Epigenetic Reprogramming by Somatic Cell Nuclear Transfer in Primates

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Key Words. Reprogramming • Nuclear transfer • Embryonic stem cells • Primates

ABSTRACT

We recently demonstrated that somatic cells from adult primates could be reprogrammed into a pluripotent state by somatic cell nuclear transfer. However, the low efficiency with donor cells from one monkey necessitated the need for large oocyte numbers. Here, we demonstrate nearly threefold higher blastocyst development and embryonic stem (ES) cell derivation rates with different nuclear donor cells. Two ES cell lines were isolated using adult female rhesus macaque skin fibroblasts as nuclear donors and oocytes retrieved from one female, following a single controlled ovarian stimulation. In addition to routine pluripotency tests involving in vitro and in vivo differentiation into various somatic cell types, prime ES cells derived from reprogrammed somatic cells were also capable of contributing to cells expressing markers of germ cells. Moreover, imprinted gene expression, methylation, telomere length, and X-inactivation analyses were consistent with accurate and extensive epigenetic reprogramming of somatic cells by oocyte-specific factors. STEM CELLS 2009;27:1255–1264

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

INTRODUCTION

Two novel strategies for deriving patient-matched pluripotent cells have evolved including direct reprogramming of somatic cells using genetic manipulations [1–3] and epigenetic reprogramming via somatic cell nuclear transfer (SCNT). Direct genetic manipulation of somatic cells to produce induced pluripotent stem (iPS) cells carries the advantage that it does not require eggs. However, it suffers serious limitations because the introduction of multiple copies of transgenes may cause insertional mutations and disrupt the normal pattern of expression and/or function of many endogenous genes. Continuous overexpression of exogenous factors is also problematic secondary to the incomplete silencing of transgenes during differentiation. Another concern is the possibility of transgene reactivation and/or spontaneous reversion of transplanted, differentiated cells back to the pluripotent state, whereby a few resident pluripotent cells may result in tumour development.

Reprogramming by oocyte-specific factors after SCNT avoids these pitfalls because it employs endogenous epigenetic pathways/processes. Indeed, SCNT and cytoplast characterization provides an invaluable system for illuminating fundamental epigenetic mechanisms of natural sequential reprogramming [4]. Recent studies indicate that both mouse and primate embryonic stem (ES) cells derived by SCNT are equivalent to ES cells derived from fertilized embryos regarding their transcriptional activity and ability to differentiate into multiple cell types in vitro and in vivo [5–7]. However, the epigenetic status of such pluripotent cells remains unclear, as many cloned animals display gross developmental abnormalities due to incomplete reprogramming [8]. As a limitation of SCNT-based reprogramming, the efficiency of deriving ES cell lines from primate SCNT embryos remains low [5]. Based on our limited data employing skin fibroblasts from an adult male monkey, as many as 150 oocytes could be required to derive one ES cell line [5].

Here we describe SCNT and ES cell derivation with nuclear donor cells derived from another monkey that resulted in high blastocyst formation, and ES cell isolation rates similar to those reported for fertilized embryos [9]. A cohort of oocytes, retrieved from one female after a single controlled ovarian stimulation, was used for SCNT with adult female monkey skin cells to produce two ES cell lines. Pluripotency evaluation included ES cell-specific marker expression and...
the ability to differentiate in vitro and in vivo into various cell types including early germ cells. Moreover, implanted gene expression, methylation, telomere length, and X-inactivation analyses demonstrated a remarkable extent of epigenetic reprogramming of somatic cells to ES cells.

### MATERIALS AND METHODS

#### Animals

Adult rhesus macaques housed in individual cages were used in this study. Animal protocols were reviewed and approved by the ONPRC Institutional Animal Care and Use Committee.

#### Oocyte Collection

Starting at day 1–4 of the menstrual cycle, females displaying regular menstrual cycles received twice-daily injections of recombinant human FSH (Organon; 30 IU, im) for 8 days and recombinant human LH (Ares Serono; 30 IU, im) on day 7–8 of the stimulation protocol. In addition, animals received a GnRH agonist (Acylcline; NIH/NICH: 0.075 mg/kg body weight, sc) and human chionic gonadotropin (hCG; Serono; 1,000 IU, im) on day 7 during the stimulation period approximately 36 hours before laparoscopic follicle aspiration and oocyte retrieval. Serum estradiol (E2) and progesterone (P4) measurements along with ultrasonographic scans were performed to monitor follicular response.

#### Somatic Cell Nuclear Transfer

Methods for monkey SCNT and embryo culture were performed as described previously [5]. Primed fibroblasts, used as nuclear donors, were established from skin biopsies of adult rhesus macaques and prepared for SCNT as previously described [5]. In brief, mature metaphase II (MII) oocytes were enucleated using the Oosight Imaging System (CRI, Woburn, MA) attached to an inverted microscope (Olympus, Tokyo, http://www.olympus-global.com) with Narishige micromanipulators. After oocyte spindle removal a disaggregated donor somatic cell was aspirated into a micropipette and placed into the perivitelline space of the cytoplasm on the side opposite of the first polar body. Cell fusion was induced by two 50 μs DC pulses of 2.7 kV/cm (Electro Square Porator T-820, BTX) in Ca2+/M-free buffer. All nuclear transfer micromanipulation and fusion procedures were conducted on microscope stage warmers (Tokai Hit) maintaining 37°C. Reconstructed oocytes were activated 2 hours post-fusion by a 5-minute exposure to 5 μM ionomycin (CalBiochem, San Diego, http://www.emdbiosciences.com) followed by a 5-hour incubation in 2 mM 6-dimethylaminopurine. Activated oocytes were cultured in 4-well dishes containing HECM-9 medium supplemented with 10% fetal bovine serum and 12 μM β-mercaptoethanol in 6% CO2, 5% O2, balance N2, at 37°C, until the embryos reached the blastocyst stage, with medium changes every other day.

#### ES Cell Derivation

Whole blastocysts or isolated inner cell masses (ICMs), recovered following immunosurgical isolation of the ICM [5], were selected based on overall morphology and plated onto feeder layers (mouse embryonic fibroblasts, mEFs) in ES cell culture media equilibrated with 3% CO2, 5% O2, balance N2, at 37°C for 5–7 days. ICM outgrowths were manually dissociated into small clumps with a microscalpel and replated on fresh mEFs. After the initial passage, colonies with ES cell-like morphology were selected for further propagation, characterization, and in vitro and in vivo differentiation as previously described [5]. Culture medium was changed daily, and the ES cell colonies were typically split every 5–7 days by manual dissociation and the collected clumps were replated onto fresh mEFs.

#### X-Inactivation and Telomere Length Measurements

Qualitative and quantitative reverse-transcription polymerase chain reaction (RT-PCR) analysis was performed using primers and probes presented in supporting information Table S2. Amplification parameters for RT-PCR were as follows: one cycle at 94°C for 2 minutes; 35 cycles at 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30-second; and one cycle at 72°C for 10 minutes.

Relative telomere length was measured using a real-time quantitative (q)-PCR method as previously described [10] using primers Tel1 and Tel2 for telomerases and 36B4 for acidic ribosomal phosphoprotein P0 used as a single-copy gene reference (supporting information Table S2). Amplification was performed using ABIPrism 7,500 sequence detection system (Applied Biosystems, Foster City, CA, http://www.appliedbiosystems.com) under the following conditions: for Tel1 and Tel2 primers—30 cycles at 95°C for 15-second and 54°C for 2 minutes; for 36B4 primers—30 cycles at 95°C for 15-second and 58°C for 1 minute. To determine the cycle threshold (Ct) value, two separate PCR runs were performed for each sample and primer pair. For each run a standard curve was generated using a reference genomic DNA isolated from in vitro fertilization (IVF)-derived ES cells diluted to 0.06–40 ng per well (fivefold dilution). Calculation of the relative telomere/single copy gene ratio (T/S value) and statistical analysis with SDS v. One.1 software (Applied Biosystems) was used to determine standard curve and Ct values. A point on the standard curve at a concentration corresponding to the average DNA concentration of the samples was used as a calibrator. The mean T/S value of skin fibroblasts and ES cells were compared and plotted against each sample.

#### Allele-Specific Expression and Methylation Analysis of Imprinted Genes

Details for quantitative expression analysis of imprinted genes can be found in the supporting information. Characteristics of the single nucleotide polymorphisms (SNPs) employed for allele-specific expression analysis, PCR primers, and conditions were described in details previously [11, 12]. Expressed alleles were determined by direct sequence analysis of the RT-PCR amplicons. For methylation analysis, gDNA was subjected to bisulfite treatment using a CpG Genome Modification Kit (Chemicon International) according to the manufacturer’s protocol. The sequence, annealing temperature, and PCR cycle number of each primer pair were as previously reported [13]. PCR products were cloned and individual clones were then sequenced with an ABI 3,100 capillary genetic analyzer (Applied Biosystems) using BigDye terminator sequencing chemistry [14]. Sequencing results were analyzed using Sequencher software (Gene Codes). For Southern blot analyses, approximately 4 μg of gDNA was digested with EcoNI and the CpG methylation-blocked enzyme BsaHI (New England Biolabs). The samples were electrophoresed through a 1% agarose gel, and transferred to a nylon membrane. The blot was then hybridized with a probe, whose template was generated by PCR of gDNA from the control tissue, using the previously reported primers [13]. The probe was produced by random priming (Megaprime DNA Labeling Systems, Amersham Biosciences, Piscataway, NJ, http://www.amersham.com) and labeled with 32P-dCTP (PerkinElmer, Life and Analytical Sciences, Boston, http://www.perkinelmer.com). The hybridization was carried out at 65°C overnight in Rapid-hyb buffer (Amersham Biosciences). The blot was washed for 30 minutes in 2XSSC/0.1% SDS and then for 20 minutes in 0.1XSSC/0.1% SDS.

#### Statistical Analysis

Embryo development data were statistically analyzed using analysis of variance. For quantitative analysis of maternally and paternally expressed imprinted genes, qRr expression, and telomere length measurements, statistical analysis with SDS v. One.1 software (Applied Biosystems) was used.
Deriving Novel Rhesus Monkey ES Cells by SCNT

We reported previously that somatic cell nuclei derived from an adult male monkey (nuclear donor 1) can be reprogrammed to the pluripotent state by SCNT [5]. Here, we investigated whether skin fibroblasts derived from female primates can also support SCNT blastocyst development and ES cell isolation. Primary cultures of skin fibroblasts isolated from two adult female rhesus macaques, nuclear donors 2 (9-year-old) and 3 (11-year-old), were fused with enucleated MII oocytes as previously described [5]. After artificial activation and in vitro culture, 43% (25/58) and 23% (3/13) embryos (nuclear donors 2 and 3, respectively) reached the expanded blastocyst stage (Table 1). In contrast, only 12.5% (3/24) SCNT embryos reconstructed with previously used male skin fibroblasts (nuclear donor 1) developed to blastocysts. These results suggest that nuclear donor cells may affect reprogramming efficiency and SCNT embryo development.

To further evaluate the extent of reprogramming, we selected 13 blastocysts based on morphology and overall appearance, produced from nuclear donor 2 and a cohort of oocytes collected from a single ovarian stimulation, to be used for ES cell isolation. Two alternative ES cell derivation methodologies were evaluated: (a) conventional ICM isolation involving immunosurgical dispersal of trophectodermal (TE) cells [5, 9] and (b) whole (intact) blastocyst culture. Isolated ICMs (n = 7) and intact blastocysts (n = 6) were plated onto feeder layers consisting of mouse embryonic fibroblasts (Table 2; [5, 9]). Blastocysts produced by IVF (16 ICMs and 21 intact blastocysts) served as a control. After 5–7 days of culture, ICMs and intact blastocysts that attached to the feeder layer and initiated three-dimensional outgrowths were manually dissociated into smaller clumps and replated onto fresh feeder layers. ICMs isolated from SCNT blastocysts failed to produce any outgrowths, while whole blastocysts gave rise to two ES cell lines (a 29% derivation rate). In contrast, IVF-derived ICMs produced 5 ES cell lines (31%), whereas only one cell line (5%) was recovered from whole blastocysts (Table 2). These outcomes indicate that significant differences between SCNT and IVF embryos exist that predicate alternative approaches for ES cell isolation. Overall, our results demonstrate that SCNT blastocyst development and ES cell isolation with selected nuclear donor cells can be as efficient as with IVF embryos [9, 15], thereby resulting in a significant reduction in the number of oocytes required to produce a single ES cell line over our previously reported efficiencies [5]. Moreover, isolation of two ES cell lines using oocytes retrieved from one female demonstrates both the technical and financial feasibility of deriving patient-matched ES cells in women for the treatment of degenerative diseases.

### Results

#### Origin and Pluripotency Analysis of ES Cells

Two novel SCNT-derived ES cell lines (designated as cloned rhesus embryonic stem (CRES)-3 and -4) grew as flat colonies typical for monkey ES cells.

To determine whether these colonies expressed key primary pluripotency markers OCT4, TRA1-60, TRA1-81, and SSEA-4, the cell colonies were exposed to immunocytochemical procedures. Positive expression of each pluripotency marker tested was confirmed in CRES-3 and CRES-4 cell lines (Fig. 1A). Detailed genetic analysis of nuclear DNA employing 40 microsatellite markers revealed complete homology of both CRES-3 and -4 to each other and to the donor fibroblasts, with no significant similarity to the oocyte donor genomic DNA (supporting information Table S1). On the other hand, mitochondrial (mt)DNA sequence analysis indicated that both CRES-3 and -4 inherited their mitochondria from the same oocyte donor female (supporting information Fig. S1). No contribution of donor somatic cell mtDNA was detected in either CRES-3 or -4. Incidentally, nuclear donor female 2 for CRES-3 and -4 was the oocyte (mtDNA) donor for CRES-1 in our previous study [5]. Cytogenetic analysis by G-banding revealed that both CRES-3 and -4 contained a normal female rhesus macaque karyotype (42, XX) with no detectable chromosomal anomalies (supporting information Fig. S2).

The key feature of pluripotent cells is their ability to differentiate into all cell types that comprise a whole organism. To examine the extent of reprogramming and pluripotency, we induced differentiation of CRES-3 and -4 cells in feeder-free suspension culture into embryoid bodies (EBs) followed by adhesion culture for several weeks. Differentiated cells exhibited a variety of different phenotypes including contracting cardiomyocytes and neurons (Fig. 1B). Furthermore, both cell lines produced teratomas when injected into severe immunocompromised mice (Fig. 1C).

#### Table 1. In vitro development of monkey somatic cell nuclear transfer embryos

<table>
<thead>
<tr>
<th>Animal</th>
<th>No. of replicates</th>
<th>No. of oocytes</th>
<th>No. of cleaved (%)</th>
<th>No. of compact morulae (%)</th>
<th>No. of blastocysts (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclear donor 1</td>
<td>n = 3</td>
<td>24</td>
<td>24 (100)</td>
<td>11 (46)</td>
<td>3 (12.5)*</td>
</tr>
<tr>
<td>Nuclear donor 2</td>
<td>n = 3</td>
<td>59</td>
<td>58 (98)</td>
<td>32 (55)</td>
<td>25 (45)**</td>
</tr>
<tr>
<td>Nuclear donor 3</td>
<td>n = 2</td>
<td>13</td>
<td>13 (100)</td>
<td>6 (46)</td>
<td>3 (23)**</td>
</tr>
</tbody>
</table>

Values with different superscript symbols within a column are significantly different, p < .01.

### Table 2. ES cell isolation from monkey SCNT blastocysts

<table>
<thead>
<tr>
<th>ES cell derivation approach</th>
<th>SCNT Isolated ICMs</th>
<th>IVF Isolated ICMs</th>
<th>Whole blastocyst</th>
</tr>
</thead>
<tbody>
<tr>
<td>Embryo source</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SCNT</td>
<td>7</td>
<td>16</td>
<td>6</td>
</tr>
<tr>
<td>Whole blastocyst</td>
<td>0 (0)*</td>
<td>5 (31)**</td>
<td>2 (33)**</td>
</tr>
</tbody>
</table>

Values with different superscript symbols within a column are significantly different, p ≤ .01.

*Isolated ICMs refers to immunosurgical isolation of the inner cell mass and subsequent plating on feeder layers as previously described [5].

**Whole blastocyst refers to the use of intact blastocysts for ES cell derivation.

Abbreviations: ES, embryonic stem; ICM, inner cell mass; IVF, in vitro fertilization; SCNT, somatic cell nuclear transfer.
Figure 1. Characterization of undifferentiated and differentiated cloned rhesus embryonic stem (CRES) cells. (A): Detection of pluripotency markers in CRES-3 and CRES-4 cells by immunocytochemistry. (Aa, Ac, Ae, Ag): Phase contrast images of undifferentiated CRES cells. The same images immunostained with OCT4 (Ab), TRA1-60 (Ad), TRA-1-81 (Af), and SSEA-4 (Ah) antibodies. (B): Immunocytochemical examination of neuronal phenotypes derived after differentiation of CRES-3 and -4. (Ba, Bc, Be): Phase contrast images. (Bb): Immunolabeled with β-III-tubulin, tyrosine hydroxylase (Bd), and MAP2 (Bf) antibodies. (C): Expression of germ cell-specific markers during in vitro differentiation of CRES cells. ES cells were differentiated in feeder-free suspension culture into EBs for 3–50 days and analyzed by reverse transcription-polymerase chain reaction. (D): Immunostaining of CRES-3-derived differentiated cultures with VASA and OCT4 antibodies after 4 weeks of differentiation. (Da–Dc): Represent the same image with phase contrast, DAPI staining (all cell nuclei), and VASA expression, respectively. (Dd–Df): Represent the same image for phase contrast, DAPI staining, and OCT4 localization, respectively. Abbreviations: EB, embryoid body; ES, embryonic stem; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; MAP2, microtubule associated protein-2.
combined immunodeficiency mice. Histological analysis of the dissected tumors identified tissues representing the three germ layers, including ectoderm derived neuroepithelium, mesoderm originated connective tissues, cartilage and smooth muscle, and cystic areas lined with cuboidal epithelium representing the endoderm (supporting information Fig. S3). In addition to their ability to differentiate into somatic lineages, mouse ES cells also contribute to germ cells upon spontaneous in vitro or in vivo differentiation in chimeras [16–19].

The ability to contribute to the germ line is an ultimate measure of pluripotency, an endpoint by which the full potential of mouse ES cells is routinely tested. However, the capacity to extrapolate this approach to ES cells from other mammals including primates, remains in question, as contribution to chimeras could be a species-specific feature of mouse ES cells only. Here, we induced differentiation of CRES-3 and -4 by suspension culture into EBs and analyzed expression of germ cell-specific markers over an extended period. As expected, OCT4 was highly expressed in undifferentiated ES cells but gradually declined after differentiation (Fig. 1C). Low level expression was detected again by day 50 of differentiation possibly indicating development of early postmigratory germ cells. A similar pattern was seen for DAZL, with expression in undifferentiated cells that was downregulated upon differentiation and reappeared at the later stages. Another marker, STELLAR was expressed in undifferentiated as well as at all stages of differentiation. In contrast, transcripts of SCP3, VASA, and GDF9, not detected in the undifferentiated ES cells, were expressed at the later stages of differentiation.

These genes are associated with germ cell/oocyte specific actions; VASA is essential for germ cell determination, SCP3 is involved in meiosis, GDF9 is an oocyte secreted factor that regulates follicle growth and maturation [20].

To corroborate these results, CRES cells differentiated in feeder-free adhesion culture were examined for expression of germ cell markers by immunocytochemistry (ICC). After differentiation for 4 weeks, cultures contained various terminally differentiated cells, were expressed at the later stages of differentiation. In contrast, transcripts of SCP3, VASA, and GDF9, not detected in the undifferentiated ES cells, were expressed at the later stages of differentiation. These genes are associated with germ cell/oocyte specific actions; VASA is essential for germ cell determination, SCP3 is involved in meiosis, GDF9 is an oocyte secreted factor that regulates follicle growth and maturation [20].

Imprinted Gene Expression and Methylation Analysis in CRES Cells

Genomic imprinting or preferential expression of one parental allele is regulated by epigenetic mechanisms including DNA methylation. Imprinted genes are known to be particularly susceptible to epigenetic alterations during in vitro embryo manipulations and ES cell culture [13, 21]. Importantly, many defects often observed in cloned animals may result from abnormal expression of imprinted genes [8]. Disruption or inappropriate expression of imprinted genes is associated with cancer and tumor development, thus, it is important to address concerns over imprinting integrity in primate SCNT-derived ES cells prior to transplantation trials.

Levels of nine maternally and nine paternally expressed genes known to be imprinted in humans (http://www.geneimprint.com) were compared between CRES-3 and -4 cells, donor fibroblasts and IVF-derived ES cells (ORMES-22) (Fig. 2). Maternally expressed PHLDA2, DLX5, ATP10A, and SLC22A18 were significantly upregulated in skin fibroblasts when compared with CRES-3, -4, and ORMES-22 cells. In contrast, several other maternally expressed imprinted genes including H19, CDKN1C, and TP73 were upregulated in ES cells, whereas expression was undetectable in fibroblasts (Fig. 2). Interestingly, the majority of paternally expressed genes including IGFI2, NDN, SNRPN, MEST, MAGEL2, and PEG3 were upregulated in ES cells compared with somatic donor fibroblasts. SCNT-derived CRES-1 and -2 cell [5] results were comparable to those seen for CRES-3 and -4 (supporting information Fig. S4). Moreover, qPCR results were in agreement with expression levels of these imprinted genes determined previously by microarray analysis (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE7748) [5].

Next, we studied the allele-specific expression of NDN, H19, IGF2, and SNRPN in CRES cells based on sequence polymorphisms within coding regions as we previously described [12]. IVF-derived ORMES-22 and -23 served as controls. First, we examined genomic DNA samples for heterozygosity by direct sequencing of PCR products and found that CRES cell lines contained at least one informative SNP for each of NDN, H19, and IGF2 (Table 3, Fig. 3). No useful sequence polymorphisms were detected for SNRPN in any CRES cell lines. Next, we sequenced cDNA samples and determined that all heterozygous (C/T) for NDN CRES cell lines expressed the T allele exclusively, the monoallelic outcome expected for an imprinted gene (Fig. 3). The parental information for nuclear donor animals that contributed skin cells for CRES cells was not available; thus precluding parent-specific expression analysis of imprinted genes in SCNT-derived ES cells. However, we analyzed heterozygous IVF-derived ORMES-22 and confirmed that only the paternal, C allele was expressed (Fig. 3). Similarly, H19 was monoallelically expressed in all four CRES cell lines and in both ORMES-22 and -23. Moreover, parent-specific analysis indicated that the maternal, G allele was expressed in both ORMES cell lines (Table 3, Fig. 3). In contrast, IGF2 was expressed from both parental alleles in informative CRES-1 and -2 and ORMES-23, based on analysis of three polymorphic sites (Fig. 3, Table 3). We also determined that SNRPN was expressed monoallelically in heterozygous ORMES-23. It was unclear which parental allele was expressed as both parents of ORMES-23 were A/G heterozygous for SNRPN (Fig. 3).

Imprinted gene expression is intimately associated with maintenance of epigenetic marks including DNA methylation. Parent-of-origin-dependent DNA methylation of CpG dinucleotides, imposed during gametogenesis within imprinting centers (ICs), facilitates discrimination between maternal and paternal alleles, resulting in monoallelic expression. These differentially methylated regions are thought to be resistant during genome-wide demethylation and remethylation waves observed during preimplantation embryo development [22]. However, abnormal DNA methylation after SCNT has been implicated in developmental failures and defects in embryos and offspring [23]. To examine if CRES cells retained differentially methylated patterns at imprinted loci, we analyzed the methylation status of IGF2/H19 and SNRPN/IGF2 ICs by methylation-sensitive Southern blot and bisulfite genomic sequencing as previously reported [13, 24].
both methylated and unmethylated alleles was observed at both loci in nuclear donor fibroblasts, ORMES-22, and CRES cell lines, reflecting maintenance of normal methylation patterns in SCNT-derived primate ES cells (supporting information Figs. S5, S6).

We also investigated the global DNA and histone methylation profiles in CRES cells and nuclear donor fibroblasts using immunocytochemical labeling with antibodies raised against 5-methyl cytidine and histone three tri-methylation at lysine 4, 9, and 27 (me3H3K4, me3H3K9, and me3H3K27). Nuclear staining for 5-methyl cytidine, me3H3K4, and me3H3K27 were comparable between all analyzed cell types indicating that genome-wide DNA and histone methylation levels at lysine 4 and 27 in pluripotent stem cells are similar to somatic cells (supporting information Fig. S7). However, we observed significant differences in me3H3K9 labeling: the strong nuclear staining was observed in skin fibroblasts while the signal in both IVF-derived ORMES-22 and SCNT-derived CRES cells was weak and patchy (supporting information Fig. S7). This observation is consistent with the conclusion that in pluripotent ES cells only a small fraction of the genome is associated with me3H3K9 modification.

Figure 2. Expression levels of selected imprinted genes in CRES-3 and CRES-4 cells. The X-axis represents: A, nuclear donor fibroblast; B, CRES-3; C, CRES-4; D, in vitro fertilization-derived ORMES-22. The Y-axis shows the relative expression levels of each imprinted gene as determined by comparison to the expression level of housekeeping control GAPDH (imprinted gene: GAPDH ratio). The mean expression level was calculated using a standard curve method followed by normalization with housekeeping GAPDH. Data represent the means ± SEM. (n = 6). *, A, • denotes significant difference (p < .05) compared with A, B, and C, respectively. Abbreviation: GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

Table 3. Summary of allele-specific imprinted gene expression in CRES cell lines

<table>
<thead>
<tr>
<th>Gene</th>
<th>NDN SNP position and type</th>
<th>H19 SNP positions and type</th>
<th>IGF2 SNP positions and type</th>
<th>SNRPN SNP position and type</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>795' C/T</td>
<td>439 A/G</td>
<td>457 C/T</td>
<td>286 C/T</td>
</tr>
<tr>
<td></td>
<td></td>
<td>371 A/C</td>
<td>405 C/T</td>
<td>775 A/G</td>
</tr>
<tr>
<td>CRES-1</td>
<td>Monoallelic</td>
<td>Monoallelic</td>
<td>—</td>
<td>Biallelic</td>
</tr>
<tr>
<td>CRES-2</td>
<td>Monoallelic</td>
<td>Monoallelic</td>
<td>—</td>
<td>Biallelic</td>
</tr>
<tr>
<td>CRES-3</td>
<td>Monoallelic</td>
<td>Monoallelic</td>
<td>Monoallelic</td>
<td>—</td>
</tr>
<tr>
<td>CRES-4</td>
<td>Monoallelic</td>
<td>Monoallelic</td>
<td>Monoallelic</td>
<td>—</td>
</tr>
<tr>
<td>ORMES-22</td>
<td>Paternal</td>
<td>Maternal</td>
<td>—</td>
<td>Monoallelic</td>
</tr>
<tr>
<td>ORMES-23</td>
<td>Paternal</td>
<td>Maternal</td>
<td>Maternal</td>
<td>Biallelic</td>
</tr>
</tbody>
</table>
| The absence of results indicates that no informative SNPs were available.

*The bp nucleotide positions correspond to Genebank accession number AY621029 for NDN, AY624137 for H19, AY624138 for IGF2, and AY643709 for SNRPN.

Abbreviations: CRES, cloned rhesus embryonic stem; ES, embryonic stem; SNP, single nucleotide polymorphism.

Both methylated and unmethylated alleles was observed at both loci in nuclear donor fibroblasts, ORMES-22, and CRES cell lines, reflecting maintenance of normal methylation patterns in SCNT-derived primate ES cells (supporting information Figs. S5, S6).

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**Telomere Length and X-Inactivation**

Telomeres are DNA-protein complexes at the ends of eukaryotic chromosomes essential for chromosomal integrity and...
normal cell growth [25]. Telomere DNA is composed of TTAGGG tandem repeats that are progressively incised with each cell division at the rate of 50–150 base pairs per cell division in human cells leading to replicative senescence [26]. Maintenance or elongation of telomeres in germ cells, early embryonic, and ES cells is sustained by ribonucleoprotein complex telomerase (TERT). Shortened telomeres have been implicated in premature aging and early death of some cloned offspring [27] indicating possible errors in restoring telomere length after SCNT. Monkey SCNT-derived CRES cell lines were examined by a real-time PCR approach [10], and the results clearly demonstrated a significant elongation of telomere length in all CRES cell lines relative to donor fibroblasts (Fig. 4A). Telomere length in SCNT-derived ES cells was comparable to IVF-derived controls, although considerable variation was apparent between ES cell lines. Furthermore, high levels of TERT expression that was detected in CRES and IVF-derived ES cells, but not in skin fibroblasts, support this observation (Fig. 4A). These data indicate an efficient reprogramming and restoration of replicative capacity of donor somatic cells to embryonic levels after primate SCNT.

Figure 3. Chromatograms showing allele-specific expression analysis of imprinted genes in CRES cells. Polymorphic nucleotides are identified by arrows. For NDN, CRES-3 was C/T heterozygous and only T allele was expressed. In vitro fertilization-derived ORMES-22, paternal, C allele was expressed. The paternal gDNA was homozygous (C/C), while the maternal gDNA was C/T heterozygous. The C allele in the ORMES-22 must have a paternal origin while the T allele was derived from the mother. Similarly, A/G polymorphism was investigated for H19 showing monoallelic expression in CRES-3. In A/G heterozygous ORMES-22, the paternal gDNA was A/G heterozygous and maternal G/G homozygous. Thus, expressed G allele in ORMES-22 is the maternal origin. IGF2 expression was biallelic in all cell lines examined and SNRPN was expressed monoallelically in heterozygous ORMES-23.

Dosage compensation in female mammals is achieved by epigenetic processes that silence gene expression from one X chromosome, a process known as X-inactivation [28]. In the mouse, both X chromosomes are presumed to be active during preimplantation embryonic development and undifferentiated mouse ES cells do not display an inactive X. Random X-inactivation occurs in differentiating mouse ES cells upon expression of the xist gene from the chromosome to be inactivated, thereby allowing this noncoding transcript to associate with the chromosome of origin and silence gene transcription [29]. In contrast to the mouse, the majority of undifferentiated human ES cell (hESC) lines show X-inactivation [30, 31] suggesting inactivation occurs early in primate development. In this study, the X-inactivation status of the female CRES-3 and -4 cell lines was assessed by measuring the level of XIST expression. All tested female samples including donor fibroblasts, undifferentiated CRES-3, -4, and IVF-derived ORMES-22 displayed strong XIST expression (Fig. 4B, 4C). In contrast, XIST transcripts were not detected in male-derived nuclear donor fibroblasts and CRES-1 and -2. Furthermore, an additional eight IVF-derived monkey ES cell lines (ORMES series [9]) were analyzed for XIST expression, with all XX cell lines possessing high levels of XIST mRNA consistent with X-inactivation (Fig. 4D). To investigate the status of X-inactivation in monkey blastocysts, an embryonic stage from which ES cells are derived, XIST mRNA levels in SCNT- and IVF-produced embryos were determined. Simultaneously, a PCR-based sexing of individual blastocysts was conducted using size differences in the genomic DNA amplicons of the X- and Y-linked zinc finger protein genes (ZFX and ZFY) as previously described [32, 33]. XIST expression was not detectable in male and female monkey blastocysts (Fig. 4E), suggesting that, similar to the mouse, both X chromosomes are active in primate preimplantation embryos. These results also indicate that epigenetic marks regulating X-inactivation are faithfully recapitulated in female primate ES cells after SCNT.
DISCUSSION

In this study, we demonstrate derivation of two primate ES cell lines from somatic cells of an adult female, reproducing our previous report that primate somatic cells can be reprogrammed to the pluripotent state by SCNT. Our results also suggest that SCNT with particular donor somatic cells can be highly efficient, yielding a near threefold increase in the blastocyst formation rate compared with other cells. Blastocyst development of 12.5% observed with the nuclear donor 1, a 9-year-old adult rhesus macaque male, was similar to our previously reported 16% efficiency with the same donor cells [5]. Culture conditions for nuclear donor skin fibroblasts derived from all three monkeys and SCNT protocols in this study were identical. Moreover, cytogenetic analysis confirmed that all three cell lines possess normal rhesus monkey karyotypes (results not shown). Thus, it is not clear why blastocyst development rates differ dramatically.

A variety of factors in nuclear donor cells have been implicated in reprogramming efficiency after SCNT, including cell type and size, population doubling time, cell cycle, and differentiation status (see for review [34]). In addition, random genetic and epigenetic changes accumulated in somatic cells in vivo or in vitro may compromise the developmental potential of donor nuclei [35]. In the mouse, reprogramming efficiency was inversely correlated with the donor cell differentiation status. For example, cloning efficiency with ES cell nuclei was up to 30 times higher than with commonly used somatic cells, such as cumulus cells and tail tip fibroblasts [36]. Another potentially confounding influence is donor cell type. Mouse cloning with cumulus cells is usually significantly higher than with tail-tip fibroblasts [37]. More direct comparisons demonstrated that blastocyst formation rates vary dramatically even if nuclear donor cells were derived from the same tissue and exposed to the same culture conditions [35, 38–41].

Figure 4. Analysis of telomere length and X-inactivation in CRES cells. (A): Relative telomere length and TERT expression in nuclear donor skin fibroblasts, CRES cell lines and in vitro fertilization (IVF)-derived ORMES-22, -23, and -7 as determined by quantitative polymerase chain reaction (qPCR). (B): X-inactivation detected by reverse transcription-PCR analysis of XIST expression in monkey nuclear donor male and female fibroblasts, CRES cells, and ORMES-22. (C): Relative XIST expression in fibroblasts, CRES cells, and ORMES-22. (D): XIST expression levels in monkey IVF-derived XX and XY ES cell lines. OR refers to ORMES cell lines [9]. (E): XIST expression in somatic cell nuclear transfer and IVF-produced male and female monkey blastocysts. The data represents the mean ± SEM (n = 4). Abbreviations: GAPDH, glyceraldehyde 3-phosphate dehydrogenase; TERT, ribonucleoprotein complex telomerase.
We demonstrate here improved ES derivation rates from SCNT embryos associated with plating intact blastocysts as opposed to isolated ICMs onto feeder layers. Previously, the majority of blastocysts was dissected for ICM isolation and only 12% (2/17) of monkey SCNT embryos resulted in ES cell lines while a limited number of intact SCNT blastocysts (n = 3) did not produce any outgrowth [5]. In revisiting this issue here, we hypothesized that exposure of SCNT blastocyst to the antibody followed by treatment with complement to selectively destroy TE cells may also affect ICM cell viability. This would be consistent with the poor TE development in SCNT blastocysts compared with IVF-derived counterparts allowing antibody penetration through the TE layer into the blastocoel cavity with access to the ICM. Based on our current SCNT blastocyst formation rate of 43% and ES cell isolation efficiency of 29%, as few as 10 or less primate oocytes could be sufficient to derive one cell line. Thus, the continued systematic optimization of SCNT approaches will likely succeed in the efficient generation of patient-specific ES cells for therapeutic applications.

We further characterized novel SCNT-derived ES cell lines that confirmed their somatic origin and pluripotency. Previously, cytogenetic analysis revealed that one of the two derived ES cell lines, CRES-1, was aneuploid [5], thus the extent of chromosomal abnormalities in primate SCNT-derived embryos and ES cells was unclear. However, both CRES-3 and 4 exhibited a normal female rhesus macaque chromosomal (42,XX) complement suggesting that cytogenetic aberrations are an unusual attribute of primate SCNT-derived ES cells.

In addition to routine pluripotency tests, we provide evidence that primate ES cells derived by SCNT are capable of differentiating into cell types expressing markers of germ cells. In vitro formation of primordial germ cells and early male and female gametes has been documented from mouse ES cells [16-18] and spontaneous or induced differentiation of hESCs can also produce cultures with germ cell-specific gene expression in an appropriate temporal sequence [20]. However, functional gametes have not yet been produced suggesting that additional studies on appropriate culture conditions for gamete formation are required. Nevertheless, these advances suggest that a patient’s somatic cells can be reprogrammed to ES cells, which subsequently can be differentiated into oocytes or sperm. Thus, infertile patients might be able to have children that are genetically their own. Additionally, hESC-derived eggs could be employed in the generation of SCNT-derived ES cells, thereby reducing the ethically problematic demand for donated human eggs.

Although it seems clear that primate SCNT-derived pluripotent cells are identical to ES cells derived from fertilized embryos in terms of transcriptional activity and potential to give rise to diverse cell types, a central question remains: are these cells epigenetically equivalent to ES cells? We found that expression levels of imprinted genes in CRES cell lines were remarkably similar to controls. Methylation analysis of IGF2/H19 and SNURF/SNRPN ICs demonstrated the presence of both methylated and unmethylated alleles in CRES cell lines, reflecting maintenance of normal differentially methylated patterns. In addition, detailed allele-specific expression based on the sequence polymorphisms demonstrated that both NDN and H19 were expressed monoallelically in CRES cells. Similar to their IVF-derived counterparts, CRES cell lines demonstrated relaxed biallelic expression of IGF2. This is in agreement with our previous findings that monkey ES cells maintain normal imprinting in NDN and SNRPN but relaxed imprinting in IGF2 [11, 13]. Expression of H19 was variable with monoallelic expression in some ORMES cell lines while other analyzed lines expressed both alleles. We also previously showed that monkey IVF produced blastocysts exhibit normal paternal expression of IGF2 suggesting that abnormal biallelic expression of this gene in ES cells is likely acquired during isolation and culture [11].

High levels of TERT expression and significant elongation of telomere length in CRES cells relative to normal donor fibroblasts indicated efficient reprogramming of proliferative potential to an early embryonic state. Our study also demonstrated that undifferentiated female CRES cells, similar to their IVF-derived counterparts underwent X-inactivation. X chromosome inactivation is random in the embryonic lineage but in the extra-embryonic lineage the paternal chromosome is preferentially silenced. Thus, reprogramming of female nuclear donor cells could be less efficient due to faulty reactivation and subsequent nonrandom inactivation of the paternal X chromosome that is active in 50% of the somatic cells. Indeed, random X-inactivation observed in the placenta of aborted cloned cattle fetuses suggest that at least some abnormalities in cloning offspring are due to aberrant recapitulation of X chromosome reactivation [42].

**CONCLUSION**

Overall, our observations are consistent with the conclusion that oocyte-induced reprogramming of primate somatic cells results in complete erasure of somatic memory and the resetting of a new ES cell-specific epigenetic state. As pointed out earlier, induction of iPSCs cells via viral-mediated integration and overexpression of multiple copies of exogenous genes raises serious safety concerns that may preclude the use of such cells for therapeutic purposes. In contrast, oocyte-induced reprogramming is based on epigenetic modifications that precede and prepare expression of endogenous pluripotency genes. Thus, SCNT provides a paradigm for identification of natural epigenetic factors in the egg that accompany nuclear reprogramming and promotes utilization of these factors for direct reprogramming.

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**DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST**

The authors indicate no potential conflicts of interest.
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