Correction

For the article “Nonhuman primate parthenogenetic stem cells,” by Kent E. Vrana, Jason D. Hipp, Ashley M. Goss, Brian A. McCool, David R. Riddle, Stephen J. Walker, Peter J. Wettstein, Lorenz P. Studer, Viviane Tabar, Kerrianne Cunniff, Karen Chapman, Lucy Vilner, Michael D. West, Kathleen A. Grant, and Jose B. Cibelli, which appeared in Suppl. 1, September 30, 2003, of Proc. Natl. Acad. Sci. USA (100, 11911–11916; first published September 22, 2003; 10.1073/pnas.2034195100), the authors note that the following statement should be added to the legends for Figs. 1A, 2A, D, and H; and 5F: “Reprinted with permission from ref. 5 (Copyright 2002 AAAS, www.sciencemag.org).”


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Nonhuman primate parthenogenetic stem cells


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Parthenogenesis is the biological phenomenon by which embryonic development is initiated without male contribution. Whereas parthenogenesis is a common mode of reproduction in lower organisms, the mammalian partheno- geneic process is viewed by some sectors of our society as ethically problematic. In nonhuman primates, there are currently three methods for deriving pluripotent stem cells: from embryos produced by in vitro fertilization (1–4), parthenogenesis (5), and from adult tissues such as cells derived from the bone marrow (6). We have previously reported the creation of a line of nonhuman primate stem cells from parthenogenetically activated eggs (5). By using this technique, ES cells were derived without the need to create or destroy a viable embryo.

Parthenogenesis, the process by which a single egg can develop without the presence of the male counterpart, is a common form of reproduction in nature. Flies, ants, lizards, snakes, fish, birds, reptiles, amphibians, honeybees, and crayfish routinely reproduce in this manner. Eutherians (placental mammals) are not capable of this form of reproduction. However, chimeras of parthenogenetic cells coupled with biparentally derived embryonic tissues have generated apparently normal offspring, and the parthenogenetic origin of several tissues has been confirmed in such chimeric animals (7). In a reported case of a human parthenogenetic chimera, contribution to several tissues has been demonstrated, including blood where 100% of the leukocytes were found to be of parthenogenetic origin (8).

Eutherian oocytes, on the other hand, can undergo parthenogenesis in vitro with variable success. When mammalian oocytes are activated (emulating the fertilization process) and transferred to a surrogate mother, they are capable of surviving to day 10 of development in the mouse, day 21 for sheep, day 29 in pigs, and day 11.5 in rabbit (9–12). The reason for this halted development is believed to be due to genetic imprinting. It has been shown that maternal and paternal genomes are epigenetically different, and that both sets are required for successful development (13–15). In parthenogenetic (activated) eggs, all of the genetic material is of maternal origin, and hence lacking paternal imprinting. It is believed that parthenotes are not capable of developing to term because they fail to develop a trophectoderm and primitive endoderm–extraembryonic tissues (9). They resemble ovarian teratomas and consist of only embryonic tissue. Androgenotes (created by the fusion of two sperm nuclei or diplodization of one sperm in the absence of female counterpart) are of purely maternal origin and develop into a structure consisting of a trophoblast and yolk sac (16). These resemble hydatidiform moles (solely trophoblastic tissue), which are formed when a sperm fertilizes an enucleated egg (17).

In the present report, we describe the parthenogenetic activation of cynomolgus macaque eggs in vitro and the derivation of a pluripotent cell line (Cyno-1). When cultured under selective conditions, these cells have divided for >2 yr, and, on induced differentiation, cell derivatives from all three germ layers were obtained.

Materials and Methods

Superovulation, Oocyte Retrieval, and Oocyte Maturation/Activation. Monkeys were injected (i.m.) with 1,000 units of pregnant mare serum gonadotropin 5 days before surgery and then injected with 500 units of human chorionic gonadotropin 24 h before surgery. For ovary isolation, monkeys were tranquilized with ketamine (10 mg/kg of body weight), intubated endotracheally, and anesthetized with isoflurane (monitored to effect: no pal-
pehal reflex, no deep pain response). Ovaries were removed by midline laparotomy incision.

Oocytes were manually harvested under a dissecting microscope. Oocyte maturation was performed in CMRL-1066 media (Sigma) with 20% FCS (HyClone), 10 units/ml pregnant mare serum (Sigma), 10 units/ml human chorionic gonadotropin (Sigma), 0.05 mg/ml penicillin, and 0.075 mg/ml streptomycin (Sigma). Eggs were incubated for 36 h at 37°C, in 5% CO2 and 20% O2. Mature metaphase II eggs were subsequently activated by incubation with 10 μM ionomycin for 8 min, followed by culture with 2 mM 6-dimethylaminopurine for 4 h. The inner cell masses (ICM) were isolated by immunosurgery as described (20) and cultured on a feeder layer of mitotically inactive mouse embryonic fibroblasts in Dulbecco’s minimal essential medium (Gibco) with 15% FCS (HyClone).

Cell Culture Conditions. Neural progenitor–ES cells were plated in flasks coated with fibronectin/laminin or fibronectin/BSA/collagen with NPMM (Clonetics, East Rutherford, NJ) and maintained at 37°C in 5% CO2. Media were changed every 3 days. Differentiation was induced by removing basic fibroblast growth factor (bFGF) and epidermal growth factor, with the addition of 200 μM ascorbic acid.

Immunohistochemical Staining. A variety of markers of stem cells and stem cell differentiation were assessed by immunocytochemistry. Antibodies and staining conditions were as follows. For surface markers, cells were incubated with primary antibodies [stage-specific embryonic antigen-4 (1:20), Developmental Hybridoma Bank], tumor rejection antigen 1-81 (1:80), and tumor rejection antigen 1-60 (a gift from P. Andrews, Sheffield, U.K.). For immunocytochemistry of embryonic markers, primary antibodies were diluted in PBS supplemented with 0.5% BSA. After washing with PBS-BSA, cells were fixed with 2% formaldehyde for 30 min and washed three times in PBS-BSA, followed by incubation with 10% normal goat serum in PBS at room temperature. Subsequently, primary antibody was added for 30 min at room temperature; cells were then washed with PBS-BSA three times, followed by incubation with secondary antibody for 30 min. Washed, stained with 4',6-diamidino-2-phenylindole (DAPI), and mounted.

Immunocytochemistry of differentiated cells, cells were fixed in 4% paraformaldehyde at room temperature for 20 min, followed by permeabilization for 2 min in 100% ethanol. After fixation, cells were washed with PBS, blocked with 10% normal goat serum in PBS at room temperature for 2 h, followed by incubation at room temperature for 2 h with nestin antibody (1:200, Chemicon), TH polyclonal 1:200 (Pel-Freez Biologicals) or TH monoclonal 1:1000 (Sigma) β-tubulin type III (TuJ1) monoclonal (1:500, Babco, Richmond, CA), in PBS. After washing, cells were incubated with a rabbit secondary antibody in PBS-BSA at room temperature for 30 min. Cells were then washed in PBS and mounted.

Alkaline Phosphatase. Alkaline phosphatase was determined as described (5). Briefly, culture medium was replaced from the plates, and cells were fixed with 4% paraformaldehyde for 20 min. Cells were washed three times in Tris-maleate buffer ([3.6 g of Trizma base (Sigma), in 1 liter of water, pH raised to 9.0 with 1 M maleic acid]) for 10 min each wash. The last wash was removed, and the staining solution ([Tris-maleate buffer: 200 μl of a 5 mM MgCl2 naphthol AS-MX phosphate (Sigma), 0.4 mg/ml; Fast red (Sigma), 1 mg/ml]) was added to the cells for 15 to 20 min. Once red colonies were detected, the reaction was stopped by adding PBS and bringing the pH to 7.4.

Antigen Profiling. Peripheral blood lymphocytes (PBLs) were isolated from whole blood by flotation on Ficoll-Hypaque. Cells were harvested from the medium:Ficoll-Hypaque interface, and the remaining red cells were hypotonically lysed. After additional washes, cells were labeled with FITC-conjugated anti-HLA-A,-B,-C (clone G46 52.6, Pharmingen) and phycoerythrin (PE)-conjugated anti-HLA-DR (clone G46.6, Pharmingen). Iso-type-matched control antibodies were included as negative controls (shaded curves in Fig. 7). Cyto-1-derived neural cells were cultured, harvested, and stained with FITC-labeled anti-HLA-A,-B,-C and PE-labeled anti-HLA-DR as above and compared with cells stained with isotype-matched control antibodies (shaded curves in Fig. 7). Treatment of Cyto-1-derived neural cells with IFN-γ involved overnight incubation with human IFN-γ (40 ng/ml). Stained cells were analyzed with a FACSScan flow cytometer and CELLQUEST software (Becton Dickinson).

Telomerase Activity Measurement. Telomerase activity was measured by using the TRANSeze kit (Intergen, Purchase, NY) as recommended by the manufacturer. Control template, buffer, and control extract were supplied by the TRANSeze kit. Extracts from the mouse feeder cells, the Cyno-1 cells (maintained on mouse feeder layer), and the differentiated Cyno-1 cells (grown for 14 days without mouse feeder layer) were normalized to the protein concentration. Heat inactivated extracts were boiled for 3 min before the assay.

Electrophysiology. The whole cell patch clamp technique was performed on differentiated stem cells that were continuously perfused with a Hepes-buffered saline (HBS) solution (containing, in mM: 150 NaCl, 10 Hepes, 2.5 KCl, 2.5 CaCl2, 1.0 MgCl2, 10 D-glucose, pH 7.4, with NaOH, osmolality 320 mmol/kg adjusted with sucrose). Tetrodotoxin (Calbiochem) was diluted from concentrated stocks into HBS and applied within 100 μM of the cell by using a linear array of fused-silica tubes (150 mm I.D., Hewlett-Packard) mounted on a manipulator. A Cs+ -based internal solution (in mM: 130 CsCl, 10 Hepes, 10 EGTA, 1 CaCl2, 4 Mg-ATP, pH 7.2 with CsOH, osmolality 305 mmol/kg adjusted with sucrose) was used in the patch electrode.

Recordings were performed at room temperature according to published procedures by using an Axopatch 1D amplifier (Axon Instruments, Foster City, CA) in voltage-clamp mode as described (18, 19). Whole-cell capacitance and series resistance were determined by fits of the capacitive transients during square-wave voltage steps by using standard software procedures contained within PCLAMP 7.0 software (Axon Instruments) and monitored throughout the recordings. Resting membrane potentials were ~70 mV. Voltage-gated currents were elicited by square-wave membrane depolarizations to 0 mV.

Results

Creation and Characterization of Monkey Parthenogenetic Stem Cells. Stem cells were created via parthenogenetic activation of eggs as described (5). Briefly, 77 eggs were isolated from the ovaries of three different cynomolgus monkeys (Macaca fascicularis, ~18 yr of age) after hormone-induced superovulation. The oocytes were then maintained in maturation medium for 36 h. Twenty-eight eggs reached metaphase II stage and were subsequently activated by incubation with 10 μM ionomycin for 8 min, followed by culture with 2 mM 6-dimethylaminopurine for 4 h. Four embryos developed to the blastocyst stage after 8 days in culture (14%) (Fig. 1A). Immunosurgically isolated ICMs (20) were cultured on a feeder layer of mitotically inactive mouse embryonic fibroblasts in Dulbecco’s minimal essential medium with 15% FCS (HyClone). Three ICM showed outgrowth within 3 days, and control extract were supplied by the TRAPeze kit. Extracts from the mouse feeder cells, the Cyno-1 cells (maintained on mouse feeder layer), and the differentiated Cyno-1 cells (grown for 14 days without mouse feeder layer) were normalized to the protein concentration. Heat inactivated extracts were boiled for 3 min before the assay.

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binding transcription factor 4 mRNA (Fig. 1 C–G) and negative for stage-specific embryonic antigen-1 and -3 (data not shown). These cells have been propagated for >2 yr maintaining their undifferentiated state. Karyotype analysis revealed 40 + 2 chromosomes, in accordance with the species of origin, M. fascicularis (data not shown).

Parthenogenic Stem Cell Differentiation. In vitro differentiation was induced by isolating the cells from the mouse feeder layer and culturing them in the presence of Dulbecco’s minimal essential medium with 15% FCS; in some instances, 1,000 units of leukemia inhibitory factor was added to the media. A large variety of specialized cell types could be generated in vitro, such as spontaneously beating cardiomyocyte-like cells and ciliated epithelium, smooth muscle cells and cytokeratin-positive cells, as well as neuronal cells (data not shown).

To assess the differentiation capacity of Cyno-1 cells, we injected them into the peritoneal cavity of immunocompromised severe combined immunodeficient mice. Eight to 15 weeks after injection, teratomas were isolated and histologically analyzed. Microscopic observations revealed the presence of mature tissues and low frequency of mitotic figures, indicating their benign nature. Furthermore, derivatives of all three germ layers were observed, including cartilage, neurons, skin, and hair follicles (ectoderm), intestinal epithelia (endoderm), and muscle and bone (mesoderm) (Fig. 2).

Telomerase activity is often correlated with replicative immortality and is typically expressed in germ cells, cancer cells, and a variety of stem cells, including ES cells, but absent in most somatic cell types (21–23). Undifferentiated Cyno-1 cells displayed high levels of telomerase activity as detected by the TRAP assay (TRAPeze kit). However, no telomerase activity could be detected in differentiated progeny of Cyno-1 cells (Fig. 3A). These data indicate a physiologically normal control of telomerase activity in Cyno-1 cells.

Genomic imprinting is initiated at gametogenesis and further modified during development. The small nuclear ribonucleoprotein polypeptide N (Snrpn) gene is an example of an imprinted gene that is expressed solely from the paternal allele (24, 25). It is monoallelically expressed from the onset of its expression at the four-cell stage (25). RT-PCR analysis confirmed the absence of Snrpn expression in Cyno-1 cells, whereas it is readily detected in inbred fibroblast cell cultures from the same species (Fig. 3B). Whereas biparental ES cells from M. fascicularis were unavailable to us for analysis, the Snrpn gene was readily detectable in biparental mouse ES cells under these same conditions (data not shown). These results indicate that the imprinting profile of at least one gene is consistent with the parthenogenic origin of the Cyno-1 cells.

Nestin-Positive Neural Precursors. Cyno-1 ES cells were plated in flasks coated with fibronectin/laminin and NPMM (supplemented with bFGF, epidermal growth factor, and neural survival medium with 15% FCS; in some instances, 1,000 units of leukemia inhibitory factor was added to the media. A large variety of specialized cell types could be generated in vitro, such as spontaneously beating cardiomyocyte-like cells and ciliated epithelium, smooth muscle cells and cytokeratin-positive cells, as well as neuronal cells (data not shown).

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factor-1). These cells begin to differentiate into a neuronal-like morphology. Within 10 days, they do not differentiate further and proliferate at a rate of 5- to 8-fold increase over a 10-day period (Figs. 4 and 5A and B). Their stage of development seems to be similar to those described by Ying et al. (26). Specifically, these cells express a high amount of nestin, which is an intermediate filament found in the developing CNS, mesenchymal tissue of the developing pancreas, and immature skeletal muscle (National Center for Biotechnology Information Locus Link).

With the removal of bFGF and epidermal growth factor and the addition of ascorbic acid, we are able to generate a high percentage of dopaminergic-like neurons (25% of TUJ+), glial, and epithelial cells. Immunocytochemistry stained positive for TUJ1, dopamine transporter (DAT), and microtubule-associated protein-2, and negative for acetylcholine transferase, dopamine-beta-hydroxylase, and NeuN (Fig. 5 C–E and data not shown). Cyno-1-derived neurons exhibited both basal and KCl-evoked synaptic release of dopamine and serotonin (Fig. 5F). Single-cell electrophysiology was also performed on these differentiated neurons. At around day 20, they begin to express voltage-
for the expression of Mafa (MHC of M. fascicularis) have analyzed the Cyno-1-derived neural cells by flow cytometry. Currents were elicited by membrane depolarizations to 0 mV every 15 s from a holding potential of −70 mV. Application of 0.5 μM tetrodotoxin inhibited >90% of these currents. (B) Inhibition was complete within 30 s of tetrodotoxin application and washed completely in <1 min.

Fig. 6. Single-cell electrophysiology. (A) Neurons derived from Cyno-1 express voltage-dependent inward currents that are blocked by tetrodotoxin. Currents were elicited by membrane depolarizations to 0 mV every 15 s from a holding potential of −70 mV. Application of 0.5 μM tetrodotoxin inhibited >90% of these currents. (B) Inhibition was complete within 30 s of tetrodotoxin application and washed completely in <1 min.

Fig. 7. Immunological profile of Cyno-1 cells. PBLs and Cyno-1-derived neural cells were analyzed by flow cytometry to quantitate expression of M. fascicularis class I (anti-HLA-A,-B,-C) and class II (anti-HLA-DR) antigens and compared with cells stained with isotype-matched control antibodies (shaded curves). PBLs express both class I and class II (DR) antigens whereas differentiated Cyno-1-derived neurons do not express either class of antigen unless treated with IFN-γ.

Discussion

We have generated a primate parthenogenetic cell line (Cyno-1) with ES cell-like properties that can be propagated in vitro in an undifferentiated state for at least 2 yr. These cells express telom-
erase activity consistent with their extended lifespan property. The in vitro derivation of large numbers of specific cell lineages from Cyno-1 cells, including the generation of unlimited numbers of dopaminergic neurons, is of particular interest. In the present context, we have demonstrated that these cells (i) express TH, (ii) release neurotransmitter (dopamine and serotonin), and (iii) are electrophysiologically active consistent with their extended lifespan property. For these reasons, we believe that neurons, differentiated from parthenogenetic stem cells, may provide an important source of therapeutic treatments. Clinical transplantation of specific fetal neurons has shown promise in the treatment of Parkinson's disease (29) and Huntington's disease (30), but obtaining such cells from animals or human fetal brain remains problematic. Neurons derived in vitro from a renewable source, such as CNS precursors (31), ES cells (32, 33), or stem cells of parthenogenetic origin, could alleviate some of the ethical and technical concerns of human cell therapy.

Although the Cyno-1 parthenogenetic stem cell seems, in all respects, to be similar to traditional ES cells, it is reasonable to question their viability and utility. They are, after all, exclusively derived from maternal DNA. When trying to understand how these parthenogenetic stem cells are capable of developing into functional tissue, it is important to remember the following characteristics of genetic imprinting. First, ES cells are isolated from the blastocyst stage, this stage exhibits low DNA methylation levels, and the effects of imprinting could be minimized (34). Second, imprinting, in some cases, has been shown to not be completely silent: there are reports of mRNA expressed from imprinted genes that should not have been transcribed (24). Third, Surani and Barton (9) suggest that parthenogenetic embryos do not develop to term because of a high frequency of errors in X chromosome inactivation that occurs in extraembryonic tissues when both X chromosomes are derived only from the female. One might conclude that the effects of imprinting have a significant effect on extraembryonic tissue, and not the ICM from which our stem cells are derived.

One could speculate that these parthenogenetically derived stem cells are capable of differentiating into high a percentage of electrophysiologically active dopaminergic neurons due to the effects of genetic imprinting. It is believed, for example, that 0.1–1% of all mammalian genes are imprinted (35). However, only ~50 imprinted genes have been identified in mice, some of which are conserved in humans (36). One of the most interesting characteristics of imprinting is that it occurs in clusters. The imprinting cluster on mouse chromosome 7 and the corresponding human chromosome 11p15.5 contains 14 imprinted genes (37). Tyrosine hydroxylase (TH), the rate-limiting enzyme of dopamine synthesis (38), is located on chromosome 11p15.5 and resides in the middle of a very well-characterized imprinted region on mouse chromosome 11p. There are in this cluster some of the best characterized imprinted genes, such as H19, insulin growth factor 2, and insulin growth factor antisense (37). Whereas tyrosine hydroxylase is biallelically expressed in mice, its imprinting status in humans has yet to be determined (37). Could this high expression level of tyrosine hydroxylase (in maternally derived parthenote cells) suggest it as an imprinted gene in nonhuman primates? Moreover, are these epigenetic modifications associated with imprinting permitting the robust expression of TH in Cyno-1-derived neural cells?

Further data analysis of functional genomic studies at the maternal precursor and differentiated stage show normal gene expression of housekeeping genes such as the mRNAs of the 60S ribosomal subunit, the glycolytic pathway, and the tricarboxylic acid (TCA) cycle. Most interesting, when the “Stemness” genes recently described by Ramalho-Santos et al. (39) were converted into their human orthologs, 96 of 216 were expressed in the parthenogenetically derived monkey neural precursor cells (J.D.H., J. C. Mychalecky, and K.E.V., unpublished results).

We report here the isolation and further characterization of nonhuman primate parthenogenetic stem cells. These cells may provide a novel tool for assessing the effects of genomic imprinting on cell differentiation and function during development in primates. Their striking differentiation capabilities (electrophysiologically active, dopamine-secreting neurons) indicate their therapeutic potential and suggest a valid alternative to biparently derived ES cells.