Cloning of non-human primates: the road "less traveled by"

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ABSTRACT Early studies on cloning of non-human primates by nuclear transfer utilized embryonic blastomeres from preimplantation embryos which resulted in the reproducible birth of live offspring. Soon after, the focus shifted to employing somatic cells as a source of donor nuclei (somatic cell nuclear transfer, SCNT). However, initial efforts were plagued with inefficient nuclear reprogramming and poor embryonic development when standard SCNT methods were utilized. Implementation of several key SCNT modifications was critical to overcome these problems. In particular, a non-invasive method of visualizing the metaphase chromosomes during enucleation was developed to preserve the reprogramming capacity of monkey oocytes. These modifications dramatically improved the efficiency of SCNT, yielding high blastocyst development *in vitro*. To date, SCNT has been successfully used to derive pluripotent embryonic stem cells (ESCs) from adult monkey skin fibroblasts. These remarkable advances have the potential for development of human autologous ESCs and cures for many human diseases. Reproductive cloning of nonhuman primates by SCNT has not been achieved yet. We have been able to establish several pregnancies with SCNT embryos which, so far, did not progress to term. In this review, we summarize the approaches, obstacles and accomplishments of SCNT in a non-human primate model.

KEY WORDS: *cloning, nuclear transfer, primate, reprogramming*

Introduction

Nuclear transfer (NT) efforts involving the nonhuman primate model commenced just prior to the turn of the millennium. By this time, the production of cloned offspring by nuclear transfer had been achieved in rodents, rabbits, and several species of domesticated animals using totipotent embryonic cells as the nuclear donors (McGrath and Solter, 1983; Prather *et al.*, 1987; Sun and Moor, 1995; Willadsen, 1986). These pioneering NT efforts provoked the start of cloning in the primate model, which is the research path that our laboratory chose; the road "less traveled by". The SCNT approach would enable production of genetically identical or genetically modified animals invaluable for biomedical research and the study of human diseases. During initial attempts to study NT in the rhesus macaque (*Macaca mulatta*) model, individual blastomeres isolated from *in vitro* fertilized embryos served as nuclear donor cells (Meng *et al.*, 1997). These blastomeres, isolated from cleavage stage embryos, were transferred into the perivitelline space of enucleated, metaphase-II stage (MII) oocytes collected following controlled ovarian stimulation (Zelinski-Wooten *et al.*, 1995). Oocyte activation was induced immediately prior to membrane fusion by a series of electrical

pulses. Resulting embryos were co-cultured on a monolayer of buffalo rat liver cells (Meng *et al.*, 1997; Zhang *et al.*, 1994). When 8- to 16-cell stage blastomeres were utilized as the nuclear donor cells, the results were very promising. Embryonic development to the blastocyst stage was comparable to *in vitro* fertilized (IVF) control embryos. Transfer of these NT embryos into surrogate animals generated four pregnancies and ultimately culminated in the live births of two healthy infants, Neti and Ditto (Meng *et al.*, 1997). Following STR analysis, used to determine the parentage of each infant, it was definitive that both Neti and Ditto were the result of successful NT technology.

SCNT was pioneered in 1996 with the production of the first live cloned offspring (Campbell *et al.*, 1996; Wilmut *et al.*, 1997). This groundbreaking achievement unveiled the possibility of "resetting" the nuclei of differentiated mammalian cells back to an embryonic state, using factors present in the unfertilized oocyte to

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help with this reprogramming process. Studies were soon underway to examine the feasibility of cloning nonhuman primates by SCNT. It remains unknown which ooplasmic factors in the oocyte contribute to nuclear reprogramming during SCNT, but it is believed that these unidentified factors are present within the cytoplasm of MII stage oocytes. Initial SCNT studies in the monkey resulted in low blastocyst yield with a majority of embryos arresting at the cleavage stage (Mitalipov *et al.*, 2002). Our follow up studies revealed incomplete nuclear remodeling, particularly failure to undergo nuclear envelope breakdown (NEBD) and premature chromosome condensation (PCC) following fusion of donor cells with enucleated oocytes (Mitalipov *et al.*, 2002; Mitalipov *et al.*, 2007). We reasoned that this phenomenon could be linked to poor subsequent development of SCNT embryos. Lack of or incomplete NEBD and PCC are generally linked to a decrease in the activity and level of maturation-promoting factor (MPF). MPF is a heterodimeric protein made up of cyclin B and cyclin-dependent kinase, which has been shown to maintain meiotic and mitotic cell phases by phosphorylating various targets within the cytoplasm, including several lamins (Peter *et al.*, 1990; Ward and Kirschner, 1990). When an oocyte is arrested at metaphase of the second meiotic division, elevated MPF activity is detectable within the oocyte (Szollosi *et al.*, 1988). During fertilization, the entry of sperm into the oocyte triggers intracellular calcium oscillations and ultimately results in MPF degradation (Szollosi *et al.*, 1988). The decrease in MPF activity releases the oocyte from meiotic arrest, enabling it to complete meiosis and initiate mitotic cleavage divisions (Susko-Parrish *et al.*, 1994). We introduced several modifications to our SCNT procedures designed to preserve MPF activity in monkey oocytes that supported improved nuclear remodeling immediately upon introduction of the donor nucleus (Mitalipov *et al.*, 2007). Moreover, SCNT embryos produced by the modified approach progressed to the blastocyst stage at much higher rates suggesting a beneficial role of nuclear remodeling on reprogramming. To date, little is known as to what role MPF and other unidentified MII ooplasmic factors may play during nuclear reprogramming of adult somatic cells. Thus, it is critical to continue investigating various aspects of SCNT to better understand this seemingly complex cytoplasmic-mediated reprogramming process. We describe here our current SCNT steps and recent efforts to produce rhesus embryonic stem cells and pregnancies from adult somatic cells.

Oocyte production

Controlled ovarian stimulation in non-human primates is routinely induced by administration of exogenous gonadotropins (Sotomaru *et al.*, 2009; Zelinski-Wooten *et al.*, 1995). In rhesus macaques, starting at menses (day 1-4 of their menstrual cycle), females receive twice-daily injections of recombinant human follicle stimulating hormone (FSH; Organon; 30 IU, im) for 8 days and recombinant human luteinizing hormone (LH; Ares Serono; 30 IU, im) on days 7-8 of the stimulation protocol. In addition, the animals receive a gonadotropin releasing hormone (GnRH) antagonist (Acyline; NIH/NICHD; 0.075 mg/kg body weight, sc) and human chorionic gonadotropin (hCG; Serono; 1,000 IU, im) on day 7 during the stimulation. Serum estradiol and progesterone measurements along with ultrasonographic scans are commonly performed to monitor follicular response. Oocytes are recovered via laparoscopic follicular aspiration, stripped of cumulus cells, and examined for determination of developmental stage (Zelinski-Wooten *et al.*, 1995). Only oocytes that have progressed to the MII stage of meiosis, evident by the presence of the first polar body in the perivitelline space, are used for nuclear transfer experiments.

In earlier reports, it was common to collect oocytes 27-32 hours post-hCG (Wolf *et al.*, 1999). In our current protocols, we recover oocytes 35-36 hours post-hCG to allow oocyte maturation and spindle migration away from the polar body, thus making it easier to visualize and enucleate the meiotic spindle. In the common marmoset, ovarian stimulation commonly entails a single, daily injection of 50 IU FSH for 11 days, followed by the administration of 75 IU hCG on the 12th day of the stimulation protocol (Sotomaru *et al.*, 2009). Germinal vesicle-stage oocytes, enclosed in cumulus cell-oocyte complexes, are recovered 16 hours post-hCG injection and placed in maturation medium for approximately 22-24 hours (Sotomaru *et al.*, 2009).

Oocyte availability has always been a major limitation in the nonhuman primate model. To help one understand just how limited this resource is, in 2009 we subjected 113 females to ovarian stimulations. Five of these stimulation cycles were cancelled prior to oocyte retrieval. On average, 43 oocytes were recovered per female, of which an average of 13 and 14 were mature MII and metaphase I stage, respectively. Only MII stage oocytes are suitable for SCNT, so this data indicates that less than one-third (13/43, 30%) of all oocytes recovered are available for SCNT. While the overall number of oocytes collected per stimulation is considerably higher compared to our results reported nearly a decade ago (Wolf *et al.*, 1999) (43 oocytes versus 16.7 oocytes), mature oocyte availability is still limited by inefficient *in vitro* maturation (IVM) protocols in the nonhuman primate model, which has been thoroughly reviewed by Zheng *et al.* (Zheng, 2007).

Oocyte enucleation

Oocyte enucleation involves the removal of chromosomes, contained within the meiotic spindle, from unfertilized MII stage oocytes. Using the naked eye or low magnification stereomicroscopes, one cannot see these individual chromosomes or even the meiotic spindles that hold these chromosomes in place. Initial attempts to visualize this nuclear DNA utilized fluorochrome bisbenzimide (Hoechst 33342) staining and ultraviolet (UV) exposure, the standard enucleation procedure at that time (Mitalipov *et al.*, 2002). Intact MII stage oocytes were incubated for 5 minutes with 5 µg/ml Hoechst 33342, placed in 30 µl of Talp HEPES media (Bavister and Yanagimachi, 1977) (TH3; with 3 mg/ml BSA) containing 5 µg/ml cytochalasin B, then incubated for approximately 10-15 minutes prior to enucleation. During the enucleation procedure, the first polar body and approximately 10% of the adjacent cytoplasm is removed via aspiration directly into the enucleation pipette. Confirmation of chromosome removal was accomplished by exposing the enucleation pipette to UV wavelengths. Low blastocyst development following such approach caused us to re-evaluate each step of the SCNT protocol. While Hoechst 33342 is considered to be a live cell DNA dye, it may adversely affect oocyte and embryo viability (Velilla *et al.*, 2002). It is likely that Hoechst 33342/UV exposure may hinder

the developmental and reprogramming potential of the primate oocytes by inducing premature oocyte activation and MPF degradation, or by adversely reacting with mitochondrial DNA (Byrne *et al.*, 2007; Mitalipov *et al.,* 2007). Several non-invasive, "blind" enucleation alternatives, including 'squish' (Simerly *et al.*, 2004) or 'one-step micromanipulation' (OSM) (Zhou *et al.*, 2006) were also tested. The main concern with these approaches is that 100% enucleation efficiency cannot be guaranteed. We tested a new, non-invasive approach, which utilizes the latest spindle imaging technology. The Oosight spindle imaging system (CRI Oosight™) makes use of the birefringence properties of the microtubules, which hold the chromosomes in place within the meiotic spindle. In clinical IVF practice, previous versions of polarized microscope (Polscope) technology have been used to examine digital images of meiotic spindles (Wang *et al.*, 2001). However, the current Oosight system allows real-time microscopy and manipulation during enucleation of meiotic chromosomes in primate oocytes (Byrne *et al.*, 2007; Sparman *et al.*, 2009). Plus, this allows us to consistently achieve 100% efficiency during this spindle removal procedure, without any adverse side-

effects. After incorporating spindle imaging technology into our SCNT protocols, a significant increase in blastocyst formation rate (from 1% to 16%) was achieved (Byrne *et al.*, 2007).

Donor cell transfer and fusion

Both the reprogramming efficiency of nuclear donor cells and subsequent embryonic development following SCNT are believed to be affected by several factors, including: the source of donor nuclei, the cell cycle stage of the recipient oocyte and the nuclear donor cell, as well as the oocyte activation method utilized. In the mouse model, the effect of different types of donor cells on SCNT cloning efficiency has been evaluated (Inoue *et al.*, 2003). Primary cultures of fetal and adult fibroblasts are commonly used as nuclear donor cells, although other cell types including myoblasts, neurons, and Sertoli cells have also been tested (Powell *et al.*, 2004). Numerous factors may affect the nuclear donor cell ability to support development following SCNT (e.g. isolation procedures and *in vitro* culture conditions, age of donor animals, etc.). In addition, it appears that lower passage cells have an advantage over higher passage cells (Powell *et al.*, 2004). Using cynomolgus monkeys, higher blastocyst development was reported following the use of fetal fibroblasts compared to other donor cell types (Okahara-Narita *et al.*, 2007). In the rhesus model, both fetal and adult fibroblast cell lines have been successfully implemented as nuclear donor cells for SCNT and routinely produce blastocysts (Byrne *et al.,* 2007; Mitalipov *et al.,* 2007; Sparman *et al.*, 2009; Zhou *et al.*, 2006). Direct comparison between three different primary cultures of adult, rhesus skin fibroblasts revealed a significant (three-fold) difference in subsequent embryonic development to the blastocyst stage (Sparman *et al.*, 2009). This finding suggests that certain donor cells may possess a higher reprogramming capability and efficiency compared to other cells, although the mechanisms for this difference are not yet clear. It is thought that the differences in epigenetic characteristics amongst different nuclear donor cells may affect the cell's reprogramming and developmental potential (Humpherys *et al.*, 2001).

During standard primate SCNT (represented in the schematic in Fig. 1), cell fusion is induced using electroporation (Byrne *et al.*, 2007; Mitalipov *et al.*, 2002). In brief, oocyte-donor cell complexes are exposed to two 50 μs DC pulses of 2.7 kV/cm in 0.25 M D-sorbitol buffer containing 0.1 mM calcium acetate, 0.5 mM magnesium acetate, 0.5 mM HEPES and 1 mg/ml fatty acid-free BSA. Approximately 30 minutes following electroporation, fusion is confirmed by the absence of the donor cell in the perivitelline space of the reconstructed embryo. Based on recent results obtained in our laboratory during the spindle-chromosomal complex transfer between MII stage oocytes (Tachibana *et al.*, 2009), we determined that exposure to the electrofusion regimen can trigger premature activation and subsequent resumption of meiosis. In order to sustain higher MPF activity before activation, we incorporated an alternative fusion technique utilizing an extract
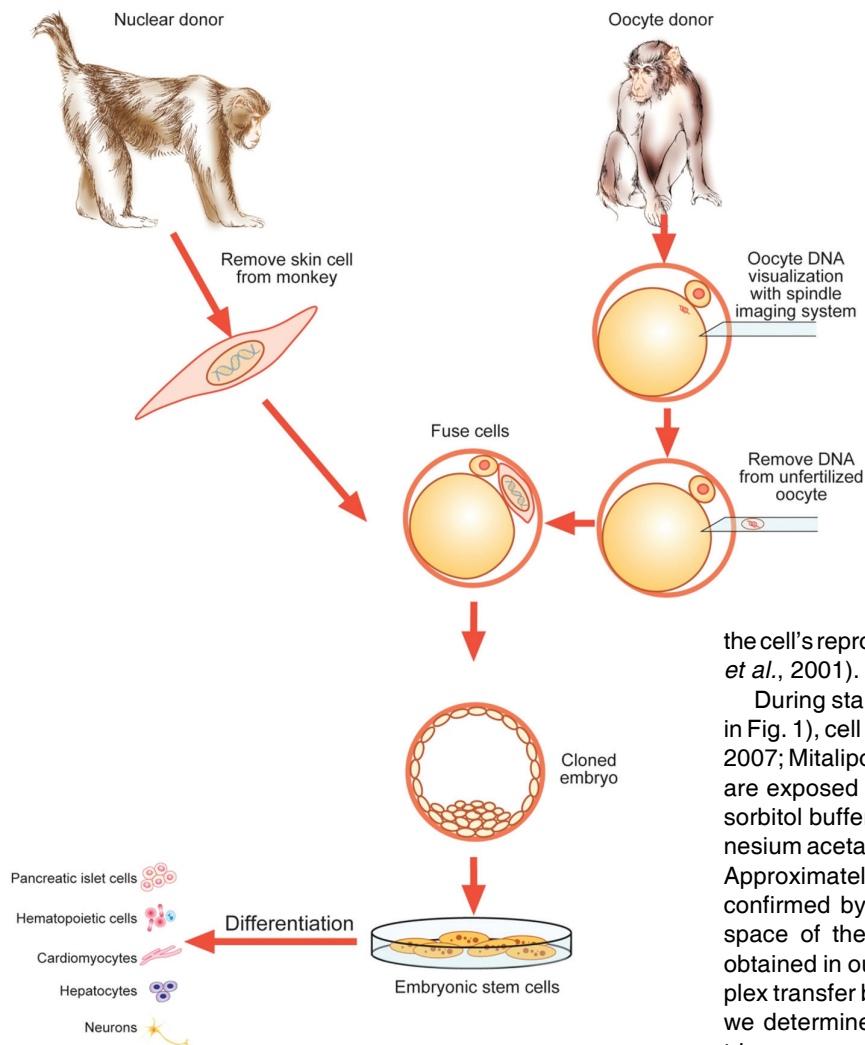

Fig. 1. A schematic of the somatic cell nuclear transfer procedure to isolate pluripotent embryonic stem cell lines.

from Sendai virus (SeV; http://www.cosmobio.co.jp) (Tachibana *et al.*, 2009). During the SCNT procedure, the nuclear donor cells (e.g. fibroblasts) are immersed in the SeV extract (30-60 seconds) prior to being transferred into the perivitelline space. Cell membrane fusion between the nuclear donor cell and the oocyte typically occurs within 20-30 minutes. Fusion rates following SeV are similar or higher than what we previously reported following the use of electroporation (Byrne *et al.*, 2007; Sparman *et al.*, 2009).

Oocyte activation

Mammalian oocytes naturally go through a process called "activation" following fertilization to help the egg prepare for subsequent development. Successful oocyte activation promotes an increase in metabolic activity and releases the oocyte from meiotic arrest, thus enabling the resumption of the mitotic cell cycle (Susko-Parrish *et al.*, 1994). During the SCNT procedure, as no sperm come into contact with the oocyte, an artificial activation method is required. Such artificial oocyte activation can be achieved by the use of chemical treatments which successfully induce calcium oscillations, much like those detected following normal fertilization with sperm. Examples of several proven methods of oocyte activation in the monkey model include treatment with ionomycin/6-dimethylaminopurine (DMAP), electroporation/cycloheximide/cytochalasin B, and ionomycin/roscovitine/cytochalasin B (Mitalipov *et al.*, 2001). In our lab, reconstructed oocytes are typically activated 2 hours post-fusion, to allow nuclear remodeling to occur (Byrne *et al.*, 2007; Sparman *et al.*, 2009). Following the standard SCNT activation protocol (Mitalipov *et al.*, 2007), all reconstructed oocytes are exposed first to 5 µm ionomycin for 5 minutes followed by a 5 hour incubation in 2 mM 6-dimethylaminopurine (DMAP). Treatment with DMAP prevents completion of meiosis and extrusion of the second polar body (Mitalipov *et al.*, 2001). We refer to this type of activation procedure as "regular" activation. One recent change to our SCNT activation protocol has been the testing of an "instant" activation technique, in which the 2 hour incubation period after fusion (prior to the start of activation) has been completely eliminated. In such a case, fused embryos are immediately activated with DMAP and placed in culture. Our recent comparison of blastocyst development following the "regular" versus "instant" activation demonstrated the beneficial effect of the latter treatment (Table 1).

Histone deacetylase inhibitor treatment

Recent studies indicate that cloning efficiency after SCNT in the mouse, pig and bovine model could be improved with trichostatin A (TSA) treatment, a histone deacetylase inhibitor (Ding *et al.*, 2008; Kishigami *et al.*, 2006; Li *et al.*, 2008). Histone deacetylation is one of the commonly studied epigenetic modifications. Inefficient nuclear reprogramming of the donor cell during SCNT may be attributed to abnormal epigenetic modification. It has been shown that treatment with TSA reduces the activity of histone deacetylase, thus resulting in an increased level of histone acetylation and enhanced regulation of DNA methylation (Yoshida *et al.*, 1990). In theory, enhancing epigenetic regulation may positively affect the reprogramming of the somatic genome

TABLE 1

DEVELOPMENT OF MONKEY SCNT EMBRYOS FOLLOWING INSTANT OR REGULAR ACTIVATION

Activation	# of replicates	# of oocytes	Fusion (%)	Pronuclei (%)	8-cell (%)[*]	Morulae (%)[*]	Blastocyst (%)[*]
Instant	7	46	45 (98%)	45 (100%)	40 (89%)	22 (49%)	9 (20%)
Regular	7	42	42 (100%)	42 (100%)	39 (93%)	16 (38%)	5 (12%)

[*]Percentages are calculated based on the number of embryos forming pronuclei.

TABLE 2

EFFECT OF TSA ON DEVELOPMENT OF MONKEY SCNT EMBRYOS

TSA	# of replicates	# of oocytes	Fusion (%)	Pronuclei (%)	8-cell (%)[*]	Morulae (%)[*]	Blastocyst (%)[*]
Control	9	90	85 (94%)	84 (99%)	81 (96%)	47 (56%)	4 (5%)[a]
37.5 nM TSA	32	243	242 (99%)	242 (100%)	229 (95)	148 (61%)	43 (18%)[b]

[a,b] Different superscripts indicate significant difference (p<0.05). [*]Percentages are calculated based on the number of embryos forming pronuclei. Both groups were activated by the "regular" activation protocol.

TABLE 3

EMBRYO TRANSFERS WITH MONKEY SCNT EMBRYOS GENERATED FROM ADULT SOMATIC CELLS

Treatment	# of recipients	# of embryos transferred	# of pregnancies (# of clinical pregnancies)
Control	12	55	0
With TSA	10	67	6 (5)[*]

Recovered oocytes were divided into two groups and both groups were activated by the "regular" activation protocol. [*]One biochemical pregnancy was confirmed by the continual rise in E2 and P4 levels.

(Li *et al.*, 2008). We tested the effect of TSA exposure during the SCNT procedure in the primate model. Fused SCNT embryos were first incubated with 37.5 nM TSA for 5 hours during DMAP treatment. Activated embryos were then transferred into embryo culture media containing 37.5 nM TSA for an additional 17-19 hours. In total, SCNT embryos were exposed to TSA for 22-24 hours. Based on our recent results, TSA treatment significantly improved the percentage of SCNT embryos that reached the blastocyst stage (Table 2). Moreover, the subsequent *in vivo* development following transfer of SCNT blastocysts into recipients was also dramatically improved in the TSA group (Table 3). While further testing could be required to optimize the proper concentration and timing of TSA supplementation, we have modified our current SCNT procedures to include the TSA.

ES cell derivation and embryo transfer

In the mid-1990's, Dr. James Thomson of the Wisconsin National Primate Research Center reported the isolation of the first embryonic stem cell (ESC) lines from rhesus macaque blastocysts (Thomson *et al.*, 1995). These primate ESCs were maintained in an undifferentiated state while cultured on feeder
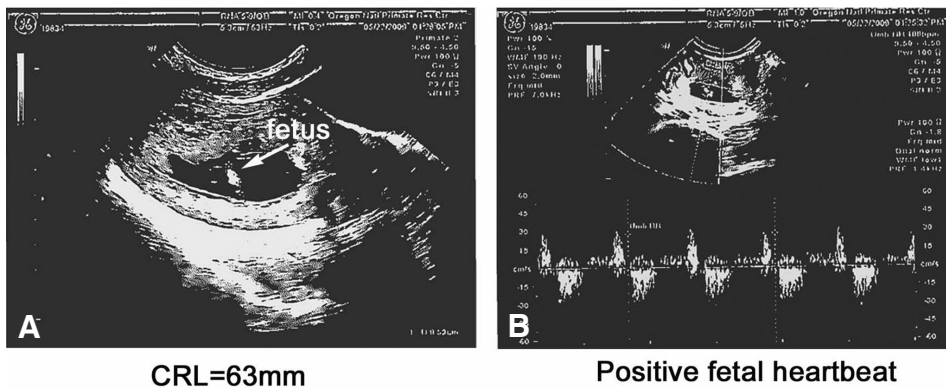
Fig. 2. Ultrasound images of the SCNT pregnancy. (A) *Ultrasonography revealed a fetus with a crown-rump length (CRL) of 63 mm in the gestational sac.* **(B)** *A positive fetal heartbeat was detected in the SCNT fetus.*

layers but retained the potential to differentiate into cells representing all three germ cell layers (endoderm, mesoderm, and ectoderm) (Thomson *et al.*, 1995; Thomson *et al.*, 1996). Prior to the establishment of rhesus ESCs, only mouse ESCs were available (Martin, 1981). Primate ESCs provided an exciting model for better understanding human development and disease *in vitro* and *in vivo*. As one would expect, following protocols and markers developed for monkey ESCs, studies were soon carried out with human embryos that culminated in the successful derivation of human ESCs (Thomson *et al.*, 1998).

Coupling of ESC research with SCNT technology also catalyzed the development of new strategies for reprogramming somatic cells and deriving patient-matched pluripotent cells (Byrne *et al.*, 2007). The ability to derive pluripotent cells that are genetically identical to individual patients, holds tremendous potential to produce cells that will not be rejected by the patient's immune system and that may help prevent numerous degenerative diseases (Drukker and Benvenisty, 2004; McKay, 2000). We employed a modified SCNT approach to produce blastocysts from adult rhesus macaque skin fibroblasts and established two ESC lines from these cloned embryos, designated as CRES-1 and CRES-2 (Byrne *et al.*, 2007). These CRES cell lines are genetically identical to each other in terms of nuclear DNA, since nuclear donor cells employed were from the same adult male. However, these cell lines contain different mitochondrial genomes since oocytes from two different females were used for SCNT. Characterization of these novel SCNT-derived ESC lines confirmed their pluripotency and origin from somatic cells (Byrne *et al.*, 2007).

As for the efficiency of this approach, in our initial study we used a total of 304 oocytes to derive two CRES cell lines; less than a 1% derivation efficiency rate. While the low efficiency rates still leave room for improvement, these results suggest that nuclear reprogramming by SCNT can support derivation of ESCs in higher primates, including humans. In a more recent study, we used adult rhesus female skin fibroblasts for SCNT and derived two additional ESC lines (CRES-3 and CRES-4) (Sparman *et al.*, 2009). Further optimizations and improvements in SCNT resulted in a nearly three-fold higher blastocyst development rate (43%) and ESC derivation rate (29%) as compared to the previous outcomes (Byrne *et al.*, 2007). Of particular interest, the oocytes

that gave rise to CRES-3 and -4 were all recovered from only one rhesus female, subjected to a single controlled ovarian stimulation. These findings reveal that it is now possible to derive a primate stem cell line from as few as 10 oocytes, or less, and that it may be economically and technically feasible to derive patient-matched ESCs for tissue replacement therapy.

The nonhuman primate is clearly an attractive research model for SCNT because of its remarkable similarity to humans, genetically and from a reproductive standpoint. The benefits of producing rhesus monkeys by reproductive cloning for biomedical research are tremendous. For instance, the production of genetically identical monkeys would significantly reduce the number of animals utilized in biomedical research. Another advantage is that we can carry out a variety of genetic manipulations with cultured nuclear donor cells, including gene targeting. Reproductive cloning with such cells would allow for the production of genetically modified primates, including gene knock-out models, to study gene function and human diseases.

At present, the production of live primate offspring following SCNT has yet to be accomplished (Mitalipov *et al.*, 2002; Simerly *et al.*, 2003). We summarize here our recent unpublished efforts in embryo transfer using rhesus blastocysts produced by SCNT with adult monkey skin cells expressing GFP (Table 3). A total of 5 pregnancies were established following transfer of 67 embryos into 10 recipients (Tables 3 and 4). Only one pregnancy resulted in a live fetus that possessed a fetal heartbeat, detected by ultrasonographic scans, while other pregnancies contained sacs without a fetus (Fig. 2). Unfortunately, this pregnancy failed to go to term and was aborted at day 81 of gestation. To determine if there was an abnormal phenotype in the aborted cloned pregnancy, we recovered fetal tissues and carried out histological and molecular analyses. The SCNT origin of the fetus was confirmed by both GFP-specific PCR and direct observation of GFP expres-

TABLE 4

SCNT PREGNANCY OUTCOMES

Recipient	# of embryos transferred	Embryonic age[*] (stage)[**]	Recipient stage[***]	# of GS	# of fetuses	Outcome
1	8	D4 (M/CM)	D2	1	0	
2	8	D4 (M/CM)	D2	1	0	
3	3	D13 (Blastocyst)	D4	1	0	
4	8	D4 (16-cell/M)	D2	1	1	aborted at 81 days of gestation
5	7	D6 (M/CM)	D3	1	0	

[*]Embryonic age, measures the actual age of the embryo, in days, from the time of fertilization (e.g. D4 = day 4 post-fertilization). [**]Stage, refers to the embryo stage of development at the time of the embryo transfer (e.g. M = Morula; CM = Compact morula). [***]Recipient stage, measures the stage of the recipient's uterus, in days, post-ovulation (D0 = day of ovulation, so D2 = day 2 post-ovulation). GS, Gestational sac
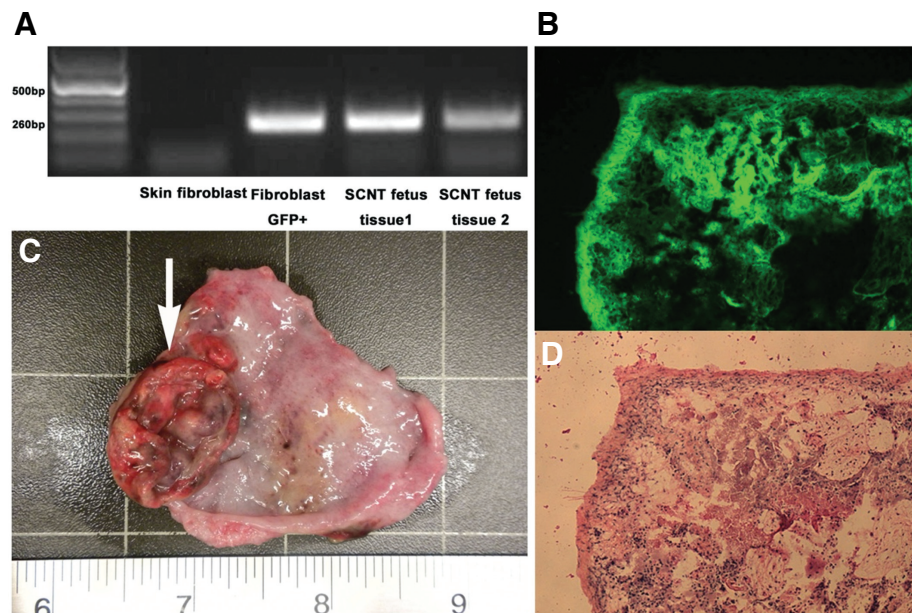
Fig. 3. Analysis of aborted SCNT fetal tissues. (A) *GFP detection by PCR amplification using eGFP specific primers.* **(B)** *Direct GFP expression in fetal tissues (40x magnification).* **(C)** *Macroscopic examination of fetal components of the aborted SCNT pregnancy (indicated by an arrow).* **(D)** *Histological examination of frozen section by HS staining (40x magnification) detecting endodermal and mesodermal components within the recovered tissue.*

sion (Fig. 3 A,B). Macroscopically, results demonstrated poor placental development with fewer chorionic villi compared to controls (Fig. 3C). Microscopically, the fetal tissue also lacked ectodermal components (Fig. 3D). These observations suggest possible insufficient development of the placental component that ultimately impaired the development of the resulting fetus.

Conclusions and future directions

Looking back at the progress made in primate SCNT in just over a decade, it is quite remarkable to see how quickly this technology has advanced, especially considering that just a few research groups are working in this area with nonhuman primates. Within only two years from the start of NT in primates, two rhesus macaque infants were already produced using embryonic cells as the nuclear donor cells. In order to demonstrate that successful nuclear transfer could be accomplished in primates using somatic cells, SCNT protocols, initially developed in other species, were tested with limited success. Introduction of a few key modifications to SCNT technology resulted in enhanced embryonic development and blastocyst yield in both cynomolgus (Ng *et al.*, 2004) and rhesus macaques (Byrne *et al.*, 2007; Sparman *et al.*, 2009).

As the first proof-of principle in primates, functional embryonic stem cell lines were established from rhesus SCNT embryos using adult skin fibroblasts (Byrne *et al.*, 2007; Sparman *et al.*, 2009). The ability to derive ESCs by SCNT holds great potential for regenerative medicine (McKay, 2000). Allogenic ESCs derived from *in vitro* fertilized (IVF) embryos would be rejected by the patient's immune system, unless the patient was treated with ongoing immunosuppressive medication (Drukker and Benvenisty, 2004). Isogenic pluripotent stem cells, genetically identical to the patient, can now be derived by the reprogramming of somatic cells using SCNT or induced pluripotent stem (iPS) cell approaches (Yu *et al.*, 2007). Recently, iPS cells were also generated from somatic cells in several nonhuman primate species, including cynomolgus monkeys (Jin *et al.*, 2009), marmosets (Wu *et al.*), and rhesus macaques (Liu *et al.*, 2008). The ability to generate pluripotent stem cells without using oocytes is a major advance in the field. However, recent mouse studies indicate that SCNT derived ESCs are superior to their iPS counterparts in terms of differentiation capacity *in vitro* and *in vivo* (Kim *et al.*, 2010; Polo *et al.,* 2010).

Therefore, comparative studies of SCNT and iPS derived stem cells must be carried out in order to further elucidate advantages and disadvantages of these two types of experimentally generated pluripotent cells.

Although no live primate infants have been produced by SCNT at this time, clinical pregnancies have been established following embryo transfer attempts using SCNT embryos in several nonhuman primate species. While therapeutic cloning has yet to be achieved in humans, our recent improvements in the protocols in the nonhuman primate model give hope for success in the future (Byrne *et al.*, 2007; Sparman *et al.*, 2009).

In conclusion, we provide here an overview of the current state of SCNT research in the rhesus macaque model. Remarkable progress has been made in adapting and optimizing SCNT protocols to produce embryos and ESCs from adult monkey somatic cells. In contrast, the *in vivo* development of monkey SCNT embryos following transfer into recipients is still limited, thus necessitating further studies to help us better understand the mechanisms of reprogramming in primates. With this, we would like to conclude with a few lines from the famous *The Road Not Taken* poem by Robert Frost:

> *Two roads diverged in a wood,*
> *and I -*
> *I took the one less traveled by,*
> *and that has made all the difference.*

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