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Original article

Comparison of methods for deriving neural progenitor cells from nonhuman primate embryonic stem cells

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Background: Feeder-free monolayer culture and the suspension culture of embryoid bodies (EB) followed by adherent culture, rosette selection, and expansion are 2 methods for deriving neural progenitor cells (NPCs). Direct comparison of these 2 methods has not yet been reported.
Objectives: To compare the influence of NPC derivation methods on the properties of NPCs derived from rhesus monkey (*Macaca mulatta*) embryonic stem cells (rhESCs).
Methods: rhESCs were used to derive NPC lines using 2 different methods. EB were produced from a suspension culture of rhESC clumps and rhESCs were cultured in feeder-free monolayers on poly-L-ornithine/laminin coated plates. NPCs were derived by exposure to induction factors. Cell morphology, neural and nonneural lineage markers were evaluated. We measured the expression of nuclear receptor tailless (TLX), which acts as repressor of glial fibrillary acidic protein (GFAP) expression.
Results: NPCs were successful derived using either method, with homogenous populations based on the expression of nestin (>97%) and Pax6 (>99%) as shown by flow cytometry. No significant difference in NPC specific markers or ability to differentiate into neurons in vitro was found between the methods. However, the expression of GFAP was >400-fold higher in cells produced by the feeder-free method. This distinction was consistent with the lower expression of TLX.
Conclusions: NPCs derived by feeder-free and EB methods share similar morphology and properties. The elevated expression of GFAP and reduced expression of TLX in NPCs derived using the feeder-free method may explain their greater heterogeneity and tendency to differentiate toward cells of an astrocyte lineage.

Keywords: Embryonic stem cells, neural progenitor cells, neural progenitor cell derivation, nonhuman primate, TLX

Cell therapies and stem cell applications are widespread topics in biomedicine. Embryonic stem cells (ESCs) are self-renewing pluripotent cells, which can differentiate into various cell and tissue types of all 3 germ lineages. Their high rate of proliferation and pluripotent characteristics make them one of the best cell sources for modeling diseases in vitro and the development of cell therapies for diseases such as Parkinson's and Huntington's.

Neural stem cells can be isolated and expanded from the subventricular zone and the subgranular zone of the dentate gyrus in the adult brain. Unfortunately, neural stem cells are difficult to obtain in large amounts from human brain tissues because limitations are not only the number of neural stem cells available, but also the ethical concerns in using human brain tissues as the cell source. Moreover, the expansion of primary neural stem cells in culture is limited and with limited plasticity after repeated passaging in vitro [1, 2]. Several groups have reported the derivation of neural progenitor cells (NPCs) from ESCs. There are 2 commonly used methods. One of the methods is by spontaneous differentiation of ESCs in suspension culture that is allowed to form an aggregated mass known as embryoid body (EB) [3, 4]. EBs consist of differentiating cells representing the 3 embryonic germ layers [5] and creating a microenvironment that promotes cell–cell interaction that plays a crucial role in the development and function of multicellular organisms. Exogenous cytokines and growth factors are used to stimulate EBs to differentiate towards a specific lineage such as retinoic acid [6, 7] and bone

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morphogenetic proteins [8, 9]. Although the EB method is commonly used to differentiate ESCs to a neural lineage, the EB is a multilayer structure with high heterogeneity and the cells would be exposed to different concentrations of growth factors that impact their subsequent differentiation [10]. By contrast, the feeder-free method allows cells to be exposed to growth factors homogeneously. However, the maintenance of rhESC without feeder remains a challenging task [11, 12]. In the present study, we investigated the effect of NPC derivation methods on the resulting NPCs using identical media for the induction of rhesus ESCs (Rhesus macaque; *Macaca mulatta*) to compare NPC properties including gene expression, homogeneity, and capability of neural differentiation in vitro.

Materials and methods

ESCs culture

rhESCs (obtained ethically and provided by Anthony Chan's laboratory at the Yerkes National Primate Center, GA, USA; any protocols involving animal care and handling were previously approved by Emory University's Institutional Animal Care and Use Committee) were maintained in media composed of knockout-Dulbecco's modified Eagle's medium (KO-DMEM) supplemented with 20% Knock-out Serum Replacement (KSR; Invitrogen, Carlsbad, CA, USA), 1 mM glutamine, 1% nonessential amino acids and 4 ng/mL of human basic fibroblast growth factor (bFGF; Chemicon, Temecula, CA, USA). Half of the ESC medium was replaced every other day. ESC colonies were passaged by mechanically dissociation into small clumps and plated on a freshly prepared mitomycin C inactivated mouse embryonic fibroblasts (MEFs).

Feeder-free adhesive culture and NPC derivation

rhESCs were mechanically passaged and grown without feeder cells on 35 mm tissue culture plates coated with 20 μg/mL poly-L-ornithine/1 μg/mL laminin (P/L) in MEF conditioned medium. MEF-conditioned medium was ES medium used for the culture of MEFs for 24 h. After 4 to 5 mechanical passages in the feeder-free culture, ESCs were dissociated using 0.25% trypsin-EDTA and then plated on P/L-coated dishes. Cells were then cultured in rhNPC induction medium with DMEM/F12 supplemented with N2 supplement (Invitrogen), 4 ng/mL bFGF, and 2 mM L-glutamine for 2 weeks. Cultures were fed with fresh medium every day. At day 14, cells were dissociated by Accutase (Life Technology, Carlsbad, CA, USA) for 5 min at 37°C followed by culturing in neural proliferation medium composed of Neurobasal A medium (Invitrogen) supplemented with 20 ng/mL bFGF, 10 ng/mL LIF (Sigma, St. Louis, MO, USA) and B27 supplement (Invitrogen). Spent medium was replaced with fresh medium every other day, and NPCs were passaged at 80%–90% confluency.

EB formation and NPCs derivation

rhESC colonies were mechanically dissected into small clumps and removed from MEF feeders. The rhESCs clumps were then cultured in suspension in 35 mm petri dishes for 6 to 7 days to form EB in ES culture medium without bFGF. Spent medium was replaced with fresh medium every other day. EBs were then transferred into 35 mm culture dishes coated with P/L (10–20 EBs per dish) and cultured in a preinduction medium composed of DMEM/F12 media supplemented with 0.5 × N2 supplement and 2 mM L-glutamine for 3 days. On day 4 of the differentiation, the cells were induced by differentiation into rhNPC by using the same induction medium as described for adherent feeder-free culture. After 6 to 7 days, rosette-like structures were manually picked up, transferred onto a P/L-coated dish, and cut into small pieces for culture. For expansion, the rosette-like structures were incubated in Accutase and then cultured in a neural proliferation medium.

Differentiation of rhesus NPCs to neurons

To determine the neuronal differentiation potential of rhNPCs derived by the 2 different methods, NPCs (about 3×10^4 cells) were plated onto P/L coated 35 mm dishes for neural differentiation [13]. NPCs were first cultured in neural differentiation medium composed of DMEM/F12 supplemented with N2 (1:100; Invitrogen) and 1 × B27 supplement (1:50; Invitrogen) for 4 days. On day 5, 200 ng/mL SHH (R&D Systems, Minneapolis, MN, USA) and 100 ng/mL FGF8 (R&D Systems) were added. On day 8, 160 μM ascorbic acid was added into the medium until day 14. Spent medium was replaced with fresh medium every other day.

Immunofluorescent staining

The cells were fixed in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS), pH 7.4, for 15 min. The fixed cells were washed for 5 min, 3

times in PBS and incubated with blocking buffer consisting of 0.2% Triton X-100 (for intracytoplasmic markers) (Sigma), 3 mM sodium azide, 0.1% saponin (Sigma), 2% bovine serum albumin (Sigma), and 5% horse serum (HyClone, Logan, UT, USA) in PBS (–) for 30 min. To detect NPC, the cells were incubated with primary antibody in PBS for 8 h. The NPC markers were nestin (Chemicon), Musashi, and SOX-2. After being washed 6 times for 5 min each with PBS, the cells were incubated with fluorescent-tagged secondary antibodies in PBS for 2 h followed by washes for 5 times with 5 min each, and the cells were then stained with 4′,6-diamidino-2-phenylindole (DAPI). After staining, the specimens were mounted on slides and examined by using fluorescence microscope.

Quantitative real-time polymerase chain reaction

Total RNA was extracted from early passages (P2 and P3) of rhNPCs cell lines using TRIzol reagent (Ambion, Carlsbad, CA, USA) and subsequently treated with Turbo DNase (Ambion). RNA concentration was determined by using a NanoDrop spectrophotometer (ThermoFisher, Waltham, MA, USA). Three micrograms of RNA were reverse transcribed using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol. The level of expression of 14 genes containing 6 NPC specific genes (*Sox2*, *Pax6*, *Nestin*, *Musashi*, *TLX*, and *NPDC1*), endoderm specific genes (*AFP*, *TTR*, and *HNF1B*), mesoderm specific genes (*GATA4*, *VEGFA*, and *RUNX1*), and astrocyte markers (GFAP and S100â) were determined by quantitative real-time polymerase chain reaction (RT-PCR) using TaqMan Gene Expression Master Mix (Applied Biosystems; ABI) and TaqMan gene expression primers on CFX96 Real-Time Detection System (Bio-Rad, Hercules, CA, USA). The relative levels of gene expression of the target RNA were normalized against GAPDH expression. The plotted data represented mean values of at least 3 independent replicates ± standard error of the mean (SEM), and the statistical significance was determined by using one-way ANOVA with a generalized linear model procedure by SAS Institute software (Cary, NC, USA).

Flow cytometry

The NPCs were dissociated into single cells using 0.25% trypsin and washed with PBS without Ca^{2+} or Mg^{2+}. The cells were harvested (1×10^6 cells per sample) in a cold fluorescence-activated cell sorting (FACS) wash buffer and were permeabilized using BD-Perm-2 for 10 min in the dark. The samples were stained with anti-Pax6 or anti-nestin antibodies at 1:100 for 30 min at 4°C in the dark followed by submersion in the appropriate Alexa Fluor 488-conjugated secondary antibody for 30 min. The control group was prepared in parallel by staining cells with secondary antibody without primary antibodies. After being washed in FACS wash buffer, the cells were fixed in 1% PFA. Flow cytometric analysis was performed on an BD FACS Calibur (BD Bioscience, Franklin Lakes, NJ, USA), and the data was analyzed using FlowJo software.

Results

Differentiation of rhesus monkey ESCs to neural progenitor cells

To compare the efficiency of deriving NPCs by feeder-free and EB derivation methods, we first examined the emergence of a neuroepithelial structure or neural rosette. In feeder-free adherent culture, the first sign of neural differentiation emerged at day 8 in the presence of N2 supplement and bFGF. The neural rosettes had a radial arrangement of elongated columnar cells as the signature morphology of neuroprogenitor cells in culture (**Figure 1D**). At day 14 in culture, these cells had developed an NPC-like bipolar structure, a typical morphology of NPCs (**Figure 1E**), and continued to be cultured for 1 week in a neural proliferation medium. In the EB method, ES cell clumps were cultured in suspension in low-adherent petri dishes to allow the formation of cell aggregates called EB (**Figure 2B**). The EBs were transferred onto P/L-coated dishes and cultured in induction medium. The development of compact columnar cells emerged from the EBs and formed neural rosette-like structures at 5 to 7 days after induction (**Figure 2C**). One of the major challenges in deriving NPCs was to eliminate non-NPCs. The rosette-like structures were identified based on morphology and mechanically isolated from the culture (**Figure 2D**). The rosette-like structures were then cut into small pieces for expansion in neural proliferation medium. More than 30 NPC lines were derived using the 2 approaches. The morphology of NPCs derived by the 2 methods was similar (**Figure 3A**). This result demonstrated that the 2 NPC derivation methods (feeder-free and EB methods) can derive NPC-like cells with no phenotypic difference in morphology.

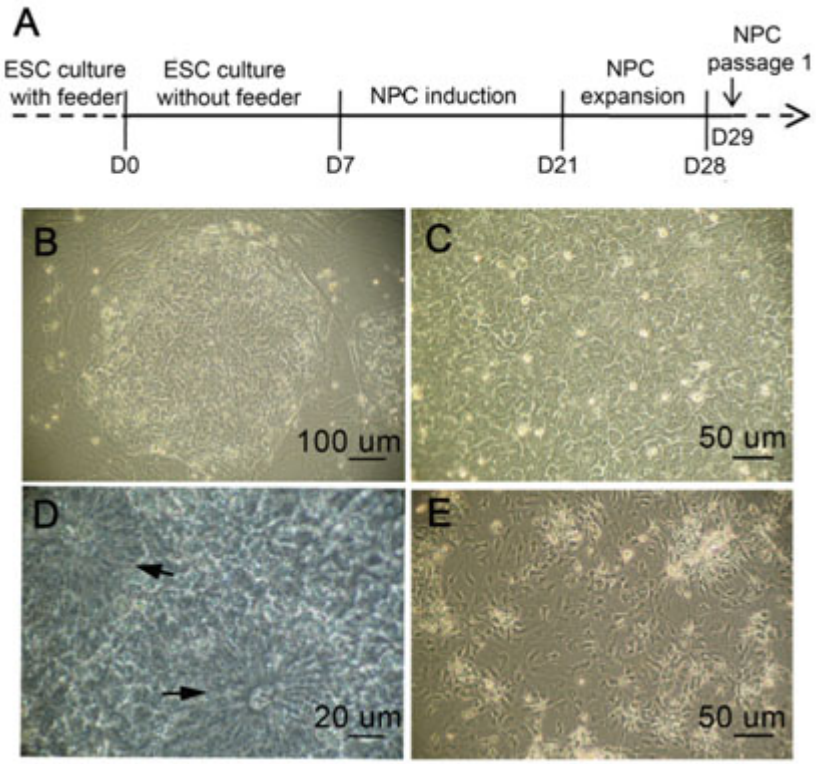

Figure 1. Induction of rhesus monkey (*Macaca mulatta*) embryonic stem cells (rhESCs) to become neural progenitors in an adherent monolayer without feeder cells. (**A**) Schematic for the monolayer cell induction method. (**B**) rhESC colony cultured on a mouse embryonic fibroblast feeder layer before transfer to feeder free conditions. (**C**) rhESC cultured in neural induction medium after 4 passages without feeders. (**D**) Neural rosette-like structure (arrows) formed at day 8 (D8) of differentiation. (**E**) rhESCs derived neural progenitor cells.

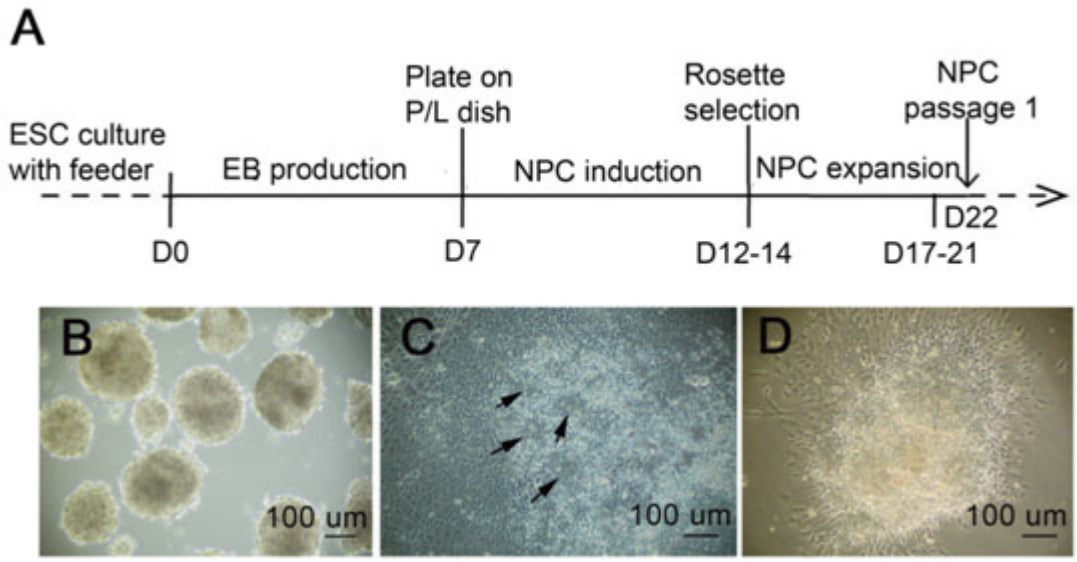

Figure 2. Derivation of neural progenitor cells from rhesus monkey (*Macaca mulatta*) embryonic stem cells (rhESCs) using the embryoid body (EB) method. (**A**) Schematic diagram of EB induction protocol. (**B**) rhESC derived EBs in suspension. (**C**) Adherent culture of EBs in an induction medium with small neural rosette-like structures (arrows). (**D**) The rosette-like structures were isolated and replated followed by second round of selection neural rosette.

Expression of NPCs specific markers of rhESC-derived NPC

Immunostaining confirmed the expression of NPC markers by NPCs derived by either method (**Figure 3A**). A similar pattern of expression of the NPC markers (Sox2, Pax6, nestin and Musashi1) and neural proliferation differentiation and control-1 (NPDC1), which is specifically expressed in the nervous system and neural cells in culture when they stop dividing and start to differentiate [14] was observed in rhNPCs derived by either method when compared with ESCs (**Figure 3B**). These results suggested that the 2 derivation methods were sufficient to generate NPCs with similar neuroprogenitor properties.

Flow cytometry revealed 99.1% of NPCs cells derived using the feeder-free method and 99.5% of NPCs cells derived using the EB method were positive for Pax6, while 97.0% of NPCs from the feeder-free method and 99.4% of NPCs cells from the EB method were positive for nestin (**Figure 3C**). These results suggested that NPCs with high homogeneity can be produced by either method.

Differentiation of rhESC-derived NPCs into neurons

To determine whether NPCs can be differentiated to neurons in vitro, FGF2 and LIF were withdrawn from the culture media, while N2, SHH, FGF8, and ascorbic acid were supplemented to enhance neural differentiation. By the end of the second week of in vitro neural differentiation, differentiated cells from EB derived NPCs developed branches and elongated spindle processes (**Figure 4B**). Cells from either method showed upregulation of neuron-specific microtubule associated protein (MAP2) expression when compared with NPCs (**Figure 4E**). Immunoreactivity further confirmed the expression of MAP2 (**Figure 4C and D**). These results suggest that NPCs derived by either method are capable of differentiating into neurons.

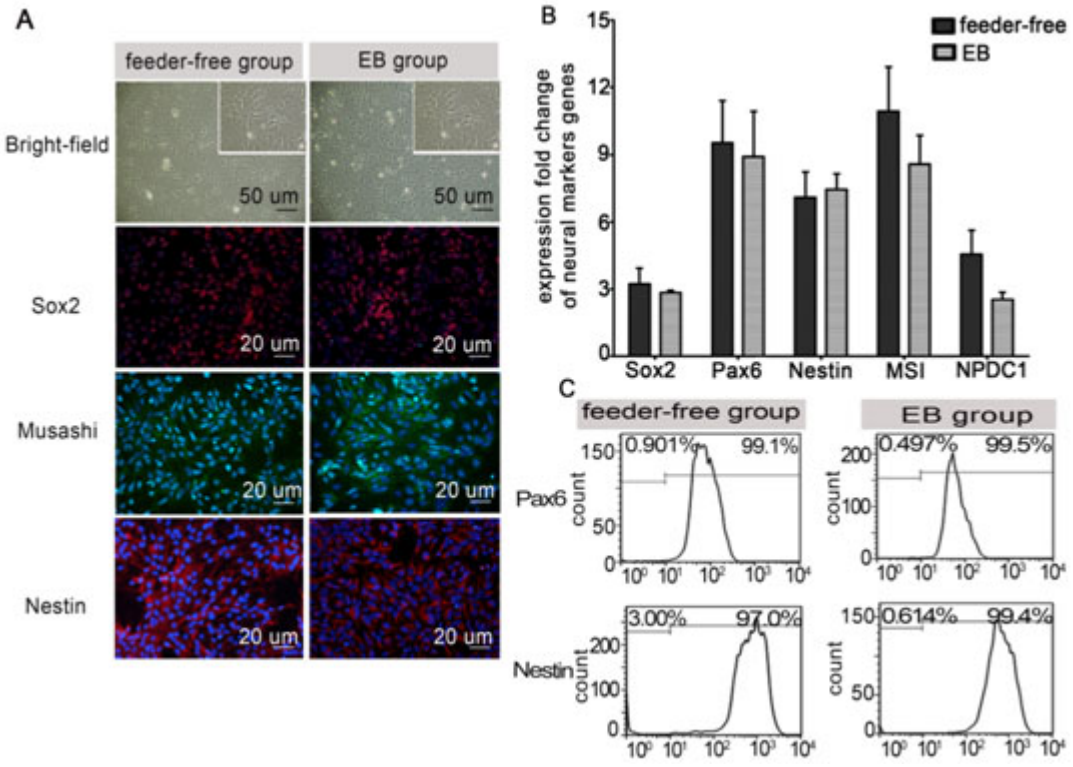

Figure 3. Characterization of rhesus monkey (*Macaca mulatta*) embryonic stem cells (rhESC)-derived neural progenitor cells (NPCs). (**A**) After derivation, the cells from both methods of derivation showed NPC morphology and stained for NPC markers; Sox2, Musashi, and nestin. (**B**) The expression of NPC-related genes was measured by real-time polymerase chain reactions. Data are the mean of fold change relative to rhESC gene expression ± SEM (samples run in duplicate, n = 3, $P < 0.05$). (**C**) Flow cytometry analysis of rhESC-derived NPCs with 99.1% and 99.5% were Pax6-positive and 97.0% and 99.4% were nestin-positive for feeder-free and embryoid body derived NPCs, respectively. The x-axes represent the fluorescent intensity and y-axes show the number of cells.

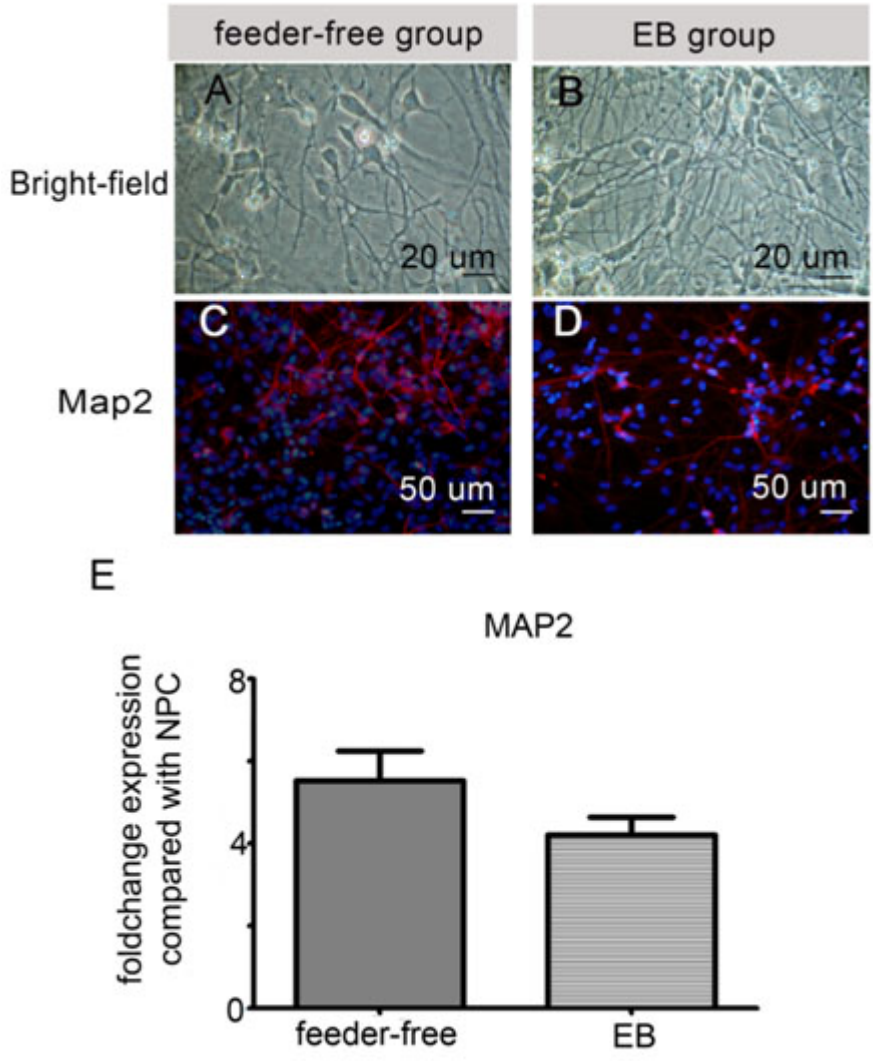

Figure 4. Differentiation of rhesus monkey (*Macaca mulatta*) neural progenitor cells (rhNPCs) into neural cells. (**A**) rhNPCs from feeder free-based and (**B**) EB-based methods showed highly branched morphology and elongated spindle processes, consistent with neuronal morphology. (**C–D**) Immunoreactivity revealed MAP2-positive cells in neural cells differentiated from feeder free and EB derived NPCs. (**E**) Quantitative real time polymerase chain reactions revealed the expression of MAP2 in neural cells derived from the 2 groups (n = 3, $P < 0.05$).

Expression of nonneural lineages and astrocyte markers in rhNPCs

To achieve a homogenous culture of NPCs is one of the major challenges for their application. To determine the homogeneity of NPCs and their commitment to neural lineage, we examined the patterns of expression of nonneural lineage markers, which include markers for mesoderm (e.g., *GATA4*, *RUNX1*, and *VEGFA*) and endoderm (e.g., *AFP*, *TTR*, and *HNF1B*). All markers tested were downregulated when compared with their level of expression in undifferentiated ESC. Although, *RUNX1* in cells from the feeder-free method was upregulated, the upregulation was not significantly different from the level of expression in ESC (**Figure 5A)**. The lack of significant upregulation of nonneuronal genes indicated that these 2 NPC derivation methods are not favoring nonneural cell lineage differentiation. The level of expression of glial fibrillary acidic protein (GFAP) in NPCs derived by the feeder-free method was significantly higher than that in NPCs derived by the EB method at early passages (**Figure 5B**). Immunoreactivity confirmed the expression of GFAP in some of the feeder-free derived NPCs (**Figure 5C**). By contrast with GFAP, an alternative astrocytic marker that is a late marker of astrocyte

development [15], S100β was not upregulated in NPCs compared with ESCs, and there was no difference between NPCs derived by either method (**Figure 5B**). These findings indicate that the feeder-free conditions promote early differentiation of astrocytes.

The expression of GFAP is related to TLX expression in rhNPC

Several studies reported that the nuclear receptor tailless (TLX) plays an important role in vertebrate brain functions [16, 17]. TLX belongs to the nuclear receptor, NE2E orphan nuclear receptor family. TLX is expressed in the neuroepithelium of developing central nervous system [16] and acts as a repressor of cell cycle inhibitors to maintain the undifferentiated state, self-renewal, and proliferation of adult progenitor cells from rodents [18]. In addition, TLX suppresses the expression of GFAP to inhibit astrocyte differentiation and to activate neuronal lineage commitment [3, 16]. Therefore, we examined if the downregulation of *TLX* resulted in the upregulation of GFAP expression in NPCs. As shown in **Figure 5D**, the expression of *TLX* in the NPCs derived by the feeder free-method was significantly lower than that of those derived by the EB method. This finding was consistent with the higher *GFAP* expression in feeder-free group, and further suggested the astrocytic tendency of feeder-free derived NPCs.

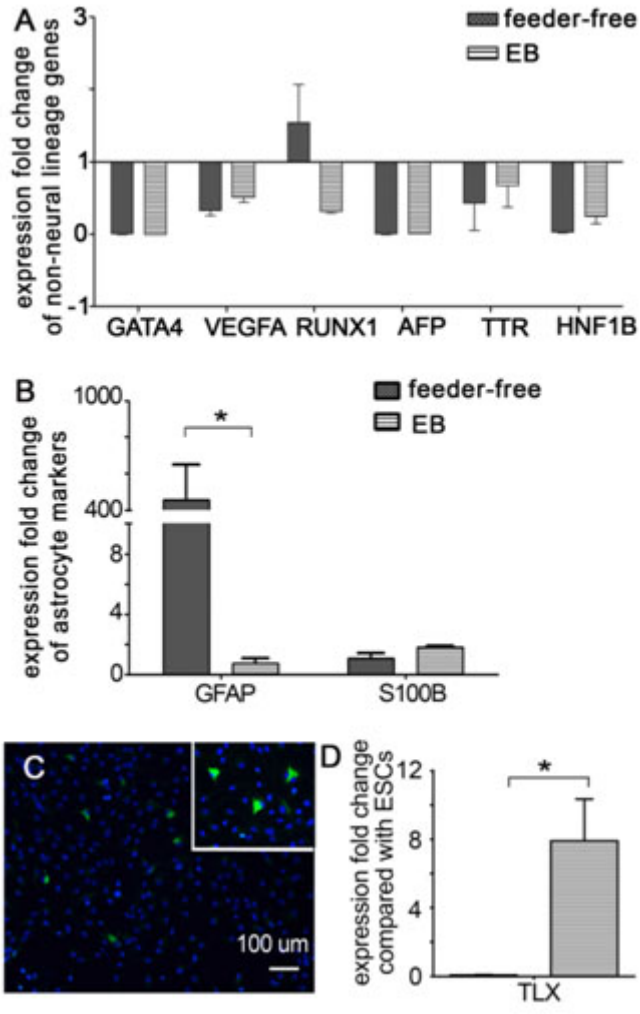

Figure 5. Expression of nonneural lineage and neural lineage markers. Quantitative real-time polymerase chain reaction (qRT-PCR) analysis indicated that 3 independent rhesus neural progenitor cell lines derived from feeder-free and embryoid body (EB) methods showed similar gene expression patterns for (**A**) nonneural lineage and (**B**) expression of astrocyte markers, GFAP and S100β. (**C**) The expression of GFAP was confirmed by immunoreactivity and on neural cells differentiated from feeder-free derived NPCs. (**D**) Nuclear receptor tailless (TLX) expression was measured by qRT-PCR in neural cells differentiated from feeder-free derived NPCs (n = 3, $P < 0.05$).

Discussion

Several methods have used to derive NPCs [19-21]. Most of these methods are focused on growth factors that are added in the induction. The present study demonstrated that rhNPCs can be derived from ESCs by 2 commonly used methods. The formation of EB followed by the expansion and culture of NPCs from a neural rosette, which is one of the most common methods, and feeder-free culture of human and nonhuman primate ESCs without serum [12, 13, 21-23].

The first sign of progenitor cells was the elongation of cells along with the formation of the rosette structures. These rosette-like cells are the source of the neuroectoderm. They express many of the markers of neuroepithelial cells, which have been identified as characteristic of NPCs [24-26]. The rosette-like structures often appeared as a group of 2 to 4 rosettes in adherent EB after 5 days of culture in an induction medium. However, these rosette-like cells could not be easily maintained in long-term culture, and rosette structures disappeared after 2 to 3 passages. The maintenance of rosette-like structures was more challenging with the feeder-free method than the EB method. We observed that cell growth, cell survival, and rosette formation in culture was highly dependent on cell density. This finding is consistent with previously published reports that the efficiency of neural induction, proliferation, and differentiation are dependent on cell density [27-30]. Our results clearly showed that rhESC-derived NPCs from either of the 2 methods had upregulated NPC-specific gene expression markers compared with the parent ESCs. These markers included: (1) Sox2, a transcription factor that is expressed in early neuroepithelium and is restricted to proliferating neural progenitors during the development of the embryonic central nervous system [31, 32], (2) Musashi 1, an RNA binding protein that regulates the translocation of target mRNA during neural development and is expressed in the progenitor cells of the CNS [33], (3) Pax6, a transcription factor that is expressed in early neuroectoderm to determine the differentiation from pluripotency to neuroectoderm fate, and to promote neurogenesis of striatal neural stem cells [34, 35], (4) nestin, an intermediated filament protein that is expressed in multipotent neural stem cells, and is required for self-renewal and survival of neural stem cells [36], and (5) neural proliferation differentiation and control-1 (NPDC1), which is specifically expressed in neural cells that have stopped dividing and initiating differentiation [14]. Our findings showed that there was no difference in the expression of these markers in NPCs derived by feeder-free or ES methods.

Although EB is composed of 3 embryonic germ layers, neural rosettes derived from EB have a higher yield of progenitor cells. Our results showed that 99.5% and 99.4% of NPCs derived from EB were positive for Pax6 and Nestin, respectively, which were slightly higher than those derived from feeder-free methods (Pax6 99.1% and Nestin 97.0%; **Figure 3C**). Quantitative real-time (qRT)-PCR data further demonstrated the downregulation of primitive endoderm genes, which were expressed during EB differentiation and included α-fetoprotein (AFP), transthyretin (TTR), and hepatocyte nuclear factor (HNF1β) [37]. AFP was expressed in yolk sac and embryonic liver [38]. The expression of TTR was also detected in visceral yolk sac endoderm and in developing fetal liver [39]. HNF1 was required for the formation of yolk sac-like structure in EBs [37]. We also investigated the expression of mesoderm-associated genes, which include those for GATA binding protein 4 (*GATA4*) and vascular endothelial growth factor A (*VEGFA*). Both mesoderm markers were significantly down regulated in NPCs derived by either method when compared with ESCs. Gata-4 and GATA-6 expression in mouse ESCs promote their differentiation into extraembryonic endoderm [40] and Gata-4 is expressed in lateral mesoderm for tissue specification and differentiation [41]. VEGF is essential for angiogenesis and a cardiovascular system [42]. Additionally, runt-related transcription factor 1 (RUNX1) is essential for definitive hematopoiesis and plays an important role in the hemogenic endothelial cells, which are differentiated endothelial cells with hematopoietic potential to hematopoietic cells and hematopoietic stem cells transition during embryogenesis [43, 44]. RUNX1 was only upregulated in feeder-free derived NPCs, but not in those derived by an EB method. Gene expression analysis found that these 2 NPC derivation methods do not favor nonneural cell lineage differentiation.

Although our results showed high yield and high homogeneity of NPC population, NPCs derived by feeder-free culture had significantly increased expression of GFAP (**Figure 5B**), but no difference in the expression of S100β was observed. S100β expression in the late stage of neural development is preceded by GFAP-expression when early astrocyte

differentiation occurs [45]. These findings are consistent with the report by Erceg et al. [11] that GFAP was detected in NPCs derived from hESCs using a feeder-free method. Human neural stem cells isolated from fetal neural tissue also express low levels of GFAP mRNA and little or no S100β [46].

By contrast, the expression of TLX, also known as NR2E1, was significantly low in feeder-free derived NPCs that exhibited a high level of expression of GFAP when compared with NPCs derived by the EB method. TLX is an orphan nuclear receptor that plays an essential regulatory role in maintaining an undifferentiated state, proliferation, and suppresses astrocyte differentiation [16, 47]. TLX expression knockout dramatically reduced the neural stem cell population, but increased GFAP staining and extended GFAP processes in the subventricular zone [3]. This finding suggested that NPCs derived by feeder-free methods may have a higher tendency toward an astrocytic lineage with lower expression of TLX and high expression of GFAP.

Both methods of NPC derivation can produce NPCs with similar characteristics including the formation of a neural rosette-like structure, cell morphology, gene expression pattern, and cell homogeneity. However, NPCs derived using the EB method took approximately 2 weeks to establish, while they took 3 to 4 weeks using the feeder-free method. Manual selection and subculture of rosette-like structures by the EB method resulted in a more homogenous population of NPCs than the feeder-free method where rosette-like structures were not isolated. In the present study, we showed the derivation of rhNPCs by 2 simple and efficient methods that allow us to derive NPCs for in vitro modeling to aid the development of cell therapy and drug discovery.

Various methods have been reported for the derivation of NPCs from monkey ESCs for different applications [45]. Various base media, growth factors, growth factor antagonists, morphogens, and supplements have been investigated to determine the best conditions to induce differentiation of ESCs into NPCs. There were attempts to supply nutrients and induce neural differentiating pathways to mimic the environment in vivo. However, further study is needed to understand the complexity of neural differentiation. In the present study, we compared the rhNPCs derived from rhESCs by using 2 commonly used methods. There was no significant difference in homogeneity and the expression of NPC markers. One interesting finding was the up-regulation of GFAP and down regulation of TLX in feeder-free derived NPCs that suggested their higher tendency or commitment to the astrocytic lineage as compared to those derived from EBs. Nonetheless, NPCs derived by either method were capable of differentiating into neurons and can be maintained in culture [13].

Conflict of interest statement

The authors declare that there is no conflict of interest in this research.

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