the exception of the cell soma. These results contribute to an understanding of the nestin and β-III-tubulin expression patterns during H9 cell neural differentiation.

2. Materials and methods

2.1. Cell culture

The human ESC cell line H9 (passage ~23) was cultured as previously described [17].

2.2. H9 cell neural differentiation

The neural differentiation of these cells was performed according to a previously described protocol [18]. Briefly, the domed human ESC colonies were transferred to human matrix-coated plates with 10 mg/cm^2 human collagen IV, 0.2 mg/cm^2 human vitronectin, and 5 mg/cm^2 human fibronectin (Sigma) in mTeSR™1 medium. After the appearance of rosette structures, the cells were maintained for five additional days in the same medium. From D7 to D14, the medium was changed to neural differentiation medium supplemented with 10 μM/ml all-trans retinoic acid (RA). The neural differentiation medium consisted of DMEM: F-12, B27 supplement (Invitrogen), 25 mg/ml human insulin, 6.3 ng/ml progesterone, 10 mg/ml putrescine, 50 ng/ml sodium selenite, and 50 mg/ml human holotransferrin (Sigma). On D14, the cells were plated on human laminin/ornithine-coated slides and maintained at least four weeks in the neural differentiation medium.

2.3. Immunofluorescence assay

The cells were subjected to an immunofluorescence assay (IFA) as previously described [19]. The following antibodies were used

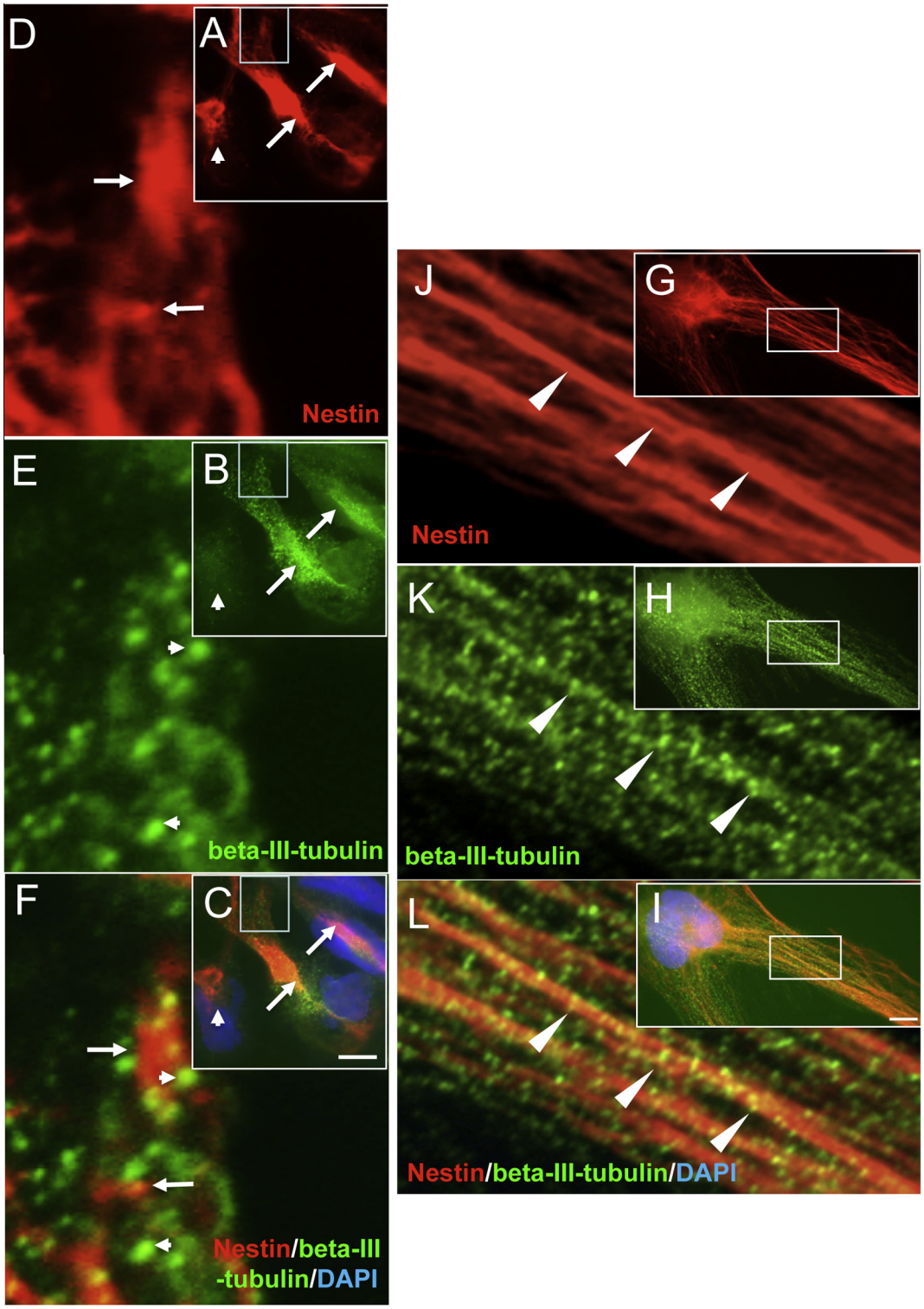

Fig. 2. Localization of nestin and β-III-tubulin in H9-derived NPCs. H9-derived NPCs were subjected to an IF assay for nestin and β-III-tubulin. (A–C) The H9-derived NPCs express nestin (A and C, red) and β-III-tubulin (B and C, green). The nuclei were stained with DAPI. Bar = 10 μM. (D–F) Higher-magnifications images of the inlets in (A–C), respectively. The arrowheads indicate the β-III-tubulin-positive signals, which were co-localized with few or no nestin signals (green). (G–I) Expression of nestin in cell processes. The nuclei were stained with DAPI. Bar = 10 μM. (J–L) Higher-magnifications images of the inlets in G-I, respectively. The colocalization of nestin and β-III-tubulin is indicated by triangles (yellow). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

for the IFA: rabbit monoclonal anti-Oct4 (1:3000, Cell Signaling Technology, Danvers, MA, USA), chicken anti-MAP2 (1:5000, Chemicon, Billerica, MA, USA), mouse anti-β-III-tubulin (1:200, Chemicon, Billerica, MA, USA), chicken anti-β-III-tubulin (1:100, Millipore, Temecula, CA, USA), rabbit anti-Nestin (1:200, Sigma), and rabbit anti-Musashi-1 (1:200, Sigma, St. Louis, MO, USA).

2.4. Immunoblotting assay

The immunoblotting (IB) assay was performed as previously described [19]. The antibodies used for the IB were the following: rabbit monoclonal anti-Oct4 (1:3000, Cell Signaling Technology, Danvers, MA, USA) and mouse anti-β-actin (1:5000, Sigma, St Louis, MO, USA).

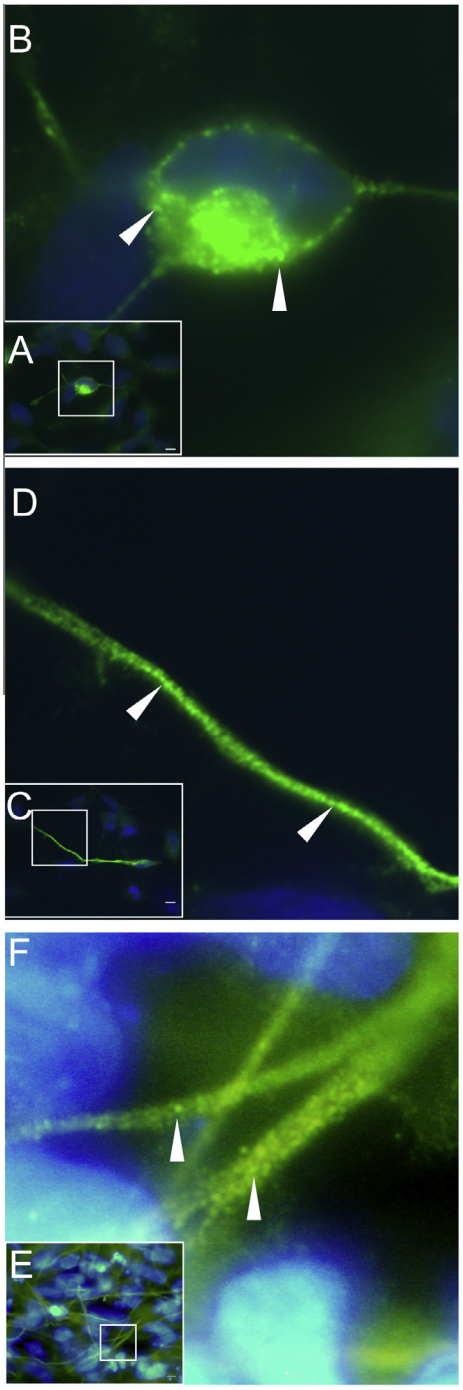

Fig. 3. Distribution of β-III-tubulin during H9 cell neural differentiation. (A–D) IFA with a mouse monoclonal anti-β-III-tubulin antibody. Expression of β-III-tubulin (green) in the cell soma (A and B) and neurite-like structures (C and D). B and D represent higher-magnifications images of the inlets in A and C, respectively. (E–F) IFA with chicken polyclonal β-III-tubulin antibodies. F represents a higher-magnification image of an inlet in E. Bar = 10 μM. The punctate distribution of β-III-tubulin is indicated by triangles. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

2.5. Micrograph

The cells were visualized under a microscope at different time points. A Zeiss Axiovert 200 M fluorescence microscope and the AxioVision (Release 4.7) software were used for the visualization.

3. Results

3.1. Induction of neural differentiation of human ESCs

Neural progenitors derived from human ESCs are potential cell sources for the study of early neural development *in vitro* [18]. To obtain NPCs from human ESCs, the neural differentiation of H9 cells was performed as previously described [16]. Undifferentiated H9 cells exhibited robust nuclear expression ofOct4 (Fig. 1A), whereas the differentiated H9 derivatives were devoid of Oct4 expression (Fig. 1B). The downregulation of Oct4 at different time points during the differentiation process was confirmed through an IB assay (Fig. 1C). The H9 derivatives were then subjected to an IF assay to analyze the expression of the NPC marker proteins nestin and Musashi-1, the immature neuron marker protein β-III-tubulin, and the mature neuron marker protein MAP2. The results demonstrated the expression of nestin and musashi-1 and a weak expression of β-III-tubulin in the NPCs (Fig. 1D and E) at D14 of the differentiation. Moreover, the presence of nestin in growth cone-like structures was also revealed (Fig. 1D, arrows). At D42 of the differentiation, the expression of both nestin and β-III-tubulin (Fig. 1F) was identified in many of the H9-derived neural cells, which indicates that these cells had reached a stage of improved neurogenesis. The coexpression of MAP2 and β-III-tubulin in the cells (Fig. 1G) further confirmed the maturation of the H9-derived neurons. These results suggest that we achieved the successful induction of NPCs and neurons from H9 cells.

3.2. Expression patterns of nestin and β-III-tubulin in NPCs

To investigate the subcellular localization of both nestin and β-III-tubulin in the derived NPCs, we performed an IFA for nestin and β-III-tubulin (Fig. 2A–L) in the NPCs during the earlier stages of the neural differentiation process. Most of the NPCs expressed high levels of nestin, which was distributed as filaments in the cells (Fig. 2A and C), some of the NPCs expressed β-III-tubulin as small particles that were distributed randomly (Fig. 2B and C) throughout the cells. Although some cells were only nestin-positive (Fig. 2A–C, arrowheads), others cells were positive for both nestin and β-III-tubulin (Fig. 2A–C, arrows). In the double-positive cells, the expression patterns of nestin (Fig. 2D–F, arrows) and β-III-tubulin (Fig. 2D–F, arrowheads) were different. However, in some of the NPCs with well-developed cell processes, nestin formed filament networks in the cell processes (Fig. 2G and I), and β-III-tubulin was distributed mainly in these processes as small particles (Fig. 2H and I). The orientation of β-III-tubulin expression is not random but seemingly similar to that of nestin expression (Fig. 2J–L). The colocalization of nestin and β-III-tubulin was also found in the NPCs, as shown through a yellow color (Fig. 2J–L, triangles). These results suggest that both nestin and β-III-tubulin may be involved in the formation of cell processes during H9 neural differentiation.

3.3. Characterization of β-III-tubulin expression in H9-derived neurons

We further addressed the subcellular distribution of β-III-tubulin in the H9-derived neurons by IFA using two different anti-tubulin antibodies. Both antibodies produced strong signals of β-III-tubulin in the H9-derived neurons (Fig. 3A–F). As the differentiation proceeded, some of the cells formed long neurite-like structures (Fig. 3C–F) that were characterized by a robust expression of β-III-tubulin (Fig. 3D and F, arrows). Moreover, a punctate distribution of β-III-tubulin was clearly found in both the cell soma and the neurite-like structures under

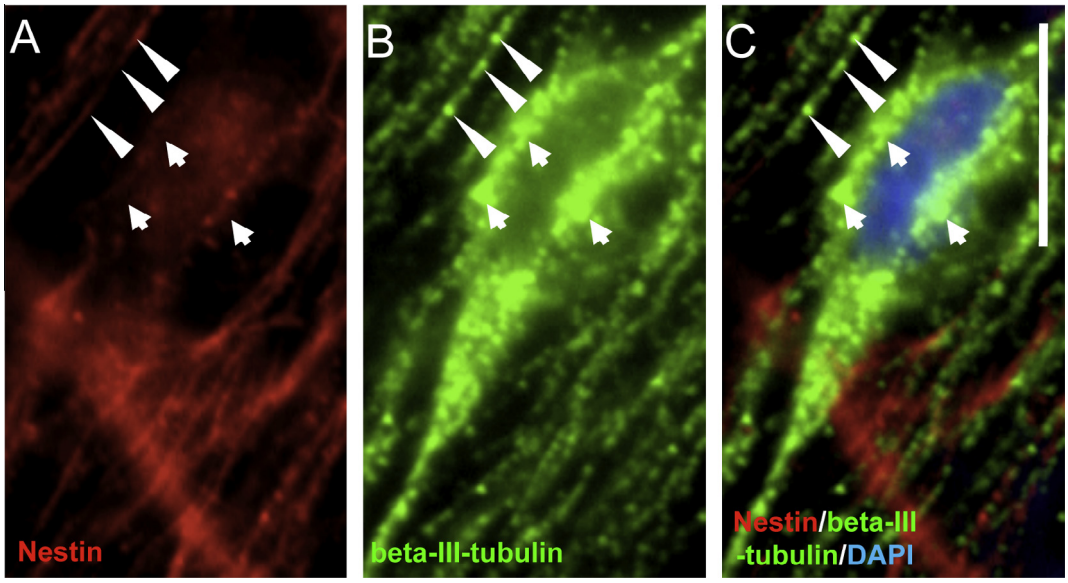

Fig. 4. Localization of nestin and β-III-tubulin in H9-derived neurons. H9-derived neurons were subjected to an IF assay for nestin (A and C, red) and β-III-tubulin (B and C, green) at D42 of the neural differentiation. The arrowheads indicate β-III-tubulin-positive structures in which few or no nestin signals were detected (B and C, green). The colocalization of nestin and β-III-tubulin in the neurite-like structures is indicated by triangles (yellow). Bar = 10 μM. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

higher magnifications (Fig. 3B, D, and F). These results suggest that β-III-tubulin may be involved in the formation of the neurite-like structures during the neuronal differentiation of H9 cells.

3.4. Expression patterns of nestin and β-III-tubulin in neurons

Because the colocalization of nestin and β-III-tubulin in NPCs was confirmed, we examined the colocalization of nestin and β-III-tubulin in neurons. We thus performed an IFA for nestin and β-III-tubulin in the H9-derived neurons at D42 of the neural differentiation. In the H9-derived neurons, we found a robust expression of β-III-tubulin distributed in both the cell soma and the neurite-like structures (Fig. 4A and C). However, few or no nestin signals were detected (Fig. 4A and C, arrowheads) in the cell soma. Moreover, the colocalization of nestin and β-III-tubulin was found mainly in the neurite-like structures (Fig. 4A and C, triangle). These results suggest that both nestin and β-III-tubulin may be involved in the formation of the neurite-like structures during H9 cell neuronal differentiation.

4. Discussion

During cell division, the cellular architecture undergoes disassembly, reorganization, and partitioning into the daughter cells [5]. These processes, which are characterized by extensive remodeling, are orchestrated by components of the cytoskeleton, which is a composite of microtubules, Ifs, and actin microfilaments [20,21]. Nestin and β-III-tubulin, which are cytoskeleton proteins, are thought to be involved in neurogenesis [5,22,23].

In our study, a successful neural differentiation was confirmed by the presence of nestin, musashi-1, β-III-tubulin, and MAP2 in the H9 derivatives (Figs. 1 and 2). In the NPCs, the robust expression of nestin was found in growth cone-like structures (Fig. 1D, arrows), which is consistent with the results of a previous report that found that nestin plays a role in growth cone guidance during axon elongation [24].

The coexpression of MAP2 and β-III-tubulin was also detected (Fig. 1G), which is in agreement with the coexpression of MAP2 and β-III-tubulin in the neuronal cells generated from human fetal tissue [25]. In addition, β-III-tubulin-positive signals were also identified in some of the NPCs (Fig. 2B, C, H and I). Moreover, the colocalization of nestin and β-III-tubulin was found mainly in the cell processes and not in the cell soma of the NPCs (Fig. 2G–I), which suggests that the expression of nestin and β-III-tubulin may be involved in the formation of cell processes during the differentiation of NPCs.

We also discovered a random β-III-tubulin punctate distribution in the cell soma of the NPCs (Fig. 2B, C, E, and F). During the formation of the cellular processes, the β-III-tubulin punctate distribution was rearranged approximately along the nestin filament networks (Fig. 2H, I, K, and L), as was indicated by the similar orientation distribution and colocalization of nestin and β-III-tubulin (Fig. 2J, K, and L, triangles) in the processes of the NPCs. This colocalization was also found in the neurite-like structures of the H9-derived neurons (Fig. 4A–C). Moreover, the neurons also exhibited a punctate distribution of β-III-tubulin in both the cell soma and the neurite-like structures (Fig. 3A–D, triangles). In addition, even those neurons subjected to IFA with chicken anti-β-III-tubulin antibodies exhibited a punctate distribution of β-III-tubulin (Fig. 3F, triangles), which appears to be a different pattern from that observed in primary mature neurons. In fact, as observed in Fig. 4A–C, the punctate distribution of β-III-tubulin in the neurite-like structures appeared to be rearranged according to theorientation of the nestin-filament-like structures and clearly exhibited incomplete thread-like or filamentous structures. Because antibody binding leads to a twofold increase in the diameter of neurofilament cores and forms globular mass attachments [26], we speculate that the punctate distribution obtained may not be a faithful representation of the β-III-tubulin distribution but might be the result of specific antibody binding to the filament cores.

During later stages of the neural differentiation, the derived neurons exhibited robust expression of β-III-tubulin in both the cell soma and the neurite-like structures (Figs. 3 and 4). In addition, a low level of or no nestin was expressed in the β-III-tubulin-positive structures in the neurons (Fig. 4A–C, arrowheads), which suggests a downregulation of nestin in the H9 cell-derived

neurons. This finding is consistent with previous results that showed that nestin is expressed during early neural development and is downregulated in mature neurons [18]. Nevertheless, although nestin is commonly replaced by proteins specific for neurons and glia in terminal differentiated cells, nestin-expressing neurons in the adult rat and human basal forebrain have been reported [11,12,26]. These findings are supported by our data on the colocalization of nestin and β-III-tubulin in the NPCs (Fig. 2I and L) and neurons (Fig. 4C). However, the functions of the colocalization of both proteins remain unclear. The *in vitro* neural differentiation of H9 cells may provide a potential tool for the investigation of the function of the interaction between nestin and β-III-tubulin.

In conclusion, our data suggest that the neural differentiation of H9 cells may provide a potential *in vitro* tool for the study of the expression patterns of the neuronal cytoskeleton during neurogenesis.

Acknowledgments

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