Rhesus Monkeys Produced by Nuclear Transfer

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ABSTRACT

Genetically identical nonhuman primates can provide a powerful animal model for gene therapy and research activities where the physiological parameters directly or indirectly under study are heritable. Here we demonstrate that nuclear transfer is a viable technology for the production of identical rhesus macaques. Oocytes recovered from gonadotropin-treated females were enucleated by aspiration of the first polar body and underlying ooplasm, then activated by cycloheximide exposure. Individual diploid blastomeres, recovered from in vitro-fertilization-produced embryos (either fresh or frozen-thawed) and used as nuclear donors, were injected under the zona pellucida of enucleated (chromosome-free) oocytes and fused by electric pulses. The reconstituted embryos were cocultured on buffalo rat liver cells before cryostorage and transfer to synchronized host mothers. Of the 9 females receiving a total of 29 reconstituted embryos, 3 became pregnant, with two live births resulting, one male and one female. The parentage of both infants was established unequivocally by genotype analysis at 7 highly variable short tandem repeat loci.

INTRODUCTION

Genetically identical, cloned animals are of tremendous importance in contemporary biology since physiological parameters often vary with regard to the genetic relationships among the animals under study [1]. Such an animal resource is analogous to the utility of inbred strains of mice or to human identical twins in biomedical research and can be used advantageously in genetic studies on the control of mammalian development, in the establishment of biomedical models for human disease [2], and in gene therapy research. The production of cloned animals by nuclear transfer has been successful in rodents, rabbits, and several domesticated species [3]. McGrath and Solter [4] were the first to describe a reliable technique in the mouse employing zygote pronuclei as nuclear donors in a virus-mediated, cell fusion protocol. Soon thereafter, the first normal lambs [5] and cattle [6] were produced by transfer of cleavage-stage blastomere nuclei into enucleated eggs. Recent breakthroughs in nuclear transfer technology have greatly increased the feasibility of propagating valuable domesticated animals, such as transgenics or animals with exceptional agricultural attributes. Sheep were produced using an established, cultured cell line of the embryonic disk from embryos on Day 9 of gestation in vivo [7] and, more recently, from cells recovered and cultured from adult mammary gland [8].

Nonhuman primates are often the preferred animal model for addressing many questions pertaining to developmental biology and biomedical research because of their genetic and physiological similarity to humans [9]. However, the ability to obtain oocytes and embryos from these species has been limited by cost concerns and access to the animal resource. More recently, in vitro fertilization (IVF) technology in the rhesus macaque has benefited from the availability of human recombinant gonadotropins for ovarian stimulation, making repeated use of females practical [10, 11]. Furthermore, the generation of presumptive embryonic stem cell lines from primates, including the rhesus macaque, provides a potential, unlimited source of donor nuclei for nuclear transfer [12]. The approach employed here to produce rhesus monkeys by nuclear transfer is schematically presented in Figures 1 and 2.

MATERIALS AND METHODS

Oocyte Recovery, IVF, and Embryo Culture

Adult female rhesus monkeys exhibiting normal menstrual cycles were down-regulated with a GnRH antagonist followed by injections of human recombinant gonadotropins as described elsewhere [10, 11]. Rhesus monkeys were pretreated for 7 days with a GnRH antagonist (Antide; Ares Serono, Randolph, MA; 1.0 mg/kg BW, s.c., once daily); the pretreatment continued during administration of either recombinant human FSH alone (R-hFSH; Ares Serono; 30 IU, i.m., twice a day) or sequential treatment with R-hFSH (for 6 days) followed by R-hFSH+h-LH (Ares Serono; 30 IU each, i.m., twice a day, for 1–3 days). Ovarian response was monitored from circulating levels of estradiol and by ultrasonography of follicle number and size. When ultrasonography revealed follicles ≥ 4 mm in diameter, R-hCG (Ares Serono; 1000 IU, i.m.) was given the next day. Twenty-seven hours after the administration of a bolus injection of R-hCG, follicles were aspirated by laparotomy or laparoscopy. The pooled aspirates from left and right ovaries were immediately transferred to the laboratory for recovery of oocytes. Oocytes were evaluated for maturity and transferred to equilibrated culture medium (TALP) [13] with 0.3% BSA overlaid with heavy white mineral oil saturated with TALP.

Rhesus monkey semen was collected by penile electroejaculation [14]. The ejaculate was diluted 1:30 with TALP-Hepes (pH 7.4) and washed twice (360 × g, 7 min). After washing, the final pellet was resuspended in TALP containing 0.3% BSA and incubated in 5% CO2:95% air at 37°C for 4 h. Sperm were activated by exposure to dibutyryl cAMP and caffeine (1 mM each, final concentration) for 1 h immediately before insemination. After a 12–16-h incubation period with sperm (2 × 105 motile cells/
ml) at 37°C, oocytes were examined using Hoffman optics (Hoffman Modulation Optics, Greenvale, NY) for the presence of pronuclei.

Buffalo rat liver cells (BRL 1442; ATCC, Rockville, MD) were thawed in a 37°C water bath, washed once (360 × g, 4 min), and resuspended in CMRL-1066 (Sigma Chemical Co., St. Louis, MO) plus 10% neonatal calf serum (NCS; Sigma). The cells were seeded in 20-μl drops in 35- or 60-mm petri dishes overlaid with mineral oil and incubated at 37°C in 5% CO₂:95% air. Monolayer drop cultures were prepared 2 days before embryo coculture [15].

Zygotes were cocultured for 2–3 days in 20 μl CMRL-1066 + 10% NCS drop cultures on BRL cell monolayers under mineral oil at 37°C in 5% CO₂:95% air. Monolayers were maintained at between 60% and 100% confluency during coculture, and the medium was changed every 24 h. Embryos were evaluated and videotaped daily using a Nikon (Tokyo, Japan) inverted microscope with interference optics.

**Nuclear Transfer**

For nuclear donor preparation, zona pellucidae of IVF-produced cleaving embryos (4–32 cells) were removed by exposure to pronase (Sigma; 5 mg/ml in TALP-Hepes containing 0.3% BSA; 45 sec) followed by trypsin (Sigma) treatment (0.05% in Ca²⁺-, Mg²⁺-free PBS; 30 sec). Individual healthy-looking blastomeres were isolated by repeated pipetting and then transferred into coculture drops to recover for at least 30 min.

Oocyte enucleation (removal of chromosomes) and activation involved initial selection of mature metaphase II (MII) oocytes, within 12 h of follicular aspiration, and removal of cumulus cells by pipetting in TALP-Hepes containing 0.3% BSA and hyaluronidase (1 mg/ml; Sigma). Oocytes were then exposed to the cytoskeletal inhibitor cytochalasin B [4] (7.5 μg/ml; Sigma) for 30 min before transfer to the stage of an inverted microscope fitted with micromanipulators for the enucleation procedure. The outer and inner diameters of the holding and enucleating pipettes were 150/35 μm and 30/25 μm, respectively. Micromanipulation was conducted in TALP-Hepes + 0.3% BSA + cytochalasin B (7.5 μg/ml) overlaid by mineral oil. Oocytes were positioned on the holding pipette with the first polar body at 4 o’clock (Fig. 2, left panel). The enucleating pipette was inserted through the zona pellucida at 3 o’clock, and the polar body along with the underlying ooplasm was aspirated into the pipette. The pipette was slowly withdrawn, forming a cytoplasmic bridge that gradually thinned and eventually broke, allowing removal of approximately 25–35% of the ooplasm immediately adjacent to the polar body without oocyte lysis. Zygotes were recovered approximately 15 h postinsemination and were washed free of supernumerary sperm and exposed to medium containing cytochalasin B [4] before enucleation.

An individual blastomere was aspirated into an enucleating pipette and placed under the zona of an enucleated oocyte (Fig. 2, right panel) or enucleated zygote. These pairs were then cultured in KSOM [16] with 10% NCS. Oocyte pairs were exposed to KSOM containing cycloheximide (7.5 μg/ml) [17], cytochalasin B (7.5 μg/ml) [18], and 10% NCS for 1 h to induce ooplasm activation; they were then placed individually in an electrical field. Membrane fusion was induced by electric pulse (2 continuous square-wave DC pulses of 2.2 kv/cm for 50 μsec in 0.3 M mannitol, 100 μM CaCl₂, 100 μM MgSO₄, and 0.5 mg/ml polyvinylpyrrolidone; BTX Electro-Square-Porator T820 Electroporation System; BTX, Inc., San Diego, CA) following alignment of the membranes to be fused, parallel to the DC electrodes. This was performed either with a holding pipette controlled by a micromanipulator or manually with a pipette. Oocyte pairs received a second and third pulse at hourly intervals. Between pulses, the pairs were held in KSOM containing cycloheximide and cytochalasin B. When enucleated zygotes were used as cytoplasts, only one electrical pulse was employed to induce membrane fusion. All reconstituted embryos were subsequently cocultured on a BRL cell monolayer as described above.

**Embryo Cryopreservation and Transfer**

The cryopreservation, storage, thawing, and transfer of reconstituted embryos to the oviducts of synchronized recipients was as described previously for IVF-produced embryos [19].

**Pedigree Analysis**

Five milliliters of whole blood was collected into EDTA-containing vacutainers and shipped to our nonhuman primate genetics laboratory in San Antonio, TX. High molecular weight DNA was extracted by a salting-out method [20], resuspended in single-strength TE (10 mM Tris-Cl, 0.2 mM EDTA, pH 7.5), and then used as template DNA in polymerase chain reactions (PCR) using commercially available primers (Research Genetics, Huntsville, AL) that

amplify short tandem repeat (STR) loci in human DNA. Primers that amplified homologous, polymorphic loci in rhesus monkeys were identified and optimized for MgCl₂ concentration and buffer pH (PCR Optimizer; Invitrogen, San Diego, CA), as reported earlier [21]. PCR cocktails consisted of 50 ng of each primer (forward and reverse); 200 μM of dATP, dGTP, and dTTP; 2.4 μM of dCTP; 2.5 μM of [³²P]dCTP; 0.5 μl Taq polymerase (Promega, Madison, WI); 50 mM KCl; 10 mM Tris-Cl (pH 8.3); and 15 ng template DNA. Total reaction volume was 15 μl. PCR was performed on an MJ-100 (MJ Research, Watertown, MA) thermal cycler in 96-well microliter trays with mineral oil overlay. Step-cycle conditions began with a 5-min, 94°C denaturation followed by 30 cycles of 1 min/94°C denaturation, 45 sec/55°C annealing, and 1.5 min/72°C extension with 2 sec autoextend per cycle and a final 10-min, 72°C extension. After 1:1 dilution with a formamide loading dye, 4 μl PCR amplification products was loaded into 6% denaturing polyacrylamide gels and electrophoresed for 2.5 h at 60 W. Gels were wrapped in cellophane, vacuum dried for 60 min, inserted into autoradiography cassettes, and exposed to Kodak XAR-5 x-ray film (Eastman Kodak, Rochester, NY) at room temperature for 4–5 days. All 8 individuals involved in ascertainment of correct parentage (putative sire and dam, both nuclear transfer infants and their host mothers, and the enucleated oocyte donors) were genotyped at 7 STR loci (D1S306, D1S215, D2S175, D3S1768, D6S284, D10S1412, D18S858). Correct parentage was ascertained by visual inspection of codominant alleles. The genotype of the sperm donor was “subtracted” from the infant’s genotypes to identify the maternal allele; then the three potential mothers (the host mother, the enucleated oocyte donor, and the donor of the oocyte nucleus) were examined for presence or absence of the maternal allele.

RESULTS

Three cytoplast sources were evaluated during initial experimentation: fresh MII oocytes obtained from gonadotropin-treated monkeys 27 h post-hCG administration and cultured until release of the first polar body; aged MII oocytes (MII oocytes that had failed to fertilize by approximately 20 h after insemination); and zygotes obtained from IVF. A total of 166 reconstituted embryos were produced by nuclear transfer using either fresh or cryopreserved 6- to 16-cell-stage blastomeres as the source of diploid nuclei (Table 1). The survival rate after micromanipulation and

<table>
<thead>
<tr>
<th>Cytoplasmic source</th>
<th>No. of repetitions</th>
<th>No. of oocytes or zygotes</th>
<th>No. enucleated (%)</th>
<th>No. fused (%)</th>
<th>No. cleaving (%)</th>
<th>Overall efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zygote</td>
<td>2</td>
<td>15</td>
<td>11 (73)</td>
<td>10 (91)</td>
<td>9 (90)</td>
<td>42</td>
</tr>
<tr>
<td>MII (aged)</td>
<td>4</td>
<td>37</td>
<td>30 (81)</td>
<td>27 (90)</td>
<td>10 (37)</td>
<td>27</td>
</tr>
<tr>
<td>MII (fresh)</td>
<td>8</td>
<td>114</td>
<td>97 (85)</td>
<td>78 (60)</td>
<td>59 (76)</td>
<td>56</td>
</tr>
</tbody>
</table>

*a* MII, metaphase II.

*b* Underwent at least one uniform cell division.

*c* Corrected for 70% fertilization efficiency.
NUCLEAR TRANSFER IN RHESUS MONKEYS

FIG. 3. Reconstituted embryos (left) after coculture with BRL cells in vitro for 2 days (X200). Rhesus monkey infants (right) produced by nuclear transfer technology.

cell fusion did not differ significantly ($p > 0.05$) among the three groups. Reconstituted embryos involving aged MII oocytes as cytoplasts showed a reduced capacity to initiate cleavage compared to the other two groups. Moreover, during 1–4 days of culture, the developmental potential of reconstituted embryos produced with aged MII oocytes ($n = 37$) was significantly lower than that of the fresh MII oocyte group ($n = 38$; 26% vs. 55% at 8- to 16-cell stage, respectively, $p < 0.05$). The overall efficiency in producing cleaving, reconstituted embryos was highest when fresh MII oocytes were used as cytoplasts. Therefore, fresh MII oocytes were selected as the cytoplasmic recipients during subsequent attempts to produce live animals. In these attempts, efforts were focused on producing live young and not necessarily on producing genetic clones.

Of the fifty-nine 2- to 16-cell-stage reconstituted embryos (Fig. 3, left) that appeared morphologically normal, 29 were transferred into the oviducts of synchronized recipients ($n = 9$) during spontaneous menstrual cycles (Table 2). One animal received 6 embryos while the remaining 8 received 2 or 3 embryos. Animals were monitored for pregnancy by measuring circulating levels of estradiol and progesterone as well as by conducting uterine ultrasonography. Three biochemical pregnancies resulted, one of which was lost at approximately 30 days of gestation. The remaining two pregnancies culminated in the birth of one male (19255) and one female (19235) at 166 and 149 days gestation, respectively (Fig. 3, right). The male weighed 440 g and is normal and healthy. The female infant weighed 300 g and is also healthy despite the premature delivery. No clinical abnormalities were observed in a postnatal examination.

The parentage of both nuclear transfer infants was ascertained by genetic typing with 7 PCR-amplified DNA markers. For the male infant (19255), the enucleated oocyte donor was excluded at 3 independent STR loci and the host mother (13646) was excluded at 2 loci as the mother (Table 3; Fig. 4). For the female infant (19235), the enucleated oocyte recipient (16426) was excluded as the mother at 5 loci, and the host mother (16150) was excluded as the mother at 6 loci. In contrast, all 7 loci included both the putative sire and the putative mother (14893), as would be expected under Mendel’s rules if they were the true parents. These data definitely indicate that the putative sire (the sperm donor) and the putative mother (the female from which the nucleus was harvested) were the true biological parents of both nuclear transfer infants, which are genetically unrelated to both their respective host mothers and to the enucleated oocyte donor.

<table>
<thead>
<tr>
<th>Stage of donor nuclei</th>
<th>4–8 cell</th>
<th>12 cell</th>
<th>8–16 cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of host mothers</td>
<td>3</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>Status of reconstituted embryos</td>
<td>frozen</td>
<td>fresh</td>
<td>frozen</td>
</tr>
<tr>
<td>Number of pregnant host monkeys</td>
<td>2</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Number of fetuses/total embryos transferred (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of live births</td>
<td>2/9 (22)</td>
<td>1/10 (10)*</td>
<td>0/10 (0)</td>
</tr>
</tbody>
</table>

* A singleton pregnancy was established but subsequently lost at about 30 days of gestation.
TABLE 3. Results of 7 STR markers used for maternity determination of nuclear transfer (NT) infants 19255 and 19235.*

<table>
<thead>
<tr>
<th>STR marker</th>
<th>NT infant 19255</th>
<th>NT infant 19235</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Dam</td>
<td>Dam</td>
</tr>
<tr>
<td></td>
<td>Enucleated</td>
<td>Enucleated</td>
</tr>
<tr>
<td></td>
<td>oocyte donor</td>
<td>oocyte donor</td>
</tr>
<tr>
<td></td>
<td>Host mother</td>
<td>Host mother</td>
</tr>
<tr>
<td>D1S306</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>D1S215</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>D2S175</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>D3S1768</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>D6S284</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>D10S1412</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>D18S858</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Total number exclusions</td>
<td>2</td>
<td>3</td>
</tr>
</tbody>
</table>

*For each locus, the paternal allele was identified as that allele shared by NT infant 19255 (left) or 19235 (right) with their father, 14609, and the remaining allele was identified as the maternal allele; the 3 potential mothers (host mother, enucleated oocyte donor, and nuclear donor) were excluded (−) if they lacked the maternal allele or were included (+) if they possessed the maternal allele; the nuclear donor (center) is the only potential mother never excluded at any locus and is therefore the mother of both infants (see Fig. 4, A and B for comparison and text for further discussion).

DISCUSSION

Successful embryo reconstitution by nuclear transfer requires nuclear reprogramming that is critically dependent upon precise interactions between the donor nucleus and the recipient cytoplasm [3, 22]. Interruptions in the normal pattern of DNA synthesis in reconstituted embryos, which is regulated by metaphase-promoting factor (MPF) activity in the recipient cytoplasm, can induce chromosomal damage and suppress subsequent development [23, 24]. Such effects of the donor cell cycle stage on developmental potential can be largely overcome by transferring the donor nucleus into preactivated cytoplasm as exemplified by the three cytoplasts utilized in this study. Although MPF levels should be low in all three cytoplasmic sources after activation, as induced by either sperm or electrical stimulation in combination with cycloheximide and cytochalasin B exposure, the fresh MII oocyte proved the most effective candidate as a cytoplast. One technical disadvantage in using the mature oocyte is the necessity of confirming complete chromatid removal. In the zygote, pronuclei are highly visible and easily removed. However, DNA removal in the MII oocyte is an essentially blind process, although a staining step can be instituted to ensure complete removal [25].

Synchronization between the nuclear donor and recipient cytoplasm or between reconstituted embryos and host mothers for embryo transfer is a significant challenge in primates, where the number of available animals is limiting. We utilized cryobiology to circumvent this problem. In evaluating embryo cryosurvival, we routinely thawed embryos destined to be used as nuclear donors at least 24 h prior to the reconstitution procedure. This allowed time for cell division and embryo recovery from cryodamage. However, some blastomers were considered unusable, on the basis of morphological abnormalities or partial lysis, despite reports that nonviable blastomers may still contain a viable nucleus [26, 27]. While our results confirm the feasibility of using frozen-thawed embryos as nuclear donors, we cannot conclude that the developmental potential of embryos reconstituted with cryopreserved nuclei is equivalent to that of embryos resulting from fresh nuclei transfer. Cryopreservation was also critical in our efforts to synchronize host mothers with developing reconstituted embryos. In the absence of a cryopreservation program, limitations in ovulation detection methods as well as inherent variability in menstrual cycle length would require maintenance of a very large colony of recipients to ensure synchrony. Of course, the cryopreservation of both the donor nucleus and the reconstituted embryo increases the risk of cryoinjury.

Another approach to overcome limitations imposed by the donor nucleus source is to use cultured cell lines from the inner cell mass [28] or embryonic disk cells [7].

FIG. 4. Autoradiographs of 2 STR markers used to identify nuclear donor female 14893 as the true mother of both nuclear transfer infants. From left to right, identities of individuals in the pedigrees at top are: host mother 13646, nuclear transfer (NT) infant 19255, sire 14609, mother (nuclear donor) 14893, NT infant 19235, host mother 16150, enucleated egg donor 16426, and female 8090 (mother of 16426). Left) Locus D3S1768 excluded enucleated egg donor 16426 for both infants 19255 and 19235, and also excluded host mother 16150 for infant 19235. Right) Locus D6S284 excluded host mother 13646 and the enucleated egg donor 16426 for infant 19235, and excluded the host mother 16150 for infant 19235.
cently, a putative embryonic stem cell line was isolated and characterized in the rhesus monkey [12]. The use of undifferentiated cell lines if totipotent not only provides unlimited numbers of nuclear donor cells but, perhaps most importantly, also permits genetic manipulations prior to embryo reconstitution. An in vitro evaluation of the developmental potential of reconstituted rhesus monkey embryos, while of interest, was impractical because of the limited number of embryos available and our primary focus on producing live young after embryo transfer. After IVF in the rhesus macaque, 50–60% of zygotes reach the hatching blastocyst stage [29]. Clearly, the in vivo developmental capability of reconstituted embryos was proven for the first time in the present study with 3 pregnancies initiated and two live births following 9 transfers. Finally, genetic analysis of these infants using STR markers provided definitive proof of the success of nuclear transfer technology.

ACKNOWLEDGMENTS

The authors recognize the contributions of Dr. Mary B. Zelinski-Wooten and members of our ART core laboratory; Manfred Alexander and Dana K. Persons for technical assistance; William Baughman and the surgical team; Dr. David Hess and the Hormone Assay services core laboratory; Vince Warren for photographic assistance; Drs. Barry Bavister, John Eppig, Robert Foote, and Richard Schultz for manuscript review; and Ares Advanced Technology, Inc. (Ares Serono) for the Antide and recombinant human gonadotropins utilized in these studies.

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