Original Research Report

Uniform Adherent Neural Progenitor Populations from Rhesus Embryonic Stem Cells

DEANNE TIBBITTS, RAJ R. RAO, SOOJUNG SHIN, FRANKLIN D. WEST, and STEVEN L. STICE

ABSTRACT

Rhesus and human embryonic stem cells (ESCs) are similar, making rhesus ESCs an appropriate preclinical allograft model for refining stem cell therapies. Use of rhesus ESC-derived neural progenitors (NPs) in preclinical applications will be enhanced if the neural derivation process is scalable and free from contaminating ESCs or nonneural cells. In this study, we have quantified temporal gene expression changes of rhesus ESC differentiated to uniform NPs using simple feeder-free adherent cultures. NPs exhibited a significant up-regulation of neural-specific genes and a down-regulation of pluripotency genes. Additionally, expression of Hu, MAP2, and Tuj1, shows that NPs can form post-mitotic neurons. This study represents a simple and scalable means of producing adherent primate NPs for preclinical testing of neural cell-based therapy.

INTRODUCTION

HUMAN AND RHESUS EMBRYONIC STEM CELLS (ESCs) share many characteristics, including morphology, surface marker expression, and developmental potential (1). Rhesus ESCs can thus play an important intermediate translational role as stem cell differentiation strategies are transitioned from rodent systems to human clinical applications. Clinical trials for human ESCs as a therapy will be greatly enhanced by data gathered first in an allograft primate transplant model. Rhesus ESCs provide the opportunity to refine differentiation protocols in a species in which allogeneic transplants of these derivatives can be used to test in vivo efficacy. The method of propagating cells of interest becomes important when considering the scalability and application of these cells in the last preclinical large animal models, such as non-human primates. For any clinical application, the ability to generate large numbers of cells is critical, use of bulk passage methods, and a feeder-free system will facilitate progress. Manual dissection of rhesus embryoid bodies (EBs) has been employed to select neural rosettes or tubes of columnar cells (2,3); however, any technique that requires manual selection and propagation may hamper scalable expansion of the cells for large animal studies.

Previously, most ESC differentiation studies derived the cell type of interest through an EB intermediate. Differentiation via this three-dimensional structure allows for cell–cell communication that is not possible in an adherent cell culture setting. Rhesus ESC-derived EBs produced multiple cell types. Kuo and colleagues (3) used the EBs to initiate differentiation of rhesus ESCs and
showed that this method produced cells expressing markers from all three germ layers. However, the difficulty in precisely controlling differentiation and the subsequent appearance of multiple cell types are inherent problems of this method (4). Dang and colleagues compared EB differentiation cultures to adherent differentiation culture and reported that cell number limitation was not a factor in adherent differentiation cultures. In addition, they showed that adherent differentiation seemed to exclude cell differentiation toward hematopoietic development. Further studies also demonstrated that adherent differentiation with mouse ESCs produced efficient neural commitment (5). In addition, we have previously demonstrated through immunocytochemistry that greater than 90% of the adherent neural progenitor cells derived from human ESC were nestin and Musashi positive and none of the cells were Oct-4 positive (6). Together, recent studies have focused on directed differentiation of ESC to a desired cell type or progenitor populations; however simultaneous quantitative gene expression analysis of both neural and nonneural genes has not been determined in rhesus monkey ESCs.

The aim of this study was to quantitate the temporal changes in gene expression of adherent cultures of rhesus monkey ESCs undergoing differentiation in conditions conducive to generating neural phenotypes. We allowed rhesus ESC to differentiate spontaneously on Matrigel for 7 days before transferring them to a neural-permissive substrate and medium that selectively propagated neural progenitors. Using real-time PCR, we demonstrate that these cells exhibit between 1.5- and 17-fold up-regulation of neural developmental gene expression and a significant down-regulation of pluripotent gene expression with little contamination from other germ layers. As expected, gene expression gradually changed over time, with the most dramatic change observed at day 17. The cells were then propagated enzymatically for three passages (nestin positive) and upon further differentiation also exhibited expression of key mature neuronal markers (HU, MAP2, and Tuj). This population of rhesus neural progenitors is uniquely suited for further studies due to the simplicity of its derivation, the uniformity of the cultures (no feeder cells), and the ease of its propagation.

MATERIALS AND METHODS

ESC culture

Rhesus ESC were routinely cultured in Dulbecco’s modified Eagle medium (DMEM)/F12 base medium (Gibco) supplemented with 20% knockout serum replacement (Gibco), 2 mM l-glutamine (Gibco), 0.1 mM nonessential amino acids (Gibco), 50 units/ml penicillin/50 µg/ml streptomycin (Gibco), 4 ng/ml basic fibroblast growth factor (bFGF) (Sigma-Aldrich), and 0.1 mM β-mercaptoethanol (Sigma-Aldrich). Cells were passaged every 3–4 days using 0.05% trypsin-EDTA (Gibco). Cells were cultured on inactivated mouse embryonic fibroblasts (MEFs) plated 3 or more days prior to use. The cells were checked for normal karyotype prior to experimental use.

Neural differentiation

Rhesus ESCs were grown for 7 days on Matrigel-coated dishes in ES growth medium. Cells were trypsinized and replated on dishes coated with poly-L-ornithine and laminin and grown for 10 days in neural-permissive medium. Neural permissive medium was comprised of DMEM/F12 supplemented with 2 mM l-glutamine, 50 units/ml penicillin/50 µg/ml streptomycin, 20 ng/ml bFGF, and 1 × N2 supplement (Gibco). 1 × N2 supplement is composed of human transferrin (100 µg/ml), bovine insulin (5 µg/ml), progesterone (6.3 ng/ml), putrescine (16.11 µg/ml), and selenite (5.2 ng/ml). These cells were passaged every 5–7 days by brief exposure to 0.05% trypsin-EDTA solution. At each time point, cells were harvested for flow cytometry and gene expression analysis. Neural progenitor cells were further differentiated by exposure to neural differentiation medium for 7 days and stained for a panel of neural markers.

Real-time PCR

In preparation for real-time PCR analysis, total RNA was isolated from the crude homogenate using the Qiashredder kit (Qiagen) and RNeasy kit (Qiagen) according to the manufacturer’s instructions. The integrity of the mRNA produced from all samples used was verified and quantified using a RNA 600 Nano Assay (Agilent Technologies) and the Agilent 2100 Bioanalyzer. Total RNA (5 µg) was reverse-transcribed using the cDNA Archive Kit (Applied Biosystems Inc.) according to manufacturer’s protocols using the MultiScribe™ Reverse Transcriptase. Reactions were incubated initially at 25°C for 10 min and subsequently at 37°C for 120 min. Quantitative PCR (Taqman™) assays were chosen for the transcripts to be evaluated from Assays-On-Demand™ (ABI) a prevalidated library of human-specific QPCR assays and incorporated into 384-well Micro-Fluidic Cards™. Two microliters of the cDNA samples (diluted to 50 µl) along with 50 µl of 2 × PCR master mix were loaded into respective channels on the microfluidic card followed by a brief centrifugation. The card was then sealed, and real-time PCR and relative quantitation was carried out on the ABI PRISM 7900 Sequence Detection System (Applied Biosystems Inc.). After excluding failed reactions (reactions called as ‘undetermined’ by the SDS
software) and genes for which fewer than two replicates existed from further analysis, δCt values were calculated. For calculation of relative fold change values, initial normalization was achieved against endogenous 18S ribosomal RNA using the ΔΔCT method of quantification (ABI). Average fold changes from three independent runs were calculated as 2−ΔΔCT. Data analysis for differential expression between the different populations was conducted in triplicate. Significance was determined by running t-tests for each gene at a 95% confidence interval (p value < 0.05) between day 0 and day 7, day 0 and day 17, and day 7 and day 17. ΔCt and fold change values were calculated within each replicate, and then averaged to produce relevant standard deviation (SD) values.

**Immunocytochemistry**

Cells were seeded onto Permanox chamber slides (Nunc) coated with Matrigel or poly-L-ornithine/laminin and fixed with 4% paraformaldehyde/4% sucrose for 20 min. Antibodies were directed against SSEA-4 (Chemicon, 1:1000), Oct-4 (Santa Cruz, 1:500), nestin (Neuromics, 1:100), smooth muscle actin (Dako, 1:50), MAP2abc (Sigma-Aldrich, 1:200), HuC/D (Molecular Probes, 1:50), and Tuj1 (Covance Research Products, 1:500). Antibodies were detected using secondary antibodies conjugated to Alexa Fluor 488 or 594 (Molecular Probes, 1:1,000).

**Flow cytometry**

Cells were fixed in 2% paraformaldehyde/2% sucrose for 15 min, washed twice with Dulbecco’s phosphate buffered saline (DPBS), and held at 4°C in 1% bovine serum albumin (BSA) in DPBS until all samples were collected. For detecting surface markers, cells were blocked in 1% BSA in DPBS and stained with either SSEA-4 (1:2000; Chemicon) or mouse immunoglobulin G (IgG) isotype control (1:2,000; Sigma). Antibody was detected using a fluorescently conjugated secondary antibody (goat anti-mouse IgG Alexa Fluor 488, 1:1,000; Molecular Probes). Cells were analyzed using a Beckman Coulter FC500. A total of 10,000 events were counted for analysis.

**RESULTS**

**Differentiation of rhesus ES cells to neural progenitors**

To generate an adherent population of neural progenitors, we took a two-step approach. Rhesus ES cells were plated on Matrigel in ES growth medium and allowed to differentiate spontaneously for 7 days. The cells were briefly exposed to trypsin and replated on a polyornithine/laminin substrate in a neural-permissive medium. To determine the percentage of cells that were differentiating in response to the treatment, we collected cells at three time points and subjected them to flow cytometry. Table 1 shows the decrease in expression of SSEA-4, a cell-surface glycolipid and marker of pluripotency, as the ES cells moved toward a neural phenotype. After 10 days in culture on polyornithine/laminin these cells had developed distinctive neural progenitor morphology (Fig. 1A,B). Immunocytochemistry revealed these cells to be nestin positive (Fig. 1C) and SSEA-4 and Oct-4 negative (Fig. 1D,E).

**Temporal characterization of gene expression using real-time PCR**

To quantitate the differentiation process further in the population of cells obtained, cells were harvested for real-time PCR analysis at day 0, day 7, and day 17. These three samples were assayed for the expression of a panel of genes that are indicative of pluripotency, ectoderm, mesoderm, or endoderm. As detailed in the Methods section, genes whose expression met specific stringent criteria (see Methods) were considered for further analysis. Table 2 summarizes genes with a significant fold change at either day 7 or day 17.

**Culture of neural progenitors**

After their derivation, the cells were passaged enzymatically every 5–7 days with >95% viability. After two passages, the cells were cryopreserved, and subsequent thawing resulted in recovery of cultures. The cells retained the same morphology after three passages as they had after derivation.

**Differentiation of neural progenitors to mature neurons**

To assess the ability of these cells to differentiate to mature neurons, the neural progenitors were grown for 7 days in a serum-free differentiation medium with reduced bFGF. The cells were stained for Tuj1, Hu, and MAP2 (Fig. 2), all markers of post-mitotic neurons. Both MAP2 and Tuj1 staining localized cytoplasmically in cell bod-
ies and neurites, whereas Hu staining was confined to the nucleus, indicating the ability of these neural progenitors to differentiate into more mature neurons.

**DISCUSSION**

To our knowledge, this is the first report of quantitative real-time PCR temporal analysis of initial neural differentiation events from a starting rhesus ESC population. The derivation of the neural progenitors was achieved using a simple adherent ES cell differentiation method. Previously most neural progenitors were derived using the EB to foster differentiation driven by three-dimensional cell–cell interactions (3,6–8). When we combined the Matrigel adherent substrate with a defined medium, rhesus ESC spontaneously differentiated for 7 days. ESCs at this 7-day stage were harvested to moni-

<table>
<thead>
<tr>
<th>Gene</th>
<th>Category</th>
<th>Expression</th>
<th>FC ± SD*</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD9</td>
<td>Pluripotent</td>
<td>↓</td>
<td>4.51 ± 0.27</td>
</tr>
<tr>
<td>CER1</td>
<td>Pluripotent</td>
<td>↓</td>
<td>89.61 ± 0.95</td>
</tr>
<tr>
<td>FGF13</td>
<td>Pluripotent</td>
<td>↓</td>
<td>2.68 ± 0.07</td>
</tr>
<tr>
<td>FOXH1</td>
<td>Pluripotent</td>
<td>↓</td>
<td>7.26 ± 0.005</td>
</tr>
<tr>
<td>EBAF</td>
<td>Pluripotent</td>
<td>↓</td>
<td>5.70 ± 0.72</td>
</tr>
<tr>
<td>FN1</td>
<td>Neural-related</td>
<td>↑</td>
<td>3.61 ± 0.25</td>
</tr>
<tr>
<td>GATA3</td>
<td>Neural-related</td>
<td>↑</td>
<td>21.96 ± 0.64</td>
</tr>
<tr>
<td>PTCCH</td>
<td>Neural-related</td>
<td>↑</td>
<td>3.89 ± 0.18</td>
</tr>
<tr>
<td>BMP4</td>
<td>Mesoderm-related</td>
<td>↑</td>
<td>3.04 ± 0.09</td>
</tr>
<tr>
<td>GSC</td>
<td>Mesoderm-related</td>
<td>↓</td>
<td>17.75 ± 0.09</td>
</tr>
<tr>
<td>HOXB4</td>
<td>Mesoderm-related</td>
<td>↓</td>
<td>4.78 ± 0.66</td>
</tr>
<tr>
<td>KDR</td>
<td>Mesoderm-related</td>
<td>↓</td>
<td>4.65 ± 0.88</td>
</tr>
<tr>
<td>TBX6</td>
<td>Mesoderm-related</td>
<td>↓</td>
<td>3.18 ± 0.39</td>
</tr>
<tr>
<td>GATA6</td>
<td>Endoderm-related</td>
<td>↓</td>
<td>13.24 ± 0.75</td>
</tr>
</tbody>
</table>

Comparative analysis was conducted between samples at day 7 (rhesus ES cells on Matrigel) and day 17 (rhesus neural progenitors on polyornithine/laminin) against day 0 (rhesus ES cells).

*FC, Fold change; SD, standard deviation.
tor changes in gene expression. Without the addition of MEF conditioned medium, Matrigel alone has been shown to be insufficient to maintain the pluripotency of human ESCs (9), thus making it an appropriate substrate for this protocol. After 7 days, the cells were transferred to a serum-free medium containing N2 and 4 ng/ml bFGF on polyornithine/laminin-coated dishes. bFGF has long been a recognized mitogen of neural precursor cells (10) and polyornithine and laminin have previously been used together to support neural progenitors (11). The cells derived from this protocol developed an appropriate neural progenitor morphology and were nestin positive (Fig. 2) and further characterized by immunocytochemistry and real-time PCR. In this study, we found that 7 days of differentiation without serum was sufficient to induce neural differentiation. An additional 10 days of neural induction resulted in further increased neural-associated gene expression and decreases in pluripotent gene expression.

The panel of real-time PCR probes and primers arrayed on the ABI microfluidics card were optimized for the human sequence of each gene; it was unknown how well any of these would recognize rhesus monkey transcripts. Overall, 28.6% of the total reactions yielded a result called ‘undetermined’ by the SDS 2.1 software. This was due to inefficient amplification of the target sequence, which is caused by either insufficient availability of cDNA or weak homology to the probe/primer set. Because an equal amount of cDNA was loaded in all wells of the card, the latter is the more likely cause of any failed reactions. We believe on the basis of the stringency of our criteria, the genes retained for final analysis reflect the gene expression profile of the population of cells undergoing neural differentiation.

As shown in Table 2, many of the genes up-regulated at day 17 have been previously described as genes related to neural development. These genes encode extracellular matrix proteins (FN1, THBS1), cell adhesion molecules (VCAM, NCAM, MCAM), transcription factors (GATA3, HEY1, LMO2, MADH2), an RNA-binding protein (Musashi-1), a transmembrane receptor (Patched), and cytoskeletal proteins (NEFL, NEDD5). Numerous studies have demonstrated the relevance of these genes to neural development. Fibronectin-1 (FN-1) expression increased 3.6- and 6.5-fold on days 7 and 17, respectively. Fibronectin is a fundamental component of the neural crest migratory pathway (12), and fibronectin-deficient mice exhibit increased neuronal apoptosis (13). In developing mice (E9), thrombospondin-1 (2.7-fold change on day 17) was found to be densely deposited between neuroepithelial cells (14).

The vascular cell adhesion molecule VCAM was more up-regulated than any other gene at day 17; its expression increased 17-fold compared to day 0 (ESC). In mice, VCAM has been shown to be co-expressed with nestin in neural progenitors of the central nervous system (15). Other cell adhesion molecules were significantly up-regulated (MCAM, 2.7-fold; NCAM, 9-fold), though to a lesser extent. MCAM is strongly expressed in the chick in both the neural epithelium of the developing forebrain and neural crest cells (16), whereas NCAM is expressed in neural progenitors as they migrate from the ventricular zone of the developing brain (17).

Neural-related transcription factors related to neural development were also up-regulated. Hey-1 has been implicated in the maintenance of neural precursors in the brain and is highly expressed in the ventricular zone of the developing brain and increased 9.7-fold on day 17. LMO2 is highly expressed in the developing mouse central nervous system (18). GATA3 is expressed in the ventral neural tube and developing mouse hindbrain (19), whereas MADH2 transcripts are present in the neuroectoderm of the forebrain and hindbrain of mouse embryos (E10.5) (20). Overall, we observed that these neural-associated transcription factors were more highly expressed after the neural phenotype was observed at day 17.

Other neural-related genes were also up-regulated. Musashi, an RNA-binding protein, was up-regulated 5-fold at day 17 and has been shown to be initially expressed in the neural tube and later restricted to the ventricular zone (21). Patched, the receptor for Sonic Hedgehog, was up-regulated 8-fold at day 17 in our study. Localization of Patched varies temporally, but in the chick expression is first restricted to the neural tube, extending from the caudal end through the diencephalon (22). Using a LacZ reporter under the control of the neurofilament light-chain (NEFL) promoter, Yaworsky and colleagues (23) were able to localize expression of the promoter to neuroepithelial cells in transgenic mouse embryos (E8.5), indicating the relevance to neural development. NEDD5 is one of a group of genes discovered in mouse neural precursor cells that are down-regulated during development of the brain (24), pointing to a role in early neural development. We found that both NEFL and NEDD5 were up significantly up-regulated at day 17. In total, 13 neural developmentally related genes were up-regulated by day 17 and SSEA4 was absent based on flow cytometry analysis.

The only gene significantly up-regulated at day 7 was BMP4, which is an important factor in nascent mesoderm induction and patterning (25). Transient expression of BMP4 at day 7 but not at day 17 is likely indicative of the mixed population of cells present at the day 7 time point. Its regression to near baseline levels at day 17 is potentially a result of directed neural differentiation in the overall population of cells.

Most genes significantly down-regulated at day 7 and day 17 are markers of pluripotent human ESCs. Many of the analyzed genes down-regulated in our study have been found to be highly expressed by human ES cells in other large-scale gene expression studies (26–28). In an
**FIG. 1.** Phase-contrast micrograph (A,B) and immunocytochemical staining (C) for nestin (green), DAPI (blue); (D) SSEA4 (green), DAPI (blue); (E) Oct-4 (green), DAPI (blue) of rhesus neural progenitor cells. Insets are rhesus ES cells stained positive for SSEA4 (D) and Oct-4 (E). Scale bar, 100 μm.

**FIG. 2.** Immunocytochemical staining for (A,B) MAP2 (green) and DAPI (blue); (C,D) Tuj1 (red), HuC/D (green), and DAPI (blue) of rhesus neural progenitors differentiated for 7 days. Scale bar, 100 μm.
array comparing hES cells and human embryonic carcinoma (EC) cells to other tumor cell lines and normal tissues. Sperger and colleagues (29) identified GABRB3 as the second most up-regulated receptor in hESCs, with DNMT3β and OTX2 as the third and twelfth most positively significant genes in hESC overall, respectively. Brandenberger and colleagues (30) identified Cerebrus (CER-1), FGF13, LeftyA, FoxH1, and Goosecoid as commonly expressed genes in their analysis of pooled mRNA from the hES cell lines. It has also been proposed that Goosecoid is directly regulated by FoxH1–dependent transcriptional activation (31). In our study, we found that all of these genes were significantly down-regulated by day 17 of differentiation. Simultaneous down-regulation of both pluripotent and candidate genes associated with mesoderm differentiation in our study would seem to support that this adherent culture system is specific for neural induction.

Other genes down-regulated in our study have also been associated with or involved in mouse ES cell maintenance or mesoderm early development. In mouse ES cells, CD9 was found to be regulated by the LIF/STAT3 pathway (32), and while it has recently been shown that LIF does not support maintenance of pluripotency in human ES cells (33), this has not nullified the use of CD9 as a marker of pluripotency (34). Upon generation of GATA-6 knockout mice, Koutsourakis and colleagues (35) found expression of GATA-6 in a subset of cells in the inner cell mass of 3.5-day preimplantation embryos, with null mutants exhibiting lethality at 5.5 days post coitus (dpc).

HoxB4 plays a role in very early hematopoiesis (36) but may be active earlier in development; Kuliev and colleagues (37) detected HoxB4 transcripts in cleavage-stage human embryos. Mesoderm-associated genes were significantly down-regulated genes at both day 7 and day 17. Tbx6 is expressed in paraxial mesoderm and is required for cells to choose between a neural or mesodermal fate (38). KDR and Flt-1 are receptors for both vascular endothelial growth factor (VEGF) and placental growth factor (39), two proteins important for angiogenesis, although they play a role in placental development as well (40).

In this study, real-time PCR using the microfluidics approach offers the opportunity to quantitatively examine a limited set of candidate genes believed to be important in early development. Large-scale gene expression profiling (cDNA and oligo microarrays) has become an important and popular tool to compare human stem cell populations (26–29,41,42) as well as to monitor differentiation (43–45), and results are confirmed via quantitative PCR or other comparable methods. However, large-scale rhesus monkey microarrays are not available, so we elected to use a more focused approach using real-time PCR. Discrepancies between optimal rhesus and human probe/primer sets may have impacted our ability to generate replicates with ‘tight’ standard deviations and eliminated some important genes from our final list. Fortunately Pou5f1 was one of these genes. Although Pou5f1 was down-regulated, it produced variability among replicates, thus not meeting our defined stringency criteria, and had to be excluded. However, on the basis of the greatly reduced SSEA4 expression, there is little doubt that there are very few pluripotent cells at day 17.

Taken together, the group of genes up-regulated at day 17 is a strong indicator of a population of neural progenitors. The lack of significant up-regulation of non-neural genes at day 17 and the down-regulation of pluripotent markers points to the selectivity of the differentiation process. For a clinical application, the consistency of the transplanted population of cells is critical, and we feel that this method of neural progenitor derivation is both simple and robust. Further experiments will be required to examine the ability of these cells to respond to terminal differentiation cues to produce therapeutically relevant products. However, on the basis of gene expression, morphology, and immunocytochemical analysis, the rhesus neural cells derived in this study are similar to neural stem-like cells derived from human ES cells (6). This simple and scalable method of producing neural progenitors represents a step forward in the progress toward a primate model for cell-based therapy of neurodegenerative diseases.

ACKNOWLEDGMENTS

We thank Don Wolf for the generous gift of rhesus ES cells and Roger Nielsen for assistance with real-time PCR. This work was supported in part by funding from the National Institutes of Health (NIH-NS44208-2) and partly by the Georgia Tech/Emory Center (GTEC) for the Engineering of Living Tissues, and ERC program of the National Science Foundation under Award Number EEC-9731643.

REFERENCES


Address reprint requests to:
Steven L. Stice, Ph.D.
Director, Regenerative Bioscience Center
Rhodes Animal Science Complex
425 River Road
The University of Georgia
Athens, GA 30602

E-mail: sstile@uga.edu

Received December 17, 2005; accepted January 12, 2006.