Supporting Materials and Methods

Ovarian stimulation, recovery of rhesus macaque oocytes, fertilization by intracytoplasmic sperm injection and embryo culture

Procedures for controlled ovarian stimulation and oocyte recovery have been described previously [1, 2]. Briefly, one to four days following the onset of menses, adult rhesus macaque females were subjected to a follicular stimulation protocol, using twice-daily intramuscular injections of recombinant human FSH (30IU ; i.m. GonalF, Serono ; http://www.serono.fr) for 7-9 days. On the last days of hormonal stimulation, ovarian morphology was recorded by ultrasonography (Kontron medical; http://www.kontronmedical.com). Monkeys responding to follicular stimulation (follicules >3mm in diameter) received an injection of recombinant hCG (rhCG; 1000 IU i.m., Organon ; http://www.organon.com) to induce oocyte maturation. Cumulus-oocyte complexes (COC) were collected from anesthetized animals by follicular aspiration after laparotomy (27-32 hours post-hCG) and placed in Hepes-buffered TALP medium (modified Tyrode solution with albumin, lactate, and pyruvate) [3] containing 0.3% bovine serum albumin (BSA) (TH3 medium) at 37°C. Oocytes were freed from COC by mechanical trituration after brief exposure (30 sec) to hyaluronidase (0.5 mg/ml). The collected oocytes were assessed for maturity and placed in CMRL-1066 medium (Invitrogen) containing 10%FBS (Hyclone; Perbio), or in chemically defined protein-free HECM-9 medium [4], at 37°C in 5% CO_2, covered with paraffin oil (Ovoil, VitroLife ; http://www.vitrolife.com). Following this incubation, only matured, metaphase II (MII)-arrested oocytes were used for fertilization by intracytoplasmic sperm injection (ICSI) [5]. Spermatozoa from rhesus macaques were obtained by penile electroejaculation [6], washed and resuspended in TH3 at a final concentration of $5X10^6$/ml. An oil-covered micromanipulation chamber with a 20µl drop of TH3 with the oocytes and a 5µl drop of 10% polyvinylpyrrolidone (Sigma) with spermatozoa was prepared. The micromanipulation chamber was mounted on an inverted microscope (DMIL, Leica, Heerbrugg, Switzerland, http://www.leica.com). An individual sperm was immobilized, aspirated into an ICSI pipette (Humagen, http://www.humagenivf.com) and injected into the cytoplasm of a MII-arrested oocyte, away from the polar body. After ICSI, injected oocytes were placed in 30µl drops of embryo culture medium covered with paraffin oil, in 35mm dishes (Falcon) and cultured at 37°C in 5% CO_2, 5% O_2 and 90% N2 in a modular incubator chamber (ICN Biomedicals,

http://www.icnbiomed.com). Initially, embryos were cultured in a complex medium, based on CMRL-1066 medium supplemented with 10%FBS, on a monolayer of buffalo rat liver (BRL) cells (25000cells/well), at 37°C, in 5%CO2. Two other culture conditions were also used: HECM-9 medium or KSOM/AA, a potassium simplex optimized medium supplemented with amino-acids [7], at 37◦C in 5% CO2, 5% O2 and 90% N2. In the case of HECM-9 and KSOM /AA media, embryos were transferred at the 8-cell stage to fresh drops of embryo culture medium supplemented with 5% FBS. Embryos were cultured for a maximum of 9 days with medium change every other day. Unless otherwise indicated, all reagents were from Sigma. All experimental procedures were approved by the Local Ethic Committee (CREEA) and complied with the European law on the care and use of laboratory animals.

Mycoplasma testing, freezing, and storage

ICM derived cell lines were tested for mycoplasma contamination using a PCR based Mycoplasma detection kit (VenorGeM®-QP ; Minerva-Biolabs ; http://www.minerva-biolabs.com) and frozen in freezing medium containing 5%DMSO (Hybrimax, Sigma), 40%FBS, and 10%KOSR, using a control rate freezer (CRYO1°C freezing container; Nalgene), and stored in liquid nitrogen.

Flow cytometric analysis of cell cycle distribution.

Single cell suspensions of LYON-ES cells were obtained by treatment with 1 mg/ml collagenase IV (37°C for 20-30 min) followed by treatment with 0.1% trypsine (37°C for 3 min). For DNA content analysis, cells were fixed in 70% ethanol, rehydrated in PBS, treated for 30 min with RNase A (1 mg/ml) and for 5 min with propidium iodide (1µg/ml). Fluorescence intensity was determined by flow cytometry on a Becton Dickinson FACscan. Data acquisition was performed with the CellQuest (Becton Dickinson) software, and the percentages of G1-, S-, and G2-phase cells were calculated with the MODFIT-LT software program (Verity Software House Inc ; http://www.vsh.com).

Cell-cycle duration:

BrdU cumulative labeling[8] allows to derive S phase and G1+G2/M durations. Cumulative BrdU labeling was performed on LYON ES cells grown on coverslips in 24-well plates in KOSR and FBS conditions. 48 hours after plating, BrdU (20 µg/ml) was added to the medium. Each time point was repeated three times. After appropriate survival periods, coverslips were fixed with 2% paraformaldehyde (PFA) in PBS at +4°C. The labeling index

(LI) values were determined as the proportion of BrdU positive cells (i.e. cells that were in S phase during the BrdU exposure) with respect to the undifferentiated pool of LYON ES cells (i.e. the Oct-4 positive cell population).

To detect both Oct-4 expression and BrdU incorporation, cells were first treated to reveal Oct-4 expression as described above. DNA was then denatured by incubation in 2N HCl, followed by wash in borate buffer, pH 8.5. Non-specific binding was blocked with 10% normal goat serum. BrdU incorporation was revealed by incubation with Alexa 488-conjugated anti-BrdU antibody (1:50 in DAKO-diluent) from Molecular Probes, for 2 hours at room temperature. Nuclei were counterstained with Hoechst 33258 (1 ng/ml).

Coverslips were examined using an oil objective microscope under UV light (as described above). Coverslips were scanned at regular spacing. From 500 to 1000 cells were observed per coverslip. A minimum of two coverslips was observed for each condition.

Cell transplantation, immunosuppression, and brain histology

For inducing neural differentiation, tau-GFP LYON-ES colonies were seeded on mitomycin C-treated MS5 stromal cells as described [9]. Rosettes were treated with Sonic Hedgehog (200ng/ml), FGF8 (100ng/ml) and FGF20 (2ng/ml), starting from day 6 of differentiation. After 13 days, rosettes were trypsinized and $0{,}5x10^5$ cells/cm^2 were re-plated into matrigel coated dishes (260µg/ml) in selective medium (neurobasal medium supplemented with glutamine, fibronectin, N2, B27, FGF2 (20ng/ml), and cultured for 3 days before grafting. Four deeply anaesthetised (4% Halothane) adult male Sprague-Dawley rats were transplanted bilaterally with 25 000 neural precursors (1µl) in 2 injection sites in the dorsal cerebral cortex. Rats were immunosupressed 3 days before implantation, and until 4 days after, with daily i.p. injections of 15mg/kg of cyclosporine A (Sandimmune ; Novartis). The rats received 10mg/kg of cyclosporine A daily (i.p.) during the survival period (from 2,5 to 8 weeks). Following a lethal injection of pentobarbitone, the rats were transcardially perfused with 4% paraformaldehyde and their brain collected and postfixed for 24 hours at 4°C, cryoprotected in sucrose (25%), and sectioned at 50µm with a microtome (HM440-E; Microm ; http://www.microm.fr). Slices were permeabilized with 0,1% triton and blocked with 10% NGS in TBS for 30 min at RT. Incubation with primary antibody was for overnight at 4°C, and incubation with fluorescently tagged secondary antibody was for 1hour at room temperature. Primary antibodies are detailed in Supplementary Table 2. Sections were mounted with 0.1% n-propylgallate (P3130; Sigma) in phosphate buffer (1M) and glycerol

(1:1) to prevent fading on fluorescent illumination [8] and analysed on a LEICA TCS SP equipped with an Argon-krypton laser, and the Leica software (TCS NT) (http://www.leica-microsystems.com). All experimental procedures were approved by the Local Ethic Committee (CREEA and complied with the European law on the care and use of laboratory animals).

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