Formation of Embryonic Bodies to Produce Neural Stem Cells from iPSCs Using the Corning® Spheroid Microplate

Application Note

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Introduction

Induced pluripotent stem cells (iPSCs) are derived from somatic cells that have been reprogrammed back into the embryonic-like state by introducing genes important for maintaining the essential properties of embryonic stem cells (ESCs). Possessing differentiation potential and self-renewal ability, iPSCs have applications in the analysis of cell fate, disease mechanisms, and drug development. Due to their ability to produce all types of human cells, iPSCs can be used for therapeutic purposes, as well. Furthermore, ethical issues associated with the production of ESCs do not apply to iPSCs because they allow the generation of patient-specific stem cell lines that can be used in clinical therapies[1].

Specific neurons and glia can be generated from iPSCs to replace lost or damaged tissues in neurodegenerative and neurological diseases and can be used to perform high throughput screening of novel drugs for these neural diseases. The first step in generating neurons and glia is induction of iPSCs into neural stem cells (NSCs). Neurons and glia derived from mouse ESCs were first reported over 35 years ago and have since been successfully generated from human ESCs. Multiple protocols for producing NSCs from ESCs have been established, as summarized by Cai and Grabel in 2007[2]. However, whether these protocols are applicable in iPSCs is still unclear, and no reports have provided a comparison of these protocols.

In this study, we chose three protocols to produce NSCs from an iPSC line, iPS1. The first protocol was embryonic body (EB) selection in defined medium. This approach used early stage EBs plated on an adhesive substrate in serum-free medium to selectively induce a neuroectoderm-derived neural progenitor population. The purity of NSCs reached 80% to 95% using this protocol in human ESCs[3]. The second protocol used commercial NSC induction medium, which could direct the differentiation of iPSCs to NSCs in adherent culture within 1 week[4]. The third protocol was very low density culture in defined medium. This protocol aimed to directly culture single ESCs at a very low density (1 to 20 cells/well) in defined medium and triggered the ESCs to differentiate into an intermediate cell type, primitive NSCs, without embryonic formation[5].

Through evaluating the strengths and weaknesses of iPSCs in terms of the purity of NSCs, gene expression levels, handling accessibility, and the duration of transformation from iPSCs to NSCs, these three protocols were compared. The first protocol, EB selection in the defined medium, utilized Corning 96-well spheroid microplates to generate early stage EBs and yielded a higher purity of NSCs with higher expression of NSC marker genes, although an additional 5 days in culture may be required. Based on the strengths and weaknesses of this protocol, we recommend the use of this approach for directing iPS1 cells into NSCs. This protocol may also have applications in other iPSC lines.

Materials and Methods

Cells and Culture Medium

The iPSCs used in this study were iPS1 cells derived from human skin fibroblasts, which were obtained from Industrial Technology Research Institute (ITRI; Taiwan). Essential 8, N2, B27, StemPro® NSC serum-free medium (KnockOut™ DMEM/F-12), GlutaMAX™-I Supplement, basic fibroblast growth factor (FGF), epidermal growth factor (EGF), StemPro Neural Supplement, and NSC induction medium were purchased from Thermo Fisher.

Microplates

Corning® 96-well black/clear round-bottom Ultra-Low Attachment spheroid microplates (Corning Cat. Nos. 4515 and 4520) and 6-well clear, TC-treated plates (Corning Cat. No. 3516) were provided by Corning Life Sciences. Nunc untreated 6-well plates were purchased from Thermo Fisher.

Plate Coating

For vitronectin coating, 6-well plates were incubated with 0.5 µg/cm^2 vitronectin (Corning Cat. No. 354238) at room temperature for 1 hr. For Poly-L-Ornithine/Laminin coating, the Poly-L-Ornithine (MilliporeSigma Cat. No. P3655) stock solution was diluted in cell culture-grade distilled water at 1:500 to prepare a 20 µg/mL working solution. The culture vessels were then incubated for 1 hr. at 37°C. After rinsing the culture vessels twice with sterile water, the culture vessels were coated with 20 µg/mL Laminin (Cat. No. L2020; Sigma) working solution and then incubated for 2 hr. at 37°C.

Immunofluorescence Staining and Flow Cytometry

The cells were rinsed twice with phosphate-buffered saline (PBS) and then fixed with 4% paraformaldehyde (Sigma) for 30 min. The cells were then permeabilized with 0.5% Triton™ X100 (Sigma) in PBS for 10 min. The fixed cells were then blocked with 5% bovine serum albumin in PBS for 30 min. at room temperature and were incubated for 1 hr. at room temperature with the primary antibodies (rabbit anti-Nestin [MilliporeSigma Cat. No. ABD69; 1:200], mouse anti-tubulin βIII [MilliporeSigma Cat. No. MAB1637; 1:200], and rabbit anti-Sox2 [MilliporeSigma Cat. No. AB5603; 1:200]). After rinsing three times with PBS, cells were incubated with the appropriate secondary antibodies, i.e., Alexa Fluor® 488 goat anti-rabbit IgG (H+L) (Thermo Fisher) and Alexa Fluor 594 goat anti-mouse IgG (H+L) (Thermo Fisher), for 1 hour.

After staining, all fluorescence images were collected under a fluorescent microscope (Carl Zeiss; Observer D1) and analyzed using AxioVision 4.8.1 microscope software. Flow cytometry analysis was performed on a CytoFLEX S Research Flow Cytometer (Beckman Coulter).

Quantitative Real-time Polymerase Chain Reaction (qPCR)

Total cellular RNA was extracted using an RNeasy® Mini kit (Qiagen Cat. No. 74104). cDNA synthesis was performed with a High Capacity cDNA Reverse Transcription kit (Thermo Fisher Cat. No. 4368814). qPCR was performed using Power SYBR Green® PCR Master Mix (Thermo Fisher Cat. No. 4367659) on a QuantStudio 3 real-time PCR machine (Thermo Fisher). In this study, the housekeeping gene β-actin was used for data normalization. All primers are summarized in Table 1.

Table 1. Specific primers for qPCR analysis

Primers	Sequences (5'-3')	Product Size (bp)
Nestin	CTCCAGAAACTCAAGCACC TCCTGATTCTCCTCTTCCA	145
TUBB3	TCCGCTCAGGGGCCTTTGGAC GCTCCGCCCCCTCCGTGTAG	108
AQP4	AGCAGTCACAGCGGAATTTCT TCTGTTCCACCCCAGTTGATG	81
Nanog	GGACACTGGCTGAATCCTTCC CTCGCTGATTAGGCTCCAACC	143
SOX2	ATGGAGAAAACCCGGTACGC TTTTGCGTGAGTGTGGATGG	112
ACTB (β-actin)	GCACCACACCTTCTACAATGA TGTCACGCACGATTTCCC	100

Production of NSCs from iPSCs

The iPS1 cell line was directed to produce NSCs using three protocols: EB selection in defined medium, application of commercial NSC induction medium in adherent culture plates, and culturing at very low density in defined medium. Details for each protocol are described below, and the schematic workflows are shown in Figure 1.

Protocol 1: EB Selection in Defined Medium

Around 50 x 50 µm iPS1 cell clusters were cut using a glass capillary or the StemPro EZPassage and suspended in Essential 8 medium in two types of plates (Corning 96-well spheroid microplates or untreated plates) for 2 days, during which early stage iPS1 EBs were formed. The EBs formed from spheroid microplates were round in shape with a uniform size (approximately 200 µm to 250 µm). EBs formed in untreated plates showed irregular shapes and various sizes, ranging from smaller than 100 µm to larger than 400 µm (Figure 2). These early stage EBs were transferred to N2B27 medium. After 5 days of induction, the EBs were transferred to adherent culture plates precoated with Poly-L-Ornithine/Laminin (PLO/Laminin) and cultured in NSC maintenance medium. The putative NSCs determined by morphology could be found after 5 days of culture in NSC maintenance medium (Figure 3).

Protocol 2: Commercial NSC Induction Medium

Clusters of iPSCs were harvested and directly cultured in NSC induction medium for 7 days in vitronectin-coated plates. It was essential to remove any differentiated and partially

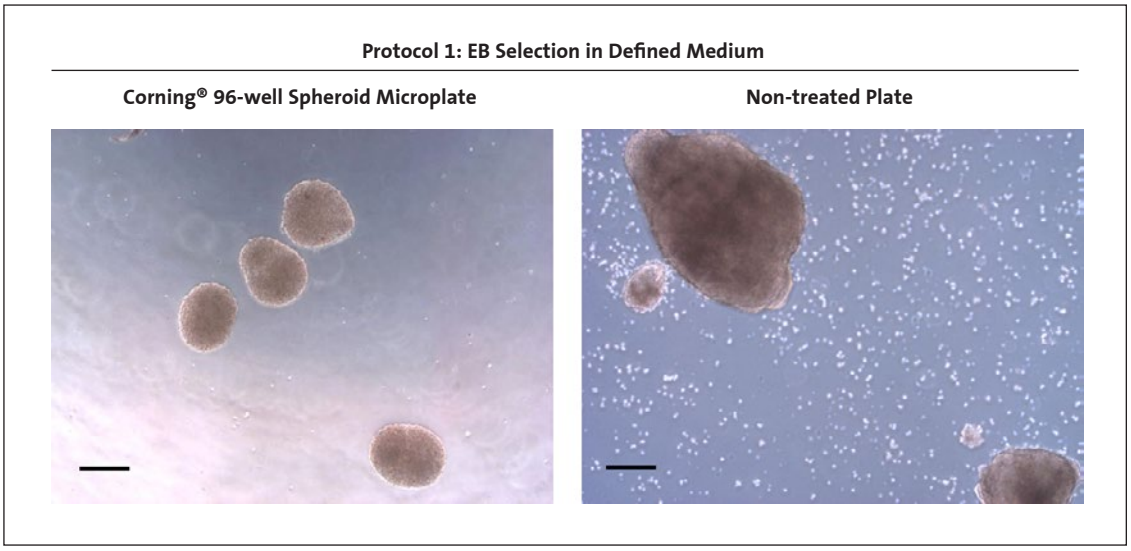

Figure 1. Early stage EBs formed from iPS1 cells using Corning 96-well spheroid microplates and untreated plates. Scale bar: 200 µm.

differentiated colonies every 2 days, as described in the user manual[4]. Putative NSCs were obtained after applying NSC maintenance medium (Figure 3).

Protocol 3: Very Low Density IPSCs in Defined Medium

Single iPS1 cells were cultured at a very low density (1 to 20 cells/well) in Essential 8 medium for 7 days. It was assumed that iPS1 cells would first produce primitive NSC spheres, which could then further differentiate to NSCs following the addition of FGF/EGF. However, the cells failed to form primitive NSC spheres after 7 days of culture in defined medium. Accordingly, this protocol did not work for iPS1 cells, and this failure may be related to the nature of this iPS1 cell line, which preferred to grow as clusters. There were no putative NSCs produced by this protocol.

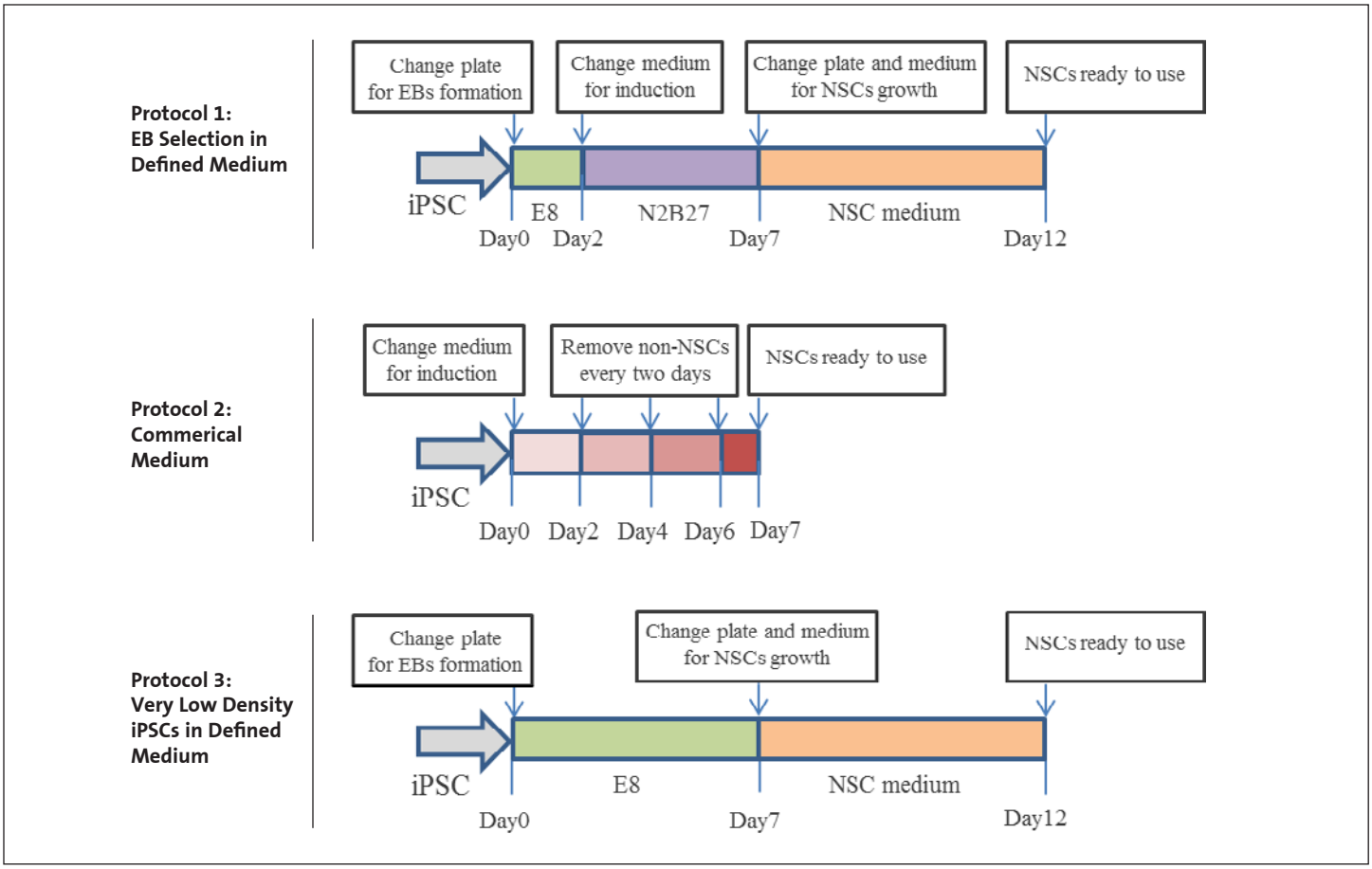

Figure 2. Schematic workflows of protocols for producing iPS1-derived NSCs

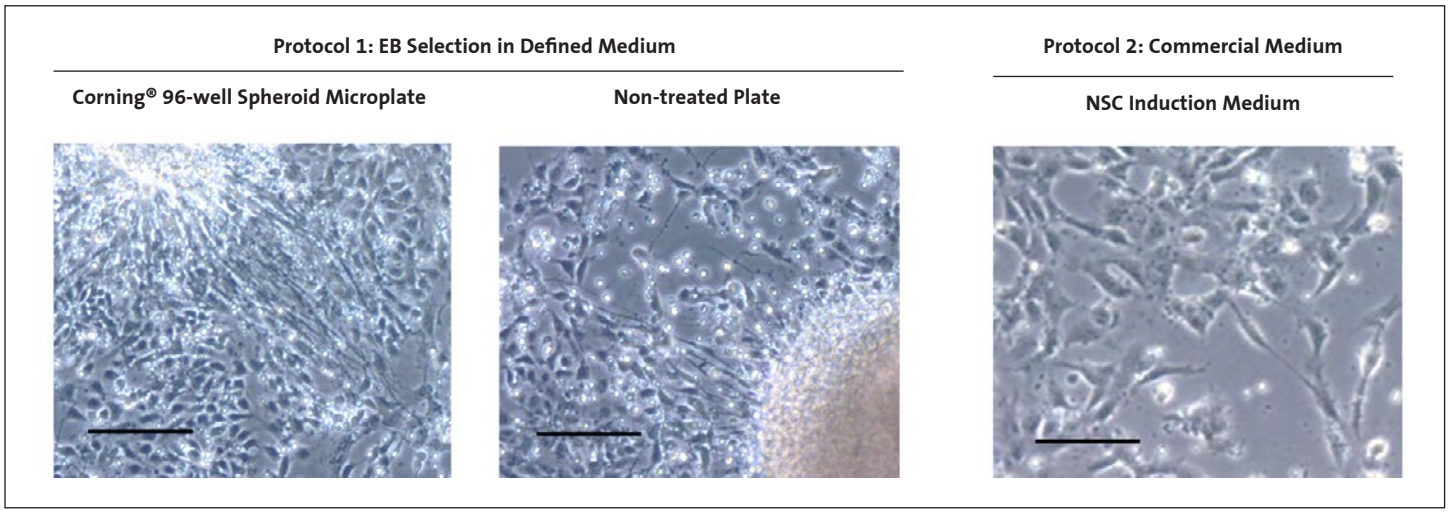

Figure 3. Morphology of putative NSCs produced from iPS1 cells by various protocols. Scale bar: 100 µm.

Immunofluorescence (IF) Staining of Specific Biomarkers for Evaluation of the Characteristics of NSCs

In order to confirm the characteristics of putative NSCs, we detected Nestin as an NSC marker and tubulin βIII as a neuronal marker by IF staining (Figure 4). The results indicated that most of the cells were positive for Nestin staining (4',6-diamidino-2-phenylindole [DAPI] counterstaining not shown), and some cells were stained for tubulin βIII. However, tubulin βIII was stained strongly in cell aggregation regions, implying that cells in aggregation regions tended to further differentiate into neurons rather than remain in the NSC state. For the EB selection method, putative NSCs from untreated plates showed more aggregation than other protocols. Plating of large EBs in adherent culture may result in incomplete cell expansion and yield more aggregation regions than plating small EBs. Controlling the size of EBs formed in Corning® 96-well spheroid microplates can yield early EBs measuring ~200 µm in size by seeding 50 x 50 µm iPS1 cell clusters in each well. Plating the ~200 µm EBs in adherent culture efficiently reduced the aggregation regions and tubulin βIII-expressing cells. For the commercial medium protocol, because the differentiated regions were removed every 2 days, only a few aggregation regions were found in cultures (Figure 4).

The IF staining results indicated that both the EB protocol (using Corning spheroid microplates) and commercial medium protocol yielded high purity NSCs with small amounts of differentiated neurons. Although the EB protocol required an extra 5 days in NSC induction medium, it was not necessary to remove the differentiated cells every 2 days. The ability to obtain high purity NSCs and the ease of handling are the strengths of EB selection protocols using Corning spheroid microplates.

Gene Expression in Putative NSCs

qPCR was applied for analysis of specific gene expression of putative NSCs produced by these protocols. The expression was normalized to that of the housekeeping gene *ACTB* (β-actin). The putative NSCs produced by these protocols showed characteristics of NSCs, including expression of *Nestin* and *SOX2* (NSC marker genes) and lack of expression of *AQP4* (a glial marker gene) and *Nanog* (a PSC marker gene). A lower level of *TUBB3* (tubulin βIII, neuronal marker) was expressed in putative NSCs, implying that some NSCs differentiated further into neurons (Figure 5). The NSCs produced by the EB protocol using untreated plates show lower expression of the NSC marker gene *SOX2*, suggesting that the purity of NSCs in culture was low, although no high expression of *TUBB3* was observed.

Compared with gene expression levels during the fifth and seventh passages of NSCs, NSC features (*Nestin* and *SOX2* expression) become slightly stronger in the commercial medium protocol and much stronger in the EB protocol using Corning spheroid microplates. The NSCs generated by untreated plates showed similar levels of gene expression for all features after a few subcultures.

This result was consistent with the IF staining results demonstrating that the NSCs generated by the EB protocol using Corning spheroid microplates and the commercial medium protocol possessed high purity NSCs. Interestingly, the NSC features of these cultures became stronger after a few subcultures, particularly for the NSCs produced by the EB protocol using Corning spheroid microplates.

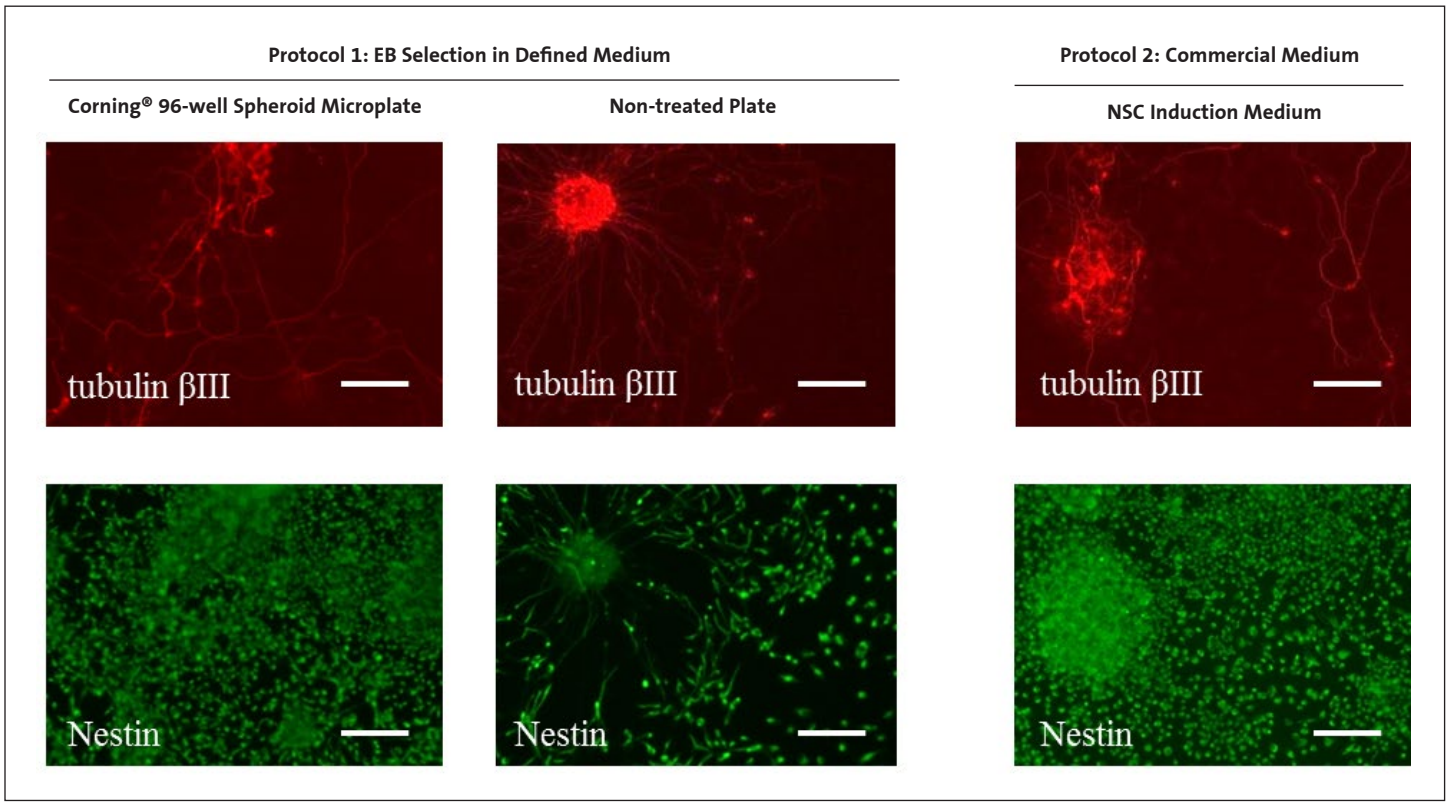

Figure 4. Immunofluorescence (IF) staining for Nestin and tubulin βIII in putative NSCs derived from iPSCs by various protocols. Scale bar: 100 µm.

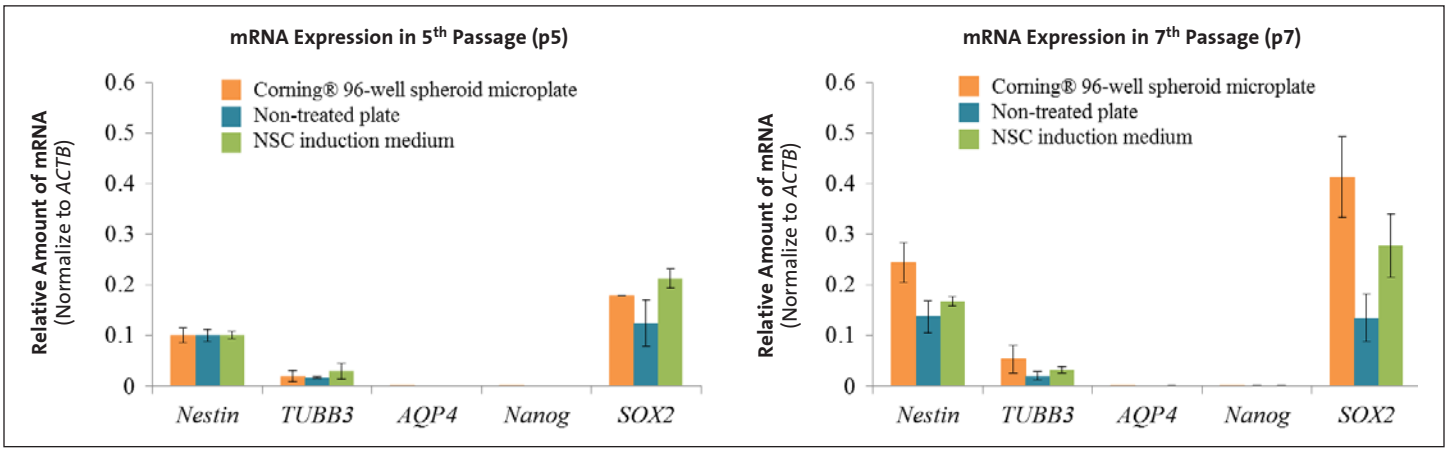

Figure 5. qPCR analysis of *Nestin*, *TUBB3*, *AQP4*, *Nanog*, *and SOX2* expression in NSCs derived from iPS1 cells by various protocols at passages 5 and 7.

Flow Cytometry Analysis of Putative NSCs

In order to quantify the NSC percentage among all derived cells, whole-cell based flow cytometry analysis was applied to cells stained with the NSC markers Nestin and SOX2. For NSCs produced by the EB protocol using Corning® spheroid microplates and the commercial medium protocol, Nestin (+) and SOX2 (+) cells accounted for more than 97% of all cells, as shown in Figures 6 and 7.

Nestin (+) and SOX2 (+) cells were defined as population 2 (P2, fluorescence level ≥10^3), and a subgroup with higher intensity signals (≥10^5) was defined as population 3 (P3).

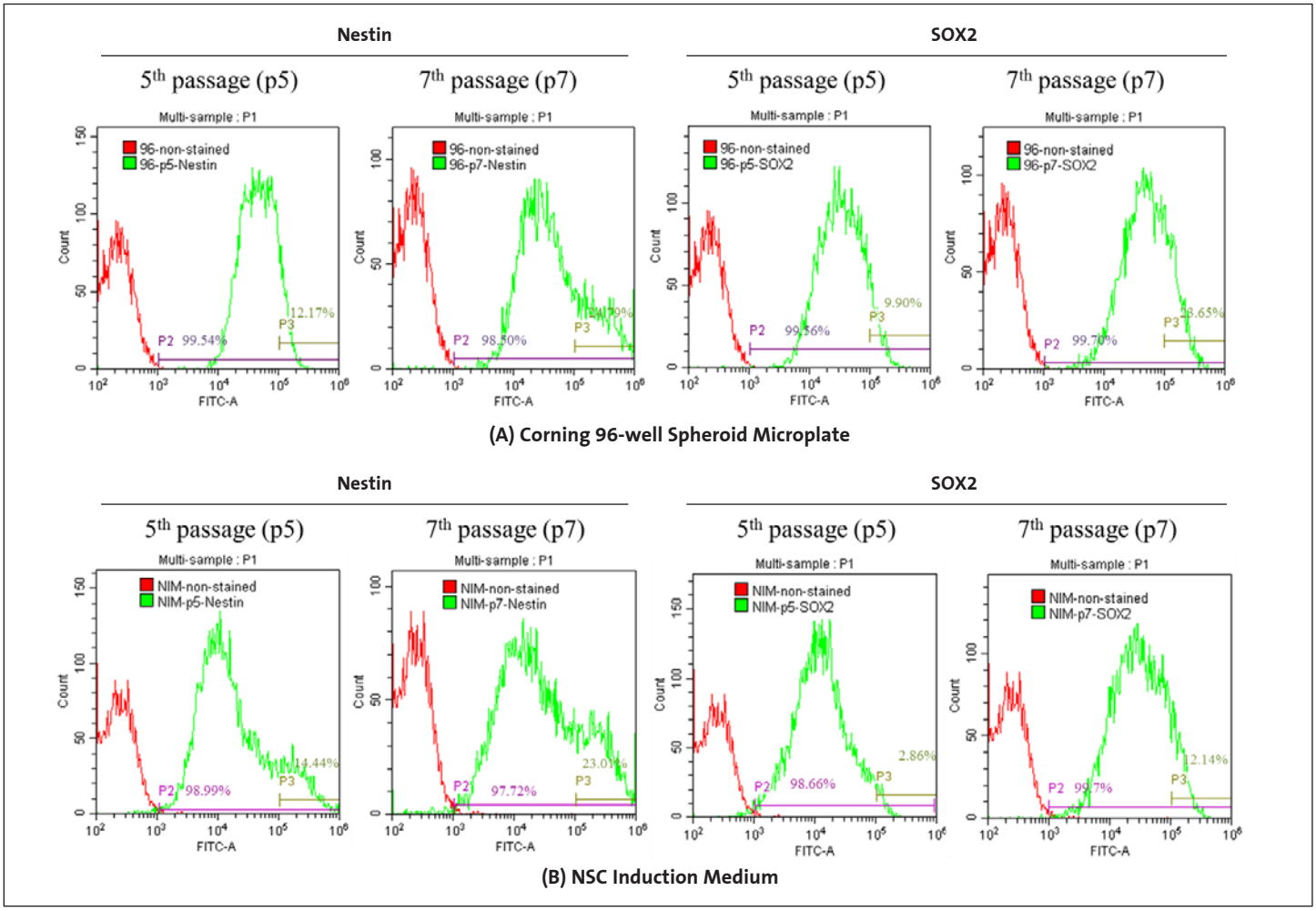

Figure 6. Representative flow cytometry histograms of Nestin and SOX2 expression in NSCs derived from iPS1 cells by various protocols at passages 5 and 7. Population 2 (P2) refers to cells with fluorescence intensity higher than 10^3, and population 3 (P3) refers to cells with fluorescence intensity higher than 10^5. 96 refers to NSCs produced using Corning 96-well spheroid microplates. NIM refers to NSCs produced by commercial NSC induction medium.

From passages 5 to 7, the percentage of P2 cells did not change significantly. Interestingly, the P3 portion became much higher, particularly for those produced by the EB protocol (Figure 7). These flow cytometry results were consistent with the results from qPCR analysis, demonstrating that the NSCs generated by the EB protocol using Corning® spheroid microplates possessed stronger NSC features after a few subcultures. These stronger features were contributed by a subgroup of NSCs that expressed higher Nestin and SOX2 levels rather than increased NSC numbers. The functional differences between these NSCs populations are not clear and will not be investigated further here.

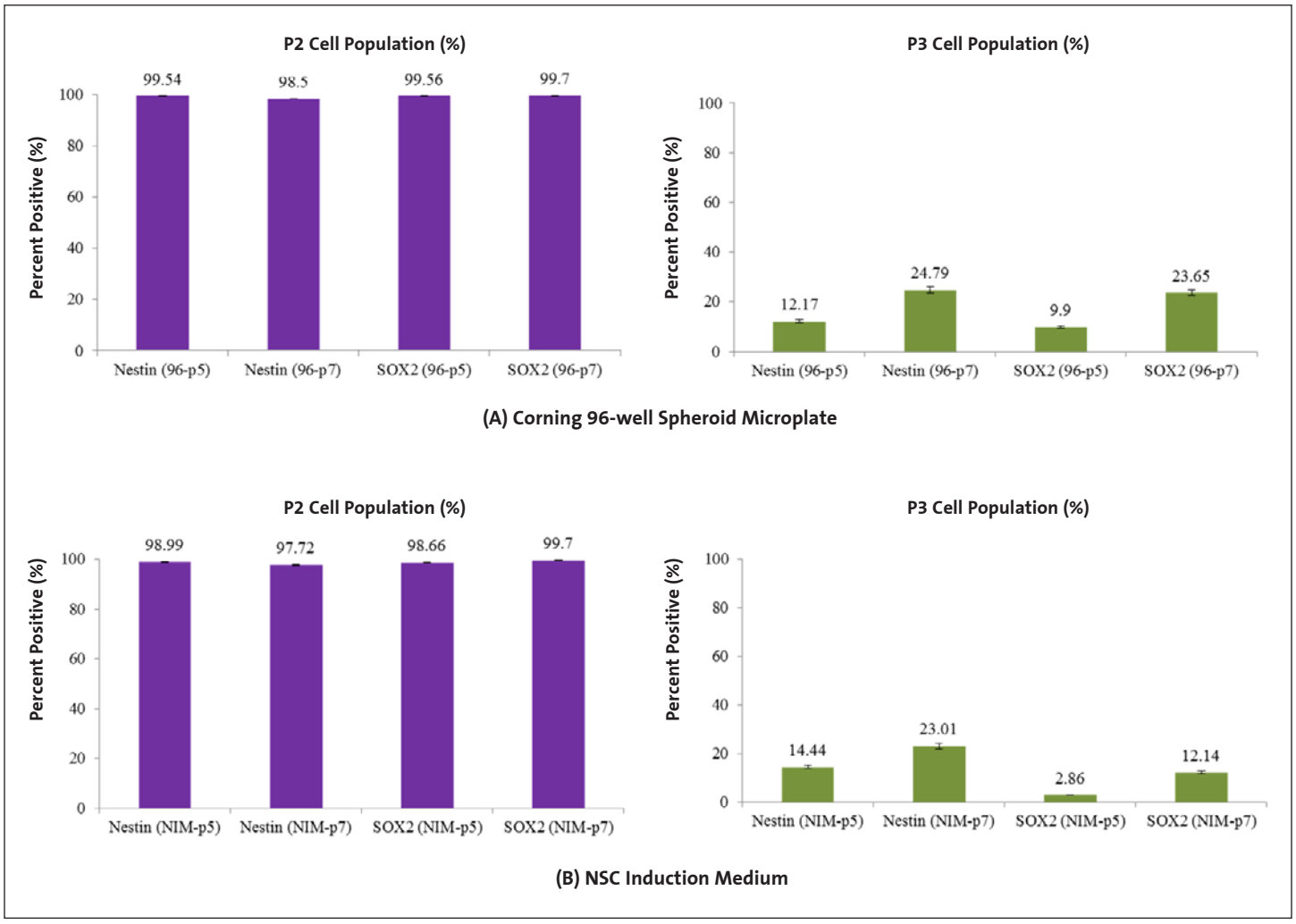

Figure 7. Expression levels of Nestin and SOX2 in NSCs derived from iPS1 cells by various protocols in passages 5 and 7. Population 2 (P2) refers to cells with fluorescence intensity higher than 10^3, and population 3 (P3) refers to cells with fluorescence intensity higher than 10^5. 96 refers to NSCs produced using Corning 96-well spheroid microplates. NIM refers to NSCs produced by commercial NSC induction medium.

Conclusions

- The iPS1 cell line could be directed to NSCs using two protocols, i.e., the EB selection protocol using Corning® 96-well spheroid microplates and the commercial medium protocol. The very low density protocol did not work for iPS1 cells.
- The strengths and weaknesses of these protocols for producing NSCs from iPSCs are summarized in Table 2.
- The Corning 96-well spheroid microplates could generate uniform EBs, and the sizes of the EBs could be controlled.
- The NSCs generated by the EB protocol using Corning 96-well spheroid microplates had a higher NSC purity and the highest NSC marker gene expression levels among all tested protocols. This protocol was highly recommended for differentiation of iPSCs into NSCs.

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Table 2. Advantages and disadvantages of the three protocols for producing NSCs from iPS1 cells

Protocol	Strengths	Weaknesses
EB selection in defined medium: Using Corning 96-well spheroid microplates	◗ Uniform and controllable EB size, which will reduce aggregation and increase NSC purity ◗ Higher NSC purity and stronger NSC features ◗ Handling accessibility (no extra effort when culturing)	◗ Time-consuming (extra 5 days)
EB selection in defined medium: Using untreated plates	◗ Low cost ◗ Handling accessibility (no extra effort when culturing)	◗ Uneven size of EBs. Larger EBs will increase the aggregation regions and reduce the purity of NSCs ◗ Time-consuming (extra 5 days)
Commercial NSC induction medium	◗ Time-saving (5 days) ◗ Can achieve high NSC purity by removing non-NSCs every 2 days	◗ Extra effort will be taken to remove non-NSCs every 2 days in order to increase the NSC purity

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