JARB
Journal of Animal Reproduction and Biotechnology

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Generation of Neural Progenitor Cells from Pig Embryonic Germ Cells

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ABSTRACT   As a preclinical study, many researchers have been attempted to convert the porcine PSCs into several differentiated cells with transplantation of the differentiated cells into the pigs. Here, we attempted to derive neuronal progenitor cells from pig embryonic germ cells (EGCs). As a result, neuronal progenitor cells could be derived directly from pig embryonic germ cells through the serum-free floating culture of EB-like aggregates (SFEB) method. Treating retinoic acid was more efficient for inducing neuronal lineages from EGCs rather than inhibiting SMAD signaling. The differentiated cells expressed neuronal markers such as PAX6, NESTIN, and SOX1 as determined by qRT-PCR and immunostaining. These data indicated that pig EGCs could provide valid models for human therapy. Finally, it is suggested that developing transgenic pig for disease models as well as differentiation methods will provide basic preclinical data for human regenerative medicine and lead to the success of stem cell therapy.

Keywords: embryonic germ cells, neuronal differentiation, pig, retinoic acid

INTRODUCTION

In 1998, the establishment of human embryonic stem cells (ESCs) paved the way for regenerative medicine by tissue engineering. This trend was accelerated by the establishment of human induced pluripotent stem cells (iPSCs) and cloned ESCs in 2007 and 2013, respectively (Choi and Lee, 2019). Accompanied by the generation of human PSCs, methods for differentiation into various types of cells have been developed (reviewed in (Tabar and Studer, 2014)). Recently, in addition to differentiation into specific cell types, putative organs composed of multi-cellular tissues, named organoid, were generated *in vitro* (reviewed in (Yin et al., 2016)). Various types of

tissues including intestinal, brain, eye and kidney have been researched. Because they more closely resemble *in vivo*-organs and tissues than a single type of cells, these organoids might provide *in vitro*-tools for drug screening, disease modeling, and organ development models. As a clinical trial of human PSCs for cell therapy as mentioned above, it makes the research of animal PSCs involving differentiation and transplantation study more important as a preliminary.

For this reason, many researchers have been attempted to convert the porcine PSCs into various types of somatic cells with transplantation of the differentiated cells into the pigs. First of all, hepatocytes have been derived from pig iPSCs and ESCs (Ao et al., 2014; Park et al., 2015). The

differentiated cells from iPSCs and ESCs have molecular and functional similarities with hepatocytes. They have abilities including lipid metabolism, glycogen storage, and LDL uptake. Moreover, several studies have tried to transplant iPSCs or differentiated cells into pig disease models. Rod photoreceptors and retinal pigment epithelial cells were produced from pig iPSCs, and then these cells were transplanted into subretinal space of pigs (Zhou et al., 2011; Sohn et al., 2015). Other groups used undifferentiated pig iPSCs to treat myocardial infarction and myocardial ischemia (Li et al., 2013; Zhang et al., 2014). The engrafted iPSCs ameliorated symptoms via differentiation into vessel cells (Li et al., 2013). However, undifferentiated PSCs have the potential to form tumors when engrafted into the body (Lee et al., 2013), it needs to be careful about the implant of undifferentiation PSCs. Recent studies showed that PGCs and skeletal myotubes could be induced from pig iPSCs (Wang et al., 2016; Genovese et al., 2017).

Neuron cells derived from PSCs such as dopaminergic neuron and retinal cells have been considered as alternative cell sources for treating neuronal diseases (Osakada et al., 2008). In the pig, neuronal cells have successfully derived from pig PSCs (Puy et al., 2010; Gallegos-Cárdenas et al., 2015). Here, we attempted to derive neuronal progenitor cells using previously derived pig embryonic germ cells (EGCs) (Choi et al., 2018). To optimize the culture conditions for inducing neuronal cells, various signaling molecules involved in neuronal development were analyzed. As a result, retinoic acid treatment, rather than inhibition of SMAD signaling, have more crucial roles in neural differentiation of pEGCs. Similar to other species, neuronal progenitor cells induced by treatment of retinoic acid expressed markers of neuronal progenitor such as PAX6, NESTIN and SOX1 as determined by immunostaining and PCR analysis.

MATERIALS AND METHODS

Animal care

The care and experimental use of pigs and mice were approved by the Institutional Animal Care and Use Committees (IACUC) at Seoul National University (Approval No.: SNU-16120-9-1 for MEFs isolation). Pregnant ICR mice were purchased from SAMTACO BIO Inc., Korea. The mice were taken care according to the standard protocol of IACUC and sacrificed by cervical dislocation after anesthesia.

Culture of pig embryonic germ cells

Pig embryonic germ cells (EGCs) previously derived were used in this study (Choi et al., 2018). According to the previous study, the EGCs were cultured with EGC media supplemented with 40 ng/mL human recombinant basic fibroblast growth factor (bFGF; R&D Systems). EGC media consisted of 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM, low glucose) and Ham's F10 media containing 15% fetal bovine serum (FBS; collected and processed in the USA), 2 mM glutamax, 0.1 mM β-mercaptoethanol, 1× MEM nonessential amino acids, and 1× antibiotic-antimycotic (all from Gibco, USA). Subculture was performed every 7 days. EGC colonies were dissociated by treating 0.25% trypsin solution for 5 min and then transferred into new feeder cells. Media were changed every 24 h and all cells were cultured under humidified conditions containing 5% CO_2 at 37°C.

In vitro differentiation into neural progenitor cells

Differentiation of neural lineage was accomplished according to the serum-free floating culture of EB-like aggregates (SFEB) methods with some modifications (Watanabe et al., 2005). Cultured EGCs were dissociated into single cells using 0.25% trypsin/EDTA solution (Welgene) and cultured in Ultra-Low attachment plates (Sigma Aldrich, MO, USA) with STEMdiff™ Neural Induction Medium (STEMCELL, Vancouver, Canada) containing 5 μM retinoic acid (RA) and SMAD signaling inhibitors (SMADi; 400 μM Noggin and 2 μM SB431542) for 5 days. After suspension culture, the dissociated cells were aggregated and formed embryoid bodies. Cultured embryoid bodies were seeded on BD Matrigel™ (BD Biosciences, NJ, USA)-coated plates and cultured for 8-11 days with STEMdiff™ Neural Induction Medium containing 10 ng/mL bFGF, RA, and SMAD inhibitors. After 8-11 days, differentiated cells were used for immunostaining or qPCR analysis.

Quantitative real-time polymerase chain reaction (qPCR)

Total RNA from individual samples was extracted using TRIzol® reagent (Invitrogen, MA, USA) according to the manufacturer's instructions. Complementary DNA was synthesized using a High-capacity RNA-to-cDNA Kit (Applied Biosystems, CA, USA) according to the manufac-

turer's instructions, producing a final volume of 20 μL. Extracted cDNA samples were amplified with DyNAmo HS SYBR Green qPCR Kit (Thermo Scientific, MA, USA) containing 1 pmol of each primer set listed in Table 1 in a 10 μL reaction volume. Amplification and detection were conducted using the ABI 7300 Real-Time PCR system (Applied Biosystems) under the following conditions: one cycle of 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 sec and annealing/extension for 1 min (annealing/extension temperatures depended on each primer set). We analyzed the dissociation curve and loaded the amplified products on gels to confirm the specificity of PCR products. The relative expression level was calculated by normalizing the threshold cycle (Ct) values of each gene to that of the *ACTB* via the Δ^{-Ct} method (Livak and Schmittgen, 2001).

Immunostaining

Differentiated cell samples were preincubated for 10 min at 4°C and fixed with 4% paraformaldehyde for 30 min. After washing twice with Dulbecco's phosphate-buffered saline (DPBS; Welgene), samples were treated for 1 h with 10% goat serum in DPBS to prevent nonspecific binding. Serum-treated cells were incubated overnight at 4°C with primary antibodies. The primary antibodies used were as follows: SOX1 (Santa Cruz Biotechnology, CA, USA; 1:50), PAX6 (Santa Cruz Biotechnology; 1:50), NESTIN (Santa Cruz Biotechnology; 1:50), and ZO-1 (Santa Cruz Biotechnology; 1:50). When used the antibodies for intracellular proteins such as SOX1 and PAX6, fixed cells were treated for 5 min with 0.2% Triton-X100 (Sigma-Aldrich, MO, USA) before serum blocking. After incubation with the primary antibody, the cells were treated for

3 h at room temperature with Alexa Fluor-conjugated secondary antibodies. Nuclei were stained with Hoechst 33342 (Molecular Probes). Images of stained cells were captured using an inverted fluorescence microscope.

Statistical analysis

All gene expression data from qPCR analyses were statistically analyzed using GraphPad Prism 6 statistical software (GraphPad Software, CA, USA). Statistical differences between datasets were determined by one-way analyses of variance (ANOVAs) followed by Fisher's least significant difference (LSD) tests. Differences were considered significant at $p < 0.05$.

RESULTS

Pig EGCs can be differentiated into neural lineage by treating retinoic acid

To apply pig PSCs for a therapeutic model, a development of methods for directed differentiation into specific lineages are important. For this reason, this study aimed to develop a method for inducing neural lineage from pig EGCs. It has been verified that several signaling including SMAD inhibition and retinoic acid are involved in differentiation into neural cells from pluripotent cells (Parsons et al., 2011). So, firstly, to find which molecules are most efficient in inducing neural cells from pig EGCs, SMAD signaling inhibitors and retinoic acid were analyzed (Fig. 1A). Embryoid bodies were successfully formed by suspension culture as presented in Fig. 1B and, after plating onto matrigel, they were cultured for 8 days (Fig. 1C). As measured by qPCR, neural-specific marker genes such as *PAX6, NES, PLAG1*, and *DACH1* were highly expressed in the retinoic acid-treated group compared with other groups (Fig. 1D). When examined with various concentrations of retinoic acid (Fig. 2A and B), the expression of neural markers was most efficiently up-regulated in 5 μM-treated groups (Fig. 2C). Five μM retinoic acid successfully induced neural lineage-related genes during suspension culture (Fig. 2D). Furthermore, bFGF treatment during adherent culture facilitated the proliferation of neural progenitor cells (Fig. 3). Induced neural progenitor cells expressed neural markers such as SOX1, PAX6, ZO1, and NESTIN as determined by immunostaining (Fig. 4). Taken together, we developed methods for inducing neural lineage from pig EGC by treating retinoic acid.

Table 1. Primer sets for neuronal markers

Genes	Primer sequence	Size
PAX6	5'– AGAGAAGACAGGCCAGCAAC –3' 5'– GGCAGAGCACTGTAGGTGTT –3'	169
Nestin	5'– TGCCTGGGGGAGGAATCTTTT –3' 5'– CTCTTCAGCCAGGTTGTCGC –3'	252
PLAG1	5'– CAGCCAAGATTGGCCACAATG –3' 5'– AGCCATGTGCCTGATGACAGA –3'	116
DACH1	5'– CAGGCTTTCGACCTGTTCCT –3' 5'– CAGTCCCCTCAAGATGCGAA –3'	126
ACTB	5'– GTGGACATCAGGAAGGACCTCTA –3' 5'– ATGATCTTGATCTTCATGGTGCT –3'	131
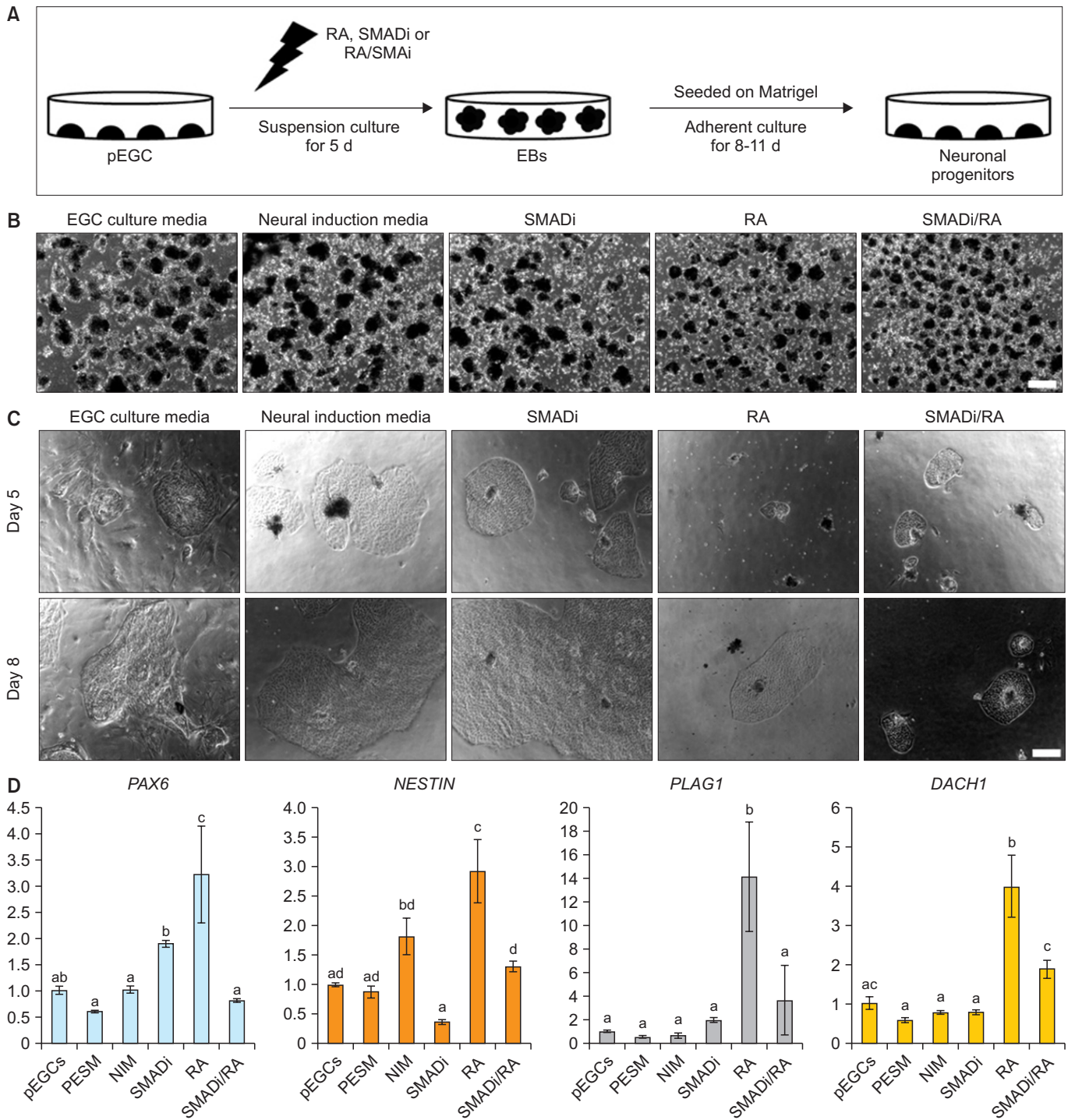
Fig. 1. Effects of culture conditions on neural differentiation in pig EGCs. (A) Differentiation of neural lineage was accomplished according to the serum-free floating culture of EB-like aggregates (SFEB) methods with some modifications. Differentiation was conducted with the porcine embryonic stem cell media (PESM) as a control media and the neural induction media (NIM). To find which molecules are most efficient in inducing neural cells from pig EGCs, SMAD signaling inhibitors and retinoic acid were selected. (B) Embryoid bodies were formed by suspension culture. (C) After plating onto matrigel, they were cultured for 8 days. (D) As measured by qPCR, genes expressed in neural progenitors were highly expressed in the retinoic acid-treated group compared with other groups. Scale bar = 400 μm.
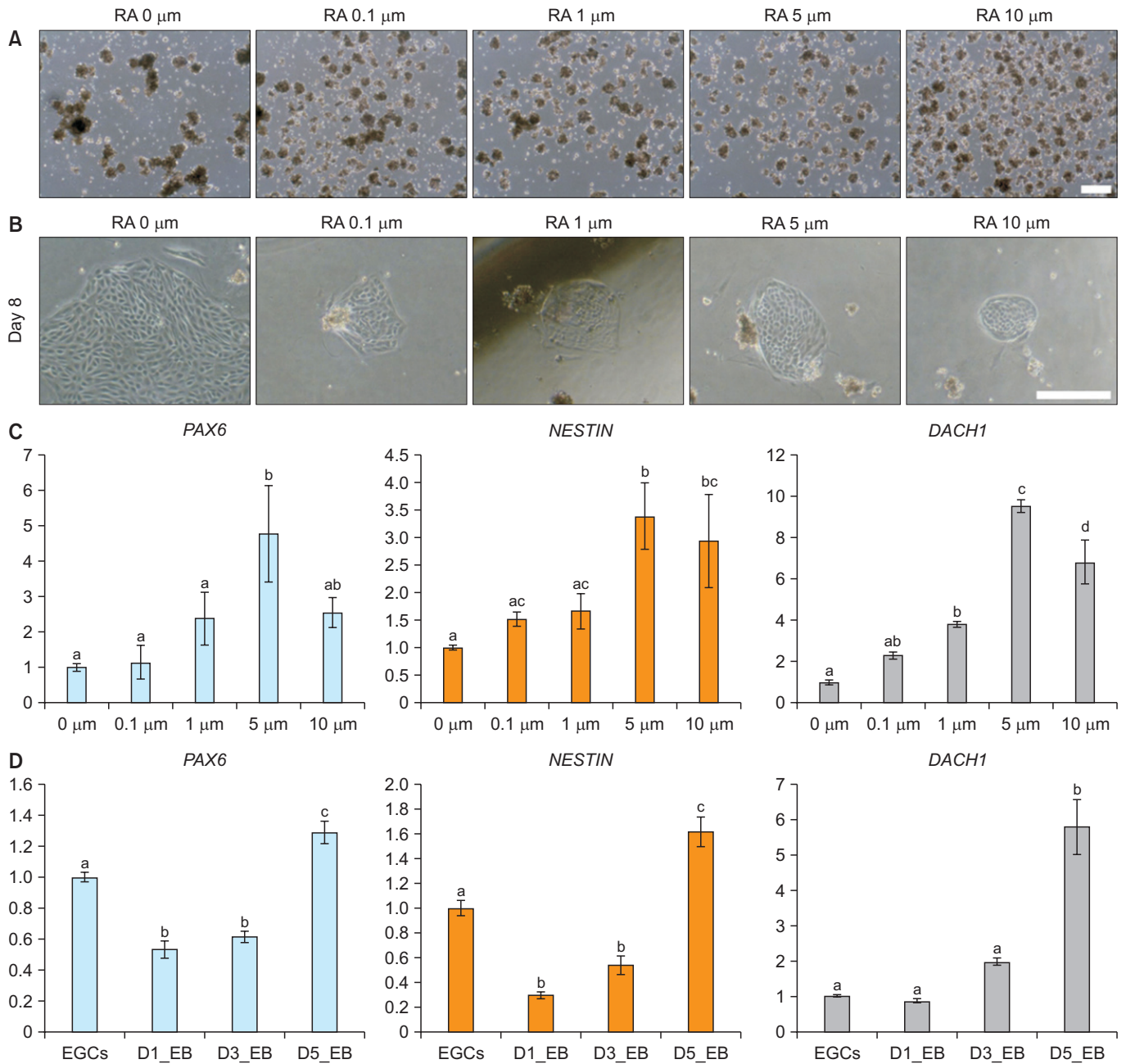
Fig. 2. Effects of RA concentration on neural differentiation in pig EGCs. (A, B) To optimize culture conditions, embryoid bodies were formed with various RA concentrations and then cultured on Matrigel for 8 days. (C) When examined with various concentrations of retinoic acid, the expression of neural markers was most efficiently up-regulated in 5 µM-treated groups. (D) Five µM retinoic acid-induced neural lineage-related genes during suspension culture. Scale bar = 400 µm.

DISCUSSION

Human pluripotent stem cell-derived neural cells such as dopaminergic neuron and retinal cells have been anticipated as alternative cell sources for cell therapy instead of fetal tissues (Osakada et al., 2008). Induction of neuronal lineage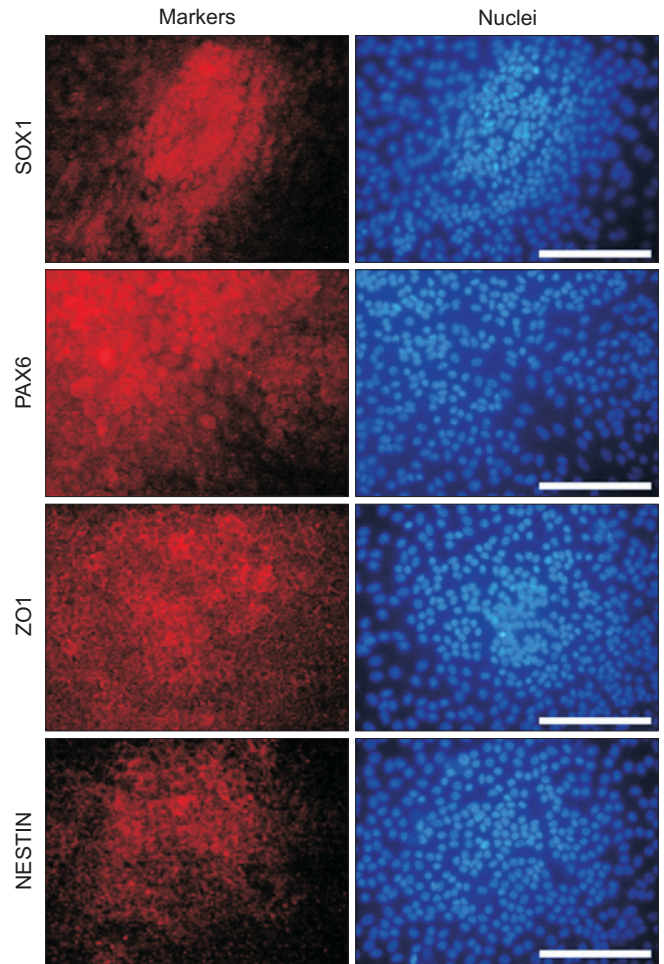s from pluripotent cells has been accomplished by co-culture with feeder cells made of PA6 stromal cells or culture with serum-free media containing signaling molecules including retinoic acid and inhibitors of SMAD signaling (reviewed in (Schwartz et al., 2008)). Depending on the combination of signaling molecules, pluripotent stem cells (PSCs) have been able to differentiate into various types of neurons including dopaminergic neurons (Lee
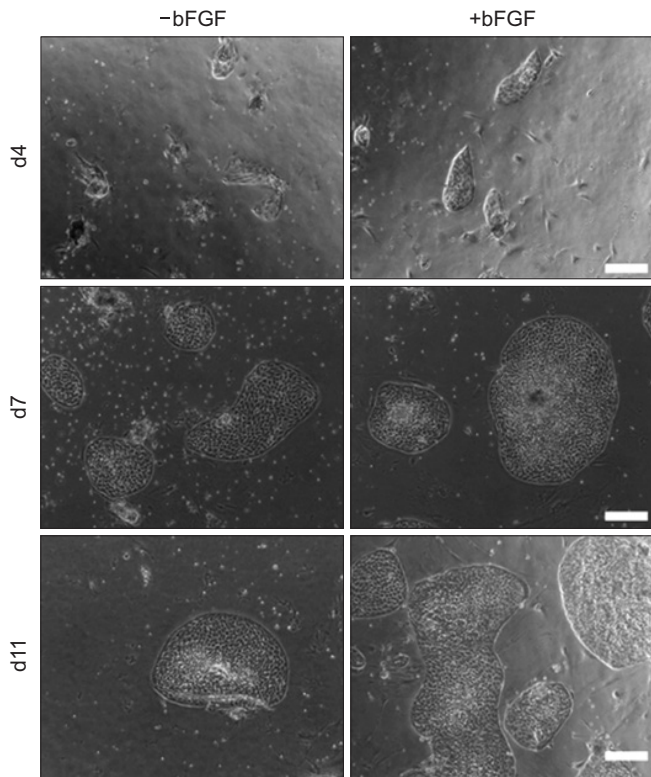
Fig. 3. Effects of bFGF on the growth of neural progenitor cells. Basic FGF treatment during adherent culture facilitated the proliferation of neural progenitor cells. Scale bar = 400 μm.

Fig. 4. Expression of neural markers as determined by immunostaining. The expression of neural markers such as SOX1, PAX6, ZO1, and NESTIN was determined by immunostaining. Scale bar = 200 μm.

et al., 2000), motor neurons (Wichterle et al., 2002), cerebral cortex (Eiraku et al., 2008) and pituitary (Suga et al., 2011) and cortical pyramidal neurons (Espuny-Camacho et al., 2013). Interestingly, neuroectodermal cells derived from PSCs could recapitulate *in vivo*-development and form multicellular organs, called brain organoid, through three-dimensional culture with extracellular matrix (Matrigel) (Lancaster et al., 2013). Because they more closely resemble *in vivo*-organs and tissues than a single type of cells, brain organoids might provide *in vitro*-tools for drug screening, disease modeling and organ development models (Yin et al., 2016).

Studies on stem cells of large animals have been proven important for preclinical researches of human disease. So, many researchers have been attempted to convert the porcine stem cells into neuronal lineages and transplant the differentiated cells into the pigs (Puy et al., 2010; Zhou et al., 2011; Gallegos-Cardenas et al., 2015). First of all, neuronal differentiation has successfully induced from *in vitro*-cultured ICM and iPSCs. During *in vitro*-culture, pig ICMs were spontaneously differentiated

into neural rosette-like structures while they couldn't be maintained during an expanded culture (Puy et al., 2010). The cells isolated from neural rosette-like structures could be developed into neurons including astrocyte and oligodendrocytes. In another study, researchers successfully induced neural progenitor cells from pig iPSCs (Gallegos-Cardenas et al., 2015). It was assessed that neural differentiation of pig iPSCs resembles that of human PSCs in terms of gene expression pattern, which means pig PSCs could provide valid models for human therapy. In addition, Zhou and colleagues tried to transplant differentiated cells into pig disease models. Rod photoreceptors were produced from pig iPSCs, and then these cells were engrafted into the subretinal space of pigs (Zhou et al., 2011). The transplanted cells have successfully resided in

the subretinal region.

In this study, we attempted to derive neuronal progenitor cells from pig EGCs according to the serum-free floating culture of the EB-aggregates (SFEB) method (Watanabe et al., 2005). Similar to other species, neuronal progenitor cells were successfully induced by treatment of retinoic acid and these cells expressed neuronal markers such as PAX6, NESTIN, and SOX1. In the case of the inhibition of SMAD signaling, because SMAD signaling was not sufficiently activated to interfere with the neural differentiation in pEGCs, it seems that the inhibitions have no significant effects on the neural differentiation. Despite the lack of *in vivo* developmental competence (Choi et al., 2018), these data indicated that specific types of somatic cells could be directly induced from pig EGCs by manipulating culture conditions. In consistent with human and mouse, the same signaling molecules were involved in neural differentiation of pig EGCs, which means pig EGCs could be applied for human researches. Recently, authentic pig ESCs having teratoma-forming capacity were derived and could directly differentiate into neural cells, pancreatic progenitors, and cardiac muscles using the culture conditions previously applied in human PSC researches, which suggests that pig model is suitable for preclinical research of human PSCs (Choi et al., 2019). To date, several transgenic pigs harboring neurological diseases such as Alzheimer's disease and spinal muscular atrophy induced by genetic modifications have been reported (Kragh et al., 2009; Lorson et al., 2011). If the development of transplantation methods into these disease-model animals were achieved apposing with the study on differentiation from PSCs, it would lead to the success of human cell therapy using stem cells.

CONCLUSION

Based on our results, neuronal progenitor cells could be derived directly from pig EGCs through the SFEB method. Treating retinoic acid was more efficient for inducing neuronal lineages from EGCs than inhibiting SMAD signaling. Although neuronal markers such as PAX6, NESTIN, and SOX1 were expressed, it would be more developed to maturate neurons such as astrocytes, dendrocytes and dopaminergic neurons, along with the functional test. Finally, the production of transgenic pig for disease models as well as differentiation methods will provide basic pre-clinical data for human regenerative medicine and lead to the success of stem cell therapy.

CONFLICTS OF INTEREST

No potential conflict of interest relevant to this article was reported.

ACKNOWLEDGEMENTS

AUTHOR CONTRIBUTIONS

Conceptualization: Choi KH, Lee CK. Data curation: Choi KH. Formal analysis: Choi KH, Oh JN. Funding acquisition: Choi KH, Lee CK. Investigation: Choi KH, Lee DK, Lee M, Jeong J, Choe GC. Methodology: Choi KH, Lee DK. Project administration: Choi KH, Lee CK. Resources: Lee CK. Validation: Choi KH, Kim SH. Visualization: Choi KH, Lee DK. Writing - original draft: Choi KH, Lee CK. Writing - review & editing: Choi KH, Lee CK.

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