Human embryo cloning via SCNT with adult fibroblast cells

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The first objective of this study was to evaluate the preimplantation embryonic potential of adult fibroblast cells from infertile male patients using bovine oocytes for somatic cell nuclear transfer (SCNT). Skin tissue was biopsied from four males suffering from azoospermia devoid of germ cells in order to establish fibroblast cell cultures for SCNT. From 92 SCNT-reconstructed bovine oocytes, 44 embryos (47.8%) developed up to late preimplantation. We conclude that fibroblast cells from these infertile male patients can promote embryonic development in interspecies SCNT. The second objective was to create human embryos using fibroblast cells from the four infertile males and oocytes from their wives for SCNT. From 27 SCNT-reconstructed human oocytes, 12 embryos (44.4%) developed up to the 4-10 cell stage about 50-55 hrs post-SCNT and 11 embryos served for intrauterine transfer in four instances. Even through blood ß-hCG levels showed negative pregnancy results for all four cases, we have documented further evidence of the creation and transfer of human embryos cloned for reproductive purposes. Human SCNT may be applicable to infertile male patients that have no alternative options for procreating their own progeny.

A variety of adult cell types have been successfully employed for somatic cell nuclear transfer (SCNT) in farm animal cloning technology (Wilmut et al. 1997; Kato et al. 2000). So far, follicular granulosa cells and skin fibroblast cells have been most efficiently and extensively utilized as cell donor sources in reproductive cloning (Wells et al. 1999; Kubota et al. 2000). Despite multiple attempts and worldwide efforts to improve the cloning efficiency for live-born progeny, the success rates for obtaining mammalian clones from adult somatic cells employed for SCNT remain still limited (Illmensee 2001).

Several risk factors for reproductive cloning in animals that may be responsible for the rather low survival rates have been discussed and proposed for further investigations. Not only epigenetic alterations in methylation of genes (Young et al. 2001; Shi et al. 2003; Santos et al. 2004), but also changes in structure of chromosomes (Betts et al. 2001; Tani et al. 2001; Wade et al. 2002) can be envisaged as critical for the cloned offspring. A number of researchers in the cloning field have pointed out that the short period of time for somatic donor cell nuclei to be properly

reprogrammed in the cytoplasm of recipient oocytes may not be sufficient enough to initiate and sustain normal clonal embryogenesis (Fulka et al. 1996; Bourc'his et al. 2001; Du F et al. 2002). Also, the volume ratio of karyoplast (from the donor cell) and cytoplast (from the enucleated oocyte) in SCNT procedures has shown to affect the developmental potential of cloned embryos (Zakartchenko et al. 1997). In addition, cloning efficiency can be influenced by cell-cycle heterogeneity of the cultured somatic cells and may be improved by increasing cell-cycle uniformity for somatic donor cells used in SCNT (Liu et al. 1997; Miyoshi et al. 2002; Wells et al. 2003).

For future advancements in reproductive cloning, it will be important to further increase our experience and knowledge about how to rejuvenate and reprogram human adult cells by modern molecular and cellular biotechnology (Illmensee 2002). In this context, we have started a novel approach using two different types of adult human cells (granulosa cells from female patients enrolled in IVF programs and fibroblast cells from infertile male patients) for SCNT into enucleated bovine oocytes (Zavos and Illmensee 2003; Illmensee et al. 2006). Development of such a cloning biological model enables us to utilize it as a bioassay to test and compare the efficiency of different human adult somatic cells their ability to promote embryonic development via interspecies SCNT.

Recently, we have documented and proven via PCR analysis and DNA sequencing that fibroblast cells from azoospermic patients when fused with enucleated bovine oocytes could promote development of interspecies embryos (Illmensee et al. 2006). In the current report, we have summarized our interspecies SCNT using fibroblast cells from four azoospermic patients devoid of germ cells. In parallel but not concurrently, we have carried out human SCNT utilizing these cultured fibroblasts and oocytes from the infertile patients' wives in order to create human cloned embryos for reproductive purposes. We recently reported on the first transfer of a cloned human embryo, using fibroblast cells from an azoospermic patient for SCNT as a possible modality for treatment of severe male infertility (Zavos and Illmensee 2006). The current clinical report describes and documents our continuous efforts of transferring cloned human embryos in-utero.

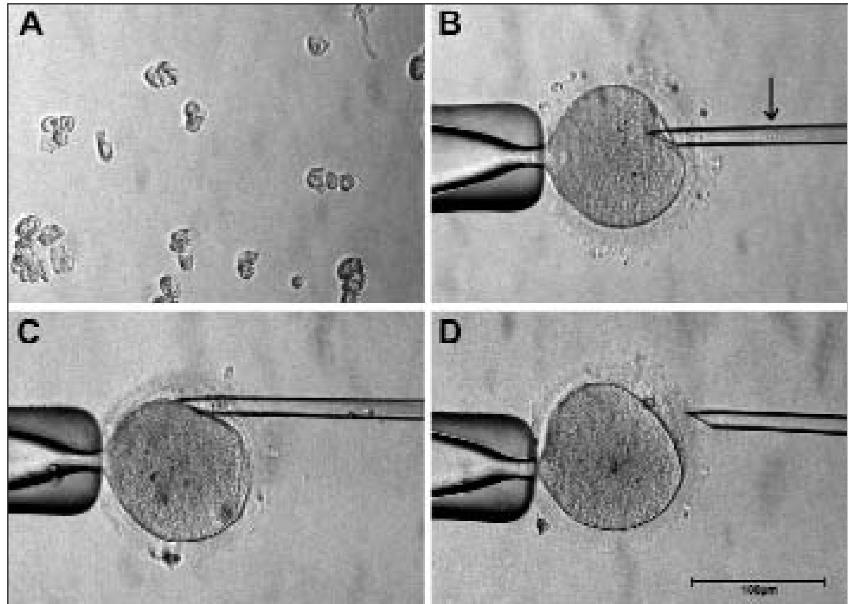


Figure 1. Human somatic cell nuclear transfer (SCNT) procedure. (A) Fibroblast cells from an infertile male patient devoid of germ cells were prepared for SCNT. (B) Microsurgical enucleation of mature oocyte by removing the polar body (arrow) and the cytoplasmic metaphase-II complex. (C) Injection of fibroblast cell into the perivitelline space between zona pellucida and oocyte. (D) Tight attachment of injected fibroblast cell to the membrane of enucleated oocyte.

Materials and methods

This study was reviewed and approved by the Institutional Review Board (IRB) of Reprogen, Ltd.

Patients

Four male patients that suffered from infertility were thoroughly investigated and diagnosed with azoospermia devoid of germ cells. The couples were

Table 1. Interspecies SCNT embryos originating from human adult fibroblast cells fused with bovine oocytes

Case	Interspecies SCNT		Parthenotes (controls)	
Number	Oocytes operated	Embryos developed	Oocytes activated	Embryos developed
1a	13	7	22	17
2	34	16	54	38
3	19	8	31	23
4	26	13	46	39
Total	**92**	**44 (47.8%)**	**153**	**117 (76.5 %)**

** Data from Zavos and Illmensee (2006).*

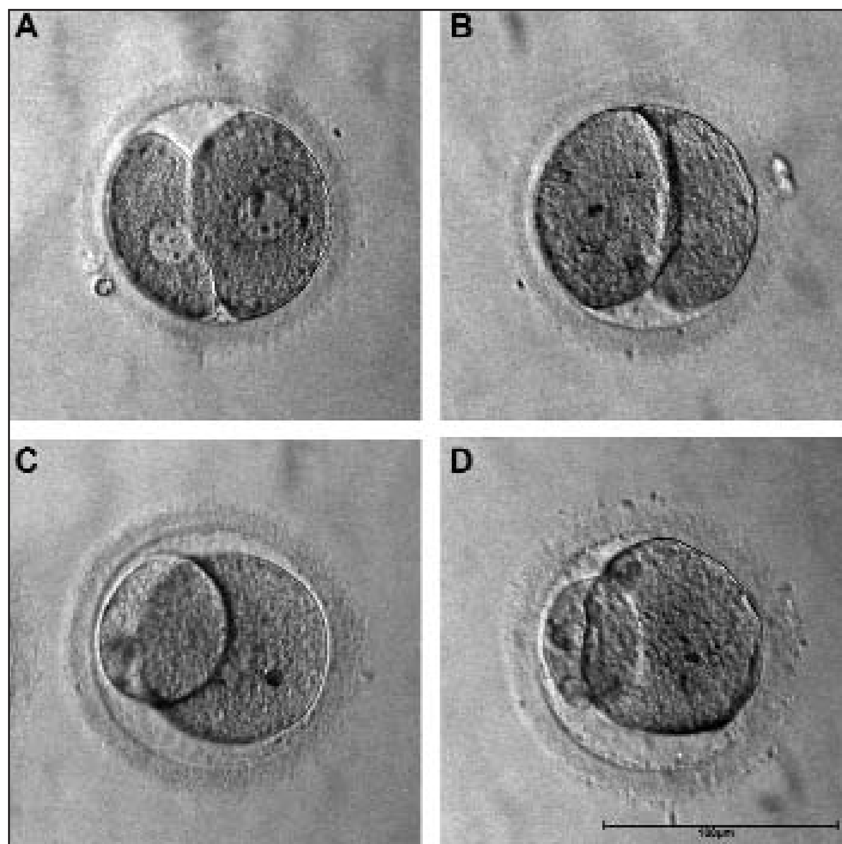


Figure 2. Development of human cloned embryos after first cleavage at approximately 20-22hrs post-SCNT. (A and B) Both embryos show two equally-sized blastomeres. (C and D) These embryos show two unequally-sized blastomeres. Note the clear appearance of nuclei in cells with homogenous cytoplasm.

provided with full explanation of all the procedures to be performed and only after giving their written consent, the procedures were carried out.

Fibroblast cell culture

Skin tissues biopsied from the male patients under local anesthesia were processed as previously described (Zavos and Illmensee 2006). Cultures of primary fibroblast cells were established in-vitro from which cells were collected and prepared for SCNT (Figure 1A).

Bovine oocytes and human cells for interspecies bioassay

For quality control purposes, single fibroblast cells from the infertile male patients and enucleated bovine oocytes were utilized for SCNT, as described previously (Illmensee et al. 2006; Zavos and Illmensee 2006).

As controls, non-treated bovine oocytes were activated chemically for parthenogenesis and cultured independently in-vitro. Development of interspecies SCNT embryos and parthenogenetic embryos was monitored daily up to seven days in culture. Developmental success rates were compared between SCNT-derived and parthenogenetically-derived (controls) embryos.

Ovarian stimulation and oocyte retrieval

The four wives of the male infertile patients were down regulated with a GnRH analogue, 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) 36hr prior to oocyte retrieval.

After transvaginal oocyte retrieval the oocyte-cumulus complexes were washed and placed in 80IU hyaluronidase (Cooper Surgical, Trumball, CT, USA) to obtain cumulus-free oocytes. They were washed and briefly incubated in IVC-One medium (In Vitro Care, San Diego, CA, USA) supplemented with 10% SSS (Irvine Scientific, Santa Ana, CA, USA) at 5% CO2 and 37o C.

Human somatic cell nuclear transfer (SCNT)

Human metaphase-II oocytes containing a polar body attached to the oocyte's membrane were used for enucleation. By penetrating a beveled micropipette (~20_m OD) through an opening in the zona pellucida (ZP), previously prepared using Tyrode solution (Irvine Scientific, Santa Ana, CA, USA), the polar body and the underlying ooplasmic material (metaphase-II complex) was aspirated into this micropipette (Figure 1B). Single fibroblast cells were inserted under the ZP (Figure 1C) and attached tightly to the oocyte membrane (Figure 1D). Human SCNT technology was carried out according to our previously described procedures (Zavos and Illmensee 2006).

Culture in-vitro and embryo transfer (ET)

Development of the human SCNT embryos was monitored daily under standard culture conditions (Zavos and Illmensee 2006). The morphological and developmental quality of these embryos was rated according to current human IVF grading standards (grade I being the best on a scale of I-IV). Based upon this quality rating, these embryos were subsequently transferred in-utero, using standard ET

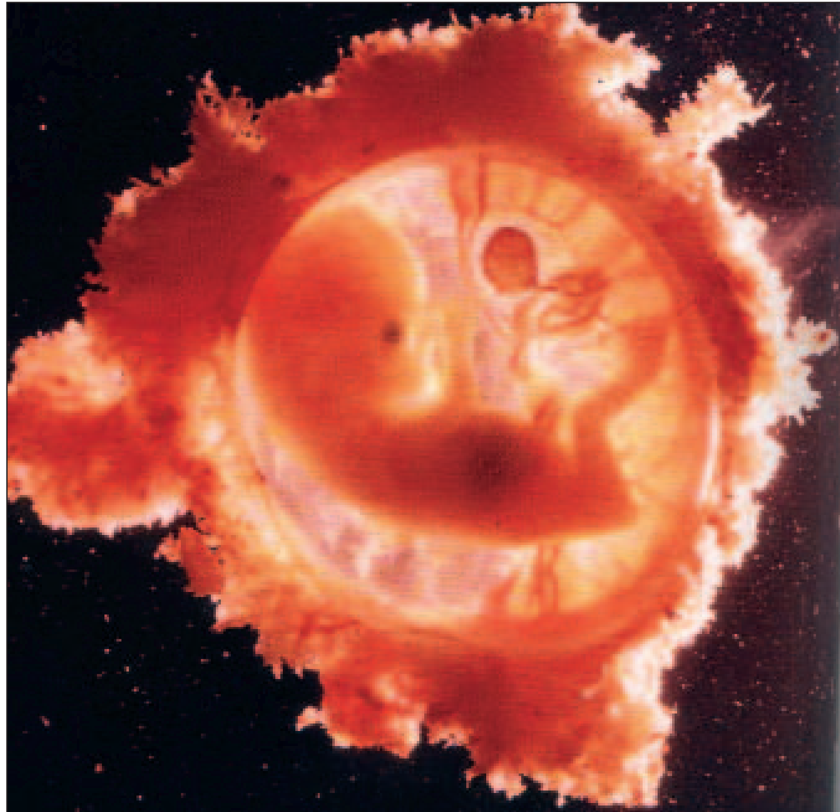
methods. The patients' luteal phase was supported via progesterone injections for two weeks following embryo transfer, after which quantitative blood ß-hCG levels were tested for pregnancy.

Interspecies SCNT

Fibroblast cells derived from skin biopsy of four infertile male patients were successfully cultured in-vitro and used for interspecies SCNT as previously described (Illmensee et al. 2006). From 92 SCNT-reconstructed bovine oocytes, 44 embryos developed up to late preimplantation (Table 1). Serving as controls for our activation and culture procedures, non-treated bovine oocytes were activated chemically and cultured in-vitro under the same conditions as the SCNT-reconstructed oocytes. From 153 parthenogenetically activated oocytes, 127 embryos developed through preimplantation (Table 1).

Human SCNT

Human oocytes and fibroblast cells were processed for microsurgical enucleation and SCNT, as described previously (Zavos and Illmensee 2006). From 27 SCNT-reconstructed oocytes, 12 embryos developed and were cultured in-vitro. The first cell division was seen about 20-22 hrs post-SCNT (Figure 2). Eleven embryos progressed further to the 4-10 cell stage at about 50-55hrs post-SCNT (Table 2). The quality of the embryos was rated and ranged between grades I to III according to current human IVF grading standards (Figure 3). Seven out of the 11 embryos showed a developmental delay of about 12-15hrs, most likely due to the rather stressful SCNT procedures imposed on the oocytes and fibroblasts. To some extent, it may also result from delayed reprogramming of the somatic donor nuclei in the cytoplasm of the enucleated oocytes in order to switch from adult to embryonic gene expression.



Chemical activation was carried out on 6 enucleated oocytes that did not show fusion with fibroblast cells, similarly to the 27 SCNT-reconstructed oocytes. No cleavage could be induced in these 6 oocytes of which 3 of them showed fragmentation about 20hrs post-activation. Parthenogenetic development as a result of enucleation failure was not observed on these oocytes, thus corroborating proper enucleation of the SCNT-reconstructed oocytes.

From the 12 SCNT embryos that advanced developmentally, 11 were considered to be morphologically suitable (grade I - III) for transfer in-utero. The four couples were given the option of having the quality of their embryos analysed further via pre-implantation genetic diagnosis (PGD). All four couples chose not to pursue any invasive embryonic analyses (PGD)

Table 2. Human SCNT embryos originating from adult fibroblast cells fused with oocytes

Case number	Oocytes operated	Embryos developed	Embryos transferred	Pregnancy
1a	3	1	1	--
2	7	4	4	--
3	8	4	3	--
4	9	3	3	--
Total	**27**	**12 (44.4%)**	**11**	**--**

***Data from Zavos and Illmensee (2006)*

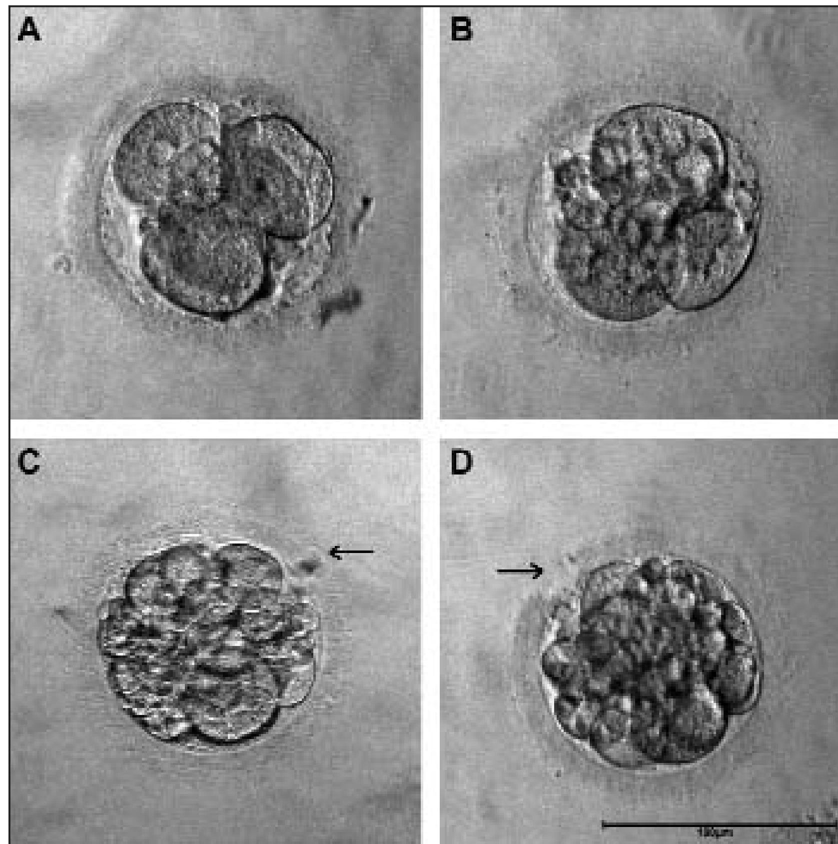


Figure 3. Development of human cloned embryos at approximately 50-55hr post-SCNT prior to intrauterine transfer. (A) 4-cell embryo (grade I) showing equally-sized blastomeres. (B) 4-cell embryo (grade II) with equally-sized blastomeres and some cellular fragments. (C and D) 10-cell embryos (grade III) with some fragments. Arrows point to the Tyrode-prepared opening in the zona pellucida.

that could possibly damage these embryos and limit their implantation potential and also because of their own religious beliefs. Incidentally, all four couples were of the Muslim religion, which customarily restricts oocyte donation and also some sort of invasive embryonic analyses, and which decisions were very much respected by all members of our scientific and clinical team. The four patients' luteal phase was supported via progesterone injections for two weeks following the embryo transfer, after which the blood ß-hCG levels showed negative pregnancy results.

Discussion

During the past years, several interspecies bioassays have been developed to test the embryonic potential of various adult cells. Bovine oocytes have been successfully employed in interspecies SCNT to test adult cells from pig, sheep, rat and rhesus monkey for their embryonic potential in-vitro (Dominko et al. 1999). Karyotyping results and molecular data using PCR and DNA technology have been published on interspecies embryos that were derived from SCNT using adult human cord fibroblast cells fused with enucleated bovine oocytes (Chang et al. 2003). We have employed such interspecies bioassays to test the embryonic in-vitro potential of human adult fibroblast cells from infertile male patients via SCNT in the bovine system. Our results have shown that these somatic cells are able to promote development leading to interspecies embryos (Illmensee et al. 2006). We have also shown via PCR amplification and DNA sequencing that these interspecies embryos created via SCNT contained human genomic and mitochondrial (mt) DNA which was identical to the human donor cell source. In addition, these interspecies SCNT embryos contained bovine mtDNA of oocyte origin. Heteroplasmy of mtDNA has been revealed in cloned animals and has been discussed in the context of possible implications for the cloned offspring (Takeda et al. 2003; St. John et al. 2004).

Recently, we have examined the embryonic potential of fibroblast cells derived from another infertile male patient suffering from azoospermia and cryptorchidism using the bovine bioassay model (Zavos and Illmensee 2006). From the resulting interspecies embryos, PCR amplification and DNA sequencing unequivocally documented that these embryos were composed of the human genomic DNA specific for the patient's fibroblast donor cell source and contained both human and bovine mtDNA. Due to these previous extensive and positive investigations and results on interspecies SCNT embryos (Illmensee et al. 2006; Zavos and Illmensee 2006), We have established very clearly that such elaborate molecular analysis was not essential and necessary and, therefore, has not been included in the present clinical report.

With regards to human SCNT, it was recently reported that human oocytes were only promoting optimal development when they were enucleated and further processed for SCNT within 1hr post-retrieval (Stojkovic et al. 2003). Furthermore, when using failed fertilized oocytes from IVF programs about 24hrs post-retrieval, they were not able to initiate proper and regular embryonic development and therefore, turned out to be inefficient for

SCNT purposes (Lavoir et al. 2005). It therefore seems crucial to utilize mature human oocytes rather quickly after retrieval for successful SCNT. We have also established that immature oocytes, on the other hand, need further culture for maturation to metaphase II (polar body extrusion) before their use in SCNT (unpublished data).

In 2003, the creation of the first human cloned embryo for reproductive purposes was reported using human enucleated eggs and heterologous human granulosa cells for SCNT (Zavos 2003). In 2006, we published results on the first intrauterine transfer of a human SCNT-derived embryo, but without achieving a pregnancy. In this case, fibroblast cells from an infertile male devoid of germ cells were employed for successful SCNT (Zavos and Illmensee 2006). Concerning these attempts and future developments in the field of reproductive cloning, it was stated that a wide perspective must be maintained on this type of work (Edwards 2003). In the context of ethical and medical considerations, critical safety issues concerning risk factors for malformations have also been emphasized for human cloning (Rhind et al. 2003; Lantham et al. 2004).

In the current clinical report, we have presented and summarized further evidence for our attempts in creating SCNT human embryos for intrauterine transfer. Even though no pregnancy was established in four cases so far, we have shown that human reproduction via SCNT and the creation and transfer of human cloned embryos may eventually be applicable in the future for patients that suffer from severe infertility and may have no other viable alternative options for procreating their own offspring.

Key words: *adult fibroblasts- assisted reproductive technologies (ART) - human embryo cloning - interspecies bovine bioassay - male infertility - somatic cell nuclear transfer (SCNT)*

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