

## Rapid Communication

# Somatic Cell Nuclear Transfer in Humans: Pronuclear and Early Embryonic Development

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### ABSTRACT

**Human therapeutic cloning requires the reprogramming of a somatic cell by nuclear transfer to generate autologous totipotent stem cells. We have parthenogenetically activated 22 human eggs and also performed nuclear transfer in 17 metaphase II eggs. Cleavage beyond the eight-cell stage was obtained in the parthenogenetic-activated eggs, and blastocoele cavities were observed in six. Three somatic cell-derived embryos developed beyond the pronuclear stage up to the six-cell stage. The ability to create autologous embryos represents the first step towards generating immune-compatible stem cells that could be used to overcome the problem of immune rejection in regenerative medicine.**

### INTRODUCTION

**E**merging embryonic stem cell-based technologies offer the potential for many novel therapeutic modalities. However, clinical implementation requires a definitive resolution of the problem of histocompatibility. The ability to generate totipotent stem cells that carry the nuclear genome of the patient using nuclear transfer (NT) techniques would overcome this last major challenge in transplantation medicine (1). It would enable the production of virtually all cell and tissue types, all carrying the nuclear genome of the patient. And since the starting somatic cell such as fibroblasts can be modified by gene targeting (2), the resulting cells would be modified as well. Clinical application may include the production of cardiomyocytes to replace damaged heart tissue or insulin producing B-cells for patients with diabetes, among many others (3). However, the realization of these therapies relies on the generation of early-stage embryos for the purpose of stem cell isolation.

The first demonstration that a somatic cell could be dedifferentiated was shown in cattle when NT-derived blastocysts were generated using cumulus cells (4). These results were later confirmed by Wilmut et al. with the cloning of an adult animal from a quiescent mammary gland-derived cell (5) and by Cibelli et al. with actively dividing fetal fibroblasts (6). Despite these and subsequent studies, protocols for NT us-

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ing somatic cells are still being developed, and the mechanisms underlying this biological phenomenon have yet to be characterized and understood.

Embryo reconstitution by NT depends upon a number of important technical and biological variables such as egg quality, enucleation and cell transfer procedures, and egg activation. Ultimately, the nucleus of the donor somatic cell must render itself to be reprogrammed by the egg, which in turn must be capable to induce all the epigenetic changes to down-regulate somatic genes and up-regulate embryonic ones.

Although NT as a means of generating stem cells has been achieved in mice (7–9) and cattle (10), the cloning of primate embryos, including humans, using somatic donor cells has been problematic and has yet to be reported. The present study describes protocols for the generation of human embryos reconstructed with somatic cells. These methods will provide the foundations for later studies on the generation of NT-derived stem cells for their further use in isogenic cell replacement therapy.

## MATERIALS AND METHODS

### *Superovulation and egg retrieval*

Egg donors were women between the ages of 24 and 32 years with at least one biologic child. They underwent thorough psychological and physical examination, including assessment by the Minnesota Multiphasic Personality Index test, hormone profiling, and PAP screening. They were also screened carefully for infectious diseases, including hepatitis viruses B and C, human immunodeficiency virus, and human T-cell leukemia virus. Criteria for acceptance into the study will be published elsewhere.

Donor ovaries were down-regulated by at least 2 weeks of oral contraceptives, followed by controlled ovarian hyperstimulation with twice daily injections of 75–150 units of gonadotropins. Pituitary suppression was maintained in some donors by concomitant twice daily administration of Synarel, beginning 3 days before discontinuing oral contraceptives and 5 days before initiating gonadotropin injections, and in other donors by injection of Antigone beginning with leading follicle diameters of 12 mm. Ovarian stimulation was calculated to minimize the risk of ovarian hyperstimulation syndrome by ensuring the serum estradiol levels of the donor did not exceed 3,500 pg/ml on the day of human chorionic gonadotropin (hCG) injection to stimulate the resumption of oocyte meiosis. Blood serum estradiol levels were measured at least every 2 days, and hCG was administered when the leading follicle reached at least 18 mm by ultrasound examination. Eggs were collected from antral follicles of anesthetized donors by ultrasound-guided needle aspiration into sterile test tubes. They were freed of cumulus cells with hyaluronidase and scored for stage of meiosis by direct examination.

### *Somatic cell isolation*

Adult human fibroblasts were isolated from 3-mm skin biopsies taken from consenting adult volunteers. Skin explants were cultured for 3 weeks in DMEM (Gibco, Grand Island, NY) plus 10% fetal calf serum (HyClone, Logan, UT) at 37°C and 5% CO<sub>2</sub>. Once cellular outgrowth was observed, fibroblasts and keratinocytes were enzymatically dissociated using 0.25% trypsin and 1 mM EDTA (GibcoBRL, Grand Island, NY) in PBS (GibcoBRL) and passaged 1:2. Fibroblasts were used at the second passage. The identity of these cells was later confirmed by immunocytochemistry, and seed stocks of these cells were frozen and stored in liquid nitrogen until use as cell donors. Cumulus cells were used immediately after egg retrieval and processed as previously described (11).

### *Egg enucleation and NT*

Prior to manipulation, eggs were incubated with 1 µg/ml bisbenzimidazole (Sigma, St. Louis, MO) and cytochalasin B (5 ng/ml; Sigma) in embryo culture media for 20 min. All manipulations were made in HEPES-buffered human tubal fluid (HTF; Irvine Scientific, Santa Ana, CA) under oil. Chromosomes were visualized with a 200× power on an inverted microscope equipped with Hoffman optic and epifluorescent ultraviolet light. Enucleation was performed using a piezo electric device (Prime Tech, Japan) specially de-

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signed to minimize the damage generated during the micromanipulation procedure. A 10  $\mu\text{m}$  I.D. blunt needle that contained mercury near its tip to be able to control the penetration capacity and accuracy of the procedure was used to penetrate gently the zona pellucida and aspirate the chromosomes and adjacent cytosol. Donor cells were maintained in a solution of 12% PVP (Irvine Scientific) in culture media and loaded into a small piezo-driven needle of  $\sim 5 \mu\text{m}$  I.D. The nucleus of the donor cells was isolated by suctioning the cells in and out through the pipette, and it was immediately injected into the cytosol of enucleated eggs. After reconstruction, eggs were returned to the incubator and activated 1–3 h later.

### *Egg activation and culture*

At 35–45 h after exogenous hCG stimulation, eggs were activated by incubating them with 5  $\mu\text{M}$  ionomycin (Calbiochem, La Jolla, CA) for 4 min followed by 2 mM 6-dimethylaminopurine (DMAP; Sigma) in G1.2 for 3 h. Eggs were then rinsed three times in HTF and placed in G1.2 (Vitrolife, Vero Beach, FL) or Cook-Cleavage (Cook IVF, Indianapolis, IN) culture media for 72 h at 37°C in 5% CO<sub>2</sub>. On the fourth day of culture, cleaving eggs resembling embryos were moved to G2.2 or Cook-Blastocyst until day 7 after activation.

### *Human subjects*

Strict guidelines for the conduct of this research have been established by Advanced Cell Technology's independent Ethics Advisory Board (EAB). In order to prevent any possibility of reproductive cloning, the EAB requires careful accounting of all eggs and embryos used in the research. No embryo created by means of NT technology may be maintained beyond 14 days of development. The EAB has also established guidelines and oversight for the donor program that provided the human eggs used in this research. This includes extensive efforts to ensure that the risks to donors are minimized, that donors are fully informed of the risks, and that their consent is free and informed. More information on this subject can be obtained at [www.advancedcell.com/ethics.html](http://www.advancedcell.com/ethics.html) [for a review of the ethical issues see (12)].

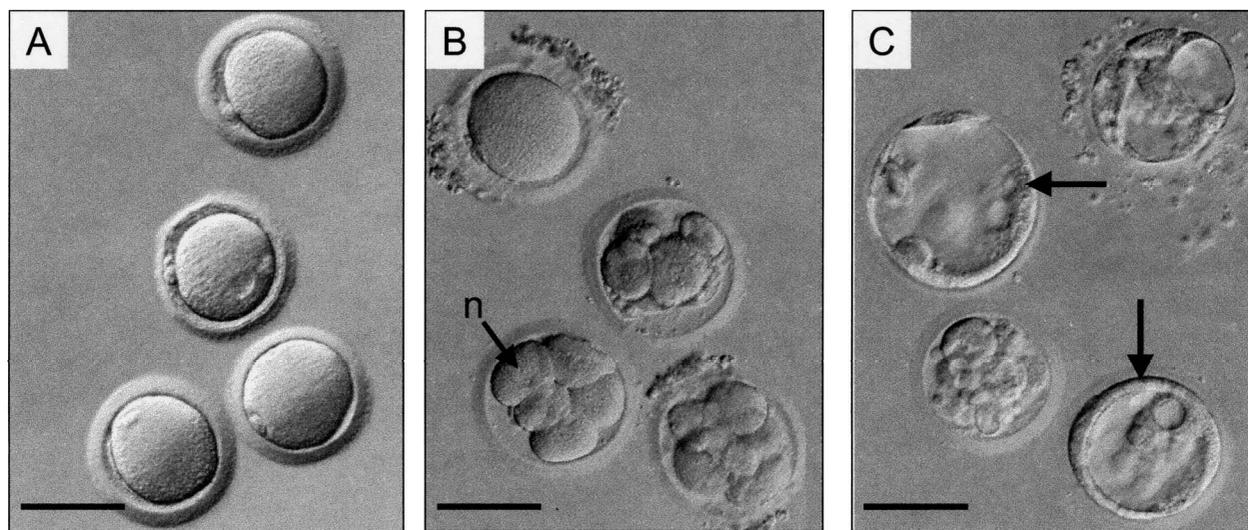
## RESULTS

A total of 71 eggs were obtained from seven volunteers (Table 1). At the time of retrieval, five eggs were at the germinal vesicle stage, and no further development was observed after 48 h in culture. Nine eggs were at metaphase (M) I stage and were systematically used for activation or NT after  $\sim 3$  h in culture. Fifty-seven eggs that were at MII stage were immediately used for NT or parthenogenetic activation experiments.

Eggs from three volunteers were used for parthenogenetic activation. A total of 22 eggs were activated at 40–43 h after hCG stimulation. Twelve hours after activation, 20 eggs (90%) developed one pronucleus

TABLE 1. MATURATION PROFILE OF HUMAN EGGS AT THE TIME OF COLLECTION

<i>Donor</i>	<i>No. of eggs</i>	<i>Germinal vesicle</i>	<i>MI</i>	<i>MI</i>
1	6	1	0	5
2	15	0	0	15
3	8	2	0	6
4	11	2	4	5
5	15	0	2	13
6	11	0	3	8
7	5	0	0	5
Total	71	5	9	57



**FIG. 1.** Parthenogenetic development in human eggs. (A) MII eggs at the time of retrieval. (B) Four- to six-cell embryos 48 h after activation. Distinguishable single-nucleated blastomeres were consistently observed (n). (C) Blastocoele cavity (arrows) were detected on day 6 and maintained in culture until day 7. Scale bars = 100  $\mu$ m.

and cleaved to the two- to four-cell stage on day 2 (day 0 = activation). On day 5 of culture, evident blastocoele cavities were observed in six of the parthenotes (30%, as percentage of cleaved eggs) (Fig. 1 and Table 2), though none of them displayed a clearly discernible inner cell mass.

Eggs from seven volunteers were used for NT procedures. A total of 19 eggs were reconstructed using nucleus from fibroblasts and cumulus cells. Twelve hours after reconstruction with a fibroblast nucleus, seven eggs (69%, as a percentage of reconstructed eggs) exhibited a single, large pronucleus, morphologically similar to those observed in eggs fertilized with sperm. Only one pronucleus with prominent nucleoli (up to 10) was observed in each reconstructed egg. Four of eight eggs injected with cumulus cells developed pronuclei, and three of those cleaved to four or six cells (Fig. 2 and Table 3).

## DISCUSSION

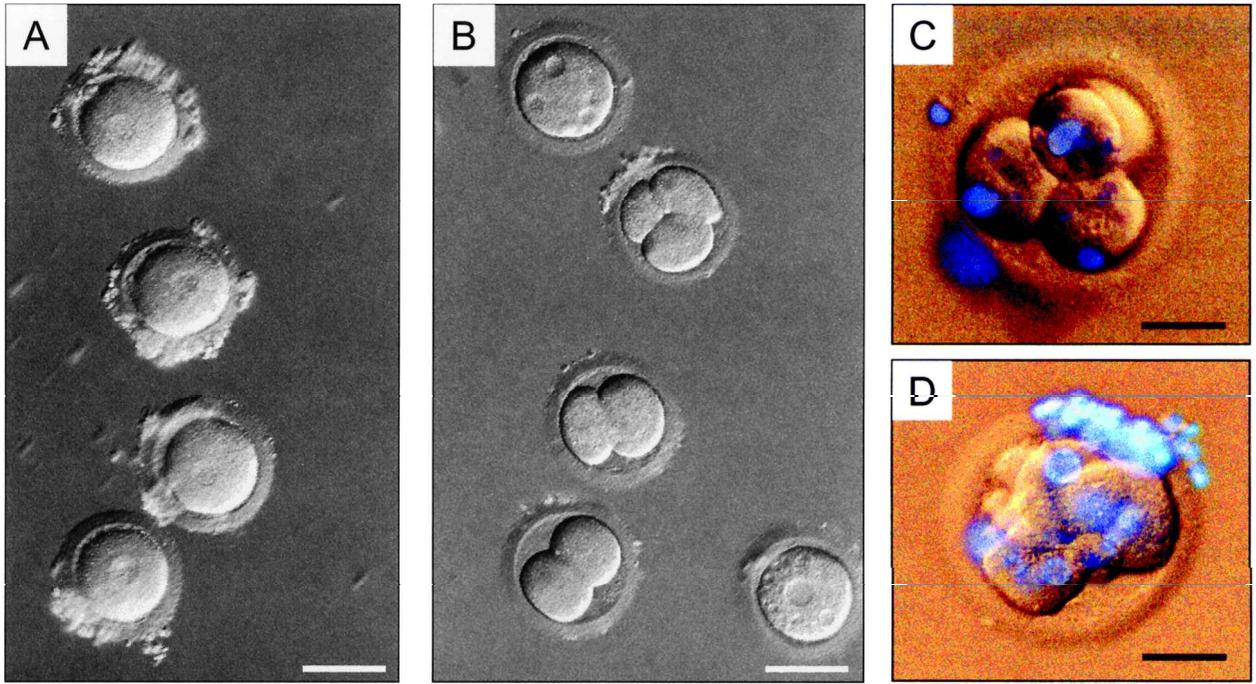
Previous studies have indicated the possibility of human parthenogenetic development. Rhoton-Vlasak et al. in 1996 (13) have shown that short incubations with calcium ionophore can induce pronuclear formation, and recently Nakagawa and collaborators (14) demonstrated that a combination of calcium ionophore and puromycin or DMAP could not only trigger pronucleus formation but early cleavage as well. A similar protocol has shown also to be applicable in nonhuman primate eggs (15). Here we show an effective

TABLE 2. PARTHENOGENETIC ACTIVATION OF HUMAN EGGS

Donor	No. of eggs	Pronucleus (%) <sup>a</sup>	Cleaved (%) <sup>a</sup>	Embryos with blastocoele cavity (%) <sup>b</sup>
1	5	4 (80)	4 (80)	0
2	14	13 (93)	13 (93)	4 (31)
6	3	3 (100)	3 (100)	2 (67)
Total	22	20 (90)	20 (90)	6 (30)

<sup>a</sup>As a percentage of activated eggs.

<sup>b</sup>As percentage of cleaved eggs.



**FIG. 2.** NT-derived human embryos reconstructed with cumulus cells. Pronuclear-stage embryos at (A) 12 and (B) 36 h, (C) a four-cell embryo at 72 h, and (D) a six-cell embryo at 72 h. In C and D the nucleus of the embryos were stained with bisbenzimidazole (Sigma) and visualized under UV light. Scale bars = 100  $\mu\text{m}$  for A and B and 50  $\mu\text{m}$  for C and D.

protocol for parthenogenetic activation of human eggs, embryonic cleavage, and the formation of a blastocoele cavity. This finding may have important implications on somatic cell cloning and offers the alternative of generating human totipotent stem cells without paternal contribution. In addition, the removal of the parthenogenetic female pronucleus and the transfer of two male pronuclei may allow the production of embryos and resulting stem cells for a male donor.

There are currently no published reports of successful somatic cell NT in humans. In this study we obtained embryonic cleavage following NT from two different cell types: adult cumulus cells and skin fibroblasts. Using cumulus cells as donors, three eggs cleaved to the two-cell, four-cell, and six-cell stages, respectively. Eggs reconstituted with cultured adult fibroblasts developed pronuclei but did not cleave. Several reasons can be attributed to the differences in development, ranging from cell cycle state, chromatin structure, DNA methylation, and embryonic origin of the cells, among others (6,16–23). We believe, however, that the difference is technical rather than biological. Fibroblast cells are double the size of cumulus cells, and as such they require more intensive manipulation for the isolation of its nucleus; this may in turn accidentally damage the nucleus, causing it to halt development at early stages.

TABLE 3. SOMATIC CELL NUCLEAR TRANSFER IN HUMAN EGGS

<i>Donor</i>	<i>Cell type</i>	<i>Reconstructed eggs</i>	<i>Pronucleus (%)<sup>a</sup></i>	<i>Cleaved (%)<sup>b</sup></i>
3	Fibroblast	2	0	0
4		5	4 (80)	0
5		4	3 (75)	0
6	Cumulus	5	3 (60)	3 (100)
7		3	1 (33)	0
Total		19	11 (58)	3 (27)

<sup>a</sup>As a percentage of reconstructed eggs.

<sup>b</sup>As percentage of pronuclear embryos.

Unlike reproductive cloning (which aims to produce an entire organism), human therapeutic cloning does not seek to take development beyond the earliest preimplantation stage. Rather, the goal is to derive primordial stem cells *in vitro*, such as embryonic stem cells from the inner cell masses of blastocysts as a source of cells for regenerative therapy (3). Animal studies suggest that these cells may eventually play an important role in treating a wide range of human disease conditions, including diabetes, arthritis, AIDS, strokes, cancer, and neurodegenerative disorders such as Parkinson's and Alzheimer's disease (24–27). In addition to generating individual or small groups of replacement cells, there is also the possibility that these cells could be used to reconstitute more complex tissues and organs, including blood vessels, myocardial “patches,” kidneys, and even entire hearts (28,29). NT techniques have the potential to eliminate the immune responses associated with the transplantation of these various tissues, and thus the requirement for immunosuppressive drugs and/or immunomodulatory protocols that carry the risk of serious and potentially life-threatening complications. It has therefore been suggested that the medical applications of NT may have significant merit and should be actively pursued (3); however, we urge that the use of NT in human reproduction is currently unwarranted.

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