Stem Cell Research (2009) 2, 178–187

available at www.sciencedirect.com

ScienceDirect

www.elsevier.com/locate/scr

REGULAR ARTICLE

Establishment and characterization of baboon embryonic stem cell lines: An Old World Primate model for regeneration and transplantation research

Calvin R. Simerly [a,1], Christopher S. Navara [a,1], Carlos A. Castro [a,1], Janet C. Turpin [a], Carrie J. Redinger [a], Jocelyn D. Mich-Basso [a], Ethan S. Jacoby [a], Kevin J. Grund [a], David A. McFarland [a], Stacie L. Oliver [a], Ahmi Ben-Yehudah [a], Diane L. Carlisle [a], Patricia Frost [b], Cecilia Penedo [c], Laura Hewitson [a], Gerald Schatten [a,*]

[a] *Division of Developmental and Regenerative Medicine, Department of Obstetrics, Gynecology, and Reproductive Sciences; Cell Biology-Physiology; and Bioengineering; Pittsburgh Development Center; Magee-Womens Research Institute, University of Pittsburgh, Pittsburgh, PA 15213, USA*

[b] *Southwest National Primate Research Center (NPRC), Southwest Foundation for Biomedical Research, San Antonio, TX 78227, USA*

[c] *Veterinary Genetics Laboratory, University of California, Davis, One Sheilds Avenue, Davis, CA 95616, USA*

Received 21 October 2008; received in revised form 23 January 2009; accepted 6 February 2009

Abstract Here we have developed protocols using the baboon as a complementary alternative Old World Primate to rhesus and other macaques which have severe limitations in their availability. Baboons are not limited as research resources, they are evolutionarily closer to humans, and the multiple generations of pedigreed colonies which display complex human disease phenotypes all support their further optimization as an invaluable primate model. Since neither baboon-assisted reproductive technologies nor baboon embryonic stem cells (ESCs) have been reported, here we describe the first derivations and characterization of baboon ESC lines from IVF-generated blastocysts. Two ESCs lines (BabESC-4 and BabESC-15) display ESC morphology, express pluripotency markers (Oct-4, hTert, Nanog, Sox-2, Rex-1, TRA1-60, TRA1-81), and maintain stable euploid female karyotypes with parentage confirmed independently. They have been grown continuously for >430 and 290 days, respectively. Teratomas from both lines have all three germ layers. Availabilities of these BabESCs represent another important resource for stem cell biologists.

Abbreviations: ESCs, embryonic stem cells; ICC, immunocytochemistry; ICM, inner cell mass; iPS, induced pluripotency; MEFs, mouse embryonic fibroblasts; nhp, nonhuman primates; OWP, Old World Primate; SCNT, somatic cell nuclear transfer.

* Corresponding author. Fax: +1 412 641 2410.
E-mail address: schattengp@upmc.edu (G. Schatten).

[1] These authors contributed equally to this work.

1873-5061/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.scr.2009.02.004

Introduction

Nonhuman primates and embryonic stem cells derived from nhps (nhpESCs) have contributed significantly to the translation of mouse stem cell findings to humans. For example, the establishment of nhpESCs (Thomson et al., 1995, 1996) provided the experimental foundation and biomedical rationale for the derivations of human ESCs 3 years later (Thomson et al., 1998). Innovative discoveries with nhpESCs continue to provide experimental evidence in support of hESC investigations (Friedrich Ben-Nun and Benvensity, 2006; Shufaro and Reubinoff, 2004; Dvash and Benvenisty, 2004). Parthenogenetic nhpESCs (Cibelli et al., 2002a) preceded the generation of parthenogenetic human ESCs (Lin et al., 2007; Mai et al., 2007; Revazova et al., 2007), which are of great utility notwithstanding the findings that they are unlikely to be exact immune matches to the female donor (Dighe et al., 2008). Therapeutic cloning has now succeeded in rhesus (Byrne et al., 2007; Cram et al., 2007), inspiring encouraging advances in humans (French et al., 2008) beyond previous credible attempts (Cibelli et al., 2002b; Lavoir et al., 2005; Stojkovic et al., 2005). Now, evaluating the clinical utility of stem cells generated by induced pluripotency (iPS) as well as direct differentiation (Zhou et al., 2008) is of keen importance. Nonhuman primate ESCs are accelerating mechanistic discoveries of diseases, e.g., nhpESCs as Alzheimer's disease models (Wianny et al., 2008). Transplantation of differentiated nhpESCs into primate models of Parkinson's disease (Takagi et al., 2005) and other disorders (Hematti et al., 2005) is providing encouraging preclinical results on the feasibility of stem cell therapies in clinical regenerative medicine, as shown in rodent models (Ben-Hur et al., 2004). Macaques, which include both rhesus (Mitalipov et al., 2006; Navara et al., 2007; Thomson et al., 1995) and cynomolgus (Suemori and Nakatsuji, 2006), represent the only family of OWPs from which nhpESCs have been established [reviewed by (Wilmut and Taylor, 2007)], though ESCs from the New World marmoset have also been derived (Thomson et al., 1996; Sasaki et al., 2005).

Notwithstanding the admirable contributions of macaque ESCs to translational and fundamental biology, several crucial questions about hESC biology which could be solved with nonhuman primates have not been answered using the available nhp stem cell lines. These questions include: Are primate ESCs pluripotent when investigated using ideally intraspecific or even interspecific chimera assays? Will they contribute to the germ line? Will pluripotent primate ESCs engraft and differentiate properly when transplanted into fetal or adult primates? Will they be rejected or will they generate teratomas or other tumors? Will primate cells generated by iPS behave similar to nhpESCs derived from embryos generated by IVF or somatic cell nuclear transfer (SCNT)? There are several reasons that these questions have not yet been solved, including severe limits in resource availability, cost, generation time, and lack of pedigreed lineages, as well as possible limitations in the nhpESCs currently available.

Results

Baboon (*Papio anubis*) embryos were produced by ICSI and cultured *in vitro* for 8–12 days postinsemination. Eleven females generated 157 successfully fertilized oocytes

Table 1 Baboon ESC established line from embryos generated by intracytoplasmic sperm injection

Oocyte No. (*n*)	Zygote No.	2 cell No.	3-to-4 cell No.	6-to-8 cell No.	16-to-32 cell No.	Morula No.	Early blastocyst No.	Expanded blastocyst No.	ESC in culture No.			Baboon ESC established line(>3 months) (blst) [zygote]
									Plated (blst) [zygote]	1 week (blst) [zygote]	1 month (blst) [zygote]	
166 (13)	157 (95%)	128 (77%)	124 (75%)	103 (62%)	83 (50%)	65 (39%)	54 (33%)	19 (11%)	16 (84%) [10%]	15 (79%) [10%]	2 (11%) [1%]	2 (11%) [1%]

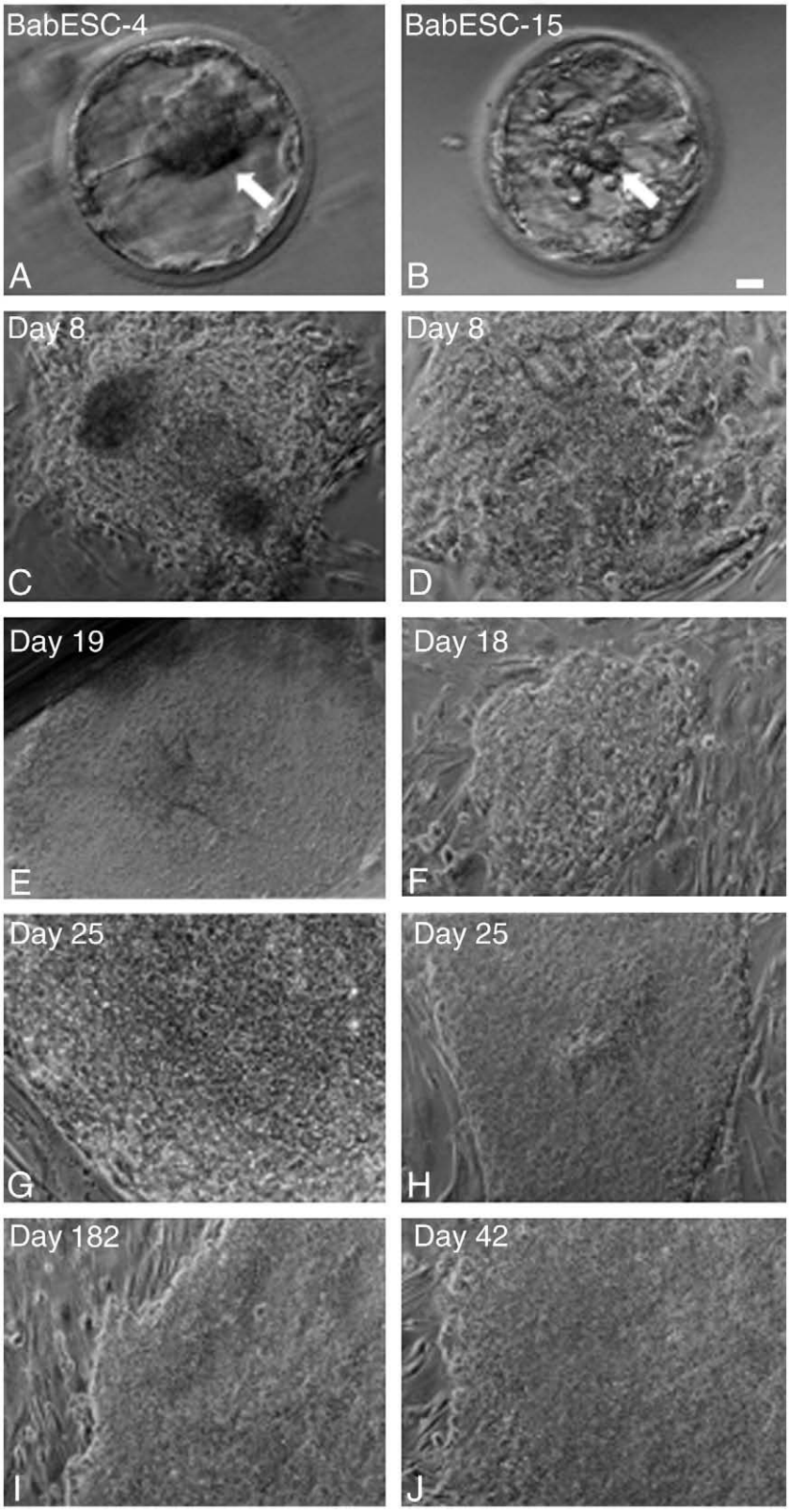

Figure 1 Derivation of baboon embryonic stem cell lines, BabESC-4 and BabESC-15. (A, B) Baboon blastocysts produced from ICSI zygotes cultured *in vitro* for 9 days (arrows: prominent inner cell masses). (C, D) Day 8 post-ICM plating, showing rapid expansion of both nascent cell lines in culture. (E, F) Colony growth observed after the first mechanical passage ~Days 18–19 post-ICM plating. (G, H) Both baboon cell lines have colonies demonstrating definitive morphological characteristics of embryonic stem cells, including large nuclear-to-cytoplasmic ratios, tightly packed cells with prominent nucleoli, and distinct colony borders. (I, J) BabESC colonies at 182 and 42 days postplating, respectively. Left column: BabESC-4. Right column: BabESC-15. Bar: 20 μm; (C–J) ×100 inverted phase contrast optics.

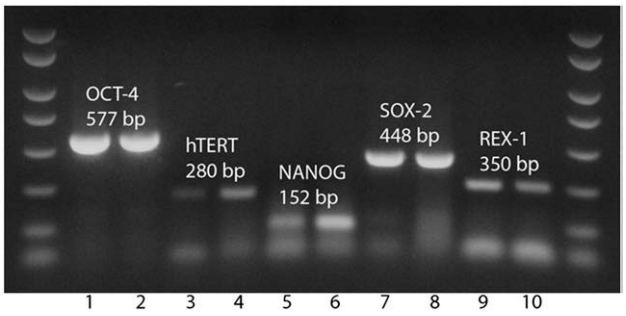

Figure 2 RT-PCR analysis of pluripotency markers in baboon ESC lines. Baboon ES colonies with good morphology were scraped, RNA was isolated, and reverse transcriptase PCR was performed using the primers described under Materials and methods. Results indicated expression of POU5F1 (Oct-4; 577 bp), hTERT (280 bp), Nanog (150 bp), Sox-2 (488 bp), and Rex-1 (350 bp). Negative controls included no template and no RT, both negative (not shown). BabESC-4, Lanes 1, 3, 5, 7, 9; BabESC-15, Lanes 2, 4, 6, 8, 10.

(Table 1), from which 19 expanded blastocysts (11%) were produced. Their inner cell masses (ICMs) were isolated by immunosurgery and plated onto inactivated mouse embryonic fibroblasts (MEFs). Sixteen (84%) ICMs plated within the first 48–72 h postisolation, with 15 (79%) surviving at least 1 week, and 2 lines (11%) expanding in culture by 1 month (Table 1). Both surviving lines were derived from Day 9 blastocysts.

The establishment of BabESC-4 and BabESC-15 cell lines is shown in Fig. 1. The Day 9 expanded blastocysts exhibit prominent ICMs (Figs. 1A and B) that attach onto inactivated MEF feeders within 48 h after immunosurgery. Visible colonies are apparent by Day 8 post-ICM plating (Figs. 1C and D), and expand in culture over 2 weeks. Both ESC colonies demonstrate typical ESC appearances, i.e., tightly packed cells with well-defined borders, large nuclear-to-cytoplasmic ratios, and prominent nucleoli (Figs. 1E–H). BabESC-4 has been continuously cultured for >431 days (Fig. 1I) while the BabESC-15 line has been in culture for >292 days (Fig. 1J). Mechanical passaging of selected colonies deemed of good morphology, performed weekly, maintains and propagates both cell lines. Both BabESC-4 and BabESC-15 have been cryopreserved and thawed, and colonies were successfully reestablished *in vitro* (Supplemental Table 1).

Baboon ESC characterization: *In vitro* assays

The established BabESC-4 and BabESC-15 cell lines demonstrate known "stemness" markers as determined both RT-PCR and immunocytochemistry (ICC) analyses (Figs. 2 and 3, respectively). RT-PCR with specific primers detected transcripts of the correct size for hTERT, Oct-4(POU5F1), Nanog, Rex-1, and Sox-2 in both BabESC-4 and BabESC-15 (Fig. 2), but did not detect transcripts in baboon fibroblast cells lines (Supplemental Fig. S1). Antibodies recognizing Oct-4 and Nanog demonstrate specific nuclear localization in colonies

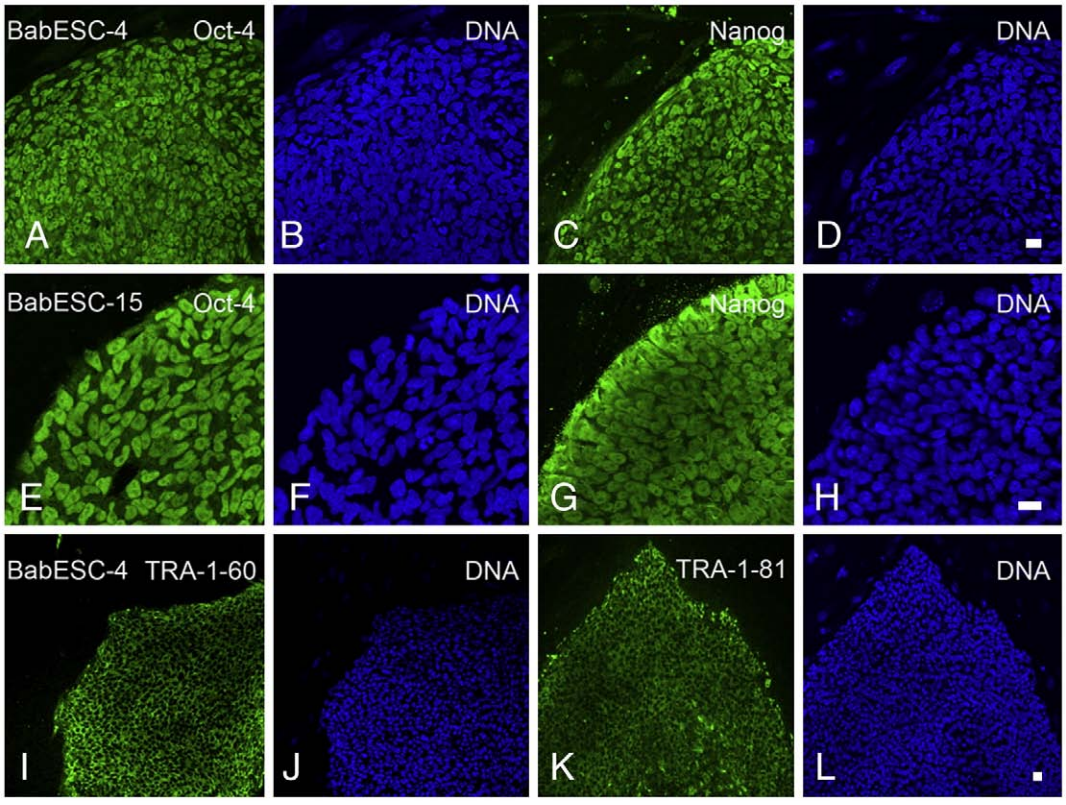

Figure 3 Immunocytochemical analysis of pluripotency markers for BabESC-4 and BabESC-15. (A–H) Baboon embryonic stem cell lines BabESC-4 and BabESC-15 express the nuclear transcription factors Oct-4 (A, BabESC-4; E, BabESC-15) and Nanog (C, BabESC-4; G, BabESC-15) as well as the cell surface markers TRA-1-60 (I, BabESC-4) and TRA-1-81(J, BabESC-4). Hoechst DNA: panels B,D, F,H, J, and L. Bars = 20 μm.

of BabESC-4 (Figs. 3A–D) and BabESC-15 (Figs. 3E–H) following ICC staining analysis. Likewise, the conserved cell surface pluripotent markers Tra-181 and Tra-160 are also identified in BabESC-4 (Figs. 3I–L) and BabESC-15 (Supplemental Fig. S2). Labeling of these two cell surface markers required different fixation protocols than those for human or rhesus ESC lines, perhaps suggesting epitope differences. The primate specific cell surface marker SSEA-4 was weaker on both baboon cell lines than those identified on macaque or human colonies, but still positive (Supplemental Fig. S2). Interestingly, SSSEA-4 staining in early baboon embryos was also weakly detected in the trophectoderm and ICM, perhaps for the reasons identified above (Supplemental Figs. S2D–G). Supplementary Table 1 summarizes the comparison of specific stemness and cell surface marker antibodies among baboon, macaque, and human lines.

Both BabESC-4 and BabESC-15 display normal female diploid cell line karyotypes with 20 autosomes and two female sex chromosomes, even when continuously cultured for >1 year (Figs. 4A and C, BabESC-4; Figs. 4B and D, BabESC-15). The pedigrees of BabESC-4 and BabESC-154 were also independently confirmed with DNA fingerprinting analysis with the presumptive gamete donors (Supplemental Table 2).

Teratoma assays and baboon ESC characterization by *in vitro* differentiation

Fig. 5 demonstrates differentiation into each of the three germ layers for BabESC-4. Figs. 5A and 5B show multiple tissue types from the three germ layers, e.g., gastrointestinal tissue, cartilage, and skin. Ectoderm derivatives, including retinal pigment, were observed (Figs. 5C and 5D). Mesoderm is in Figs. 5E and 5F, e.g., striated muscle (Fig. 5E) and cartilage undergoing ossification (Fig. 5F). Endodermal derivatives like respiratory ciliated epithelium organizing a tracheal–bronchiolar structure (Fig. 5G) and early development of gastrointestinal tissue (Fig. 5H) were observed.

The pluripotency of BabESC-15 has been established by the generation of a teratoma and by spontaneous differentiation of colonies *in vitro* (Supplemental Fig. S3). We identified prominent ectoderm and mesodermal germ lines in a single teratoma following tissue sectioning (Fig. S3A–D). Ectoderm derivatives included early brain development (Fig. S3A), mature brain tissue with pigmented cells (Fig. S3B; pc, arrows), and classical choroid plexus tissue (Fig. S3C). Mesoderm derivatives were identified as cartilage (Fig. 3SD; ca) with adjacent areas of choroids plexus (Fig. S3D; cp) and pigmented cells (Fig. S3D; pc, arrow) observed. Evidence of early gastrointestinal tissue (endoderm) was observed but not yet fully confirmed (our unpublished observation).

Alteration of the feeder cells to STOs on a modified stem cell medium containing no hLIF, Activin A, and reduced bFGF (4 ng/ml) permitted BabESC-15 cells to differentiate within 2 weeks. Some colonies expressed cytokeratin-11, an ectodermal lineage marker of differentiation (Fig. S3F, green), while others appear as differentiated neuronal cells

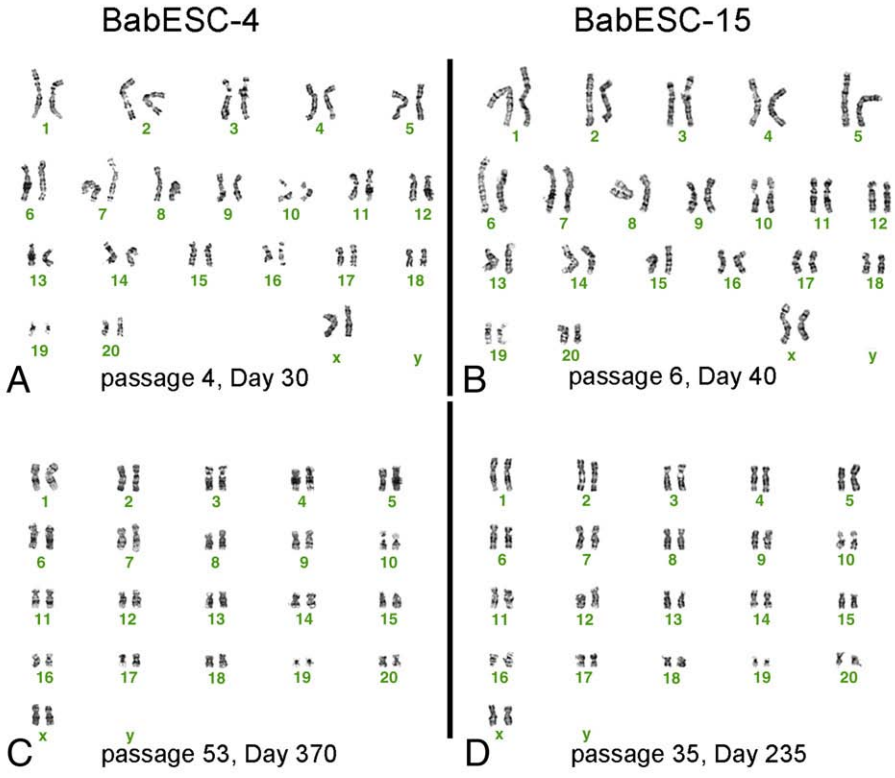

Figure 4 Karyotypes of BabESC-4 and BabESC-15 cell lines by Giemsa chromosomal banding. BabESC-4 (panel A, passage 4, Day 30; panel C, passage 53, Day 370) and BabESC-15 (panel B, passage 6, Day 40; and panel D, p35, Day 235) are both normal female diploid lines, with 20 autosomal pairs and 2 female sex chromosomes.

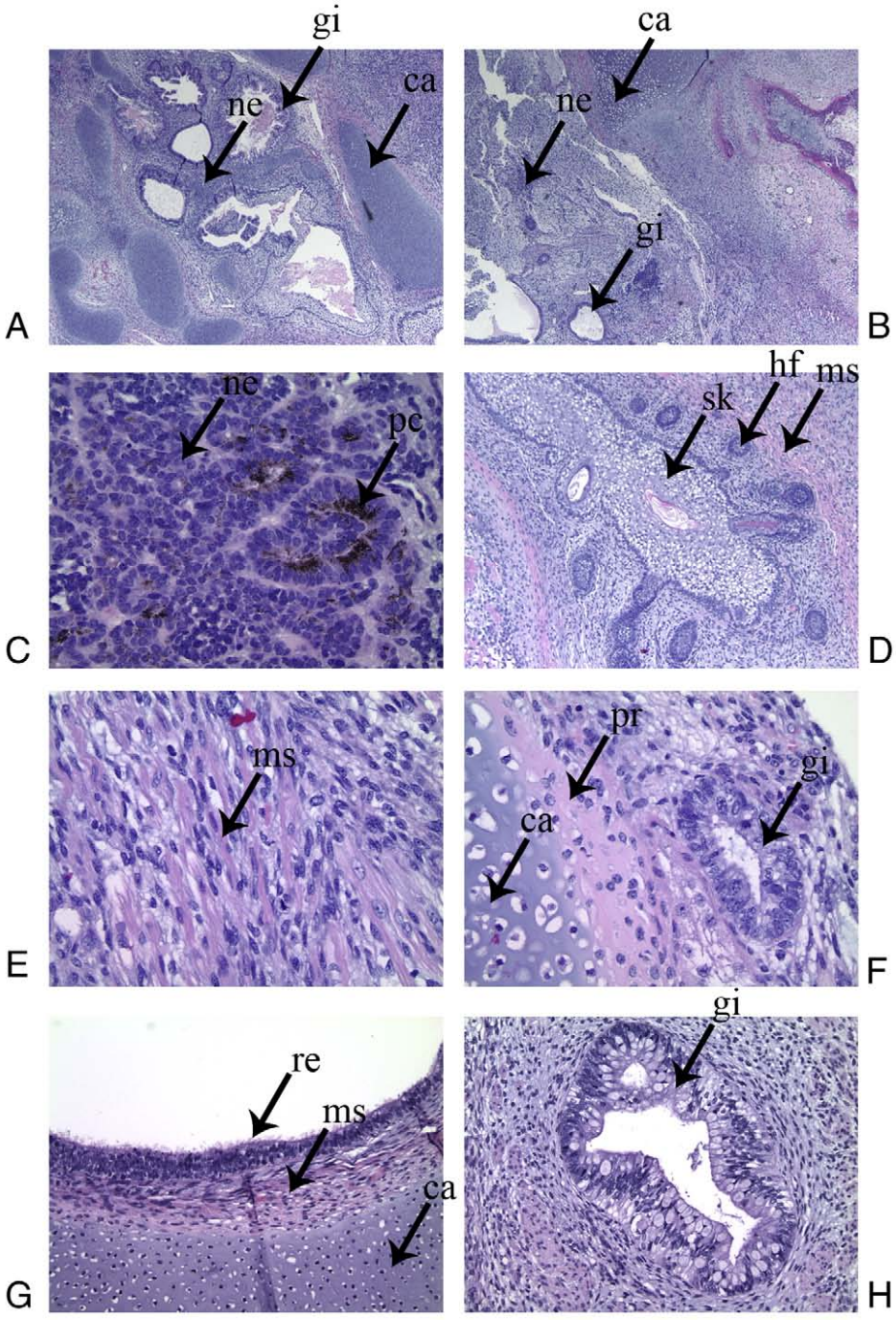

Figure 5 BabESC-4 teratoma differentiation into all three germ layers. (A, B) All three germ layers were present. ne, neuroectoderm; gi, gastrointestinal tissue; ca, cartilage. (C, D) Ectoderm, e.g., retinal pigment (C: pc, arrow), skin (D: sk, arrow), and hair follicle (D: hf, arrow). (D) muscle (ms, arrow). (E, F) Mesoderm, e.g., striated muscle (E: ms, arrow) and cartilage (F; ca, arrow). (F) Pr, periostium; gi, gastrointestinal tissue. (G, H) Endoderm, e.g., respiratory ciliated epithelium (G: re, arrow) and gastrointestinal epithelium (H: gi, arrow). (G) Muscle, ms, arrow; cartilage, cs, arrow. Magnifications: (A, B) ×4; (C) ×40; (D) ×10; (E, F) ×40; (G,H) ×20.

prominently expressing β-III tubulin (Fig. S3G, red; Fig. S3E, DNA, blue; merged image, Fig. S3H).

Discussion

The derivation and characterizations of baboon PSCs here have several biomedical justifications including providing an OWP research resource for translational medicine with characteristics either complementary or superior to macaques. Baboons are the closest human relatives among the OWP; they are extensively studied in transplantation research since their physiology, size, and organ compatibilities more closely model human conditions, and they have been extensively pedigreed for genetic and environmental parameters which generate complex disease phenotypes including diabetes, heart disease, and obesity. Their size and

relative docility support noninvasive MRI imaging studies of postimplantation development as well as tracking the fates of transplanted PSCs. Macaques, and especially certain strains of rhesus macaques (e.g., MaMu), are used extensively for invaluable HIV-AIDS studies (Braun and Johnson, 2006). Finally, baboons represent a preclinical research resource that is readily available and relatively cost-effective to complement the nearly inexhaustible demands on macaques.

Baboon ESCs join the rhesus and cynomolgus ESCs in expressing pluripotency markers identical to hESCs (e.g., Oct-4, Nanog, Rex, hTert) and unlike mouse ESCs (e.g., not SSEA-1; Laslett et al., 2003). They form all three germ layers in teratomas, grow continuous in culture after extensive numbers of passages, display stable diploid karyotypes, and differentiate *in vitro*. Genetic pedigree analysis permits independent confirmations of accuracy for quality control, which is not possible with anonymously donated hESCs. Genetic and immunological matching after transplantation is enabled due to the availability of parents of these lines and also to the ability to generate full-sibling offspring for further investigations.

Pluripotent stem cells from NHPs have been obtained from macaques and marmosets, and now here in baboons. A consideration of NHP diversity is worthwhile as well as evaluations of their potential contributions for regenerative studies. Nonhuman primates include apes (greater apes such as chimpanzees, gorillas, and orangutans; lesser apes such as gibbons), OWPs (e.g., macaques, baboons, vervet, or green monkeys), new world monkeys (NWP; e.g., marmosets, capuchins, spider, squirrel, and owl monkeys), and the prosimians such as lemurs. Notwithstanding their genetic proximity to humans and the sequencing of the chimpanzee genome (Culotta, 2005; Pennisi, 1995; Wildman, 2002), investigations with apes are restricted appropriately, though rationales for establishing ape PSCs include important neuroprogenitors, "-omics" comparisons, and immunological and infectious disease research rationales. On the other end of the spectrum, prosimian stem cells might offer interesting resources for taxonomic and evolutionary biologists. Because the primary justifications for NHP research are fundamental biology and translational medicine, PSCs from OWPs and NWPs can be predicted to continue to generate the greatest dividends based on research investments. Among the NWPs, marmoset stem cell research has led to the first ESC lines (Thomson et al., 1996), as well as interesting transplantation studies (Iwanami et al., 2005; Oka et al., 2004). However, because of genetic commonality among OWPs and humans, as well as the enormous experimental resources established especially for macaques, OWPs are likely to remain as the most prominent NHP models for PSC studies. PSCs have been established from rhesus (Mitalipov et al., 2006; Navara et al., 2007; Thomson et al., 1995) and cynomolgus (Suemori and Nakatsuji, 2006; Vrana et al., 2003), and, in personal communications, pig-tail (Hayes, 2008) macaques. Also, important findings regarding PSCs have been reported for differentiation (Boyd et al., 2008; Elkabetz et al., 2008; Kawasaki et al., 2002a; 2002b; Mizuseki et al., 2003; Nakatsuji et al., 2008; Stadtfeld et al., 2008; Trounson, 2006; Vaca et al., 2006); therapeutic improvements after transplantation (Takagi et al., 2005; Takahashi, 2006); histocompatability assays (Dighe et al., 2008; Rajesh et al., 2007); and epigenetics (Fujimoto et al., 2005, 2006; Mitalipov et al., 2007; Mitalipov, 2006; Rugg-Gunn et al., 2005a, 2005b, 2007; Zhang et al., 2007) (Brons et al., 2007).

Both the baboon ESC lines presented here and the rhesus ESCs differ in certain growth characteristics from hESCs, and slight modifications of culture conditions are employed. From our experience in growing human and nhp lines side-by-side in the laboratory, the nhpESCs lines appear to have stricter requirements on feeder layer density in order to maintain compact colony morphology and well-defined borders (~26 000 cells/cm^2 for nhps versus 40–60 000 cells/cm^2 for humans). Also, nhpESCs require greater adherence to passage time (7–8 days maximum) compared to hESCs and manual passaging methods are required. Notwithstanding these minor differences, they are far more similar to hESCs than mouse ESCs, and therefore represent an important translational resource.

In conclusion, here we describe the derivation and characterizations of embryonic stem cell lines from pedigreed baboon embryos. The babESCs display stringent criteria, including long-term self-renewal and pluripotency as demonstrated in teratoma assays as well as *in vitro* differentiation and by expression of pluripotency molecular markers. Early passages have been banked and independent genetic testing confirms identity. Because baboons are investigated extensively in transplantation research and because, unlike rhesus macaques, they are readily available for responsible investigations, these new baboon embryonic stem cells are proposed to represent another important resource in the armamentarium for stem cell biology.

Materials and methods

Regulations

All experiments involving animals were approved by the Institutional Animal Care and Use Committees (IACUCs) from the Southwest National Primate Research Center of the Southwest Foundation for Biomedical Research, the Magee-Womens Research Institute, and the University of Pittsburgh. Also, this work complies with NIH Guidelines for Research Involving DNA Molecules under the direction of the University of Pittsburgh Institutional Biosafety Committee. All work with lentivirus and lentivirus-transduced cells complied with BLS-2+ laboratory practices.

Baboon-assisted reproductive technology (ART)

Embryos were generated *in vitro* from gametes obtained from fertile baboons using ICSI, and developed to the blastocyst stage as modified (Hewitson et al., 1998). Briefly, hyperstimulation of female baboons was started on Days 1–2 of menses onset by a single daily subcutaneous injection of 0.25 μg/ml gonadotropin-releasing hormone (GnRH) antagonist (Antide; Ares Serono, Randolph, MA), 60 IU recombinant human follicle stimulating hormone (r-FSH; Organon, Inc., West Orange, NJ), and 60 IU recombinant human luetinizing hormone (r-HLH; Organon). A final intramuscular injection of 5000 IU of recombinant human chorionic gonadotropin (r-hCG; Serono) was administered on Day 10 and mature follicles were aspirated by laparoscopy 30 h post-hCG as

previously reported (Bavister et al., 1983). Mature oocytes were fertilized by intracytoplasmic sperm injection (ICSI) (Hewitson et al., 1998) using motile sperm separated on a PureCeption sperm gradient (Sage In-Vitro Fertilization, Inc., Trumbull, CT) as described by the manufacturer. Fertilization success was defined as zygotes with 2 polar bodies and 2 pronuclei. Culture to the expanded blastocyst stage was accomplished in CMRL-1066 medium (Boatman, 1997) with 10% fetal calf sera (Hyclone Laboratories, Inc., Logan, UT) on monolayers of buffalo rat liver cells (BRL 1442; American Type Culture Collection, Rockville, MD).

Baboon embryonic stem cell derivations

Inner cell mass cells were isolated from expanded blastocysts using immunosurgery (Navara et al., 2007). ICMs were plated onto mitomycin C-treated mouse embryonic fibroblasts (MEF; Chemicon, Millipore Corporation, Billerica, MA) in 80% Knockout Medium; 20% Knockout Serum Replacement; 1 mM L-glutamine; 0.1 mM nonessential amino acids; 1% penicillin/streptomycin; 12 ng/ml of bFGF; 10 ng/ml of Activin A and 10 ng/ml hLIF (all components from Invitrogen [Carlsbad, CA] except hLIF [Chemicon] and Activin A [Sigma]) (Navara et al., 2007). After 10–14 days, established colonies of ESCs were mechanically passaged onto new MEFs for expansion with culture medium changed every 48 h.

Pluripotency markers detected by immunocytochemistry

Putative baboon ESCs were assayed for the standard pluripotency markers (Oct-4, Nanog, SSEA-1, SSEA-4, Tra 1-60, and Tra 1-81) on undifferentiated colonies after fixation by 2% paraformaldehyde (Electron Microscopy Services, Hatfield, PA) in PBS for 40 min or absolute methanol (-20 °C; 10 min; Sigma; Navara et al., 2007). Control cell staining included differentiated colonies and baboon primary fibroblast lines which were consistently negative. Baboon embryos from the 8-cell stage to early arrested blastocysts were fixed as above after removal of the zona pellucida with brief acid Tryode's incubation. Primary antibodies were added overnight at 4 °C and secondary antibodies applied for 1 h at 37 °C after extensive washing in PBS+0.5% goat serum. DNA was detected with Hoechst 33342 added in the penultimate PBS rinse for 5 min before mounting in antifade (Vectashield; Vectors Lab, Burlingame, CA).

Pluripotency markers detected by RT-PCR

Pluripotent BabESCs were collected by scraping and pelleted at 200 *g* for 5 min. RNA was isolated using Trizol (Invitrogen) and cDNA was prepared using the Improm-II Reverse Transcription System (Promega, Madison WI) according to the manufacturer's directions. Primers used were hTERT, forward gtgtgctgcagctcccatttc and reverse gctgcgtctgggctgtcc, Oct-4, forward cgaccatctgccgctttgag and reverse ccccctgtcccccattccta, Nanog forward ctgtgatttgtgggcctgaa and reverse tgtttgccctttgggactggt, Rex1 forward gcgtacgcaaattaaagtccaga and reverse cagcatcctaaacagctcgcagaat, and Sox-2, forward cccccggcggcaatagca and reverse tcggcgccggggagatacat. Baboon skin fibroblast cells and rhesus pluripotent ES cells (line 4706; Navara et al., 2007) were run as controls.

Pluripotency demonstrated in teratomas

Approximately 5×10^5–5×10^6 baboon ESC cells of good morphology were injected into the testis of 8- to 12-week-old NOD-SCID mice (Jackson Labs) using a sterile 31 G needle to produce teratomas (Navara et al., 2007). Tumor growths were determined by palpation at approximately 10–12 weeks after injection, excised, and fixed in 4% paraformaldehyde prior to paraffin embedding. Sections were stained with hematoxylin and eosin.

Directed *in vitro* differentiation

BabESCs were plated into a 6-well tissue culture plates (Fisher Scientific, Pittsburgh, PA) with STO feeders in a modified embryonic stem cell medium composed of 80% Knockout DMEM, 20% Knockout Serum Replacement, 1 mM L-glutamine, 0.1 mM NEAA, 1% Pen/Strep , 4 ng/ml bFGF[65–68]. Colonies were fed every other day. After 2 weeks, colonies were fixed in 2% formaldehyde and immunostained using primary antibodies to cytokeratin-11 (1: 200; Abcam, Cambridge, MA) and beta-III tubulin (1: 200; Covance, Berkeley, CA).

ESC cytogenetic characterization

Slides with prepared BabESCs were banded using trypsin and Giemsa stains (Barch, 1997). Metaphase spreads were analyzed with the Cytovision Workstation and Genus image processing software (Applied Imaging, Grand Rapids, MI) to arrange baboon chromosomes into matching pairs (Pearson et al., 1979). A routine mitotic cell count was 20 metaphases, analyzing chromosomes band by band in 3 cells, 2–3 photos, and 2–3 karyotypes (American College of Medical Genetics, 2006). We performed karyotype analysis on continuous cultures at the following passage and days postplating: BabESC-4, passage 4, Day 30, and passage 53, Day 370; BabESC-15, passage 6, Day 40, and passage 35, Day 235.

Independent external genetic testing for pedigreed confirmations

DNA genotyping was performed independently at the Veterinary Genetics Laboratory, University of California–Davis under the direction of Dr. Cecelia Penedo (Supplemental Table 2).

Acknowledgments

We are grateful to the leadership and research colleagues at the Southwest NPRC including Drs. John VandeBerg, Tom Folks, William Cummins, Cheryl DiCarlo, and Karen Rice; John Tobler, Joe Jimenez, Felicia Aguillon, Kyle Ponce, Michael Washington, Annessa Raiford, Wade Hodgson, Terry Naegelin, and Antonio Perez for project coordination and animal care support; Drs. Thomas Starzl and David Cooper of the University of Pittsburgh for helpful suggestions; Virginia Penascino for laboratory support and Angela Palermo for administrative support, both at the Magee-Womens Research Institute and Foundation; and Dr. Roger Pedersen at Cambridge University and Dr. Peter Donovan at University of

California–Irvine. The support of this research by grants from the National Institutes of Health is gratefully acknowledged.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.scr.2009.02.004.

References

American College of Medical Genetics, 2006. Standards and Guidelines: Clinical Genetics Laboratories.

Barch, M.J., 1997. The AGT Cytogenetics Laboratory Manual. Lippincott-Raven, New York.

Bavister, B.D., Boatman, D.E., Leibfried, L., Loose, M., Vernon, M. W., 1983. Fertilization and cleavage of rhesus monkey oocytes in vitro. Biol. Reprod. 28, 983.

Ben-Hur, T., Idelson, M., Khaner, H., Pera, M., Reinhartz, E., Izik, A., Feubinoff, B.E., 2004. Stem Cells 22, 1246–1255.

Boatman, D.E., 1997. Responses of gametes to the oviductal environment. Hum. Reprod. 12, 133.

Boyd, A.S., Wu, D.C., Higashi, Y., Wood, K.J., 2008. A comparison of protocols used to generate insulin-producing cell clusters from mouse embryonic stem cells. Stem Cells 26, 1128–1137.

Braun, S.E., Johnson, R.P., 2006. Setting the stage for bench-to-bedside movement of anti-HIV RNA inhibitors-gene therapy for AIDS in macaques. Front. Biosci. 11, 838–851.

Brons, I.G., Smithers, L.E., Trotter, M.W., Rugg-Gunn, P., Sun, B., Chuva de Sousa Lopes, S.M., Howlett, S.K., Clarkson, A., Ahrlund-Richter, L., Pedersen, R.A., Vallier, L., 2007.

Byrne, J.A., Pedersen, D.A., Clepper, L.L., Nelson, M., Sanger, W.G., Gokhale, S., Wolf, D.P., Mitalipov, S.M., 2007. Producing primate embryonic stem cells by somatic cell nuclear transfer. Nature 450, 497–502.

Cibelli, J.B., Grant, K.A., Chapman, K.B., Cunniff, K., Worst, T., Green, H.L., Walker, S.J., Gutin, P.H., Vilner, L., Tabar, V., et al., 2002a. Parthenogenetic stem cells in nonhuman primates. Science 295, 819.

Cibelli, J.B., Lanza, R.P., West, M.D., Ezzell, C., 2002b. The first human cloned embryo. Sci. Am. 286, 44–51.

Cram, D.S., Song, B., Trounson, A.O., 2007. Genotyping of Rhesus SCNT pluripotent stem cell lines. Nature 450, E12–E14.

Culotta, E., 2005. Genomics. Chimp genome catalogs differences with humans. Science 309, 1468–1469.

Dighe, V., Clepper, L., Pedersen, D., Byrne, J., Ferguson, B., Gokhale, S., Penedo, M.C., Wolf, D., Mitalipov, S., 2008. Heterozygous embryonic stem cell lines derived from nonhuman primate parthenotes. Stem Cells 26, 756–766.

Dvash, T., Benvenisty, N., 2004. Human embryonic stem cells as a model for early human development. Best Pract. Res. Clin. Obstet. Gynaecol. 18, 929–940.

Elkabetz, Y., Panagiotakos, G., Al Shamy, G., Socci, N.D., Tabar, V., Studer, L., 2008. Human ES cell-derived neural rosettes reveal a functionally distinct early neural stem cell stage. Genes Dev. 22, 152–165.

French, A.J., Adams, C.A., Anderson, L.S., Kitchen, J.R., Hughes, M. R., Wood, S.H., 2008. Development of human cloned blastocysts following somatic cell nuclear transfer with adult fibroblasts. Stem Cells 26, 485–493.

Friedrich Ben-Nun, I., Benvensity, N., 2006. Human embryonic stem cells as a cellular model for human disorders. Mol. Cell. Endocrinol. 252, 154–159.

Fujimoto, A., Mitalipov, S.M., Clepper, L.L., Wolf, D.P., 2005. Development of a monkey model for the study of primate genomic imprinting. Mol. Hum. Reprod. 11, 413–422.

Fujimoto, A., Mitalipov, S.M., Kuo, H.C., Wolf, D.P., 2006. Aberrant genomic imprinting in rhesus monkey embryonic stem cells. Stem Cells 24, 595–603.

Hayes, E. (2008). Personal communication.

Hematti, P., Obrtlikova, P., Kaufman, D.S., 2005. Nonhuman primate embryonic stem cells as a preclinical model for hematopoietic and vascular repair. Exp. Hematol. 33, 980–986.

Hewitson, L., Takahashi, D., Dominko, T., Simerly, C., Schatten, G., 1998. Fertilization and embryo development to blastocysts after intracytoplasmic sperm injection in the rhesus monkey. Hum. Reprod. 13, 3449–3455.

Iwanami, A., Kaneko, S., Nakamura, M., Kanemura, Y., Mori, H., Kobayashi, S., Yamasaki, M., Momoshima, S., Ishii, H., Ando, K., et al., 2005. Transplantation of human neural stem cells for spinal cord injury in primates. J. Neurosci. Res. 80, 182–190.

Kawasaki, H., Suemori, H., Mizuseki, K., Watanabe, K., Urano, F., Ichinose, H., Haruta, M., Takahashi, M., Yoshikawa, K., Nishikawa, S.-I., et al., 2002a. Generation of dopaminergic neurons and pigmented epithelia from primate ES cells by stromal cell-derived inducing activity. Proc. Natl. Acad. Sci. USA 99, 1580.

Kawasaki, H., Suemori, H., Mizuseki, K., Watanabe, K., Urano, F., Ichinose, H., Haruta, M., Takahashi, M., Yoshikawa, K., Nishikawa, S., et al., 2002b. Generation of dopaminergic neurons and pigmented epithelia from primate ES cells by stromal cell-derived inducing activity. Proc. Natl. Acad. Sci. USA 99, 1580–1585.

Laslett, A.L., Filipcayk, A.A., Pera, M.F., 2003. Characterization and culture of human embryonic stem cells. Trends Cardiovasc. Med. 13, 295–301.

Lavoir, M.C., Weier, J., Conaghan, J., Pedersen, R.A., 2005. Poor development of human nuclear transfer embryos using failed fertilized oocytes. Reprod. Biomed. Online 11, 740–744.

Lin, G., OuYang, Q., Zhou, X., Gu, Y., Yuan, D., Li, W., Liu, G., Liu, T., Lu, G., 2007. A highly homozygous and parthenogenetic human embryonic stem cell line derived from a one-pronuclear oocyte following in vitro fertilization procedure. Cell Res. 17, 999–1007.

Mai, Q., Yu, Y., Li, T., Wang, L., Chen, M.J., Huang, S.Z., Zhou, C., Zhou, Q., 2007. Derivation of human embryonic stem cell lines from parthenogenetic blastocysts. Cell Res. 17, 1008–1019.

Mitalipov, S.M., 2006. Genomic imprinting in primate embryos and embryonic stem cells. Reprod. Fertil. Dev. 18, 817–821.

Mitalipov, S., Kuo, H.C., Byrne, J., Clepper, L., Meisner, L., Johnson, J., Zeier, R., Wolf, D., 2006. Isolation and characterization of novel rhesus monkey embryonic stem cell lines. Stem Cells 24, 2177–2186.

Mitalipov, S., Clepper, L., Sritanaudomchai, H., Fujimoto, A., Wolf, D., 2007. Methylation status of imprinting centers for H19/IGF2 and SNURF/SNRPN in primate embryonic stem cells. Stem Cells 25, 581–588.

Mizuseki, K., Sakamoto, T., Watanabe, K., Muguruma, K., Ikeya, M., Nishiyama, A., Arakawa, A., Suemori, H., Nakatsuji, N., Kawasaki, H., et al., 2003. Generation of neural crest-derived peripheral neurons and floor plate cells from mouse and primate embryonic stem cells. Proc. Natl. Acad. Sci. USA 100, 5828–5833.

Nakatsuji, N., Nakajima, F., Tokunaga, K., 2008. HLA-haplotype banking and iPS cells. Nat. Biotechnol. 26, 739–740.

Navara, C.S., Mich-Basso, J.D., Redinger, C.J., Ben-Yehudah, A., Jacoby, E., Kovkarova-Naumovski, E., Sukhwani, M., Orwig, K., Kaminski, N., Castro, C.A., et al., 2007. Pedigreed primate embryonic stem cells express homogeneous familial gene profiles. Stem Cells 25, 2695–2704.

Oka, S., Honmou, O., Akiyama, Y., Sasaki, M., Houkin, K., Hashi, K., Kocsis, J.D., 2004. Autologous transplantation of expanded neural precursor cells into the demyelinated monkey spinal cord. Brain Res. 1030, 94–102.

Pearson, P.L., Roderick, T.H., Davisson, M.T., Garver, J.J., Warburton, D., Lalley, P.A., O'Brien, S.J., 1979. Report of the committee on comparative mapping. Cytogenet. Cell Genet. 25, 82–95.

Pennisi, E., 1995. FDA panel OKs baboon marrow transplant. Science 269, 293–294.

Rajesh, D., Chinnasamy, N., Mitalipov, S.M., Wolf, D.P., Slukvin, I., Thomson, J.A., Shaaban, A.F., 2007. Differential requirements for hematopoietic commitment between human and rhesus embryonic stem cells. Stem Cells 25, 490–499.

Revazova, E.S., Turovets, N.A., Kochetkova, O.D., Kindarova, L.B., Kuzmichev, L.N., Janus, J.D., Pryzhkova, M.V., Revazova, E.S., Turovets, N.A., Kochetkova, O.D., et al., 2007. Patient-specific stem cell lines derived from human parthenogenetic blastocysts: HLA homozygous stem cell lines derived from human parthenogenetic blastocysts. Cloning Stem Cells 9, 432–449.

Rugg-Gunn, P.J., Ferguson-Smith, A.C., Pedersen, R.A., 2005a. Epigenetic status of human embryonic stem cells. Nat. Genet. 37, 585–587.

Rugg-Gunn, P.J., Ferguson-Smith, A.C., Pedersen, R.A., 2005b. Human embryonic stem cells as a model for studying epigenetic regulation during early development. Cell Cycle 4, 1323–1326.

Rugg-Gunn, P.J., Ferguson-Smith, A.C., Pedersen, R.A., 2007. Status of genomic imprinting in human embryonic stem cells as revealed by a large cohort of independently derived and maintained lines. Hum. Mol. Genet. 16 (Spec. No. 2), R243–R251.

Sasaki, E., Hanazawa, K., Kurita, R., Akatsuka, A., Yoshizaki, T., Ishii, H., Tanioka, Y., Ohnishi, Y., Suemizu, H., Sugawara, A., et al., 2005. Establishment of novel embryonic stem cell lines derived from the common marmoset (*Callithrix jacchus*). Stem Cells 23, 1304–1313.

Shufaro, Y., Reubinoff, B.E., 2004. Therapeutic applications of embryonic stem cells. Best Pract. Res. Clin. Obstet. Gynaecol. 18, 909–927.

Stadtfeld, M., Brennand, K., Hochedlinger, K., 2008. Reprogramming of pancreatic beta cells into induced pluripotent stem cells. Curr. Biol. 18, 890–894.

Stojkovic, M., Stojkovic, P., Leary, C., Hall, V.J., Armstrong, L., Herbert, M., Nesbitt, M., Lako, M., Murdoch, A., 2005. Derivation of a human blastocyst after heterologous nuclear transfer to donated oocytes. Reprod. Biomed. Online 11, 226–231.

Suemori, H., Nakatsuji, N., 2006. Generation and characterization of monkey embryonic stem cells. Methods Mol. Biol. 329, 81–89.

Takagi, Y., Takahashi, J., Saiki, H., Morizane, A., Hayashi, T., Kishi, Y., Fukuda, H., Okamoto, Y., Koyanagi, M., Ideguchi, M., et al., 2005. Dopaminergic neurons generated from monkey embryonic stem cells function in a Parkinson primate model. J. Clin. Invest. 115, 102–109.

Takahashi, J., 2006. Stem cell therapy for Parkinson's disease. Ernst Schering Res. Found. Workshop 229–244.

Thomson, J.A., Kalishman, J., Golos, T.G., Durning, M., Harris, C.P., Becker, R.A., Hearn, J.P., 1995. Isolation of a primate embryonic stem cell line. Proc. Natl. Acad. Sci. USA 92, 7844–7848.

Thomson, J.A., Kalishman, J., Golos, T.G., Durning, M., Harris, C.P., Hearn, J.P., 1996. Pluripotent cell lines derived from common marmoset (*Callithrix jacchus*) blastocysts. Biol. Reprod. 55, 254.

Thomson, J.A., Itskovitz-Eldor, J., Shapiro, S.S., Waknitz, M.A., Swiergiel, J.J., Marshall, V.S., Jones, J.M., 1998. Embryonic stem cell lines derived from human blastocysts. Science 282, 1145–1147.

Trounson, A., 2006. The production and directed differentiation of human embryonic stem cells. Endocr. Rev. 27, 208–219.

Vaca, P., Martin, F., Vegara-Meseguer, J.M., Rovira, J.M., Berna, G., Soria, B., 2006. Induction of differentiation of embryonic stem cells into insulin-secreting cells by fetal soluble factors. Stem Cells 24, 258–265.

Vrana, K.E., Hipp, J.D., Goss, A.M., McCool, B.A., Riddle, D.R., Walker, S.J., Wettstein, P.J., Studer, L.P., Tabar, V., Cunniff, K., et al., 2003. Nonhuman primate parthenogenetic stem cells. Proc. Natl. Acad. Sci. USA 100 (Suppl. 1), 11911–11916.

Wianny, F., Bernat, A., Huissoud, C., Marcy, G., Markossian, S., Cortay, V., Giroud, P., Leviel, V., Kennedy, H., Savatier, P., et al., 2008. Derivation and cloning of a novel rhesus embryonic stem cell line stably expressing tau-green fluorescent protein. Stem Cells 26, 1444–1453.

Wildman, D.E., 2002. A map of the common chimpanzee genome. Bioessays 24, 490–493.

Wilmut, I., Taylor, J., 2007. Stem cells: primates join the club. Nature 450, 485–486.

Zhang, Y., Li, J., Villemoes, K., Pedersen, A.M., Purup, S., Vajta, G., 2007. An epigenetic modifier results in improved in vitro blastocyst production after somatic cell nuclear transfer. Cloning Stem Cells 9, 357–363.

Zhou, J.M., Xing, F.Y., Shi, J.J., Fang, Z.F., Chen, X.J., Chen, F., 2008. Quality of embryonic bodies and seeding density effects on neural differentiation of mouse embryonic stem cells. Cell Biol. Int. 32, 1169–1175.