



RESEARCH ARTICLE

Plant-derived human recombinant growth factors and serum albumin maintain stemness of human-induced pluripotent stem cells

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Abstract

Stem cells are an important therapeutic source for recovery and regeneration, as their ability of self-renewal and differentiation offers an unlimited supply of highly specialized cells for therapeutic transplantation. Growth factors and serum are essential for maintaining the characteristics of stem cells in culture and for inducing differentiation. Because growth factors are produced mainly in bacterial (*Escherichia coli*) or animal cells, the use of such growth factors raises safety concerns that need to be resolved for the commercialization of stem cell therapeutics. To overcome this problem, studies on proteins produced in plants have been conducted. Here, we describe the functions of plant-derived fibroblast growth factor 2 (FGF2) and human serum albumin in the maintenance and differentiation of human-induced pluripotent stem cells (hiPSCs). Plant-derived FGF2 and human epidermal growth factor EGF were able to differentiate hiPSCs into neural stem cells (NSCs). These NSCs could differentiate into neuronal and glial cells. Our results imply that culturing stem cells in animal-free culture medium, which is composed of plant-derived proteins, would facilitate stem cell application research, for example, for cell therapy, by reducing contamination risk.

KEYWORDS

animal component-free growth factor, induced pluripotent stem cells, neural stem cells, plant-derived growth factor, stem cell application

1 | INTRODUCTION

Stem cells are an important therapeutic source for repair and regeneration because their capacity for self-renewal and differentiation offers a virtually unlimited supply of highly specialized cells for therapeutic transplantation (Biehl & Russell, 2009). In particular, the application of appropriate treatment according to

the degree and timing of damage to the nerve cells can be expected to be associated with a positive prognosis for the treatment of diseases of the nervous system. Since induced pluripotent stem cells (iPSCs) and neural stem cells (NSCs) play an important role in repairing peripheral nerve damage, researchers are using iPSCs and NSCs to promote regeneration after peripheral nerve damage (Wang et al., 2017).

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In the culture of iPSCs or NSCs intended to be used in cell therapy, growth factors are essential for maintaining the characteristics and inducing differentiation of stem cells. However, since the main systems for producing growth factors are based on bacterial (*Escherichia coli*) or animal cells, safety issues arise such as contamination with endotoxin from *E. coli* and the potential presence of immunogenic compounds, viruses, and pathogens derived from animal cells (Kunova et al., 2010; Mamat et al., 2015). Thus, the use of such growth factors may jeopardize the safety required for the commercialization of stem cell therapeutics. To solve these safety issues, a system for generating and using proteins produced in plants has been suggested as an alternative (Kunova et al., 2010; Musiychuk et al., 2013). Plant-based proteins including vaccines, enzymes, antibodies, growth factors, and serum albumin have demonstrated the benefits such as being cost-effective, having reduced risk, and allowing a rapid scale-up (Buyel et al., 2017; Chen & Davis, 2016; Moon et al., 2020).

Thus, animal component-free medium containing plant-derived growth factors and serum would reduce the contamination risks in stem cell transplantation. In this study, we evaluated the effectiveness of plant-derived growth factors and serum albumin in the maintenance and differentiation of human iPSCs.

2 | METHODS AND MATERIALS

2.1 | Plant-derived human recombinant proteins

Human recombinant FGF2 (Cat#E002) and EGF (Cat#F001) were provided by NBM Inc. (Poudel et al., 2019). Recombinant human serum albumin (HSA; Cat#A002) was produced in rice suspension cells (*Oryza sativa* L. cv. Dongjin) and provided by NBM Inc.

2.2 | Cell culture

Human fibroblast BJ cell-derived iPSCs were generated and characterized as described previously (Bang et al., 2018).

2.3 | Generation of iPSCs culture medium

FTDA medium was prepared according to the composition described by Frank et al. (2012). HiPSCs were cultured in an FTDA medium containing serum albumin and growth factors derived from *E. coli* (E-hiPSCs) or plant (P-hiPSCs).

2.4 | Cell counting

Cells were counted in every culture with an automated cell counter (Bio-Rad) and counter slides (Bio-Rad) with trypan blue (Sigma-Aldrich) in triplicate.

2.5 | Alkaline phosphatase staining

Cells cultured on a six-well culture dish were fixed using fixation solution (Stemgent) for 10 min at room temperature (RT) on a shaker. Fixed cells were washed by Dulbecco's phosphate-buffered saline (DPBS; Hyclone) for 5 min at RT on a shaker. A 1:1 mixture of solutions A and B (Stemgent) was added and the cells were incubated for 2 min at RT on a shaker. Then, solution C (Stemgent) was added and the cells were incubated for 30 min at RT on a shaker. The stained cells were washed with DPBS (Hyclone).

2.6 | Total RNA extraction

Total RNA was extracted using an RNeasy mini kit (Qiagen) following the supplier's instructions.

2.7 | Complementary DNA (cDNA) synthesis

Total RNA (1 µg) was reverse-transcribed into cDNA using a high-capacity cDNA reverse transcription kit (Applied Biosystems).

2.8 | Polymerase chain reaction (PCR)

All reverse-transcription polymerase chain reactions (RT-PCRs) used Ex Taq polymerase (TaKaRa Bio Inc.) and were performed for 25–28 cycles for all markers. Gene expression levels were evaluated by quantitative RT-PCR using SYBR Green with low ROX (Enzymomix) in a Roche real-time PCR system (Roche). The primer sequences are listed in Table S1.

2.9 | Immunocytochemistry

Cells were fixed in 4% paraformaldehyde for 10 min at RT and washed with DPBS (Hyclone). For permeabilization, 0.5% Triton X-100 (Sigma-Aldrich) in DPBS was added for 10 min at RT and the cells were blocked with 2% bovine serum albumin (Gibco) in DPBS for 1 h at RT. The cells were then rinsed and incubated with primary antibody overnight at 4°C and washed with 0.2% Tween 20 in DPBS. After washing, cells were incubated with secondary antibody for 1 h at RT and washed with 0.2% Tween 20 in DPBS. For nuclear staining, cells were incubated in 4'-6-diamidino-2-phenylindole (DAPI) for 1 min at RT and washed with 0.2% Tween 20 in DPBS. Information on antibodies is provided in Table S2.

2.10 | Genomic DNA isolation and bisulfite treatment

Genomic DNA was isolated using a G-spin total DNA extraction kit (Intron). Genomic DNA (1 µg) was bisulfite converted using an

Epi-ectect Bisulfite Kit (Qiagen) according to the manufacturer's instructions.

2.11 | Karyotyping

Chromosomal karyotype analysis was conducted by Korea Research of Animal Chromosomes. G-banding, used for chromosome identification, is the most common technique used to identify abnormalities due to chromosome number, translocations, deletions, inversions, and amplifications (Bickmore, 2001; Schreck et al., 2001).

2.12 | Mycoplasma testing

Mycoplasma contamination detection was conducted using an e-Myco™ Mycoplasma PCR detection kit (Intron). PCR was performed according to the manufacturer's manual.

2.13 | In vitro differentiation of E-hiPSCs and P-hiPSCs

hiPSCs were plated on a four-well plate coated with Matrigel (Corning). Two days later, the medium was replaced with Dulbecco's modified eagle medium (DMEM) culture medium containing 10% FBS and the cells were cultured with daily medium changes for 10 days.

2.14 | Teratoma formation for in vivo differentiation

hiPSCs were transplanted into immunodeficient mice and all mice were sacrificed at 10 weeks after transplantation. The teratomas were dissected, fixed with Bouin's solution, and embedded in paraffin. Paraffin sections were stained with hematoxylin and eosin (H&E).

2.15 | H&E staining

The sections were stained with hematoxylin (Sigma-Aldrich) and immersed in eosin (Sigma-Aldrich). Sections were dehydrated in alcohol solutions (50%, 70%, 80%, 95%, and 100% alcohol), mounted with mounting medium (Fisher Scientific), and examined under a microscope.

2.16 | Induction of differentiation into NSCs

For embryoid body (EB) formation, iPSC colonies (E-hiPSCs and P-hiPSCs) were removed from cell culture plates with accutase (Sigma-Aldrich) and transferred in noncoated 60 mm culture dishes (SPL Life

Science Co.). The EBs were maintained in mTeSR1 (StemCell Technologies) with Y-27632 (Selleckchem) for 4 days. For rosette formation, EBs were plated in a T-25 flask (SPL Life Science Co.) coated with 0.2% gelatin in neural induction medium consisting of DMEM and Ham's F-12 medium (1:1) (DMEM/F12; Corning) with N2 supplement (Gibco), B27 supplement (Gibco), 10 ng/ml EGF (Peprotech), and 10 ng/ml human FGF2 (Peprotech or NBM Inc.) for 10–14 days. Rosettes were mechanically isolated and transferred into noncoated 12-well plates for neurosphere formation in rosette floating medium consisting of DMEM/F12 with N2 supplement and 10 ng/ml FGF2 for 7 days. Neurospheres were harvested with a pipette tip and replated on six-well plates coated with laminin (Sigma-Aldrich) in culture medium consisting of DMEM/F12 with N2 supplement, B27 supplement, Gem21 supplement (GeminiBio), and 10 ng/ml FGF2 (Peprotech or NBM Inc.). Cells were detached with 0.25% trypsin-EDTA (Gibco) and trypsin was inhibited with trypsin inhibitor (Sigma-Aldrich). The cells were centrifuged at 300g for 4 min and replated on a Matrigel-coated 24-well plate in NSC induction medium, which contained DMEM/F12, N2, B27, and Gem21 supplements, and FGF2 (Peprotech or NBM Inc.). Established NSCs derived from E-hiPSCs and P-hiPSCs were cultured in NSC culture medium.

2.17 | Differentiation into neuronal and glial cells

For neuronal differentiation, 1×10^4 NSCs were plated on a laminin-coated four-well plate and cultured in neuron differentiation medium consisting of DMEM/F12, N2 and B27 supplements, and 300 ng/ml cyclic adenosine monophosphate (Sigma-Aldrich) for 4–5 weeks. For induction of early astrocyte differentiation, 1×10^4 NSCs were plated on a laminin-coated four-well plate and cultured in astrocyte differentiation medium consisting of DMEM/F12, N2 supplement, B27 supplement without vitamin A (Gibco), Glutamax (Gibco), 10 ng/ml CNTF (Peprotech), and 20 ng/ml BMP4 (Prospec) for 3 weeks.

2.18 | Statistical analysis

All data are presented as means \pm SEM. All statistics were calculated using independent *t* tests or analysis of variance (ANOVA) with the least significant difference tests for post hoc analysis. A value of $p < .05$ was considered significant. *It must be corrected to <0.05 and not $<.05$.

3 | RESULTS

3.1 | Culture of induced pluripotent stem cells in medium containing plant-derived growth factors and human serum albumin

The hiPSCs generated from BJ cells were initially stabilized with mTeSR, a commercial medium, and cultured in medium containing *E. coli*-derived FGF2 and HSA or plant-derived FGF2 and HSA.

Observations of cell morphology at each passage confirmed that hiPSCs cultured in medium containing *E. coli*-derived FGF2 and HSA (E-hiPSCs) and hiPSCs cultured in medium containing plant-derived FGF2 and HSA (P-hiPSCs) maintained the specific shape of hiPSCs (Figure 1a). Also, there was no significant difference in the number of living cells between hiPSCs cultured in the two media (Figure 1b). These data suggest that the effects of plant-derived growth factors and HSA on hiPSC proliferation and maintenance are similar to those of *E. coli*-derived growth factors and animal-derived serum.

3.2 | Characterization of P-hiPSCs

We performed various assays including quality assurance and quantity control assays to confirm the characteristics of E-hiPSCs and P-hiPSCs. Alkaline phosphatase positivity indicated pluripotency of E-hiPSCs and P-hiPSCs (Figure 2a) (Štefková et al., 2015; Takahashi et al., 2007). RT-PCR and quantitative RT-PCR analyses showed that P-hiPSCs expressed pluripotency genes such as *OCT4*, *SOX2*, and *NANOG* at levels equivalent to those of E-hiPSCs. (Figure 2b,c). Immunocytochemistry analysis also showed that both E-hiPSCs and P-hiPSCs were positive for *OCT4/SSEA1*, *SOX2/TRA-1-100*, and *NANOG/TRA-1-81* (Figure 2d,e). Karyotype analysis performed to confirm chromosomal integrity showed that E-hiPSCs and P-hiPSCs maintained their normal karyotype (Figure S1). Finally, the mycoplasma detection analysis performed to confirm sterility showed that all cells were free of mycoplasma (Figure S2).

To confirm the *in vitro* differentiation ability of E-hiPSCs and P-hiPSCs, we induced spontaneous differentiation and confirmed that E-hiPSCs and P-hiPSCs differentiated into the cell types of all three germ layers (Figure 3a). Next, we injected E-hiPSCs and P-hiPSCs into NOD/SCID mice, respectively, to verify their *in vivo* differentiation ability. Two weeks after injection, we visually confirmed that teratomas were formed (data not shown). Histological analysis performed 10 weeks after injection showed that teratomas formed by injection of E-iPSCs and P-iPSCs each contained endodermal, mesodermal, and ectodermal tissues (Figure 3b,c).

3.3 | Establishment of NSCs from P-hiPSCs

We induced differentiation of established hiPSCs into NSCs using NSC induction medium and culture medium containing *E. coli*-derived or plant-derived growth factors. We refer to NSCs derived from E-hiPSCs and P-hiPSCs by differentiation in medium containing *E. coli*-derived growth factors as EE-NSCs and EP-NSCs, respectively. Similarly, we refer to NSCs derived from E-hiPSCs and P-hiPSCs by differentiation in medium containing plant-derived growth factors as PE-NSCs and PP-NSCs, respectively.

We performed molecular and cellular analysis of these NSCs to confirm their characteristics. Cell morphologies of the neural lineage were observed regardless of the origin of growth factors in the NSC induction medium (Figure 4a,b). RT-PCR analysis showed that the four types of NSC lines expressed NSC-specific genes such as *SOX1*, *SOX2*, *PAX6*, and *NESTIN* (Figure 4c). Immunocytochemistry analysis to

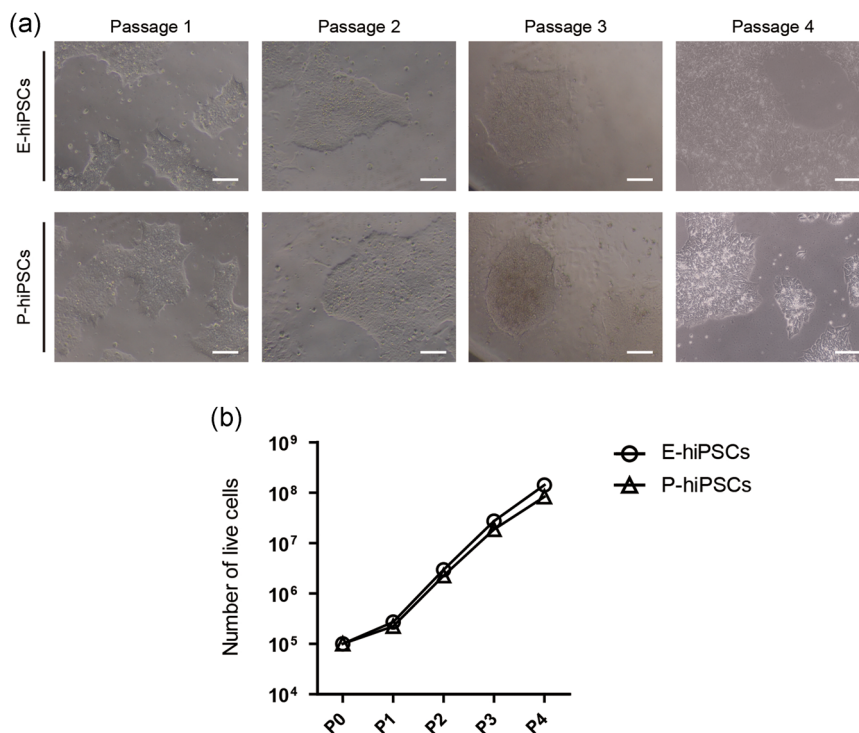
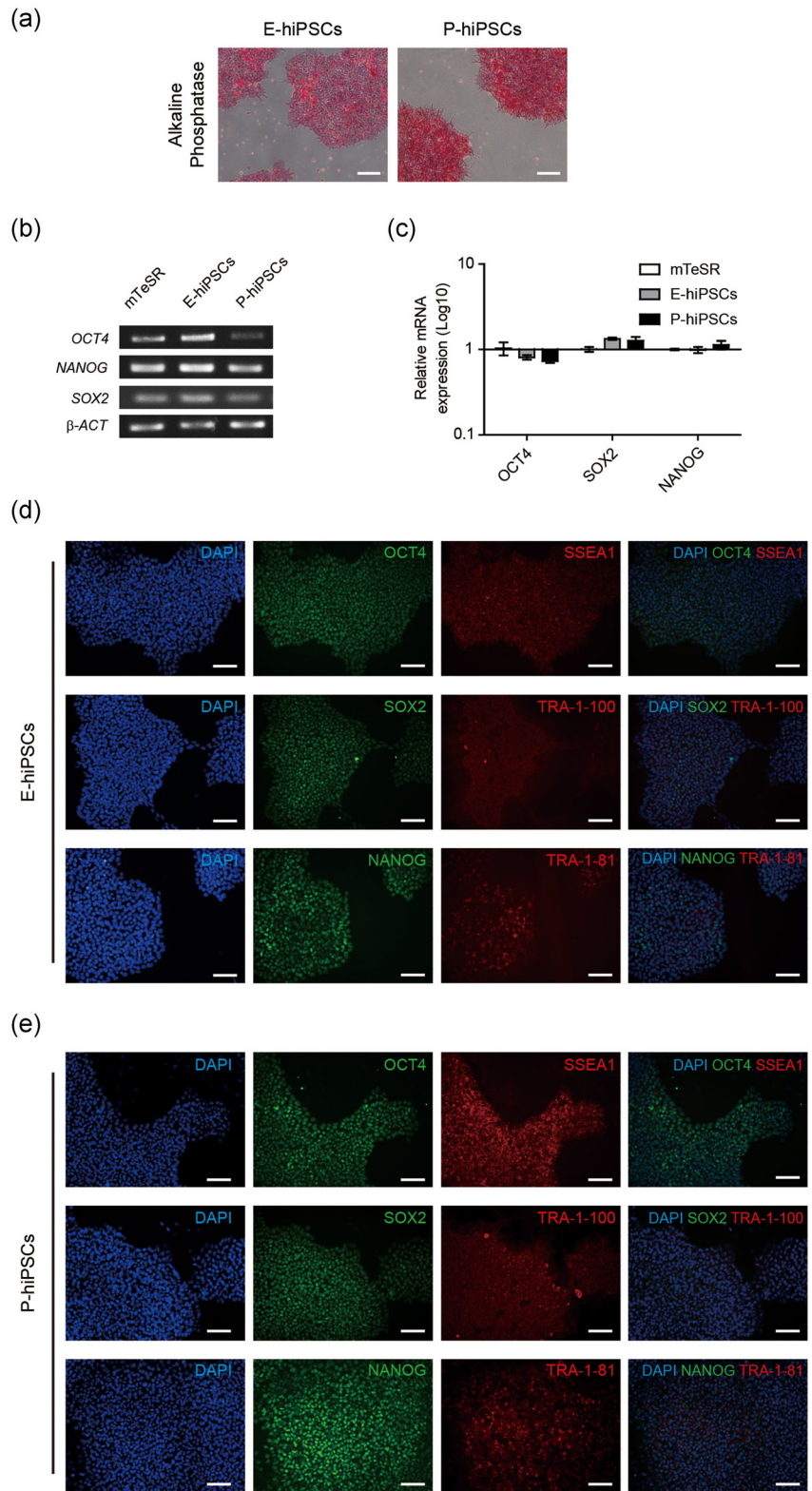


FIGURE 1 Culture of induced pluripotent stem cells in medium containing plant-derived growth factors. (a) Morphologies of hiPSCs in culture medium containing *Escherichia coli*-derived and plant-derived growth factors and serum albumin with increasing passages. (b) Growth curves of E-hiPSCs and P-hiPSCs. Scale bar = 250 μ m

FIGURE 2 Expression of pluripotency markers in E-hiPSCs and P-hiPSCs. (a) Alkaline phosphatase staining. Scale bar: 250 μ m. (b) Reverse-transcription polymerase chain reaction (RT-PCR) analysis showing mRNA expression of pluripotency markers. β -ACTIN mRNA was used as a loading control. (c) Quantitative RT-PCR analysis of mRNA levels of the pluripotency genes *OCT4*, *SOX2*, and *NANOG*. Graphs represent changes after normalization to mTeSR. (d, e) Immunofluorescence images of *OCT4*-SSEA1-positive, *SOX2*-TRA-1-100-positive, and *NANOG*-TRA-1-81-positive cells. Nuclei were counterstained with DAPI. Scale bar = 250 μ m



confirm the differentiation capacity of the established NSCs indicated that all four types were able to differentiate into neuronal and glial cells (Figure 5a,b). Overall, these data indicate that plant-derived growth factors enable differentiation of hiPSCs into NSCs from maintenance of NSCs characteristics.

4 | DISCUSSION

In 2012, Frank et al. developed culture conditions in which chemically defined factors enable maintaining human PSC (hPSC) lines (Frank et al., 2012). These culture conditions facilitate the application of

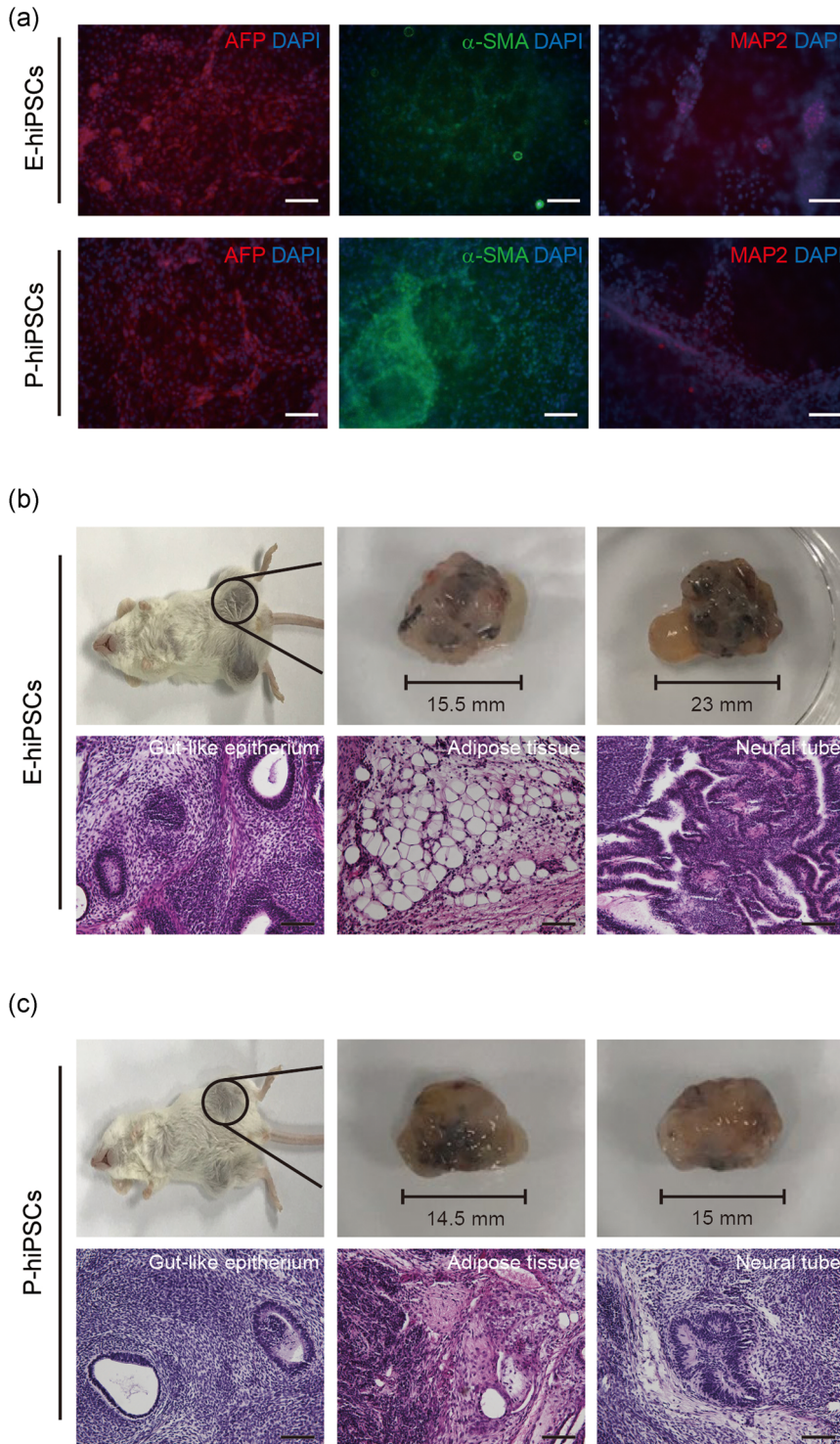


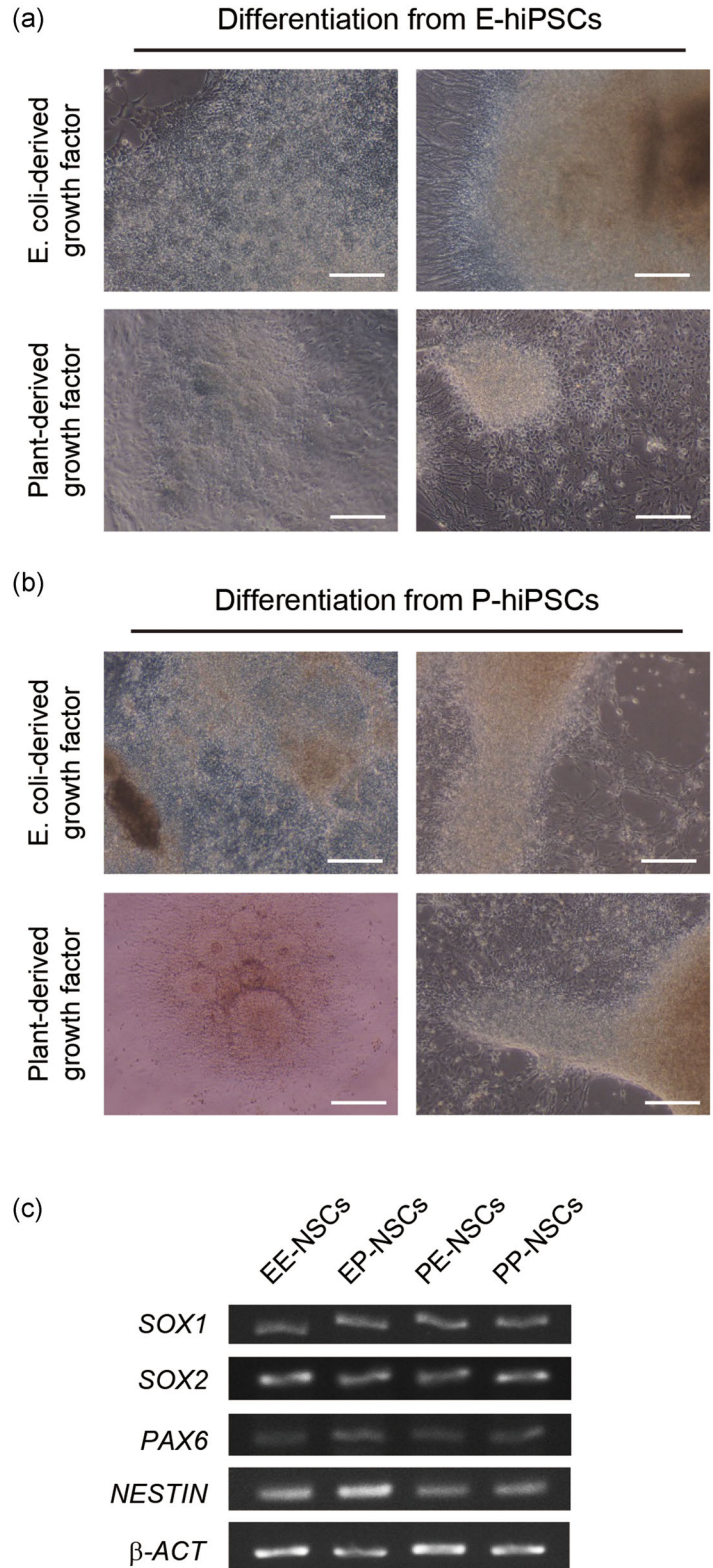
FIGURE 3 In vitro and in vivo differentiation of E-hiPSCs and P-hiPSCs. (a) In vitro differentiation. Immunofluorescence images of cells positive for alpha-fetoprotein (AFP) (endoderm), α -smooth muscle actin (α -SMA) (mesoderm), and MAP2 (ectoderm). Scale bar = 250 μ m. (b,c) In vivo differentiation. Teratoma formations and hematoxylin and eosin (H&E) staining of teratoma tissue sections. Scale bar = 100 μ m

stem cells as therapeutic agents because they promote robust self-renewal and preserve pluripotency of hPSCs while being simpler and more cost-effective than conventional hPSC cultures (Frank et al., 2012). However, since reagents used for cell culture are derived from animals, cells cultured under these conditions cannot be used for cell therapy because the risk of infection from animal-derived pathogens cannot be completely excluded (Huang & Macdonald, 2009; Inzunza et al., 2005; Lee et al., 2005). Therefore, it is necessary to prevent the

side effects that may be caused by using animal-derived reagents and to improve the conditions of stem cell culture and differentiation methods.

Recently, plant-derived proteins have emerged as an alternative for biopharmaceuticals including vaccines, enzymes, antibodies, and therapeutic proteins because of their cost-effective, high volume, low risk, and rapid expansion advantages (Buyel et al., 2017; Chen & Davis, 2016; Kunova et al., 2010; Moon et al., 2020; Musiychuk et al.,

FIGURE 4 Induction of differentiation of E-hiPSCs and P-hiPSCs into neural stem cells. (a) Differentiation of E-hiPSCs. (b) Differentiation of P-hiPSCs. (c) Reverse-transcription polymerase chain reaction analysis showing mRNA expression of neural stem cell (NSC)-specific genes in EE-NSCs, EP-NSCs, PE-NSCs, and PP-NSCs. β -ACTIN mRNA was used as a loading control. Scale bar = 250 μ m



2013). Here, we show that plant-derived growth factors allow to maintain the pluripotency of hiPSCs and to induce differentiation into NSCs. In our study, iPSC maintenance medium produced by using plant-derived FGF2 and HSA and that containing *E. coli*-derived factors had similar efficiency in maintaining hPSC characteristics.

Therefore, we believe that stem cells cultured in a medium containing animal-free growth factors and serum albumin will be more suitable for applied research such as cell transplantation and therapeutics. Moreover, we also verified the efficiency of plant-derived growth factors in differentiation into and maintenance of NSCs.

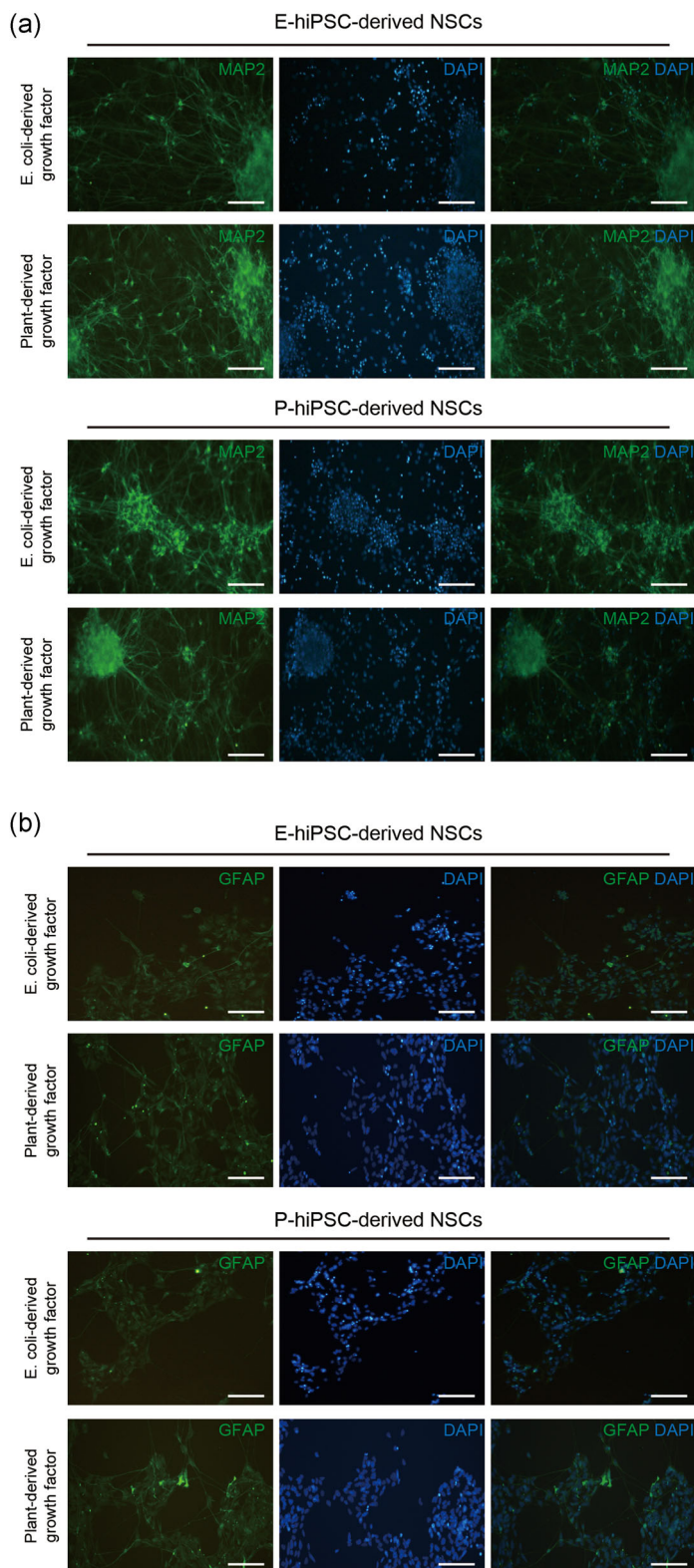


FIGURE 5 Differentiation of EE-NSCs, EP-NSCs, PE-NSCs, and PP-NSCs into neuronal and glial cells. (a) Immunofluorescence images of MAP2-positive neuronal cells differentiated from NSCs. (b) Immunofluorescence images of GFAP-positive glial cells differentiated from NSCs. Scale bar = 250 μ m

In conclusion, in this study, we produced hiPSC and NSC culture media containing plant-derived growth factors and serum albumin to reduce the risks of using existing cell cultures for applied research. We verified the feasibility of using these plant-derived proteins in applied research by verifying their functionality in the culture of hiPSCs and their differentiation into NSCs. This study can be a starting point for the

commercialization and optimization of stem cell therapeutics by improving cost-effectiveness and safety through stem cell culture and differentiation methods that rely on the use of plant-derived proteins. However, despite reducing the risk of contamination based on plant-derived products, research is still needed to eliminate the inherent toxicity of the product itself. In addition, therapeutic applications such as

iPSC-based cell transplantation require safety as well as efficacy, which requires additional research.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

AUTHOR CONTRIBUTIONS

Yukyeong Lee carried out all the experiments, analyzed the data, and wrote the manuscript. Hye J. Lee contributed to the generation of NSCs. Seokbeom Ham and Dahee Jeong performed tissue embedding. Min-seong Lee performed injection to generate teratomas. Kinarm Ko contributed to the conception and design of the experiments, financial support, manuscript writing, and final approval of the manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

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