

POSSIBLE THERAPY OF MALE INFERTILITY BY REPRODUCTIVE CLONING: ONE CLONED HUMAN 4-CELL EMBRYO

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□ *This study was conducted to evaluate the preimplantation embryonic potential of adult somatic cells from an infertile man using an interspecies bioassay for quality control and also to create human embryos via somatic cell nuclear transfer (SCNT). Skin tissue was biopsied from infertile man to obtain fibroblast cells. These cells were fused with both enucleated bovine oocytes obtained commercially and human oocytes obtained from his wife. SCNT-reconstructed oocytes were cultured in-vitro. Interspecies SCNT embryos were prepared for PCR and DNA analysis. From 13 SCNT-reconstructed bovine oocytes, 7 embryos developed (54%). DNA sequencing of these interspecies embryos showed the presence of human genomic DNA specific for the fibroblast cells of the man. From three SCNT-reconstructed human oocytes, one developed to the 4-cell stage and was subsequently transferred into the patient's uterus. Blood β -hCG levels showed a negative pregnancy result. Human fibroblast cells from an infertile patient can promote embryonic development in interspecies SCNT. This is the first evidence of the creation and transfer of a human cloned embryo for reproductive purposes. Even though no pregnancy was established, human reproduction via SCNT may be possible and applicable in the future for patients with severe male or female infertility that have no other alternative options for procreating their own offspring.*

Keywords male infertility, human somatic cell nuclear transfer, SCNT, embryo cloning, interspecies bovine bioassay, micro-PCR, DNA sequencing

INTRODUCTION

In 1997, Scottish researchers reported that a cloned sheep was created via somatic cell nuclear transfer (SCNT) using an adult somatic donor cell [23]. A few years later, other cloned animals were produced from adult fibroblast cells [17]. Several cloned calves were also generated using a variety of different adult donor cell types for SCNT purposes [16]. There are some risk factors for reproductive cloning in animals [11]. Not only epigenetic alterations in methylation of genes, but possibly also point mutations

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and changes in structure of chromosomes can be envisaged as critical for the cloned offspring [19, 22]. The short period of time for somatic donor cell nuclei have to be properly reprogrammed in the cytoplasm of recipient oocytes may not be sufficient enough to initiate and enable normal clonal embryogenesis. Cloning efficiency in goat and cattle can be significantly improved by exposing donor nuclei from blastomeres of a cloned embryo again to the cytoplasm of recipient oocytes during a second recloning step, thus prolonging the exposure time for genomic remodeling [24, 27].

For future applications in reproductive cloning, it will be important to further advance our understanding of how to rejuvenate and reprogram human adult cells by modern molecular bioengineering [12]. We have initiated a novel approach using adult human granulosa cells for SCNT into enucleated bovine oocytes [13]. Development of such a cloning biological model could be utilized as a bioassay to test and extend the efficiency of human adult somatic cells for their ability to promote embryonic development via interspecies SCNT.

We have evaluated the embryonic potential of fibroblast cells from an azoospermic patient devoid of any germ cells in interspecies bioassays using the bovine model. In parallel, we carried out human heterologous SCNT utilizing these cultured fibroblasts and oocytes from the female patient in order to create human cloned embryos for reproductive purposes.

MATERIALS AND METHODS

Patients

A 35-year-old, nulligravida woman after proper pituitary regulation and desensitization was down regulated with GnRH analogue (GnRh-a), tryptorelin acetate (Decapeptyl, Ferring GmbH, Kiel Germany) and underwent ovarian hyperstimulation with FSH-hMG according to a long standard protocol. Ovulation was triggered with 10,000 IU of hCG (Profasi; Serono, Seoul, S. Korea) 36 hr before oocyte retrieval. Her 32-yr-old husband was a patient suffering from azoospermia due to cryptorchid testes. From this patient, skin tissue was biopsied and grown in-vitro to obtain primary fibroblast cultures. The couple was provided with a full explanation of all the procedures to be performed, and only after giving their consent were the procedures carried out. The Institutional Review Board of Reprogen Ltd. (Limassol, Cyprus) reviewed and approved all of the experimental procedures employed.

Fibroblast Cell Culture

Following skin biopsy of the man under local anesthesia, the tissue material was transferred into a small Nunclon petri dish (VWR International,

West Chester, PA) containing PBS without Ca^{2+} and Mg^{2+} (Invitrogen Corp, Chicago, IL), and minced up into very small pieces. These tissue pieces were transferred to another small petri dish containing a preincubated 0.25% Trypsin and EDTA solution (Invitrogen Corporation, Chicago, IL) and incubated for 20 min at 5% CO_2 and 37°C. After incubation, the contents of the petri dish were rinsed by repeated pipetting (about 20 times) using a sterile Pasteur pipette and transferred into a 15 mL conical Falcon tube (VWR International, West Chester, PA) and allowed to sediment. The supernatant was removed and transferred to another Falcon tube containing preincubated DMEM culture medium supplemented with 10% SSS, centrifuged at 500 rpm for 3 min, and the pellet was resuspended in DMEM solution and transferred to two petri dishes containing DMEM supplemented with 1.25% non-essential amino acid solution, 1.25% L-glutamine, 1.25% pyruvic acid solution, 245 μL penicillin/streptomycin solution and 10% SSS and incubated at 5% CO_2 and 37°C. Cultures of primary fibroblast cells were established in-vitro.

For further use of fibroblast cells in SCNT, the culture medium was removed from one petri dish, replaced initially with PBS without Ca^{2+} and Mg^{2+} and then with 0.25% Trypsin and EDTA solution. After a 2 min incubation in 5% CO_2 at 37°C, the cells were detached from the petri dish, aspirated into a fine glass pipette and transferred through several washing steps in microdrops of HTF + HEPES solution supplemented with 5% SSS under properly equilibrated mineral oil. These cells served as donor cells for SCNT.

Bovine Oocytes and Human Cells for Interspecies Bioassay

The interspecies human-bovine SCNT bioassay was performed accordingly [26]. Briefly, matured bovine oocyte-cumulus complexes were incubated in 300 IU/mL hyaluronidase (Sigma-Aldrich), for removal of granulosa cells. Denuded oocytes were washed and processed for micromanipulation and SCNT. Single fibroblast cells from the infertile patient were utilized for SCNT as described below. After electrical fusion and chemical activation, the SCNT-reconstructed oocytes and developing interspecies embryos were cultured in IVC-One medium (IVC, San Diego), supplemented with 10% SSS for 6 days at 5% CO_2 , 5% O_2 and 38.5°C. As controls, non-treated bovine oocytes were activated chemically for parthenogenesis and cultured independently in-vitro under the same conditions.

PCR Amplification and DNA Sequencing on Interspecies Embryos

Interspecies-cloned embryos were prepared for analysis of human genomic DNA using 10 different microsatellite markers (Applied Biosystems'

Amp FLSTR Profiler Plus Kit) for human DNA identification. The 10 different loci were D3S1358, D5S818, D7S820, D8S1179, D13S317, D18S51, D21S11, FGA, vWA, and amelogenin. Analysis was carried out using two specific human and two specific bovine primers for amplification of mtDNA fragments from the mitochondrial D-loop region. The two human primers were: D-loop u: 5'-ttcatggggaagcagatttg-3' (primer 1), and D-loop l: 5'-tgtgctatgtacggtaaaggctt-3' (primer 2).

The two bovine primers were: D-loop u: 5'-aaatgtaaacgacgacggccag-taatcccaataactcaacac-3' (primer 1), and D-loop l: 5'-aacaggaacagctatgac-cactcatctaggcatttc-3' (primer 2). PCR amplification and DNA sequencing was carried out at the Veterinary Institute for Molecular Biology, University of Goettingen, Germany.

Ovarian Stimulation and Oocyte Retrieval

Oocyte retrieval was performed transvaginally. Three oocyte-cumulus complexes were then placed in 80IU hyaluronidase (Cooper Surgical, Trumbull, CT, USA) using standard ICSI protocols. After removal of the cumulus, 2 oocytes were at metaphase II and one oocyte at metaphase I (immature). For further maturation of the metaphase-I oocyte, it was placed in IVC-One medium supplemented with 5% maternal serum, 10% SSS, 0.01IU/mL LH and 0.02IU/mL FSH. After 6hrs of incubation at 5%CO₂, 5%O₂ and 37°C, the metaphase-I oocyte matured to metaphase II by extruding the first polar body and was washed and processed similarly.

Human Somatic Cell Nuclear Transfer (SCNT)

Following 1 hr of incubation in IVC-One medium supplemented with 10% SSS, the cumulus-free oocytes were transferred into individual microdrops of HTF + Hepes + 5% SSS under mineral oil. From another microdrop containing acidified Tyrode's solution, a small amount was aspirated into a blunt micropipette (~14µm OD). A hole was made in the zona pellucida by touching the acidified Tyrode containing pipette to the zona pellucida and expelling some of the Tyrode's solution. The oocytes were washed and transferred into another microdrop containing HTF + Hepes +5% SSS, supplemented with 5g/mL cytochalasin B under mineral oil for enucleation.

Metaphase-II oocytes containing a polar body that was not entirely separated from the oocyte and still remaining attached to the oocyte's membrane were used for enucleation. This was done to ensure that the meiotic spindle was still directly beneath the polar body, thereby avoiding the need to use fluorescent staining, which may jeopardize the development of oocytes. By penetrating a beveled micropipette (~20µm OD) through the opening in

the zona pellucida, created using the acidified Tyrode's solution, the polar body and underlying ooplasmic (metaphase-II complex) was aspirated into this micropipette. The clear structure of the polar body and the metaphase-II material could be unequivocally distinguished from the granular ooplasm of which a very small amount was aspirated into the micropipette to ensure that metaphase-II had been removed (enucleation) from the oocyte. Following enucleation, the oocytes were removed from the manipulation dish and preincubated in HTF + 5%SSS for 1 hr under 5% CO₂ at 37°C conditions. The enucleated oocytes were transferred into a mineral oil-covered microdrop of HTF + Hepes + 5%SSS medium containing cells that were previously prepared from the fibroblast culture of male patient.

Only medium-sized cells with a ruffled, pseudopodial cell surface and without a visible nucleus were picked up individually into a beveled micropipette (~20 µm OD), injected through Tyrode-made hole of zona pellucida and attached firmly to the oocyte surface. The oocytes were incubated for about 20 min, then transferred individually into a horizontally-placed fusion chamber filled with isoosmolar fusion buffer and properly orientated so that the injected cell sticking to the oocyte membrane was placed at either 12:00 or 6:00 position. The electrical fusion protocol was 36 V with two pulses of 25 µsec, using an Eppendorf multiporator. Oocytes were removed from the fusion chamber, washed and incubated as described above. Successful fusion was monitored after 20 min of incubation as evidenced by the absence of injected cell. The manipulated oocytes were incubated for 1 hr before activation. These oocytes were incubated in culture medium supplemented with 7% ethanol for 5 min, washed, incubated in culture medium supplemented with 10 µg/mL cycloheximide and 5 µg/mL cytochalasin B for 5 hrs. After the activation procedure, oocytes were washed and transferred to IVC-One medium supplemented with 10% SSS and cultured in-vitro under 5% CO₂, 5% O₂ at 37°C.

Culture In-vitro/Embryo Transfer (ET)

The three SCNT-reconstructed oocytes were cultured in-vitro in microdrops covered with equilibrated mineral oil. Embryonic development was monitored every 12hrs and embryo quality was assessed. Two of 3 manipulated oocytes showed no signs of cleavage. The third oocyte proceeded to the 4-cell stage 60 hrs post SCNT. The morphological and developmental quality of this embryo was rated as Grade 1. Based upon this quality rating, as well as the PCR data from corresponding human-bovine SNCT embryos, this embryo was considered morphologically optimal, and without any further invasive analysis, it was subsequently transferred into the patient's uterus using standard ET methods. The patient's luteal phase was supported

via progesterone injections for two weeks following embryo transfer, after which quantitative blood β -hCG levels were tested for pregnancy.

RESULTS

Primary fibroblast cultures derived by skin biopsy from the infertile man were successfully established in-vitro. After 4 days of incubation, the primary cultures showed cellular growth and morphology of typically shaped fibroblasts and some large epithelial cells in a semi-confluent manner. To reveal their developmental and embryonic capacity for SCNT we tested these fibroblasts in our interspecies bioassay [26]. Enucleated bovine oocytes were injected with single fibroblast cells that were isolated from primary culture and prepared for cell transfer. Injected bovine oocytes were fused electrically and activated chemically and then cultured in-vitro for up to 6 days. In this interspecies bioassay, from 13 SCNT-reconstructed bovine oocytes, 7 embryos developed and advanced to various stages of preimplantation (Figure 1). From 22 parthenogenetically activated oocytes (controls), 17 embryos developed during preimplantation (Table 1).

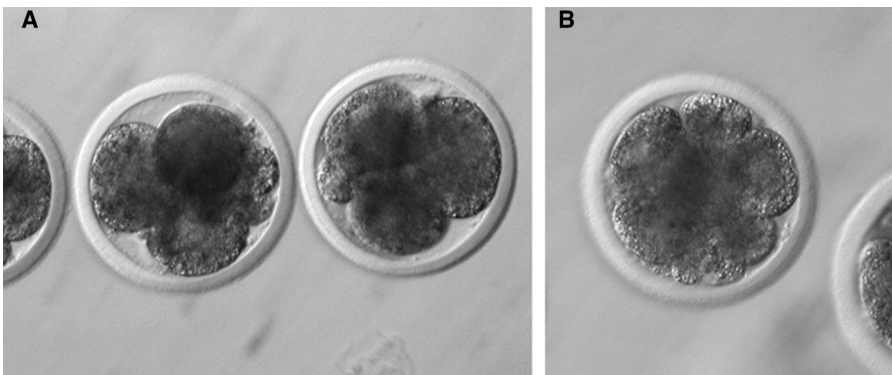


FIGURE 1 Interspecies-cloned embryos at various stages of preimplantation development, created from enucleated bovine oocytes fused with human adult fibroblast cells from the infertile man: (a) 4-cell stage, (b) morula stage.

TABLE 1 Preimplantation development of interspecies embryos derived from SCNT using enucleated bovine oocytes fused with human fibroblast cells from the man. Bovine parthenogenetically developed embryos (parthenotes) served as controls

	Interspecies SCNT	Parthenotes (controls)
Oocytes activated (n)	13	22
Embryos developed (n)	7	17
Success rate (%)	54	77

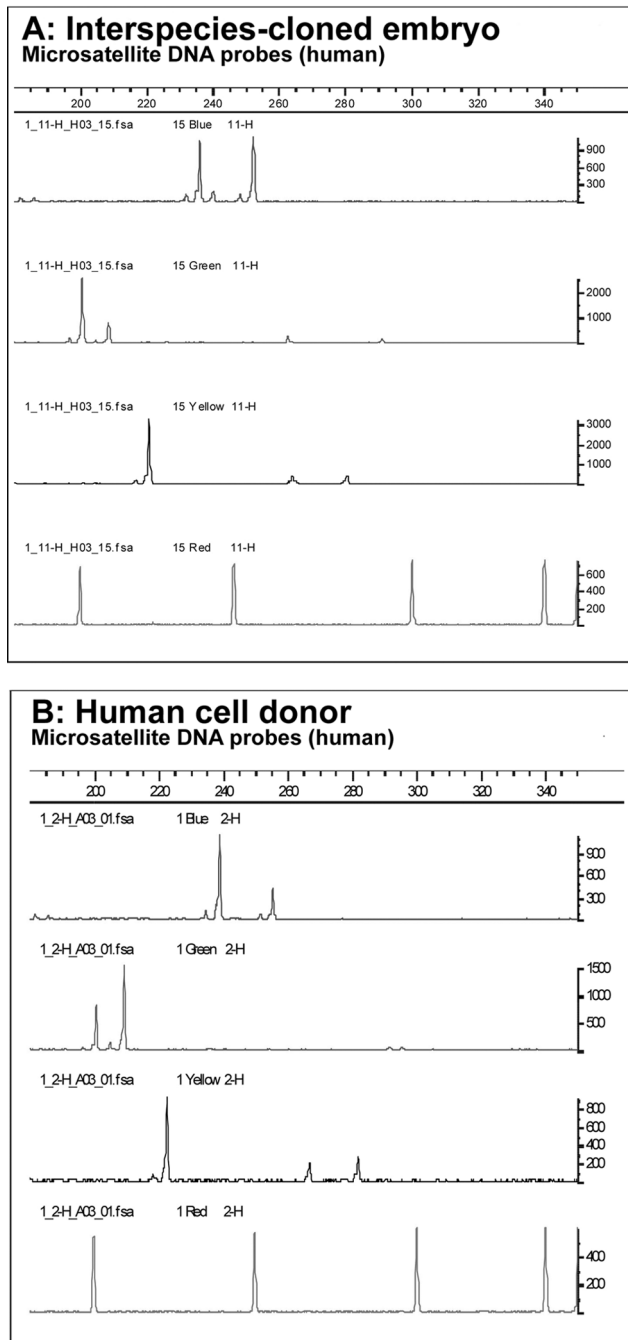


FIGURE 2 Human genomic DNA sequence analysis of (a) interspecies-cloned embryo (morula) and (b) human adult fibroblast (HF) donor cells used for SCNT. Note the identical DNA sequence profiles concerning peak positions for interspecies embryo (morula) and HF donor cells. Some variations in peak levels result from different sample analysis. Three chromosomal microsatellite probes were used for PCR amplification. FGA (chromosome 4q28), D21S11 (chromosome 21), D13S317 (chromosome 13q22–31). A standard marker (ABI Applied Biosystem ROX Reference Dye) served as internal control.

Interspecies embryos were analyzed individually for the presence of human genomic DNA, human- and bovine-mtDNA using species-specific microsatellite markers of genomic DNA and species-specific primers of mtDNA for PCR amplification. The DNA sequencing revealed that human genomic DNA was present in interspecies embryos (Figure 2). In addition, human and bovine mitochondrial (mt) DNA was conjointly detected in the interspecies embryos (Figure 3). With this interspecies bioassay, fibroblast cells derived from the male infertile patient were capable of initiating and promoting embryonic development.

From the patient's hormonally stimulated female partner, we obtained 3 oocytes, which were processed for micromanipulation and SCNT. After their culture in-vitro, embryonic development was monitored every 12 hrs and embryo quality was assessed. One of 3 oocytes showed cell division with two equally-sized blastomeres and proceeded to the 4-cell stage at 60 hrs

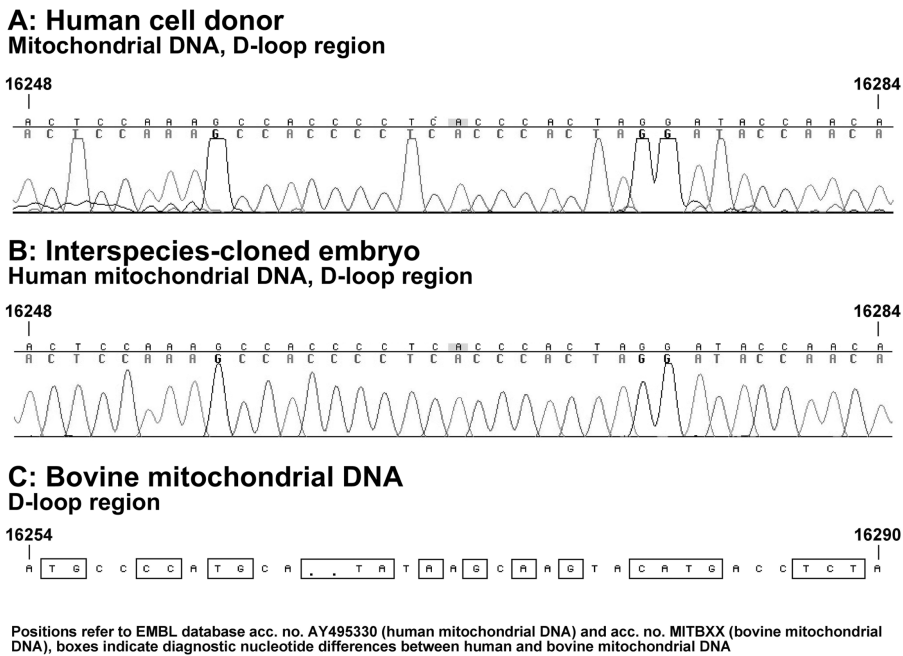


FIGURE 3 DNA sequence analysis of human and bovine mitochondrial (mt) DNA from interspecies-cloned embryo (morula) derived from enucleated bovine oocyte fused with human fibroblast cell. For the detection of human and bovine mtDNA, species-specific primers were utilized to amplify parts of the mtDNA D-loop region, respectively. The amplicons were sequenced using specific internal primers. (a) partial human mtDNA D-loop region amplified from the human donor cells. (b) partial human mtDNA D-loop region amplified from the interspecies-cloned embryo (morula). Both mtDNA sequences show identical profiles. (c) partial bovine mtDNA D-loop region amplified from the interspecies-cloned embryo (morula). The boxes indicate sequence differences between bovine and human mtDNA.



FIGURE 4 Human embryo at the 4-cell stage at 60 hrs post-SCNT. The quality of the embryo was rated as grade 1 according to current human IVF grading standards (grade 1 being the best on a scale of 1–4). The cloned human embryo was composed of four equally-sized blastomeres showing regular cytoplasmic morphology and with very few tiny fragments at the site of microsurgical operation through the zona pellucida.

post-SCNT. The quality of the embryo was rated as Grade 1. The cloned human embryo was composed of four equally-sized blastomeres showing regular cytoplasmic morphology and with very few tiny fragments at the site of microsurgical operation through the zona pellucida (Figure 4).

In our previous interspecies studies [14], PCR amplification and DNA sequencing that interspecies embryos contained human genomic and mitochondrial (mt) DNA identical to the human donor cell. The nuclear presence of the patient's fibroblast donor cell in the interspecies embryos was confirmed by PCR and DNA analysis (Figure 2), in addition to the presence of human mtDNA (Figure 3). The human cloned embryo was considered suitable for transfer. Moreover, the couple did not wish to pursue any invasive analyses that could damage the embryo because of their own religious beliefs. Based upon its high morphological quality, the embryo was subsequently transferred into the patient's uterus. The patient's luteal phase was supported via progesterone injections for two weeks following the embryo transfer, after which the blood β -hCG levels showed a negative pregnancy result.

DISCUSSION

The current success rates for obtaining mammalian clones from adult cells employed for SCNT remain rather limited. Several interspecies bioassays have been developed to test embryonic potential of various somatic

adult cells. Bovine oocytes were successfully employed in interspecies SCNT to test adult somatic cells from pig, sheep, rat and rhesus monkey for their cloning potential as nuclear donor types [5]. Interspecies embryos could develop up to the blastocyst stage. American researchers reported on interspecies SCNT using bovine oocytes and human lymphocytes or oral mucosal epithelial cells for cloning [18]. However, their data were briefly presented without providing specific information about fusion and activation of bovine oocytes or characteristics of neither the human somatic donor cells, nor presenting any documentation of the obtained embryos to furnish proof for their origin from the donor cells' genome. Korean researchers published strong molecular data and karyotyping results on interspecies embryos that were derived from SCNT of human cord fibroblast cells fused with enucleated bovine oocytes [3].

We also have used such interspecies bioassay to test the embryonic capacity of human follicular granulosa cells via SCNT in the bovine system. These somatic cells are able to initiate and promote development leading to interspecies embryos [26]. We have examined the embryonic potential of fibroblast cells derived from the infertile man in the bovine bioassay model. Using PCR amplification and DNA sequencing on these interspecies embryos, we could unequivocally document that they were composed of the human nuclear genomic DNA and contained both human and bovine mtDNA. Heteroplasmy in mtDNA has been revealed in cloned animals and implicated in the context of possible cloned offspring [20, 21].

Chinese researchers have reported on interspecies SCNT using rabbit oocytes and human fibroblast cells [4]. They documented very clearly at the molecular level that the SCNT embryos originated from the genomic DNA of the human fibroblast cell type [4]. Korean researchers have fused human cumulus cells into autologous human enucleated oocytes resulting in cloned human blastocysts from which an embryonic stem (ES) cells could be established in culture [9]. Along these lines, this group has recently published impressive results on the creation of patient-specific ES cells derived from human SCNT blastocysts [10]. Immunological compatibility between the patient and the autologous ES cells was confirmed by sharing the corresponding identity of the major histocompatibility complex required for immune matching. Their efforts represent a very promising approach towards future opportunities for applying SCNT technologies in creating cloned human embryos and ES cells for therapeutic medicine [13].

Alternatively, cloned human embryos may also be obtained via embryo splitting. In this respect, Hall et al. [8] have split abnormal polyploid 2- to 8-cell stage embryos donated from IVF cycles and created twin embryos, some of which developed to the 32-cell stage. However, none of these non-viable split embryos developed beyond this stage and were discarded [7, 8]. In a

commentary referring to these experiments and to cloning via embryo splitting in general, Jones et al. [15] acknowledged the merits of these attempts for future application in reproductive medicine.

We have created the first human cloned 4-cell embryo for reproductive purposes using human enucleated eggs and heterologous human granulosa cells for SCNT which resulted in an 8–10 cell embryo [25]. This embryo was cryopreserved for later embryo transfer into a surrogate carrier. These findings began to set the tone for a scientifically interesting debate [1] in which Edwards [6] stated that a wide perspective must be maintained on this type of research. We created another human cloned embryo derived from heterologous SCNT using fibroblast cells from an infertile male patient devoid of any germ cells and oocytes from his wife for reproductive cloning. This embryo developed to the 4-cell stage at 60hrs post-SCNT. Concerning its developmental status, this embryo was about 12hrs behind the regular time schedule of embryos routinely obtained from IVF or ICSI. This delay may have resulted from initial adaptation of the somatic cell nucleus within the cytoplasm of the recipient oocyte that can lead to delayed and aberrant nuclear reprogramming during initial embryogenesis of cloned embryos [2].

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