

Derivation and Cloning of a Novel Rhesus Embryonic Stem Cell Line Stably Expressing Tau-Green Fluorescent Protein

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ABSTRACT

Embryonic stem cells (ESC) have the ability of indefinite self-renewal and multilineage differentiation, and they carry great potential in cell-based therapies. The rhesus macaque is the most relevant preclinical model for assessing the benefit, safety, and efficacy of ESC-based transplantations in the treatment of neurodegenerative diseases. In the case of neural cell grafting, tracing both the neurons and their axonal projections in vivo is essential for studying the integration of the grafted cells in the host brain. Tau-Green fluorescent protein (tau-GFP) is a powerful viable lineage tracer, allowing visualization of cell bodies, dendrites, and axons in exquisite detail. Here, we report the first rhesus monkey ESC line that ubiquitously and stably expresses tau-GFP. First, we derived a new line

of rhesus monkey ESC (LYON-ES1) that show marker expression and cell cycle characteristics typical of primate ESCs. LYON-ES1 cells are pluripotent, giving rise to derivatives of the three germ layers in vitro and in vivo through teratoma formation. They retain all their undifferentiated characteristics and a normal karyotype after prolonged culture. Using lentiviral infection, we then generated a monkey ESC line stably expressing tau-GFP that retains all the characteristics of the parental wild-type line and is clonogenic. We show that neural precursors derived from the tau-GFP ESC line are multipotent and that their fate can be precisely mapped in vivo after grafting in the adult rat brain. *STEM CELLS* 2008;26:1444–1453

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

Embryonic stem cells (ESC) are capable of indefinite self-renewal and multilineage differentiation. One of the most important potential applications of human ESC is cell-based therapy. Before the clinical application of human ESC transplantation can be attempted, extensive studies assessing the benefit, safety, and efficacy of embryonic stem (ES)-derived cell transplantation in preclinical nonhuman primate models will be necessary, particularly in the case of neurodegenerative diseases [1].

ESC lines established in nonhuman primates (rhesus, cynomolgus, and marmoset monkeys) [2–8] have characteristics similar to those of human ESC, proving them to be an invaluable preclinical research tool. Like their human counterparts, monkey ESC are able to differentiate into many clinically relevant cell types, including hematopoietic cells [9], hepatocytes [10], insulin-producing cells [11], cardiomyocytes [12], and neural-derived cells [7, 9, 13–18]. Following transplantation, nonhu-

man primate ESC and their derivatives have been shown to survive, differentiate, and integrate into the host tissue of various animal models (rodents and monkeys), particularly the brain [19–23].

Tracing the cells in vivo is essential for studying the fate and the interaction of the grafted cells with their host environment. In the case of neural cell grafting, this makes it necessary to be able to reliably label both the parent neurons and their axonal projections in the recipient brain. Such a label is possible via tau-green fluorescent protein (tau-GFP) fusion protein expression. By binding the GFP to microtubules, tau-GFP tagging reveals the detailed morphology of cell bodies, dendrites, and axons [24, 25]. Here, we report the generation and characterization of the first rhesus monkey ESC line that ubiquitously and stably expresses a tau-GFP fusion protein. This cell line retains all the characteristics of the parental wild-type line and is clonogenic. We show that neural precursors derived from the tau-GFP ESC line are multipotent and that their integration can be monitored in vivo after grafting in the adult rat brain.

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MATERIALS AND METHODS

ES Cell Derivation and Culture

Zona pellucidae of blastocysts were removed by brief exposure (45–60 seconds) to 0.5% pronase in TH3 medium. Expanded blastocysts, possessing large and distinct inner cell masses (ICMs), were subjected to the immunosurgical method. Zona-free blastocysts were exposed to anti-monkey antiserum (Sigma-Aldrich, St. Louis, <http://www.sigmaaldrich.com>) for 30 minutes at 37°C. After washing in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, <http://www.invitrogen.com>) supplemented with 20% fetal bovine serum (FBS; Perbio Science France, Brebieres, France, <http://www.perbio.com>), embryos were incubated in guinea pig complement reconstituted with DMEM (1:5, vol/vol) for an additional 30 minutes at 37°C. Partially lysed trophectodermal cells were dispersed by gentle pipetting with a flame-pulled Pasteur pipette. ICMs were then rinsed three times with DMEM supplemented with 20% FBS. Isolated ICMs were plated onto Nunc (Nunc, Rochester, NY, <http://www.nuncbrand.com>) four-well dishes containing a feeder layer of mitomycin-C-treated mouse embryonic fibroblasts (MEFs) and cultured in knockout (KO)-DMEM containing 10% FBS/10% KO-serum replacement (SR) (Invitrogen) supplemented with 4 ng/ml basic fibroblast growth factor (bFGF; AbCys, Paris, <http://www.abcysonline.com>), human recombinant leukemia inhibitory factor (hrLIF; 1,000 IU/ml), 1% nonessential amino acids (Invitrogen), 2 mM L-glutamine (Invitrogen), 0.1 mM β -mercaptoethanol. ICMs that attached to the feeder layer and initiated outgrowth were manually dissociated into small cell clumps with a microscalpel and replated onto new MEFs. When blastocysts exhibited undistinguishable trophectoderm and ICM, immunosurgery was not performed, and the whole blastocysts were cultured on MEFs after digestion of zona pellucida with pronase.

Colonies with ESC-like morphology were selected for further propagation, characterization, and freezing. During the early stage of ESC derivation, the medium was supplemented with hrLIF (1,000 IU/ml), and half of the medium was changed every other day. For expansion and maintenance, ESC were cultured in KO-DMEM containing 20% KO-SR and 4 ng/ml bFGF. Mechanical passaging of the undifferentiated colonies was performed manually every 5–7 days by cutting the colonies into large clumps using a flame-pulled Pasteur pipette. ESC colonies were replated onto dishes with fresh feeder layers. Care was taken to ensure that the differentiated areas were eliminated during passaging. Cultures were maintained at 37°C, 5% CO₂.

Lentiviral Infection and Cell Sorting

We used two simian immunodeficiency virus (SIV)-based vectors, GAE-CAG-eGFP/WPRE (a gift from F.L. Cosset), which harbors the sequence encoding the enhanced green fluorescent protein (eGFP), and GAE-CAG-tau-GFP/WPRE, which harbors the sequence encoding tau-GFP [25], both driven by the cytomegalovirus enhanced chicken β -actin (CAG) promoter. GAE-CAG-tau-GFP/WPRE was generated by replacing the BamHI/EcoRV restriction fragment containing the eGFP cassette in GAE-CAG-eGFP/WPRE by a EcoRV blunt restriction fragment of the pTP6 vector (a gift from Tom Pratt) containing the tau-GFP cassette. We previously described the lentiviral production [26]. Briefly, 293T cells were transfected with a mixture of DNAs containing 10 μ g of the pGRev plasmid encoding the vesicular stomatitis virus glycoprotein (VSV-G) envelope; 10 μ g of pSIV3+ plasmid encoding the gag, pol, tat and rev proteins; and 13 μ g of the R4SA-CAG-eGFP-W plasmid or the R4SA-CAG-tauGFP-W plasmid, using the calcium phosphate precipitation technique. The following day, cells were refed 7 ml of DMEM and further cultured for 24 hours. The supernatant was then collected, cleared by centrifugation (3,000 rpm, 15 minutes), and passed through a 0.8 μ m filter. Prior to infection, LYON-ES1 cells were treated with collagenase IV (1 mg/ml) for 5 minutes at 37°C. Clumps of undifferentiated cells were manually selected and transferred to fresh medium (200 μ l) containing SIV-eGFP or SIV-tau-GFP in the presence of 6 μ g/ml polybrene (Sigma-Aldrich). Cells were incubated for 3 hours at

37°C before being replated on fresh feeder cells. To select for GFP-positive LYON-ES cells, GFP- and tau-GFP-expressing LYON-ES cells were treated with collagenase IV (1 mg/ml) (Invitrogen) for 20 minutes and trypsinized, and the single cell preparation was resuspended in phosphate-buffered saline (PBS) before being processed in a FACSVantage SE (Becton, Dickinson and Company, Franklin Lakes, NJ, <http://www.bd.com>). The viability of LYON-ES1 cells after fluorescence-activated cell sorting was >98% as assessed by trypan blue exclusion. The percentage of GFP-positive cells was analyzed using a FACSCanto 2. Data acquisition was performed with Diva software (Becton Dickinson).

Cloning

LYON-ES cells were dissociated to single cells for 7 minutes with trypsin (0.025%)/EDTA (0.1 g/l) (Invitrogen) and washed by centrifugation, and individual cells were selected by direct observation under a stereomicroscope and transferred by micropipettes to individual wells of 96-well plates containing MEFs and medium supplemented with 20% KO-SR and 10 ng/ml bFGF. As physiologic oxygen has been reported to enhance clone recovery in human ESC lines [27, 28], cultures were maintained at 5% O₂ and 5% CO₂, at 37°C. Emerging clones were first passaged into 24-well plates, subsequently amplified into 35-mm well plates, and frozen in liquid nitrogen. All clones were found to be GFP-positive using fluorescent microscopy.

In Vitro Differentiation of LYON-ES Cells

For the formation of embryoid bodies (EBs), LYON-ES1 colonies were treated with collagenase IV (1 mg/ml) for 30 minutes, and clumps of cells were cultured in suspension in KO-DMEM containing 20% KO-SR (Invitrogen) and supplemented with 1% nonessential amino acids (Invitrogen), 2 mM L-glutamine (Invitrogen), and 0.1 mM β -mercaptoethanol, without bFGF. Medium was changed every other day. For spontaneous differentiation, LYON-ES cells were cultured to subconfluence for 15 days, with a daily medium change. For neural differentiation, neuroepithelial-like cells spontaneously emerging in culture were selected manually and cultured in Euromed-N medium (Euroclone, Pero, Italy, <http://www.euroclone.net>) supplemented with 20 ng/ml bFGF (Abcys) and 20 ng/ml epidermal growth factor (EGF; Abcys). For neuronal differentiation, LYON-ES-derived neural cells were trypsinized (0.025%)/EDTA (0.1g/l) (Invitrogen) and replated into four-well plates (Nunc) coated with Matrigel (BD Biosciences, San Diego, <http://www.bdbiosciences.com>) in Euromed-N medium supplemented with bFGF (10 ng/ml), modified N2, and B27 (Invitrogen). Half the volume of medium was replaced every 2 days. After 7 days, the medium was changed to Euromed-N mixed with neurobasal medium (Invitrogen) (1:1) supplemented with 0.5 \times N2 and B27, bFGF (5 ng/ml), brain-derived neurotrophic factor (BDNF) (20 ng/ml) (Sigma-Aldrich), and ascorbic acid (200 μ M). After an additional 7 days in these conditions, medium was switched to neurobasal medium supplemented with B27 and BDNF (10 ng/ml) without N2 or bFGF. For glial differentiation, LYON-ES-derived neural cells were cultured in Euromed-N medium supplemented with 10% FBS (HyClone; Perbio).

Immunofluorescence and Alkaline Phosphatase Staining

Cells were fixed with 2% paraformaldehyde in PBS at 4°C for 1 hour and permeabilized in Tris-buffered saline (TBS) + 0.1% Triton X-100 (three times, 10 minutes each time). Nonspecific binding was blocked with 10% normal goat serum (Jackson ImmunoResearch Laboratories, West Grove, PA, <http://www.jacksonimmuno.com>) for 20 minutes at room temperature. Cells were incubated overnight at 4°C, with primary antibodies (supplemental online Table 2) diluted in Dako diluent (Dako, Glostrup, Denmark, <http://www.dako.com>). After three rinses in TBS, cells were exposed to affinity-purified goat anti-mouse, anti-rat, or anti-rabbit immunoglobulin G (IgG) or immunoglobulin M (IgM) conjugated either to indocarbocyanine or to cyanin (Cy3 and Cy2, respectively; Jackson ImmunoResearch Laboratories) for 1 hour at room temperature followed by nuclear staining

with 1 ng/ml Hoechst 33258 for 3 minutes. After three rinses in TBS, coverslips were mounted on slides. Coverslips were examined using an oil objective microscope under UV light to detect cyanin (filter, 450–490 nm), indocarbocyanine 3 (filter, 550–570 nm), and Hoechst 33258 (filter, 355–425 nm). Alkaline phosphatase activity was revealed using the alkaline phosphatase substrate kit (Ref. 86R; Sigma-Aldrich) according to the manufacturer's instructions.

Karyotype Analysis

LYON-ES1 cells were treated with colcemid (0.08 $\mu\text{g}/\text{ml}$) (Sigma-Aldrich) for 2 hours. Cells were then trypsinized, resuspended in 0.075 M KCl, and incubated for 10 minutes at room temperature. The cells were then fixed with fresh Carnoy's fixative (methanol/glacial acetic acid, 3:1) and dropped onto ice-cold slides. Chromosome spread was Giemsa-banded. Images were captured on a Leica microscope (Leica, Heerbrugg, Switzerland, <http://www.leica.com>) using the Mosaic imaging system (Explora Nova, La Rochelle, France, <http://www.exploranova.com>). At least 20 metaphase spreads were counted for each passage, and 14 banded karyotypes were evaluated for chromosomal rearrangements.

Telomerase Activity

Telomerase activity was determined using the TRAPEze Telomerase Detection Kit (Chemicon, Temecula, CA, <http://www.chemicon.com>) according to the manufacturer's instructions. Briefly, cell extracts were obtained from one 35-mm culture dish. Protein concentrations were normalized using the Coomassie Blue-stained protein assay reagent bovine serum albumin standards (Pierce, Rockford, IL, <http://www.piercenet.com>). Heat-inactivated controls were obtained by incubating the samples at 85°C for 10 minutes. Aliquots (1 μg) of the cell extracts were used for polymerase chain reaction (PCR). The PCR products were electrophoresed on a 12.5% nondenaturing polyacrylamide gel, and telomerase activity was detected by ethidium bromide staining.

Semiquantitative Reverse Transcription-PCR

Total RNA was prepared with a Qiagen RNeasy kit (Qiagen, Valencia, CA, <http://www1.qiagen.com>). Standard reverse transcription reactions were performed with 1 μg of total RNA primed with random primers using SuperScript II first-strand synthesis system (Invitrogen). PCR was carried out using the following parameters: denaturation at 94°C for 45 seconds, annealing at the suitable annealing temperature (supplemental online Table 1) for 1 minute, and polymerization at 72°C for 2 minutes. The sequence, annealing temperature, and cycle number of each pair of primers are listed in supplemental online Table 1. An extension step of 7 minutes at 72°C was added at the end of the cycles. Each PCR was performed under linear conditions. Reactions without reverse transcriptase were performed to control for contaminations with genomic DNA, using β -actin primers. PCR products were analyzed on a 1.5% agarose gel and visualized with ethidium bromide.

Teratoma Formation

Colonies of LYON-ES cells were selected manually and inoculated beneath the testicular capsule of 7-week-old severe combined immunodeficient (SCID) males (CB17/SCID; Charles River Laboratories, Wilmington, MA, <http://www.criver.com>). Five to 10 weeks later, mice were euthanized, and lesions were surgically removed. Teratomas were fixed in 4% PFA overnight at 4°C, sunk in 10% sucrose for 24 hours and in 20% sucrose for 24 hours, and embedded in OCT embedding medium (CellPath, Powys, U.K., <http://www.cellpath.co.uk>). Cryosections (20 μm) were washed three times for 10 minutes in TBS and processed for immunofluorescent staining (described above). Primary antibodies are detailed in supplemental online Table 2. Oil red O staining was used to label adipose-like cells, and alizarin red staining was used to mark cartilage. Detailed procedures are presented in the supplemental online Materials and Methods.

RESULTS

Establishment and Characterization of the LYON-ES1 Cell Line

Thirty-four rhesus monkey blastocysts produced by intracytoplasmic sperm injection were used. Seven embryos exhibited a small or undistinguishable ICM; thus, immunosurgery was not performed, to reduce the risk of cell loss. All of them formed outgrowths. Twenty-seven ICMs were isolated by immunosurgery, of which 22 formed outgrowths and generated five lines that were expanded for more than 12 passages and frozen. One cell line, named LYON-ES1, established without performing immunosurgery, could be easily maintained and showed rapid expansion in culture. Within the first days of derivation, cells showing an ESC-like morphology emerged (Fig. 1A). These putative ESC were manually selected and subcultured on a fresh feeder layer (passage 1 at day 6). After 1 day in vitro, colonies of small, tightly packed cells proliferated from the transferred clumps (Fig. 1B). These colonies were split after 2 days and passaged every 4–7 days. The LYON-ES1 cell line was initially derived in a medium containing 10% FBS and 10% serum replacement (KO-SR) and supplemented with bFGF and hrLIF. Under those conditions, ES cells showed low amplification rates associated with a sustained level of spontaneous differentiation, despite the manual removal of differentiated cells. After 4–5 passages, the cells were adapted to medium containing 20% KO-SR and fibroblast growth factor 2 (FGF2) (4 ng/ml). Those conditions resulted in a decrease in the incidence of spontaneous differentiation and an increase in proliferation. Under those conditions, cell morphology was homogeneous, with colonies composed mainly of ES-like cells (Fig. 1C). The morphology of LYON-ES1 cells is identical to that reported for other human and nonhuman primate ESC lines: they formed packed and tight colonies (Fig. 1C) and showed a high nucleus/cytoplasmic ratio and clearly distinguishable nucleoli (Fig. 1D). Amplification and routine passaging of the cells were performed using a manual dissociation technique so as to avoid the emergence of chromosomal abnormalities induced by enzymatic treatment [29]. The LYON-ES1 cell line has been cultured for more than 60 passages while maintaining an undifferentiated state. Immunohistochemistry and reverse transcription (RT)-PCR were used to analyze the expression of pluripotency markers. LYON-ES1 cells express the transcription factors Oct-4 and Nanog and the cell surface markers stage specific embryonic antigen (SSEA)4, TRA-1–60, TRA-1–81, and CD90 (Fig. 2A) and show strong alkaline phosphatase activity (Fig. 2A; data not shown). Normal diploid 42XX karyotype and high telomerase activity were maintained after prolonged culture (supplemental online Fig. 1A, 1B). After passaging every 5 days, LYON-ES1 cells showed sustained proliferation rates in long-term cultures. Bromodeoxyuridine cumulative labeling indicated a total cell-cycle duration of 9 hours when cells were cultured in 20% KO-SR and FGF2 (4 ng/ml). Cell cycle duration increased dramatically (15.5 hours) when cells were cultured in the medium routinely used for culturing monkey ES cells (i.e., containing 20% FBS) (supplemental online Fig. 1C) [2, 26, 30], suggesting that FBS containing medium is not optimal for monkey ES cell expansion. The distribution of self-renewing LYON-ES cells in cell-cycle phases, as analyzed by flow cytometry, indicated that the fractions of cells in G1, S, and G2/M phases are 23%, 58%, and 19%, respectively (Fig. 2B). Differentiation of LYON-ES1 cells was accompanied by a dramatic increase in the fraction of cells in G1 (Fig. 2B). LYON-ES1 cells have cell cycle characteristics that are typical of primate ES cells [26, 31].

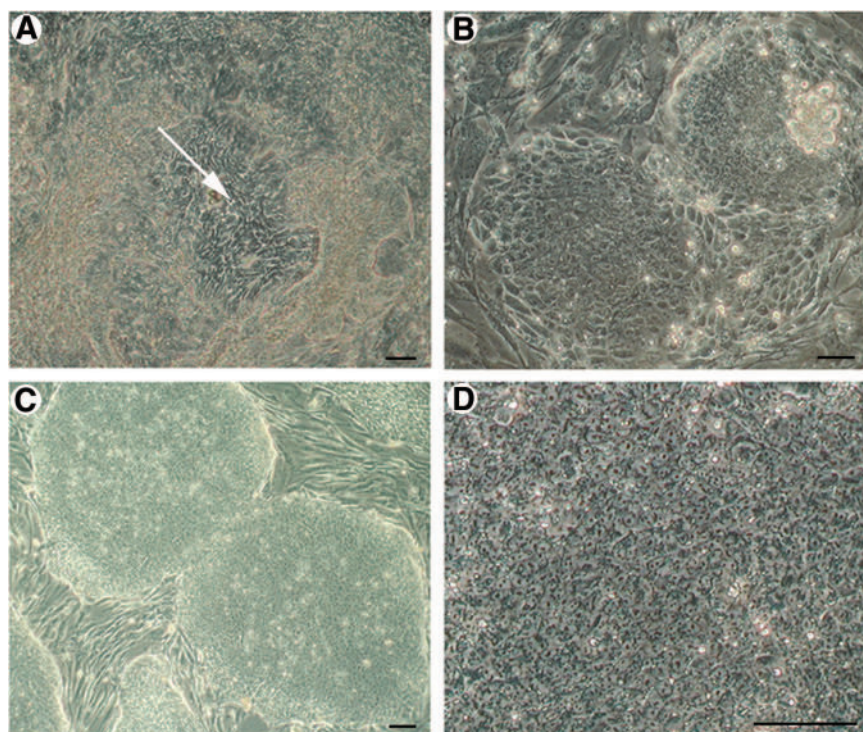


Figure 1. Derivation of the LYON-ES1 cell line. (A): Outgrowth of inner cell mass (white arrow) 10 days after initial plating of the embryo. (B): Resulting colonies 1 day after the first dissociation. Note the high nucleus/cytoplasm ratio and prominent nucleoli. Established LYON-ES1 cell line at passages 7 (C) and 28 (D). Scale bars = 100 μm (A–D).

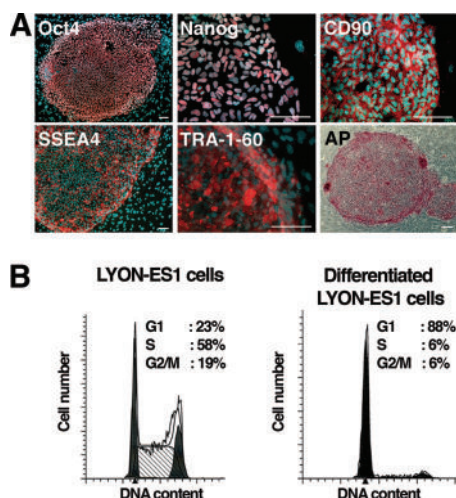


Figure 2. Characterization of the LYON-ES1 cell line. (A): Immunofluorescent staining for Oct4, Nanog, CD90, SSEA-4, and TRA-1-60 and staining for AP. (B): Histograms showing cell-cycle distribution of LYON-ES1 cells and LYON-ES1-differentiated derivatives as measured by flow cytometry. Scale bars = 100 μm (A). Abbreviation: AP, alkaline phosphatase.

LYON-ES1 Cells Are Pluripotent In Vitro and In Vivo

The capacity of LYON-ES1 cells to form differentiated cell types was assessed in vitro either by culture in subconfluent conditions or by formation of EBs (Fig. 3A). The expression of specific markers of primate ESC, ectoderm, endoderm, and mesoderm was analyzed using semiquantitative RT-PCR (Fig. 3B). Expression of oct4, Nanog, and Rex1 gradually decreases during differentiation. All tissue-specific markers were expressed in EBs and showed increased expression levels with time (Fig. 3B). After more than 40 passages, LYON-ES1 cells retained the potential to differentiate into derivatives of the three germ layers in vitro (data not shown).

Pluripotency of the LYON-ES1 cells was also assessed in vivo through teratoma formation. Injection of LYON-ES1 cells in the testes of SCID mice consistently results in teratoma formation. Immunohistochemistry shows that tumors included derivatives of the ectoderm, which are positive for glial fibrillary acidic protein (GFAP) and Nestin, and contained rosettes of neuroepithelium (Fig. 3C, upper panels). Derivatives of the endoderm stained for glucagon, GATA4, or hepatocyte nuclear factor 3 β (HNF3 β) (Fig. 3C, middle panels) were observed. The teratomas also contained mesodermal tissues such as muscle-like structures stained for desmin; cartilage-like tissue, revealed by alizarin red staining; and adipose-like cells, revealed by oil red O staining (Fig. 3C, lower panels). The range of differentiation observed within the teratomas of high-passage LYON-ES cells (passage 58) is comparable to that observed with low-passage LYON-ES cells (passage 7) (data not shown). Proliferating cells were often observed, as shown by Ki67 expression. The expression of the ES-cell markers Oct4 and TRA-1-60 was not detected (data not shown), as has also been shown with human ES cells [32], suggesting that proliferating cells correspond to precursors of immature tissue or even differentiated cells, as has recently been shown in human ES cell-derived tumors [33]. These data show that the LYON-ES1 cell line shows all the characteristics of a genuine monkey ES cell line and retains its undifferentiated and pluripotent properties after long-term culture.

Derivation of a LYON-ES1 Cell Line Stably Expressing Tau-GFP over Long-Term Cultures and Throughout Differentiation

Stable expression of GFP fused to the microtubule-associated protein tau (tau-GFP) was obtained via lentiviral infection of LYON-ES1 cells with a SIV-based lentiviral vector expressing tau-GFP under the control of the CAG promoter (described in Materials and Methods). As lentiviral infection enabled transduction of a small percentage of monkey ES cells [26], cell sorting was implemented to obtain a pure population of tau-GFP expressing LYON-ES cells. Tau-GFP enables the cell morphol-

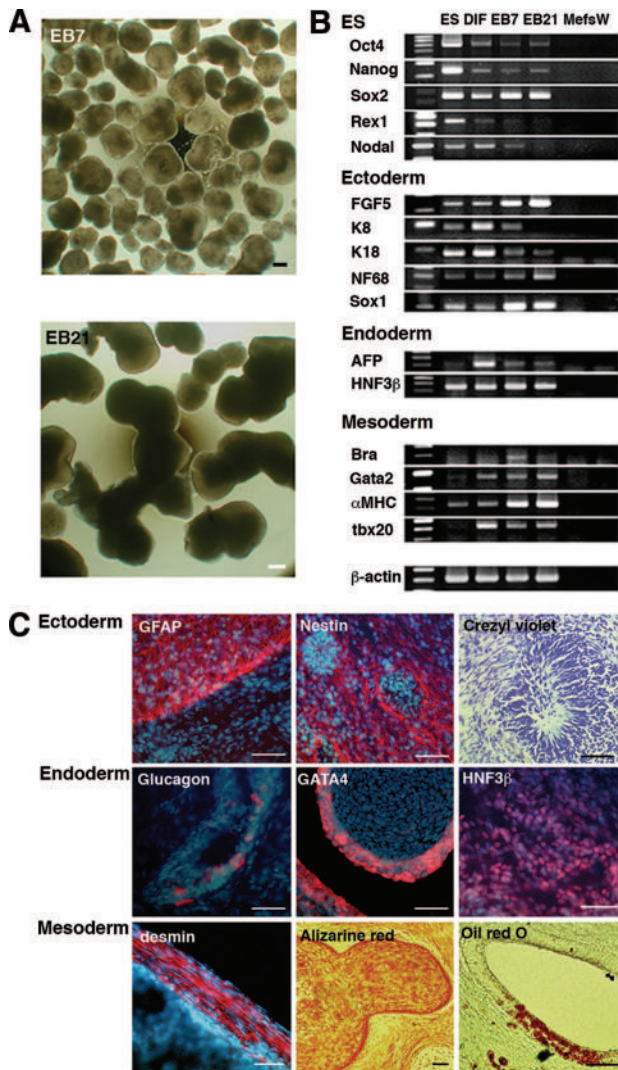


Figure 3. Pluripotency of LYON-ES1 cells in vitro and in vivo. (A): EB7 and EB21 derived from LYON-ES1 cells. (B): Reverse transcription-polymerase chain reaction analysis of ES cell and lineage marker expression in LYON-ES1 cells, DIF, EB7, and EB21. (C): Teratoma sections 6 weeks after injection of LYON-ES1 cells in the testes of SCID mice, showing derivatives of the ectoderm, endoderm, and mesoderm. Scale bars = 500 μ m (A, C). Abbreviations: AFP, alpha feto protein; Bra, Brachyury; DIF, spontaneously differentiated cells; EB7, day 7 embryoid bodies; EB21, day 21 embryoid bodies; ES, embryonic stem; FGF5, fibroblast growth factor 5; GFAP, glial fibrillary acidic protein; HNF, hepatocyte nuclear factor; K, keratin; Mefs, mouse embryonic fibroblasts; MHC, myosin heavy chain; W, water control.

ogy of living LYON-ES cells to be visualized in exquisite detail. The fluorescence was distributed evenly throughout the cytoplasm but was excluded from the nucleus (Fig. 4A). tau-GFP fluorescence was stable for more than 45 passages (Fig. 4D) and remained detectable several weeks after fixation (data not shown). Tau-GFP LYON-ES cells showed characteristics similar to those of the parental wild-type LYON-ES1 cells and to those of genetically modified LYON-ES cells stably expressing eGFP (supplemental online Fig. 2A, 2B): they expressed the pluripotent stem cell markers Oct4, Nanog, Rex1, sox2, and alkaline phosphatase, as well as the cell surface markers SSEA4 and TRA-1-60 (supplemental online Fig. 3A; data not shown). The karyotype and the level of telomerase activity of tau-GFP

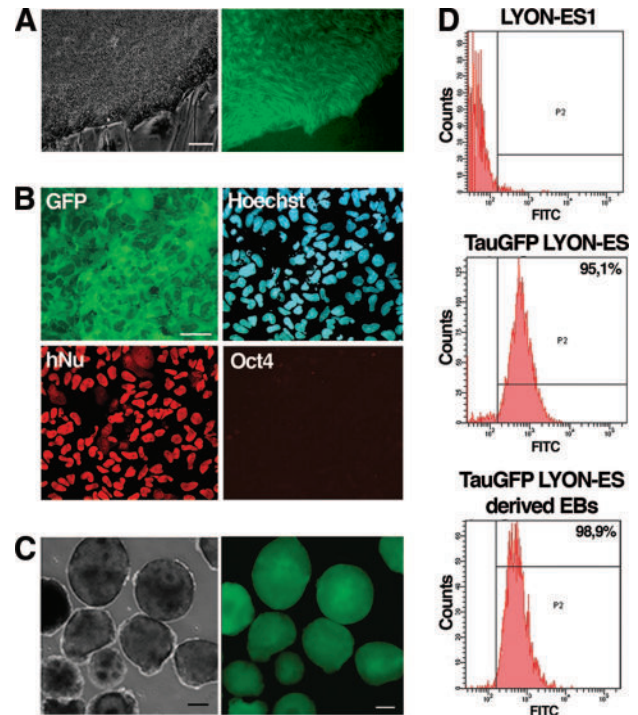


Figure 4. Stable tauGFP expression in LYON-ES cells and their in vitro differentiated derivatives. (A): Phase-contrast and corresponding live fluorescence images of tauGFP LYON-ES cells. (B): Immunofluorescent staining for GFP, hNu, and Oct4 in spontaneously differentiated tauGFP LYON-ES cells at passage 45. (C): Phase-contrast and corresponding live fluorescence images of tauGFP day 5 EBs. (D): Proportion of tauGFP-positive cells as measured by flow cytometry in living LYON-ES1 cells, tauGFP LYON-ES cells (passage 47), and tauGFP LYON-ES-derived EBs (passage 45). Abbreviations: FITC, fluorescein isothiocyanate; GFP, green fluorescent protein; hNu, human nuclear antigen; P₂, proportion; tauGFP, tau-green fluorescent protein.

LYON-ES cells were similar to those of the parental wild-type LYON-ES cells (supplemental online Fig. 3B, 3C).

Tau-GFP LYON-ES Cells Are Pluripotent and Clonogenic

The pluripotency of the transgenic LYON-ES cells was assessed via culture in suspension and in subconfluent conditions. Fluorescent microscopy observations showed that tau-GFP expression is retained during differentiation in vitro (Fig. 4C), even after numerous passages (Fig. 4B, 4D). RT-PCR and immunohistochemical analysis showed that tau-GFP LYON-ES cells give rise to cells expressing ectoderm (Sox1, nestin, GFAP, and β III tubulin), endoderm (HNF3 β and alpha feto protein), and mesoderm (α MHC, tbx20, and desmin) markers (data not shown).

The injection of tau-GFP LYON-ES cells in the testes of SCID mice resulted in teratomas that contained solid tissues and fluid-filled cystic masses, comprising derivatives of the three germ layers (Fig. 5A, 5C, 5D; data not shown), as in the case of the wild-type LYON-ES cell line. Strong tau-GFP fluorescence was readily visible 10 weeks after the injection, before any immunohistochemical amplification (Fig. 5A). Immunostaining with an anti-human nuclear antigen antibody, which specifically labels cells of primate origin, showed that all the cells of monkey origin expressed tau-GFP (Fig. 5B). These data indicate that tau-GFP expression is maintained during differentiation in vivo.

To get a homogeneous ES cell line, we isolated clones of tau-GFP-expressing cells. Tau-GFP-expressing cells were man-

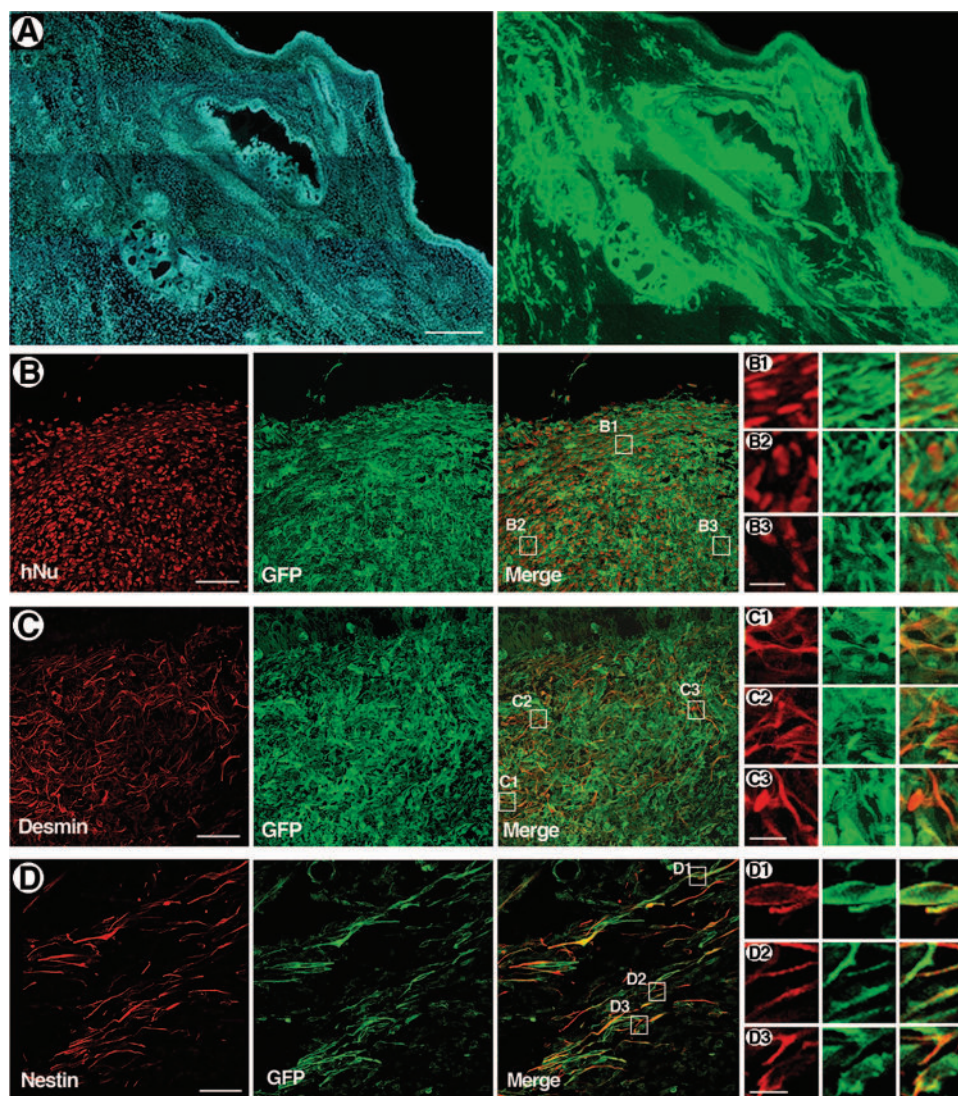


Figure 5. Analysis of teratoma sections 10 weeks after injection of tau-GFP LYON-ES cells in the testes of SCID mice. **(A):** Low magnification of tau-GFP-labeled teratoma stained with Hoechst (left) and GFP (right). Confocal images show coexpression of tau-GFP and hNu **(B)**, coexpression of tau-GFP and desmin in derivatives of the mesoderm **(C)**, and coexpression of tau-GFP and nestin in derivatives of the ectoderm **(D)**. **(B1–B3)**, **(C1–C3)**, and **(D1–D3)**: High magnifications of the fields shown in the merge images **(B)**, **(C)**, and **(D)**, respectively. Scale bars = 500 μm **(A)**, 50 μm **(B–D)**, and 10 μm **(B1–B3, C1–C3, D1–D3)**. Abbreviations: GFP, green fluorescent protein; hNu, human nuclear antigen.

ually selected and cultured individually in 96-well plates. Five clones were isolated from the tau-GFP LYON-ES parental cell line. The average cloning efficiency was 0.65%, similar to that obtained with the eGFP-expressing LYON-ES cells (data not shown) and with human ES cells [34, 35]. All clones expressed tau-GFP in living cells, as was the case for eGFP-expressing clones (supplemental online Fig. 4A; unpublished results). The fluorescence was retained after an extended period of culture (more than 12 passages). Although differences in tau-GFP expression levels were noted between different clones, transgene expression within individual clones was uniform. When tau-GFP-expressing clones were induced to differentiate in nonadhesive or subconfluent culture conditions, all the resulting cells expressed tau-GFP (supplemental online Fig. 4B, 4C).

Tau-GFP LYON-ES clones maintained the characteristics of the parental (tau-GFP) LYON-ES cell line. The cell morphology was typical of LYON-ES cells, and they exhibited a high level of alkaline phosphatase activity, as well as continuing to express the markers of pluripotency Oct4, Nanog, and Sox2 and the cell

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surface markers SSEA4, TRA-1–60, TRA-1–81, and CD90 (supplemental online Fig. 4A, 4D).

Pluripotency of the tau-GFP clones was assessed *in vivo* via teratoma formation. We injected three tau-GFP-expressing clones, and three GFP-expressing clones as controls, in the testes of SCID mice. Teratomas with pronounced differentiation into multiple somatic tissues were found in the injected testes analyzed 10 weeks after grafting. They retained tau-GFP expression, as revealed by fluorescent microscopy (data not shown). Similar results were obtained with the eGFP LYON-ES clones (unpublished results). These data indicate that tau-GFP LYON-ES cells are pluripotent *in vitro* and *in vivo* and are clonogenic and that transgene expression does not alter the properties of LYON-ES cells.

Tau-GFP Expression Is Retained Throughout Neural Differentiation *In Vitro*, and *In Vivo* After Transplantation in the Rat Brain

One of the major advantage of the tau-GFP label is that it makes it possible to follow the fate of neural cell precursors and their

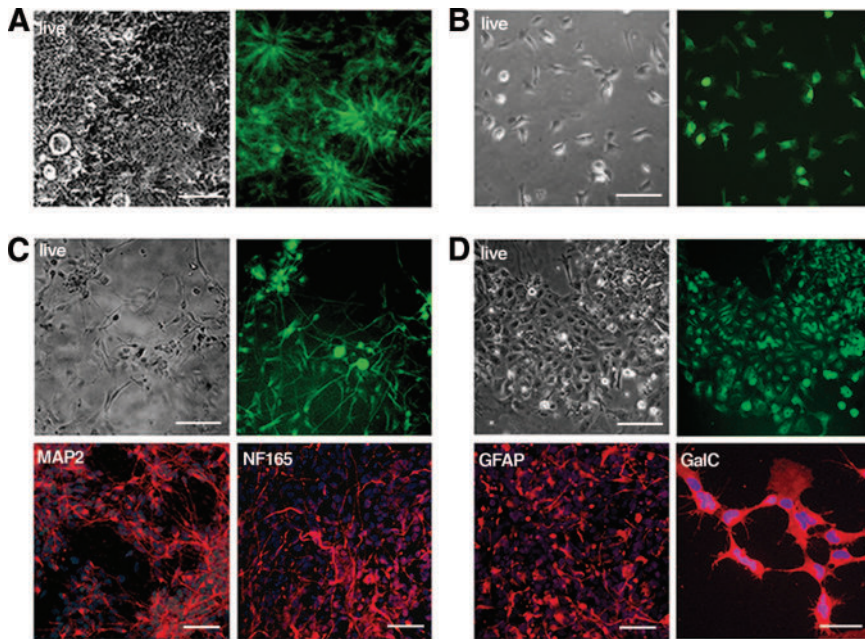


Figure 6. Tau-Green fluorescent protein (GFP) LYON-ES-derived neural precursors retain tau-GFP expression and are multipotent. Shown are phase-contrast and corresponding live fluorescence images of rosettes (A) and neural precursors (B) at passage 6, derived from tau-GFP LYON-ES cells. (C): Differentiation of tau-GFP neural precursors into MAP2- and NF165-positive neurons. (D): Serum-induced differentiation of tau-GFP neural precursors into GFAP- or GalC-expressing cells. Scale bars = 100 μ m (A, B) and 50 μ m (C, D). Abbreviations: GalC, galactocerebroside; GFAP, glial fibrillary acidic protein; MAP2, microtubule-associated protein-2; NF165, neurofilament 165.

derivatives in exquisite detail *in vitro* and *in vivo* [25]. Tau-GFP expression also provides a detailed labeling of dendritic and axonal morphologies, revealing important details concerning the connections formed by neurons derived from tau-GFP-expressing ESC after grafting in the brain.

To determine whether tau-GFP expression is retained throughout neural differentiation *in vitro*, we cultured tau-GFP LYON-ES cells in subconfluent conditions. This resulted in extensive spontaneous differentiation, notably into neuroepithelial rosettes labeled with tau-GFP (Fig. 6A). These rosettes were selected manually and replated in basal medium (Euromed-N plus N2 supplement) in the presence of FGF2 plus EGF. The resulting neural precursors were further propagated for analysis. They expressed tau-GFP, as revealed by fluorescence microscopy in living cells (Fig. 6B), and expressed the neural precursor markers *sox2*, *nestin*, *emx2*, *sox1*, *FGF5*, and neurofilament (NF) 68 and the radial glia markers *BLBP* and *Glast* (data not shown). After induction of neuronal differentiation (described in Materials and Methods), cells with tau-GFP-expressing processes appeared in culture and expressed the neuronal markers β III tubulin, microtubule-associated protein-2, and NF165 (Fig. 6C; data not shown). Following exposure to serum, neural precursors retained the tau-GFP fluorescence and differentiated into GFAP-positive astrocytes and galactocerebroside-positive oligodendrocytes (Fig. 6D). These results show that tau-GFP LYON-ES neural precursors have the capacity to differentiate *in vitro* into all three fundamental neural lineages (neurons, astrocytes, and oligodendrocytes) while retaining tau-GFP expression.

Finally, we investigated the behavior of tau-GFP LYON-ES-derived precursors *in vivo*, upon transplantation into the adult rat brain. Tau-GFP LYON-ES cells were induced to differentiate into neural precursors, which were subsequently injected in the dorsal cerebral cortex of adult rats (supplemental online Materials and Methods). Eighteen or 28 days after transplantation, the brains were fixed, and sections were analyzed histologically. We observed excellent graft acceptance and sustained survival with cyclosporine A immunosuppression. Tau-GFP was readily detected by GFP immunostaining in animals sacrificed 18 and 28 days after transplantation (Fig. 7A–7G). Grafts consisted of large numbers of tau-GFP-positive cells at the injection site, with some cells migrating laterally, away from

the core of the injection site (Fig. 7A–7C). Some neurons grow extended axonal projections over several millimeters, crossing the interhemispheric border as seen in Figure 7C. Immunostaining with the anti-hNA antibody showed that all the monkey cells maintained tau-GFP expression after integration in the brain (Fig. 7E). Only a few tau-GFP cells expressed the proliferative marker *Ki67* (Fig. 7F), indicating that most neural precursors withdraw from the cell cycle *in vivo*. Double immunostaining for GFP and GFAP or β III tubulin showed that tau-GFP neural precursors differentiate along the neuronal and the astroglial pathways after being transplanted in the rat brain (Fig. 7G, 7H). These data indicate that neural precursors derived from tau-GFP LYON-ES cells can survive, differentiate, and retain tau-GFP expression in the adult brain environment several weeks after transplantation.

DISCUSSION

In this report, we describe the derivation of a new rhesus ES cell line stably expressing tau-GFP. First, we generated a new line of ESC in the rhesus monkey (LYON-ES1) and demonstrated that they express markers and have cell cycle characteristics typical of primate ESC [2–4, 26]. LYON-ES1 cells are pluripotent, giving rise to derivatives of the three germ layers after culture in subconfluent conditions or in suspension *in vitro* and *in vivo* through teratoma formation. LYON-ES1 cells have been maintained in culture for more than 60 passages, retaining all their undifferentiated characteristics and a normal karyotype, and show high telomerase activity, consistent with their extended lifespan property. Taken together, these observations indicate that the LYON-ES1 cell line meets all the standard criteria for a pluripotent monkey ES cell line.

Using lentiviral vectors, we then generated LYON-ES cells that ubiquitously and stably express tau-GFP. Monkey ES cells have been labeled with GFP using lipofection or electroporation [36, 37]. Transduction of monkey ES cells with a lentiviral vector encoding GFP has been reported only in cynomolgus monkey [38]. However, GFP expression was not ubiquitous, and the undifferentiated properties of the infected cells were not described. Here, we show that despite lentiviral infection and

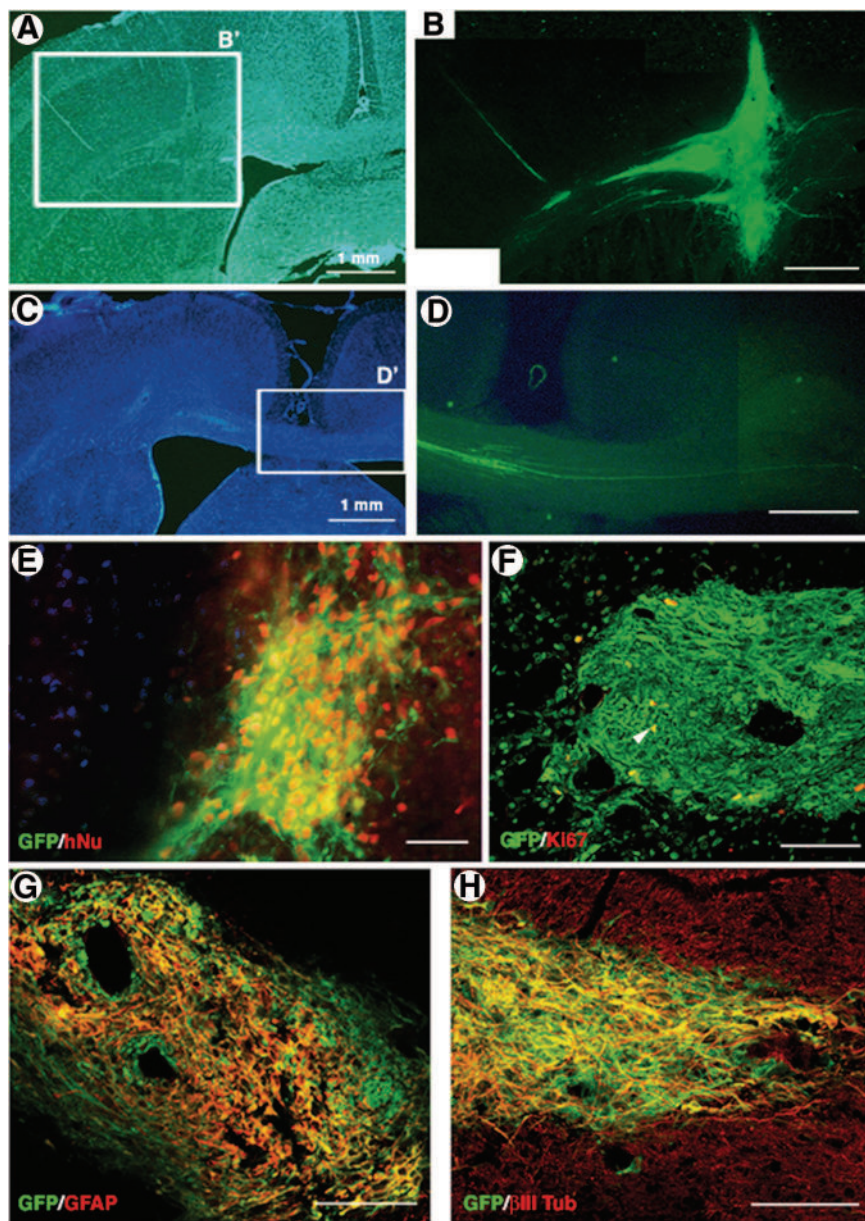


Figure 7. Integration of tau-GFP LYON-ES-derived neural precursors 28 days after transplantation in the adult rat brain. (A, C): Low-power microphotographs of Hoechst stained brain sections. (B): High magnification of field B', showing the site of the grafted cells. (D): Higher magnification of the field D', showing tau-GFP-expressing axons crossing the interhemispheric border (C). (E): Colocalization of tau-GFP and hNu. (F): Few tau-GFP cells expressed the proliferative marker Ki67 (yellow; indicated by arrowhead). (G, H): Coexpression (yellow) of tau-GFP and neuronal marker microtubule-associated protein-2 (G) and astroglial marker GFAP (H). Scale bars = 500 μ m (A–D) and 50 μ m (E–H). Abbreviations: β III Tub, β III tubulin; GFAP, glial fibrillary acidic protein; GFP, green fluorescent protein; hNu, human nuclear antigen.

cell sorting, tau-GFP-expressing cells retained the undifferentiated characteristics of the parental wild-type LYON-ES cell line and the ability to differentiate into the derivatives of the three germ layers *in vitro* and *in vivo*, while retaining transgene expression. Thus, genetic modification of LYON-ES cells is compatible with maintenance of their undifferentiated and pluripotent properties.

We demonstrated that tau-GFP labeling can readily be detected *in vitro* in living LYON-ES cells and their differentiated derivatives using fluorescent imaging and is stable after long-term culture. *In vivo*, the fate of the labeled cells can be mapped during their differentiation in teratomas and during their integration in the adult rat brain, and most importantly, the cells remained identifiable several weeks after injection without using immunohistochemical detection. Tau is a microtubule-binding protein principally expressed in neurons [39], and it is conceivable that ectopic expression of tau-GFP might compromise cell function by interfering with microtubule assembly and could potentially predispose grafted animals to neural pathologies. Indeed, disruption of normal tau function is associated with

neurodegenerative disorders, such as Alzheimer's disease [40]. However, ESC and transgenic lines carrying the tau-GFP transgene have been produced in the mouse, with no report of altered behavior [24, 25]. Consistent with these observations in the mouse, our data show that LYON-ES cells stably expressing tau-GFP have the ability to differentiate *in vitro* into multipotent neural precursors, survive, and colonize the brain after transplantation *in vivo*. This suggests that tau-GFP labeling did not have any obvious consequences on the potentialities of the labeled cells. As the gene is introduced as a random integration event, it is possible that this insertion event modifies an unknown genomic locus and could induce an alteration in cell behavior or characteristics. In our study, we have shown that tau-GFP LYON-ES cells proliferated in a manner similar to their wild-type counterparts and retained a normal karyotype after transduction. More importantly, all tau-GFP generated clones stably expressed tau-GFP and retained tau-GFP expression after *in vitro* and *in vivo* differentiation. These clones presented characteristics similar to those of the parental tau-GFP ES cells, suggesting that the site of integration of the transgene

did not produce a silencing of tau-GFP expression and did not have any deleterious effects on the phenotype or the potentialities of the cells. The efficiency of cloning was comparable to the results obtained with eGFP-expressing LYON-ES cells and to previous results with nontransduced human ES cells [34]. This suggests that transduction of tau-GFP transgene does not alter the potential of monkey ES cells to be cloned. It cannot be ruled out that more subtle cell-type-specific phenotypes could be identified if more detailed analysis were to be undertaken. Thus, experiments using tau-GFP LYON-ES clones should be designed for studying possible effects of the transgene after the induction of differentiation in vitro and in vivo.

CONCLUSION

The tau-GFP LYON-ES cell line is the first tau-GFP-expressing ES cell line derived in primates, including human. Although further experiments using tau-GFP LYON-ES cells should be designed for establishing their functional integration in vivo, we anticipate that the tau-GFP LYON-ES cells will prove a powerful tool for a wide variety of applications, including developmental studies aimed at tracing the fate and confirming the

pluripotency of monkey ESC in chimeras, as well as the development of neural transplantation technologies in monkey.

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DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicate no potential conflicts of interest.

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Derivation and Cloning of a Novel Rhesus Embryonic Stem Cell Line Stably Expressing Tau-Green Fluorescent Protein

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