Review

Derivation and characterization of monkey embryonic stem cells
K-Y Francis Pau* and Don P Wolf

Address: Primate Embryonic Stem Cell Program, Division of Reproductive Science, Oregon National Primate Research Center, Oregon Health & Science University West Campus, Beaverton, Oregon, USA

Email: K-Y Francis Pau* - pauf@ohsu.edu; Don P Wolf - wolfd@ohsu.edu

* Corresponding author

Abstract

Embryonic stem (ES) cell based therapy carries great potential in the treatment of neurodegenerative diseases. However, before clinical application is realized, the safety, efficacy and feasibility of this therapeutic approach must be established in animal models. The rhesus macaque is physiologically and phylogenetically similar to the human, and therefore, is a clinically relevant animal model for biomedical research, especially that focused on neurodegenerative conditions. Undifferentiated monkey ES cells can be maintained in a pluripotent state for many passages, as characterized by a collective repertoire of markers representing embryonic cell surface molecules, enzymes and transcriptional factors. They can also be differentiated into lineage-specific phenotypes of all three embryonic germ layers by epigenetic protocols. For cell-based therapy, however, the quality of ES cells and their progeny must be ensured during the process of ES cell propagation and differentiation. While only a limited number of primate ES cell lines have been studied, it is likely that substantial inter-line variability exists. This implies that diverse ES cell lines may differ in developmental stages, lineage commitment, karyotypic normalcy, gene expression, or differentiation potential. These variables, inherited genetically and/or induced epigenetically, carry obvious complications to therapeutic applications. Our laboratory has characterized and isolated rhesus monkey ES cell lines from *in vitro* produced blastocysts. All tested cell lines carry the potential to form pluripotent embryoid bodies and nestin-positive progenitor cells. These ES cell progeny can be differentiated into phenotypes representing the endodermal, mesodermal and ectodermal lineages. This review article describes the derivation of monkey ES cell lines, characterization of the undifferentiated phenotype, and their differentiation into lineage-specific, particularly neural, phenotypes. The promises and limitations of primate ES cell-based therapy are also discussed.

Review

Embryonic stem (ES) cells were first derived from the inner cell mass (ICM) of inbred mouse embryos in 1981 by Martin [1] and Evans and Kaufman [2]. Recently, ES cells were successfully derived from non-human primate and human embryos [3-5]. The National Institutes of Health listed 64 human ES cell lines available for research in 2001 [6]; however, only a few had been characterized and studied. Similarly, less than 7 of the more than 20 monkey ES cell lines have been well characterized apart from establishing pluripotency and genetic stability [3,4,7-9]. Even in the mouse, most ES cell studies have been performed with a single inbred mouse cell line (strain 129). The extent of diversity among primate ES cell lines is currently unknown.

Primate and mouse ES cells are similar in their ability to self-renew and differentiate into cells representing all three embryonic germ layers [see [10-14] for review]. They are different in cell/colony morphology, growth requirements and molecular signatures defining various developmental stages. For example, leukemia inhibiting factor (LIF), a component of the LIF-gp130-STAT3 signaling pathway [12], is able to sustain undifferentiated murine, but not primate, ES cell growth [13]. Thus, primate ES cells are routinely cultured on a mouse feeder layer, i.e., mitotically suppressed mouse embryonic fibroblasts. The use of feeder cells greatly limits the scaled up production of undifferentiated primate ES cell populations and the potential contamination by mouse cells is a safety concern when considering transplantation.

Safety is one of the most important issues for ES cell-based therapy. In addition to mouse cell contamination, tumor formation or other somatic cell transformations from grafted ES cell progeny must be considered. The likelihood of transplanting pluripotent ES cells as a therapeutic approach is low because of this problem. In contrast, their differentiated progeny, which also survive and integrate well in host tissues, are unlikely to form tumor cells. Although differentiated ES cell progeny can be produced in large quantity, enrichment and purification of the desired phenotypes for transplantation must be ensured. Clearly, the efficacy of ES cell-based transplantation must be tested rigorously with each and every phenotype, preferably in non-human primate models.

Derivation of monkey ES cell lines

Existing primate ES cell lines have not been directly compared by different laboratory groups. A comparative undertaking is important for several reasons. First, it helps to standardize a protocol with simplified growth requirements for primate ES cell lines, or to derive a primate ES cell line that replicates faster, is more amenable to subcloning and can be maintained in the absence of feeder layers or conditioned medium. Second, it characterizes cell line-specific developmental potential for studies of primate development or in drug discovery. Finally, it is prudent for investigators to characterize several lines before transplantation efforts begin. The quality of ES cells among different cell lines, especially the ability to self-renew and differentiate into a desired lineage, must be ascertained, at least by *in vitro* assessment, before selection for transplantation studies.

Rhesus monkey ES cell lines are available (contact:wolfd@ohsu.edu) (Table 1) with eight "R" cell lines (4 male and 4 female) derived from *in vivo* flushed blastocysts [3] and seven cell lines derived from *in vitro* produced blastocysts (*ORMES-1* to *-7*). The latter are under development at Oregon National Primate Research Center (ONPRC) [9]. Briefly, day 9 fully expanded blastocysts were treated with a rabbit anti-rhesus spleen cell antiserum (provided by Dr. James Thomson, Wisconsin NPRC) for 3 h followed by exposure to a guinea pig complement for 30 min [3,15]. This "immunosurgery" procedure [15] effectively destroyed all trophoblastic cells, exposing the ICM. After remnants of the trophoblastic layer were cleared, individual ICM cells were plated onto mouse feeder layers in ES cell medium. The expanded colonies were dissociated into small clumps of cells and replated onto new feeder layers. After the first passaging, colonies with ES cell-like morphology were selected for propagation, characterization and storage. Most lines (Table 1) are karyotypically normal with a diploid set of 42 chromosomes, however, ORMES-1 and -2 are stable, balanced translocations (12,17 and 4,19, respectively; denoted [t]). One ES cell line (366.4) has been stably transfected with a reporter gene, eGFP, by Dr. James Thomson. This eGFP-366.4 line has been enriched for GFP positive cells by fluorescence-activated cell sorting (FACS) in our laboratory. The pluripotent potential of several lines has been tested *in vivo* following teratoma formation in severely compromised immunodeficient (SCID) mice or *in vitro* forming embryoid bodies (EB), nestin-positive progenitor cells (NPC) and various glial and neuronal phenotypes (see below).

Characterization of undifferentiated primate ES cells

Primate ES cells are routinely cultured on mouse feeder cells because the molecular pathway and the key molecules required to maintain pluripotency are unknown. Our laboratory has adopted a manual method to select undifferentiated monkey ES cells for propagation and differentiation studies [16]. The procedure can enrich a monkey ES cell population to near homogeneity as indicated by the high percentage of cells expressing several pluripotent ES cell markers (Table 2). This unique repertoire of markers includes the stage-specific embryonic antigens (SSEA) 3 and 4, glycoproteins TRA-1-60 and TRA-1-81, specific enzymes such as alkaline phosphatase and telomerase, and transcriptional factors Oct4, Rex1 and GDF-3 that are rapidly down regulated upon differentiation [17,18]. The expression of markers such as SSEA-1 or Sox-1 in primate ES cell populations indicates that some of cells may have undergone differentiation. While none of these markers alone carries absolute predictive value for pluripotency, their presence as a group is highly informative (Table 2).

The ultimate identification of, and hence ability to compare, pluripotent ES cell lines might be based on genome wide expression analysis. By applying DNA microarray technology, a set of "stemness" genes has been tentatively identified in different murine stem cell populations

Table 1: Availability and characterization of rhesus monkey ES cell lines

Cell Line	Karyotype	Passages	Differentiation Potential
R366.4	42 XY, normal	6 to 60	Teratoma, EB, NPC, glial and neuronal phenotypes
R394.2	42 XX, normal	3 to 39	Teratoma, EB, NPC, glial and neuronal phenotypes
R475	42 XX, normal	5 to 16	Teratoma
R278.5	42 XY, normal	3 to 18	Teratoma
R420	42 XX, normal	3 to 16	Teratoma
R367B	42 XY, normal	2 to 8	Teratoma
R460	42 XY, normal	4 to 8	Teratoma
R456	42 XX, normal	3 to 7	Teratoma
ORMES-1	42 XY, 12/17 t	2 to 30	EB, NPC, glial and neuronal phenotypes
ORMES-2	42 XY, 4/19 t	2 to 16	EB, NPC, glial and neuronal phenotypes
ORMES-3	to be determined	2 to 10	to be determined
ORMES-4	to be determined	2 to 10	to be determined
ORMES-5	to be determined	2 to 10	to be determined
ORMES-6	42 XX, normal	2 to 10	to be determined
ORMES-7	to be determined	2 to 10	to be determined
GFP-366.4	42 XY, normal	47 to 57	EB, NPC, glial and neuronal phenotypes

Table 2: A conventional marker panel for identifying undifferentiated monkey ES cells

ES Cell Markers	Description	ES Cell Specificity
SSEA-3	embryonic stage-specific; glycolipid surface antigen	Primate only
SSEA-4	embryonic stage-specific; glycolipid surface antigen	Primate only
TRA-1-60	keratan sulphate-associated cell surface antigen	Primate only
TRA-1-81	keratan sulphate-associated cell surface antigen	Primate only
Oct4	POU-domain transcription factor	Mouse and Primate
APtase	alkaline phosphatase	Mouse and Primate
Rex1	transcription factor	Mouse and Primate
GDF-3	growth-differentiation factor 3	Mouse and Primate
Control markers		
SSEA1	embryonic stage-specific; glycolipid surface antigen	Mouse only
AFP	alpha fetoprotein, endoderm	Mouse and Primate
CTn-1	cardiac troponin-1, mesoderm	Mouse and Primate
Sox1	transcription factor, ectoderm	Mouse and Primate
HCG	human chorionic gonadotropin; trophectoderm	Primate only

[19,20]. A similar approach has been initiated in parthenogenetic monkey ES cells [21]. Extending the analysis to genes that are up- or down-regulated upon differentiation [22-24] allows identification of putative genes associated with pluripotency that, in turn, could lead to the isolation of factors critical to the propagation of undifferentiated ES cells in a defined medium without the confounding contamination of serum or feeder cells.

Differentiation of monkey ES cells

Primate ES cells spontaneously differentiate when cultured in serum-containing medium but in the absence of feeder cell support. This process is hastened by aggregating ES cells in hanging drops, in suspension or in overgrowth cultures [25]. In all cases, ES cells retain the ability to differentiate into phenotypes representing the three embryonic germ layers. In general, the hanging drop or suspension approach is appropriate when chemicals are used to induce lineage-specific differentiation. This is primarily because both approaches result in the formation of pluripotent embryoid bodies (EBs), spherical ES cell aggregates. EBs form within days, allowing chemical induction to occur early in differentiation without the interference of serum or feeder cells. In contrast, the overgrowth approach usually takes weeks, enabling the selection of lineage-specific cells by morphological assessment. For example, one interesting possibility based on studies conducted in mice is the derivation of gametes from ES cells. ES cell-derived gonadal germ cells were obtained either by prolonged culture of overcrowded ES cells forming primitive oocytes [26] or by EB formation and induction forming spermatogonial cells [27,28].

Note that it is unknown whether the gender orientation of ES cell differentiation is genetically determined and/or protocol-dependent. Certainly, epigenetic influences, including cell-to-cell and cell-to-molecule interactions at critical times, can bias ES cell differentiation toward a single lineage. It has been postulated that ES cells in culture will differentiate toward the neural lineage by default [29], i.e., without genetic or epigenetic influences. In practice, spontaneous differentiation of ES cells rarely produces cells of a single lineage, although the proportion of neural cells may be greater than other lineage-specific cells.

In our laboratory, monkey ES cell-derived EBs have been induced and differentiated into glial and neuronal phenotypes (ectodermal lineage) [16] as well as insulin-producing, pancreatic beta cell-like phenotypes (endodermal lineage) [see companion article by Dr. Linda Lester]. We have also identified cardiomyocytes (mesodermal lineage) after spontaneous differentiation of a monkey ES cell line (ORMES-2) [unpublished observation]. Here, we focus on the neural differentiation of monkey ES cells.

Neuronal differentiation by neural induction and selection
Following a neural selection protocol [30], monkey ES cells aggregated in suspension culture formed EBs in serum-containing medium within several days (Fig. 1A). After EBs were cultured in the serum-free N1 medium for 7–10 days, they were maintained in a N2 medium supplemented with FGF2 for two weeks and plated onto polyornithine/laminin surfaces [see ref. [16] for detailed methods]. Immunostaining for neural progenitor markers showed that >90% of cells in the population were nestin+ and Musashi1+ (Fig. 1B). These results indicate that the neural selection protocol effectively eliminated cells of non-neural lineages, allowing cells to differentiate into neural progenitors that maintain their differentiated characteristics for extensive periods. Neural progenitor cells can be preserved by low temperature storage. For neuronal phenotype differentiation, nestin+/Musashi1+ progenitor cells were dissociated into small clumps, plated onto polyornithine/laminin-coated surfaces, and cultured in N2 medium without FGF2 supplement (i.e., FGF2 removal and adherent culture) for 2–8 weeks. The differentiated cells exhibited numerous neuronal processes, as corroborated by the detection of the neuronal markers TujIII, NeuN or MAP2 (Fig. 1C). They also expressed neurotransmitter markers, i.e., tryptophan hydroxylase (TPH, the rate-limiting enzyme for serotonin synthesis, Fig. 1D), choline acetyltransferase (ChAT, the rate-limiting enzyme for acetylcholine synthesis, Fig. 1E), and tyrosine hydroxylase (TH, the rate-limiting enzyme for catecholamine synthesis, Fig. 1F). The percentage of TPH+, ChAT+ and TH+ phenotypes in the differentiated population was estimated at >90%, >50% and <3%, respectively. Yield of the

TH+ phenotype was slightly increased by supplementing L-Dopa in the N2 medium (Fig. 1G). After continued culture without FGF2 for 4–8 weeks, some neuronal cells expressed markers of mature neurons such as alpha-nicotinic acetylcholine receptors (Fig. 1H) and estrogen receptor-beta (Fig. 1I). Thus, FGF2 removal alone is very effective in differentiating nestin+/Musashi1+ progenitor cells into neuronal phenotypes, predominantly toward the serotonergic lineage. Either this neural selection protocol limits TH+ phenotype differentiation or this process requires epigenetic influences other than FGF2 removal alone.

Based on the evidence that local environmental cues in the adult brain can direct progenitor cells to differentiate into host tissue-specific phenotypes [31-33], monkey ES cells (from the ORMES-1 line) were subjected to neuronal differentiation in the presence of crude extracts isolated from adult monkey striatal tissues at the time of FGF2 removal. The striatum, a forebrain structure responsible for movement coordination, contains dopaminergic neuronal terminals whose cell bodies are located in the midbrain. Control cells were supplemented either with cerebral cortical extracts or with nothing. The cells were cultured under these conditions for 2–4 weeks. Medium and extracts were refreshed daily. Cells in the cultures supplemented with extracts appeared more widely spread and greater in numbers within 2 weeks (data not shown). Cell morphology was distinct among the 3 groups (Fig. 2A,2C and 2E). Many cells expressed the neuronal filament Tuj (Fig. 2B,2D and 2F). More TH+ neuronal cells were observed in the culture supplemented with striatal tissue extracts (Fig. 2F) compared to the culture with cortical extracts (Fig. 2D). Only a few cells stained positive for TH without extract supplement (Fig. 2B). These results support the concept that the striatum contains the chemical cues required to direct the differentiation of monkey ES cell-derived nestin+ progenitor cells into TH+ neuronal cells.

A conceptually different method for TH+ neuronal differentiation is to direct pluripotent ES cells into primitive neuroectoderm-like precursor cells by stromal cell (i.e., PA6 cells) co-culture [34], HepG2 cell-conditioned medium [35], or adherent culture [36]. Monkey neuroectoderm-like, precursor cell populations contain nearly homogenous nestin+ and Musashi1+ progenitor cells that can be differentiated into neuronal phenotypes [37]. Sequential additions of growth factors necessary for embryonic midbrain development, i.e., sonic hedgehog (SHH), FGF8, FGF2, brain-derived neurotrophic factor (BDNF) and ascorbic acid, enrich mouse TH+ cell populations to >50%. These TH+ cells exhibit midbrain dopaminergic neuronal properties [38]. Transplantation of enriched dopaminergic neurons into the striatum of 6-
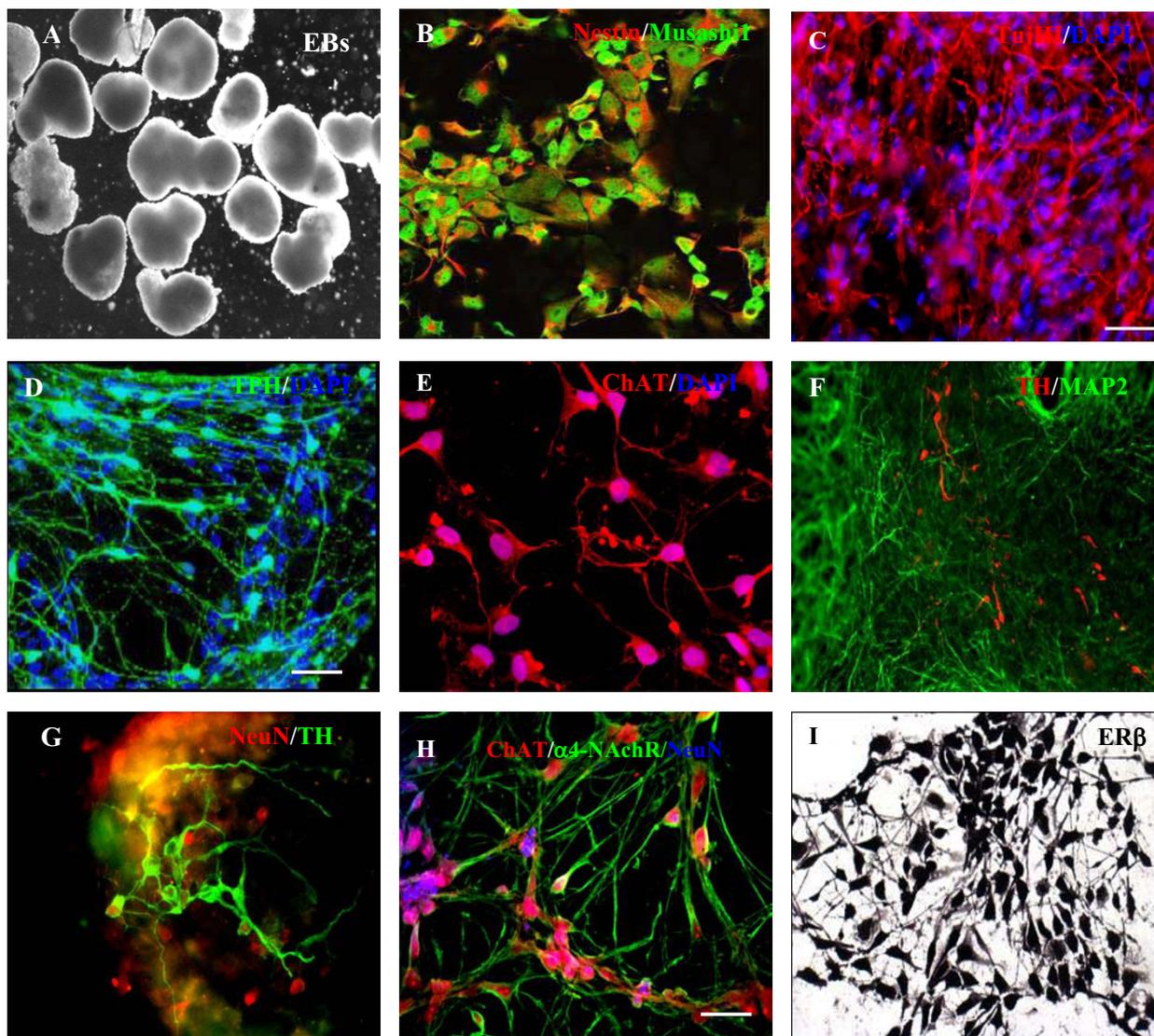
Figure 1
Neuronal differentiation of monkey ES cells into embryoid bodies (EBs, panel A), progenitor cells expressing nestin and musashi1 (panel B), neuronal cells expressing the neural filament TujIII (panel C) and neurotransmitter enzymes tryptophan hydroxylase (TPH, panel D), choline acetyltransferase (ChAT, panel E) and tyrosine hydroxylase (TH, panel F and G). Receptors of mature neurons such as α4-nicotinic receptors (panel H) and estrogen receptor β (panel I) were also observed. The image in panel A was taken directly from an inverted microscope. Images in panels B-H were taken after fluorescence-based immunocytochemistry. The image in panel I was taken after staining with the classic, non-fluorescent procedure using biotinylated antiserum and diaminobenzidine in the presence of nickel sulfate (shown in dark brown or black). Scale bars, when applied, are equivalent to 40 μm.

hydroxy-dopamine (6-OHDA)-lesioned mice or rats, rodent models of Parkinson's disease exhibiting dopamine-dependent deficiency in symmetrical movement, alleviated apomorphine-induced movement asymmetry [38-41].

Glial cell differentiation after adherent culture of nestin+/Musashi1+ progenitor cells

The majority of monkey ES cells subjected to the neural selection protocol differentiate into neuronal cells. However, approximately 10–15% of cells in the differentiated
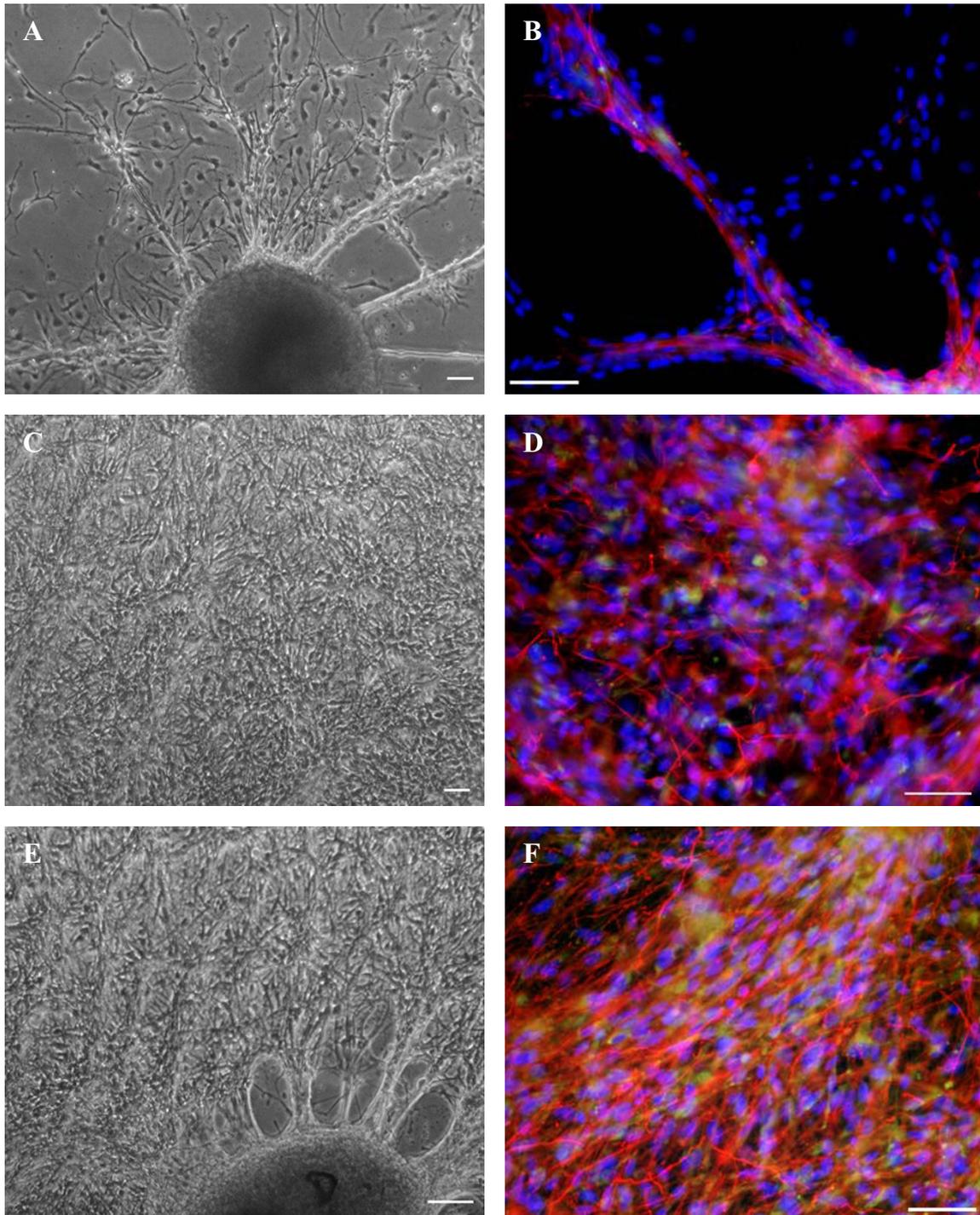
Figure 2
Neuronal differentiation of monkey ES cells under the influence of monkey brain tissue crude extracts. Images were taken before (live) and after (fixed) immunostaining for TH (green) and TujIII (red). Co-expression of TH and TujIII appears in yellow. Cell nuclei were counterstained with DAPI (blue). A and B, without extracts; C and D, with cortical extracts; E and F, with striatal extracts. Scale bars are equivalent to 50 μm.

population express the glial cell marker, glial fibrillary acidic protein (GFAP) [16], suggesting that a subpopulation of phenotypes retains the characteristics of the glial lineage. In a separate experiment, nestin[+]/Musashi1^{+} progenitor cells were produced by neural selection of monkey ES cells in overcrowding cultures. During the expansion phase (prior to differentiation), nestin[+]/Musashi1^{+} progenitor cells were plated onto adhesive culture dishes and maintained in the N2 medium supplemented with FGF-2 for up to 6 months. Throughout this period, cells, apparently originated from the nestin[+]/Musashi1^{+} progenitor cells, attached to the surface of the culture dish and multiplied. The morphology and growth pattern of these cells (Fig. 3A) bear resemblance to Schwann cell outgrowths from cultured nerve explants isolated from perinatal monkeys [42]. When isolated and plated onto separate dishes, these ES-derived cells formed monolayers, aggregated cell bundles and floating spherical bodies, especially on laminin-coated surfaces (Fig. 3B). Since Schwann cells are characterized by genes such as p75, S-100, GFAP, O4, GalC, SCIP, MBP, PLP and P0) [[43] for review; also see Fig. 4], we examined the expression of these markers in the differentiated cells and cell aggregates by immunocytochemistry and RT-PCR. The differentiated population contained phenotypes expressing nearly all tested markers of Schwann cells (Fig. 3C,3D). Of particular interest is the expression of myelin-associated proteins, MBP and PLP, in these glial phenotypes (Fig. 3E,3F). The expression of myelin proteins has also been reported in phenotypes derived from neuronal differentiation of human ES cells [44].

While the identity and function of monkey ES cell-derived glial progeny remain to be elucidated, they represent a mixture of phenotypes expressing markers typical of specific stages of Schwann cell development (Fig. 4). These markers can be divided into 3 major groups. The first group of genes and proteins, including p75, GFAP and SCIP, has a temporal expression pattern, starting during the development of immature Schwann cells and terminating in mature, myelinating cells. The second group contains S100, O4 and GC, which are expressed during the progenitor or immature stage. The expression of these genes and proteins continues in mature, myelinating Schwann cells. The third group includes myelin-associated genes and proteins such as MBP and PLP that are expressed only in the mature, myelinating Schwann cells. As shown in Fig. 4, monkey ES cell-derived glial phenotypes expressed protein markers indicative of all 3 major Schwann cell developmental stages. The expression of several gene markers has been confirmed by RT-PCR (Fig. 4, insert), including p75, GFAP, MBP and PLP. The expression of endothelin receptor A (edtlRA) and integrin β1 (itgβ1), molecules that may play important roles in Schwann cell maturation and adhesion [43], was also

observed. Quantification of marker expression in these monkey ES cell-derived Schwann cell phenotypes, i.e., counting the number of cells expressing each marker, was not performed. To delineate the temporal pattern of gene and protein marker expression will require a chronological study based on a quantitative assay. Nevertheless, the results demonstrate that monkey ES cells can be differentiated into a mixture of phenotypes that can be classified as cells in the Schwann cell lineage, including those expressing myelinating proteins. Such phenotypes have the potential to be enriched, defined, and developed into a suitable cell source for studying the safety, efficacy and feasibility of replacement therapy with myelinating cells in animal models of demyelinating disorders, such as multiple sclerosis and spinal cord injuries [45-52].

Perspectives and limitations of ES cell research and applications

Since the isolation of human ES cells [5], we have witnessed an explosion in ES cell research based on the expectation that these cells will become a valuable source for replacement therapy to cure cellular degenerative diseases. At the same time, adult stem cells isolated from various tissues carry similar expectations for regenerative medicine, given that de-differentiation (from tissue-specific stem cells to pluripotent stem cells) and trans-differentiation (between tissue-specific stem cells) become possible [53-55].

ES cell-based therapy is a viable approach to cure degenerative conditions such as Alzheimer's disease, Parkinson's disease, multiple sclerosis, spinal cord injury, diabetes and cardiac dystrophy, to name a few. The road to achieve these goals will be long, and the challenge ahead is daunting. Some of the more immediate limitations in primate ES cell research and applications include the following:

(1) The optimization of undifferentiated ES cell culture is key to standardize successful culture of undifferentiated primate ES cells and to scale-up the production of differentiated progeny for transplantation. The solution may rely on the discovery of "stemness" genes and their signaling pathways. This requirement cannot be circumvented by using mouse feeder cell-conditioned medium [56] or human cell co-cultures [57], but these alternatives are helpful in animal research. A feeder layer- and serum-free culture system for human ES cells employing a combination of serum replacement and growth factors [58] has been reported. Validation of this method in different primate ES cell lines will be valuable. Methods to quantitatively identify and isolate undifferentiated ES cells from spontaneously differentiated phenotypes during culture must be established. Moreover, the quality of pluripotent ES cells over multiple passages, such as chromosomal normalcy and differentiation potential, must be maintained.
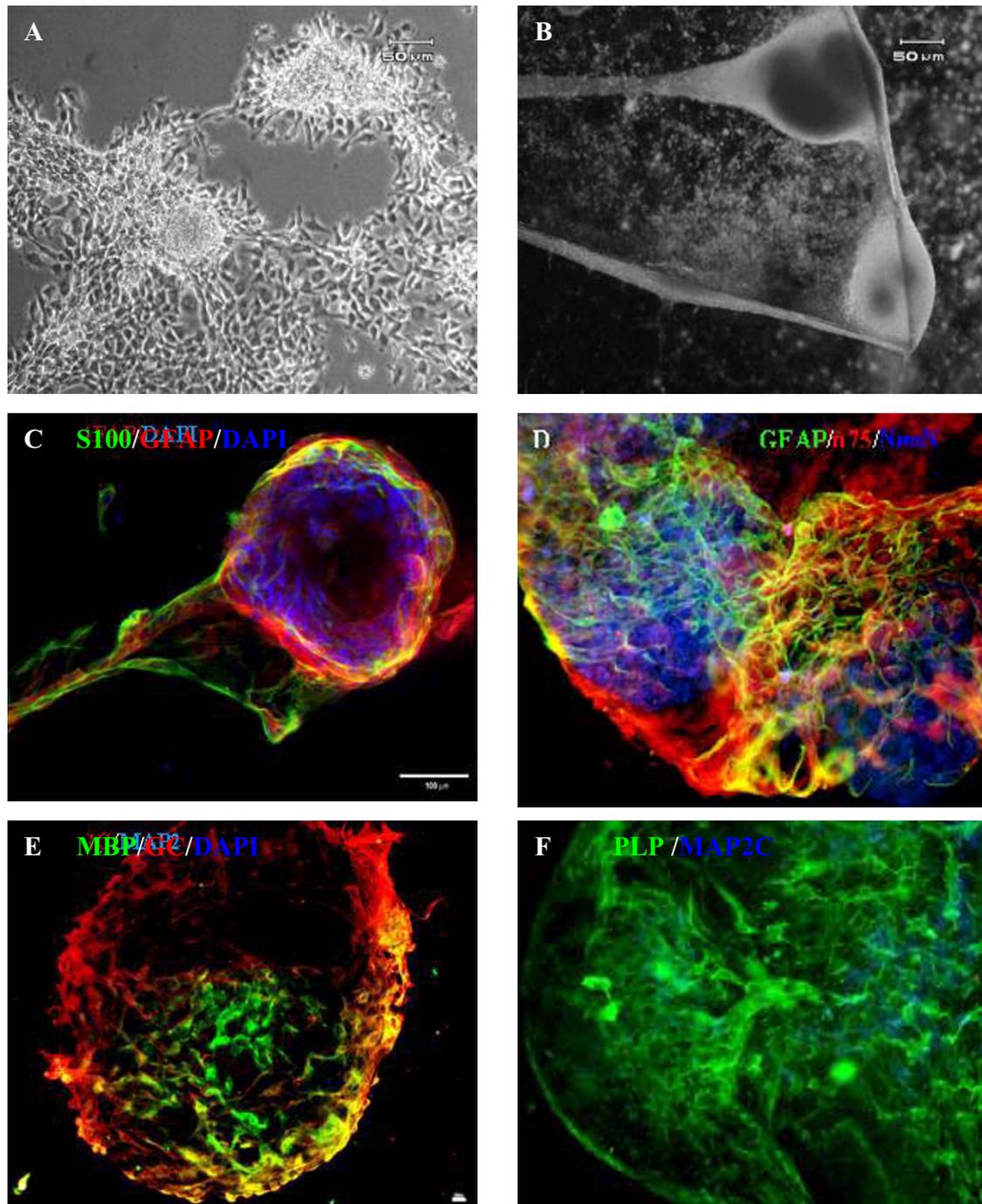
Figure 3
Differentiation of monkey ES cells into glial phenotypes. Panel A, morphology in culture; Panel B, aggregated glial cell bundles and spheres cultured on laminin-coated glass coverslips; Panel C, expression of Schwann cell markers S100 and GFAP; Panel D, expression of Schwann cell markers GFAP and p75 (NGFR); Panel E, expression of myelin protein MBP and Schwann cell marker GC; Panel F, expression of myelin protein PLP and neuronal marker MAP2C. Panel G shows the temporal pattern of Schwann cell molecular signatures during development and a mixture of glial phenotypes derived from monkey ES cells expressing these Schwann cell markers as determined by immuocytochemistry (panel H) and RT-PCR (panel I).
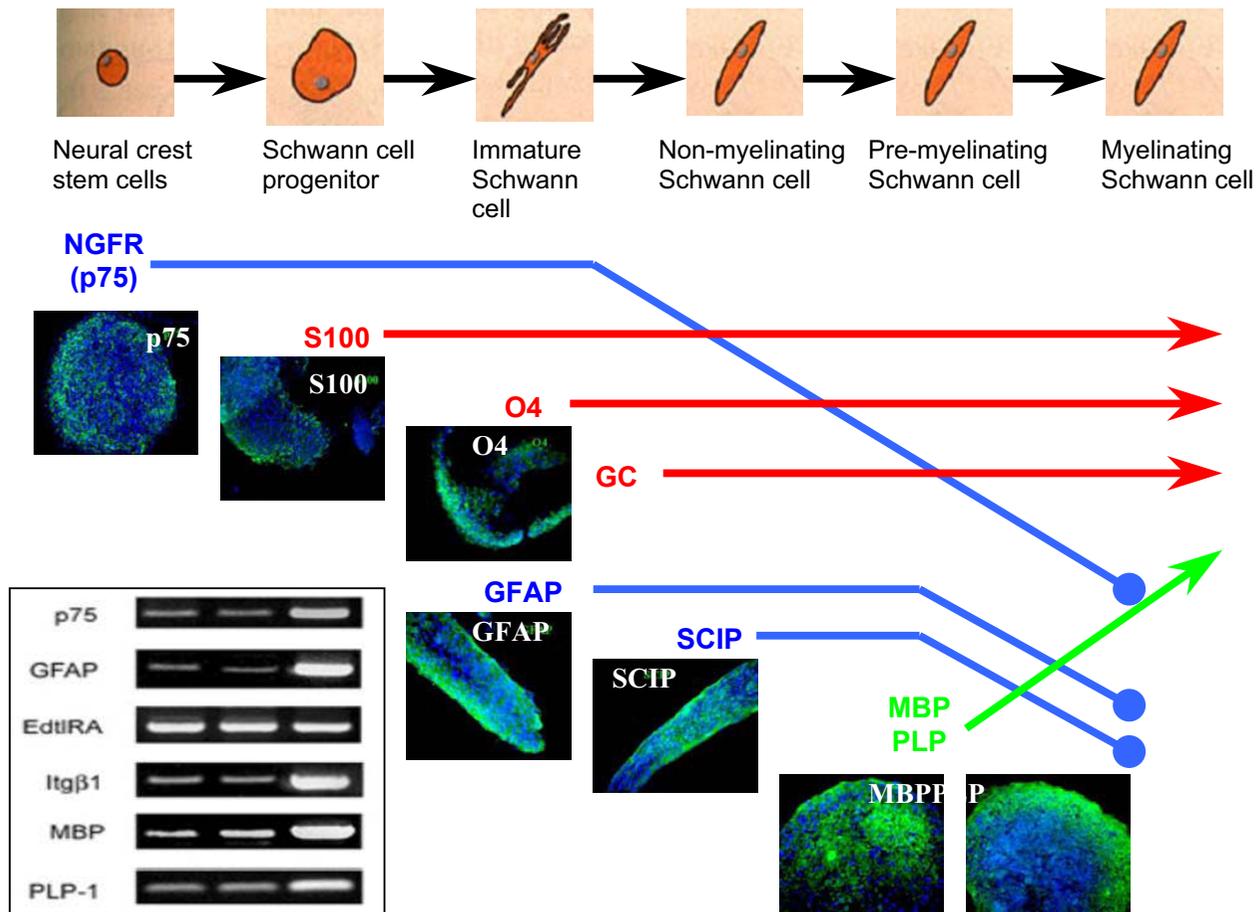
Figure 4
A schematic presentation of Schwann cell markers expressed during various developmental stages. These markers are tentatively divided into 3 groups. Group 1 are genes and proteins, including p75, GFAP and SCIP (shown in blue), that express temporally during the development between immature Schwann cells and matured, myelinating cells. Group 2 markers include S100, O4 and GC (shown in red) that express during the progenitor or immature stages and continue into matured, myelinating Schwann cells. Group 3 markers include myelinating genes and proteins such as MBP and PLP (shown in green) that express only in the mature stage of myelinating Schwann cells. Monkey ES cell-derived glial phenotypes formed cell bundles and aggregates that expressed markers of all 3 groups (shown in immunofluorescence green images; nuclei were counter-stained with DAPI in blue color), except GC, which expressed in some, but not all, samples (image not shown). The insert shows gene markers detected by RT-PCR in these monkey ES cell-derived glial phenotypes.

(2) A characterization and comparison among primate ES cell lines is essential to define line specificity. The full potential of ES cell applications can not be realized by a few cell lines studied by a small number of laboratories. A concerted effort to create an ES cell bank with well-characterized cell lines and standardized culture procedures for distribution to scientists will greatly facilitate primate ES cell research. Creating new monkey ES cell lines for unbiased characterization should provide invaluable information about ES cell line diversity that is unable to be

performed at the present time in the United States with federal funding on human ES cell lines.

(3) Phenotype-specific differentiation and enrichment protocols are critical to the application of ES cells for therapy. Differentiation protocols derived from mouse ES cell research must be applied to primate ES cells as soon as feasible, as it is most certain that modifications will have to be made to overcome species differences. Enrichment of the desired phenotype for transplantation will probably

be essential, with the quality of primate ES cells or progeny ensured after enrichment.

(4) Genetic manipulations such as functional gene knock-ins, including disease-specific genes, differentiation-promoting genes and immunocompatible genes are highly desirable. Recent success in gene transfer by homologous recombination [59] and lentiviral vectors [60] is encouraging, but these procedures need to be applied to other cell lines, by other laboratories, and improved for better efficiency. The quality and potential of genetically modified primate ES cells should be tested rigorously in animal models.

(5) Development of animal models for transplantation studies is a crucial step toward successful clinical applications. In many cases, results using rodent models cannot be translated to human patients. Currently there are no discriminative non-human primate models of cellular degenerative diseases because gene-knockout and cloning procedures in the monkey are both time consuming and technically challenging. A few experimental models, such as MPTP-induced hemiparkinsonian monkeys [61], are therefore extremely valuable for ES cell-based therapeutic studies. The development of additional disease models with nonhuman primates is urgent.

Conclusions

At least 15 rhesus monkey ES cells lines are available at the Oregon National Primate Research Center. These cell lines have been characterized by their chromosomal normalcy, growth patterns and differentiation potential. The pluripotency of undifferentiated monkey ES cells is defined by a panel of molecular signatures that include embryonic surface antigens, enzymes and transcriptional factors. Highly purified nestin+/Musashi1+ progenitor cells can be produced by a neural selection protocol. These progenitor cells are multipotent, if not pluripotent, capable of differentiating into insulin-producing endodermal cells, various neuronal phenotypes and glial cells expressing Schwann cell markers and myelinating proteins. Differentiation into certain populations, such as the serotonergic phenotypes, is induced by the selection protocol. Differentiation into other lineages, such as the dopaminergic phenotypes, is either inhibited or requires additional epigenetic influences such as those present in extracts of the dopaminergic striatum. However, enrichment of dopaminergic phenotypes by directional differentiation of mouse ES cells followed by appropriate growth factor induction has been successful. The potential exists for the differentiation and enrichment of monkey ES cells into dopaminergic neurons or myelinating glial phenotypes that serve as suitable cell sources for transplantation studies in animal models of Parkinson Disease, multiple sclerosis and spinal cord injury. Such translational stud-

ies, particularly in nonhuman primate models, are critical steps to understand the safety, feasibility and efficacy of ES cell-based therapy for the treatment of neural degenerative diseases.

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