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TECHNOLOGY DEVELOPMENT

Development of Human cloned Blastocysts Following Somatic Cell Nuclear Transfer (SCNT) with Adult Fibroblasts

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ABSTRACT

Nuclear transfer stem cells (NTSC) holds considerable promise in the field of regenerative medicine and cell-based drug discovery. In this study, a total of 29 oocytes were obtained from three young (20-24 y) reproductive egg donors who had been successful in previous cycles. These oocytes, deemed by intended parents to be in excess of their reproductive needs, were donated for research without financial compensation by both the egg donor and intended parents after receiving informed consent. All intended parents successfully achieved ongoing pregnancies with the oocytes retained for reproductive purposes. Mature oocytes, obtained within 2 h following transvaginal aspiration, were enucleated using one of two methods, extrusion or aspiration, after 45 min incubation in Cytochalasin B. Rates of oocyte lysis or degeneration did not differ between the two methods. Somatic cell nuclear transfer (SCNT) embryos were constructed using two established adult male fibroblast lines of normal karyotype. High rates of pronuclear formation (66%), early cleavage (47%) and blastocyst (23%) development were observed following incubation in standard IVF culture media. One cloned blastocyst was confirmed by DNA and mtDNA fingerprinting analyses and DNA fingerprinting of two other cloned blastocysts indicated they were also generated by SCNT. Blastocysts were also obtained from a limited number of parthenogenetically activated oocytes. This study demonstrates, for the first time, that SCNT can produce human blastocyst stage embryos using nuclei from a differentiated adult somatic cell, and provides new information on methods that may be needed for a higher level of efficiency for human therapeutic cloning.

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INTRODUCTION

The consistent and efficient generation of both heterologous and autologous stem cells using somatic cell nuclear transfer (SCNT) has the potential to revolutionize our understanding and development of treatments for degenerative diseases [1-10]. The recent voluntary withdrawal of publications, amid allegations of scientific impropriety, that claimed successful human therapeutic cloning [11], suggests a reassessment of the critical pathways to performing successful SCNT with human oocytes is required. Somatic cell nuclear transfer in mammals was first performed in the early 1980s and numerous approaches have been employed to achieve the four major steps involved in the nuclear transfer procedure [see reviews, 12-14]. These four steps are; preparation of recipient cytoplasts (enucleated oocytes); preparation of donor DNA; transfer of the donor DNA into the cytoplast; and resumption of embryonic development by parthenogenetic activation; all of which must be

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completed without damaging the donor nucleus or the host oocyte/ cellular integrity.

Derivation of cloned human embryos from SCNT techniques remains in its infancy with the only reliable publication describing the generation of a single cloned blastocyst, which did not produce a human embryonic stem cell (hESC) line [15]. Importantly, this involved an undifferentiated hESC donor cell, rather than an adult cell. Lu et al (2003) have also reported to have cloned human embryos using fetal fibroblasts and granulosa cells, although the reliability of the finding is uncertain since no DNA fingerprinting of the cloned blastocysts was provided [16]. Other preliminary reports have generated only early cleavage stage nuclear transfer embryos [17, 18] using cytoplasts obtained from both in vitro and in vivo maturation and aged human IVF oocytes that had failed to fertilize [18, 19]. Clearly, a number of significant hurdles need to be addressed if SCNT is to lead to the successful application of cell-based therapies utilizing autologous hESC lines. The scarcity of donated mature human metaphase II oocytes available for research is a significant impediment. Using an alternative source of failed-to-fertilize human oocytes obtained at 16-18 h post insemination [19] or 48 h after retrieval [20] resulted in cleavage abnormalities and early-embryonic arrest, primarily as a consequence of aneuploidy and spindle defects. These types of oocytes also appear to be more difficult to enucleate and fuse when compared to freshly recovered human oocytes [20]. The use of aged oocytes has been reported to support embryonic nuclear remodeling and reprogramming to varying degrees in the rabbit [21], mouse [22] and bovine [23]. However, failed-to-fertilize human oocytes, may show irreversible cytoplasmic aging effects that do not adequately support the effective nuclear remodeling and reprogramming of somatic cells [19, 24]. Similar failed-to-fertilize oocytes also cleave poorly after NT in non-human primates [25].

The aim of the present study was to examine the development of human cloned embryos derived from adult fibroblasts. Restoration of developmental competence to the donor nuclei was determined by evidence of remodeling (pronuclear formation) and reprogramming embryo development (blastocyst formation).

METHODS

Human Ethics

All experiments were approved by an Independent Review Committee, (Independent Review Consulting, Inc., San Anselmo, CA, USA) in accordance with the HHS Policy for Protection of Human Research Subjects (45CFR46) and the National Academy of Sciences Guidelines for the Human Embryonic Stem Cell Research [26] and appear also to comply with the recently released guidelines from the International Society for Stem Cell Research [27]. All donated oocytes were obtained from three egg donation cycles performed at the Reproductive Sciences Center (RSC), a fertility center located in La Jolla, CA, that is fully compliant with the Society for Assisted Reproductive Technologies (SART) and American Society Reproductive Medicine (ASRM) guidelines. Its laboratory is licensed by the State of California and American Association of Tissue Banks (AATB), accredited by the College of American Pathologists (CAP) and registered with the Food and Drug Administration (FDA). The clinical procedures and operative facility have been inspected and accredited by the American Association of Ambulatory Health Care (AAAHC). All facilities are subject to inspection by the various agencies.

Following operative and clinical procedures, excess-to-reproductive needs oocytes were donated without direct linkage information so that the SCNT researcher was unaware of the identity of any oocyte donor. However, the SCNT researcher maintains a record of every gamete, somatic cell, embryo donation or product of SCNT that has been donated, created

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or used. This record is sufficient so that if necessary the provenance and disposition of such materials could be determined.

Materials

All chemicals used for the production of nuclear transfer (SCNT) and parthenogenetically activated (PA) embryos were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA) unless otherwise stated. All embryo manipulations were performed at 37^0C except for the process of electrical fusion, which was performed at room temperature. All culture-ware underwent standard quality control testing prior to use and culture media were pre-incubated to 37^0C and when necessary, pre-equilibrated in a humidified atmosphere of 6% CO_2, 5% O_2, 89% N_2 in a dual gas incubator (Innova CO-170, New Brunswick Scientific, Edison, NJ, USA).

Preparation of Karyoplasts

Primary adult fibroblast cell lines were isolated from a 2-3 mm skin biopsy sample taken from two healthy, adult male donors (AF1 and AF2) using standard procedures approved in the SCNT research ethics application (S*ee Human Ethics section*).

Cell lines were established and maintained in Minimum Essential Medium (MEM) α Medium containing Glutamax™, ribonucleosides and deoxyribonucleosides supplemented with 100 U/ml Penicillin, 100 µg/ml Streptomycin, 55 µM 2-mercaptoethanol, (Gibco, Invitrogen Co., Carlsbad, CA, USA) and 20% (v/v) human serum (pooled from patients undergoing fertility treatment at RSC). All serum used tested negative for the following diseases; HIV 1, HIV 2, HTLV 1, HTLV 2, RPR, Hep. C Antibody, Hep B Surface Antigen, Anti-Hep B Core Igm, Anti-Hep B Core Ab Igg, CMV Igg/Igm, Chlaymdia and GC Cultures.

For cytogenetic analysis, donor cell lines were incubated in MEM medium with 0.1 mg/ml of Colcemid (KaryoMAX® , Gibco Invitrogen Co., Carlsbad, CA, USA) for 3–4 h, trypsinized, re-suspended in 0.075 M KCl, and incubated for 20 min at RT, then fixed in 3:1 methanol acetic acid. Chromosome number and size were determined using Giemsa– stained metaphase spreads.

For cell cycle analysis, actively dividing fibroblasts AF1 and AF2 were harvested (TrypLE™) to a single cell suspension, washed and re-suspended in DPBS. A total of $1x10^6$ cells were re-suspended in 0.25ml of ice cold GM buffer (6 mM Glucose; 0.14 M NaCl; 5.4 mM KCl; 1.1 mM Na_2HPO_4-$12H_2O$; 1.1 mM KH_2PO_4 and 0.5 mM EDTA) before adding 0.75 mL of chilled 95% Ethanol. Samples were sent on ice to Phoenix Flow Systems, Inc. (San Diego, CA, USA) where cells were stained with 50 µl/ml Propidium Iodide/RNAse A solution, acquired on a Becton Dickenson LSR Flow Cytometer and analyzed with MultiCycle AV DNA analysis software. A control sample was included in the analysis.

Fluorescent In-situ Hybridization (FISH) Analyses

FISH was performed with probes for chromosomes 13, 18, 21, X and Y in accordance with the manufacturer's codenaturation and posthybridisation instructions accompanying the hybridisation kit (Multi-Vysion PGT Fluorescent Probe Kit, Vysis, Abbott Molecular Inc., Des Plaines, IL, USA)

Fibroblast cells were analyzed with a fluorescence microscope (BX61TRF, Olympus America Inc., Melville, NY, USA) equipped with single bandpass filters for red (chromosome 13), aqua (chromosome 18), green (chromosome 21), blue (chromosome X) and gold (chromosome Y). The images were analyzed with Cytovision software (v3.6, Applied Imaging Corp., SA Jose, CA, USA).

Actively dividing fibroblasts (passage 3-5; 60%-80% confluent) without serum starvation were used as donor karyoplasts. Single cell suspensions of the donor cell lines (one or two) were prepared with recombinant trypsin

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(TrypLE™ Express, Gibco, Invitrogen Co., Carlsbad, CA, USA) on the day of SCNT.

Oocyte Donation

Prior to any procedures, oocyte donors and oocyte recipients (intended parents) were independently counseled, and informed consent obtained for the anonymous donation of those oocytes considered by the intended parents to be in excess of their reproductive needs. Typically, intended parents elected to donate those oocytes in excess of 10-12 per transvaginal aspiration [28, 29].

Oocyte donors, all of whom had at least one previous successful donation cycle, underwent routine controlled ovarian hyperstimulation involving a combination of FSH and LH containing gonadotropins using either a standard GnRH agonist long protocol (leuprolide acetate, Lupron®, TAP Pharmaceutical Products Inc., Lake Forest, IL, USA) or a GnRH antagonist protocol (cetrorelix, Cetrotide®, Serono, Inc. Rockland, MA, USA) following suppression with an oral contraceptive [30].

Beginning on Day 3 of their treatment cycle, donors received recombinant FSH 150-225 IU/day (Gonal-f®, Serono, Inc. Rockland, MA, USA) and hMG 75-150 IU/day (Menopur®, Ferring Pharmaceuticals, Inc., Suffern, NY, USA) for 9-10 days. Dosages were adjusted based on ovarian response as assessed by serum estradiol concentrations and ultrasound imaging of the ovaries. When a GnRH antagonist was utilized, a flexible start protocol was employed with the antagonist initiated when the largest ovarian follicle present reached a mean diameter of at least 12 mm. When at least two follicles had a mean diameter of 18 mm, ovulation was induced with human chorionic gonadotropin (hCG) 5,000 or 10,000 IU (American Pharmaceutical Partners, Inc., East Schaumburg, IL, USA). Cumulus-oocyte complexes (COCs) were retrieved by ultrasound-guided transvaginal aspiration 34-36 h later (Day 0) from follicles ranging in size from about 14-20 mm.

Preparation of Cytoplasts

The cumulus matrix was removed from oocytes (1-2 h post aspiration (hpa) by repeated gentle pipetting in 80 U/mL of recombinant human PH20 hyaluronidase enzyme as per the manufacturers instructions (Cumulase™, Halozyme Therapeutics, Inc., San Diego, CA, USA) in HTF-HEPES with EDTA and Glutamine medium (InVitroCare, Inc., Frederick, MD, USA) supplemented with 5% (v/v) Human Serum Albumin (HSA, InVitroCare, Inc., Frederick, MD, USA).

Cumulus-free oocytes were then incubated in 5 μg/mL cytochalasin B (CYTB) in HTF-HEPES for 45 min with Hoechst 33342 (6 μg/mL) added to the medium for the last 15 minutes of incubation to enable visualization of nuclear material under ultraviolet light (UV). Conditions for the enucleation of oocytes using the aspiration method were based on a preliminary study using 8 failed-to-fertilize or MI oocytes which showed that increasing the incubation time in CYTB decreased the level of oocyte lysis.

At 3-4 hpa, oocytes were placed in HTF-HEPES under Paraffin oil (Ovoil™, Vitrolife, Inc., Englewood, CO, USA) and enucleated using one of two methods: extrusion or aspiration. Using the first method, the zona pellucida was pierced with a sharp beveled glass pipette to make a slit and the adjacent cytoplasm containing the metaphase II (MII) chromosomes, and sometimes the first polar body, were extruded through the slit in the zona pellucida by applying pressure to the oocyte with the needle[15]. With the alternative method, the MII chromosomes were aspirated with a small volume of cytoplasm into a sharp beveled glass pipette (OD 20-25 μm). The removal of DNA was confirmed by DNA staining and brief visualization under UV light (see Figure B, supplemental data).

Somatic Cell Nuclear Transfer

A single fibroblast donor cell 10-15 μm in diameter was inserted under the zona pellucida so that it remained in contact with the cytoplast.

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This size range selected was based on the work of Boquest et al. (1999), who showed that the highest proportion (up to 95%) of cells of this diameter represented the G1(+G0) stages of the cycle cycle [31].

When the first polar body was not removed due to location of the metaphase II plate the donor fibroblast was inserted in a position that provided at least 90^0 of separation from the polar body. This allowed the correct parallel alignment of donor cell/ cytoplast with the fusion electrodes and avoided interference of the polar body with the fusion process.

NT cytoplasts-fibroblast pairs were equilibrated (5 min) in fusion medium consisting of 0.3 mol/l sorbitol, 0.1 mmol/l $MgSO_4$, 0.5 µmol/l $CaCl_2$, and 3% (w/v) HSA. Fusion was induced in a cell fusion chamber (0.5 mm gap) with two electrical pulses (1.7 kV cm^{-1} for 15 µs x 2 pulses administered 0.1s apart) generated from a CF-150B impulse generator (Biological Laboratory Equipment, Maintenance and Service Ltd., Budapest, Hungary).

All embryos were examined morphologically after 20 min and non-fused embryos were fused again. Embryos where the first polar body had not been removed at the time of enucleation were examined carefully to ensure that only fusion of the donor cell had occurred (ie the first polar body was still present after fusion).

Parthenogenetic Activation

Reconstructed SCNT and metaphase II (PA) embryos were parthenogenetically activated 2-3 h after electrical fusion (5 hpa) with calcium ionophore A-23187 (CI, 10 µM, 4 min) in HTF-HEPES, washed three times in fresh HTF-HEPES before being transferred to either the low glucose and phosphate free formulation of IVC-TWO™ (InVitroCare, Inc., Frederick, MD, USA) supplemented with 8 % (v/v) HSA and 2 mM 6-dimethylaminopurine (6-DMAP) or 10 µg/ml cycloheximide (CHX) and 2.5 µg/ml cytochalasin D (CYTD) for a further 3-4 h incubation under paraffin oil in a high humidity atmosphere of 6% CO_2, 5%O_2, 89% N_2 for 4 h.

Embryo Culture

Following a 3-4 h incubation (8-9 hpa) in 6-DMAP or CHX/CYT D, SCNT and PA embryos were washed thoroughly with IVC-TWO supplemented with 8 % (v/v) HSA and transferred in group culture conditions into one well per treatment of an In Vitro Fertilization 4 Well Plate (Falcon, Becton Dickinson and Co., Franklin Lakes, NJ, USA) containing IVC-TWO with 8% HSA under paraffin oil and maintained at 37^0C in a humidified atmosphere of 5% CO_2, 6% O_2, 89% N_2 . On Day 2, embryos were transferred to IVC-TWO with 9% HSA and then on late Day3/early Day 4 to phosphate-free, and elevated levels of glucose formulation of IVC-THREE™ (InVitroCare, Inc., Frederick, MD, USA) with 12% HSA. The rates of pronuclear formation, early cleavage and blastocyst formation were evaluated stereo-microscopically and photographed throughout *in vitro* culture.

Total Blastocyst Cell Count.

Prior to embryo lysis for microsatellite analyse, SCNT and parthenogenetic embryos were stained with Hoechst 33342 (6 µg/mL) and photographed under UV light to determine total cell number.

Microsatellite Analyses

Groups of 5-10 oocyte donor cumulus and fibroblast donor cells, SCNT and PA blastocysts were sent on ice in 5 µL of lysis buffer to Genesis Genetics Institute (Detroit, MI, USA) in coded vials for independent microsatellite and mtDNA analyses. Single stranded human genomic DNA was extracted using specifically designed and tested single cell techniques [32] and 15 autonomous DNA markers and the sex chromosomes were amplified using the PowerPlex 16 System kit (Promega Corporation, Madison, WI, USA). Genescan analysis on the amplified fragments was performed for the following microsatellite markers: Penta E (15q), D18S51 (18q21.3), D21S11 (21q11-21q21), TH01 (11p15.5), D3S1358 (3p), FGA (4q28),

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TPOX (2p23-2pter), D8S1179 (8q), vWA (12p12-pter), Amelogenin (Xp22.1-22.3 and Y), Penta D (21q), CSF1PO (5q33.3-34), D16S539 (16q24-qter), D7S820 (7q11.21-22), D13S317 (13q22-q31), D5S818 (5q23.3-32). These were run on an ABI 3130 Genetic Analyzer, and allele calls determined by GeneMapper version 4.0 using bin sets optimized for that instrument (Applied Biosystems, Foster City, CA, USA).

Mitochondrial Analyses

Following microsatellite analyses, DNA from the samples submitted in coded vials was used to amplify mtDNA hypervariable region II (HVII). The PCR forward primer spans bases 15-34, and the reverse primer spans bases 431-412 for homo sapiens mitochondrion, complete genome (NC_001807.4) and reaction conditions were essentially as those previously described [33, 34], except that -21M13 and 28M13Rev tails were added to the forward and reverse PCR primers, respectively, for use in subsequent sequencing reactions. Separate sequencing reactions were performed on both strands of each PCR product by priming off the appropriate M13 tail, using ABI Prism's BigDye Terminator version 3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA). Unincorporated dye-labeled products were removed by spin column purification, using Sephadex G-50 superfine (GE Healthcare, Uppsala, Sweden). The eluate was run on an ABI 3130 Genetic Analyzer, and base calls determined by Sequence Analysis 5.1 software (Applied Biosystems, Foster City, CA, USA).

RESULTS

Preparation of Donor Karyoplasts

Cytogenetic analysis of established fibroblast cell lines (Figures 1A and B) showed 95% AF1 (n=20) and 100% AF2 (n=20) of cells examined maintained a diploid 22 chromosomes + XY karyotype. (Figures 1C (AF1) and D (AF2)). This was confirmed with FISH analysis of both adult male fibroblast cell lines which demonstrated a diploid pattern for chromosomes 13, 18 and 21 and presence of X and Y chromosomes (Figure 1E (AF1) and F (AF2).

Cell Cycle Analyses

The distribution of cycling AF1 and AF2 adult somatic cells existing in the various phases of the cell cycle as determined by flow cytometry is shown in Figure A (see supplemental data). The prominent DNA peaks (in blue) show the majority of adult somatic cells at a given point in time were in G1 (+G0) where values of 63.4% of AF1 (Fig. A (a)) and 53.6% of AF2 (Fig A (b)) were determined. Compared to values for other phases of the cell cycle S phase (light green) and G2 +M (green) where 18.7% AF1 and 30.4% AF2 were in S Phase and 17.9% AF1 and 16% AF2 were in G2+M phase. The presence of cellular debris was determined at 2.7% and 2.2% for AF1 and AF2 respectively. Fig. A (c) is control DNA.

Oocyte Donation

From March to September 2006, three oocyte donors (20-24 y) and their intended parents agreed to participate in the SCNT research project. For this part of the SCNT project, 29 excess-to-reproductive-needs oocytes were anonymously donated within 1-2 h of transvaginal aspiration.

Preparation of Cytoplasts

The first polar body (PB) was observed in 90% (26/29) of oocytes indicating that most had matured to the Metaphase II (MII) stage of development (Table 1). Two methods of enucleation were employed following incubation in CYTB, extrusion and aspiration (Figure 2, A2). Additional figures of the aspiration process can be seen in the supplemental data. Figure B (a1-a3) shows metaphase II enucleation with the polar body and (b1-2) for metaphase II enucleation without removal of the polar body. The PB was only removed (12/21, 57% of enucleation attempts) if in close proximity to the metaphase plate. The aspiration method removed the least amount of associated cytoplasm. Enucleation was confirmed with UV fluorescence following incubation of the oocyte

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in Hoechst 33342 (Figure 2, A3 and Figure B a1-a3 and b1-b2).

Out of 23 enucleated, 7 (33 %) SCNT embryos either lysed after enucleation or following electrical fusion or showed no evidence of pronuclear formation and degenerated after 24 h of culture (Figure 2, C4). There was no difference between the methods of enucleation in terms of rates of degeneration or lysis.

Somatic Cell Nuclear Transfer

Following SCNT, 14 (66%) or 5/8 (63%) AF1 and 9/13 (69%) AF2) reconstructed embryos showed evidence of DNA remodeling (pronuclear formation) at 6-7 hrs after chemical activation (Table 1; Figure 2, C1, C2, C4). On Day 3, 10 embryos (47% or 4/8 (50%) AF1 and 6/13 (46%) AF2), with five or more cells were observed (Figure 2 C3, C4) and 5 (50% of multi-cellular embryos) of those (2/10 (20%) AF1 and 3/13 (23%) AF2) formed blastocysts on Day 5/6 (Figure 2 C5, C6, See supplemental data Figure C, a, b, c).

Blastocysts were also observed in the small number of metaphase II oocytes that underwent parthenogenetic activation (n=5) from CI/DMAP (2 Bl, n=4) and CI/CHX/CYTD (1 Bl, n=1) activation groups.

Total Blastocyst Cell Count

Total cell counts were performed on three parthenogenetic and five SCNT blastocysts obtained following nuclear transfer with both AF1 and AF2 donor cells as determined by DNA staining with Hoechst 33342 prior to preparation for microsatellite analysis. Cell numbers for parthenogenetic embryos ranged from 35-54 and 41-72 for SCNT blastocysts (see supplemental data (Figure D (a) parthenogenetic blastocyst (Coded reference K1) and (b) SCNT blastocyst (Coded reference K8) following nuclear transfer with AF2 (Coded reference C5 and C6) donor cell to oocyte derived from Egg donor 3 (Coded reference F1 and F2).

Microsatellite Analyses.

A total of 16 coded samples were sent for independent microsatellite analysis, and included duplicate samples of cumulus cells from the two egg donors (Egg donor 1 (Coded reference A1 and A2) and 3 (Coded reference F1 and F2)) and fibroblast cells from the two somatic cell donors (AF1 (Coded reference B3 and B4) and AF2 (Coded reference C5 and C6), five SCNT blastocysts, four parthenogenetic embryos and one blank sample (no DNA) to check for contamination due to handling. Two of the SCNT blastocysts (9F, K7) and two parthenogenetic samples failed to amplify and gave no reliable data and the blank was negative (data not shown). The microsatellite analyses of the three remaining (Coded reference 8E) and (Coded reference 10G and K8) blastocysts were consistently matched with the DNA from each of the AF1 (Coded reference B3 and B4) and AF2 (Coded reference C5 and C6) adult fibroblast donors respectively. Assuming allele drop-out (ADO) of TPOX (8) allele, blastocyst 8E from Egg Donor 1 (Coded reference A1 and A2) can be explained using alleles from adult fibroblast donor AF1 (Coded reference B3 and B4) alone; citing ADO of D13S317 (8) allele, blastocyst 10G from Egg Donor 1 (Coded reference A1 and A2) can be explained using alleles from fibroblast Donor AF2 (Coded reference C5 and C6) alone and Blastocyst 8K from Egg Donor 3 (Coded reference F1 and F2) can be explained using alleles from AF2 (Coded reference C5 and C6) alone, assuming that alleles THO1 (9.3), D13S317 (13) and TPOX (8) peaks are due to un-visualized alleles in the AF2 (Coded reference C5 and C6) donor cell samples (see Figure 3a and b). The original fingerprinting sequence data and allele calls for each of the cloned blastocysts are provided in the supplemental data (Figures F-L). Microsatellite analysis of parthenogenetic blastocyst (Coded reference K1) and morula (Coded reference K6) can be explained using alleles from Egg Donor 3 (Coded reference F1 and F2) alone (see supplemental data; Figures E, M and N).

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Mitochondrial DNA Analyses

Following successful microsatellite analyses, DNA from the samples submitted in coded vials was used to amplify the mtDNA HVII region. Separate sequencing reactions from both strands of each PCR product are shown in the supplemental data (See Table A and electropherogram data of mtDNA hypervariable region II; Figures Q-S). Reliable data was not obtained from all SCNT blastocysts due to the amount of material available and variations in the amount of amplified DNA. However SCNT blastocysts (Coded reference K8) showed the variations T at 33 bp, G at 34bp and 8C at 270bp in forward sequencing of HVII, and can be explained by the mitochondrial DNA sequence of Egg Donor 3 (Coded reference F1 and F2) and matched that of the parthenogenetic embryos (Coded reference K1 and K6) from the same egg donor.

DISCUSSION

This study demonstrates, for the first time, that SCNT can be utilized to generate cloned human blastocysts using differentiated adult donor nuclei remodeled and reprogrammed by human oocytes. Evidence of successful SCNT was shown with DNA fingerprinting analyses of three SCNT cloned blastocysts where embryo genomic DNA was that of the donor fibroblast cell line and were not fragmented oocytes or of parthenogenetic origin [35, 36]. Examination of mtDNA sequence from the HVII region showed that one SCNT blastocyst (Coded reference K8) could be explained to egg donor 3 (Code reference F1 and F2) mtDNA and was identical to the parthenogenetic embryos (Coded reference K1 and K6) obtained in the same experimental series.

The study describes a method allowing the derivation of human NT blastocysts using young oocytes and established adult male fibroblast lines of normal karyotype. The rate of SCNT blastocyst formation are similar to the development rates seen in standard IVF cycles (fertilization rate of 70%-80% of oocytes collected and 40%-60% of fertilized oocytes develop into blastocysts) [37-39] and to a previous nuclear transfer study with undifferentiated hESC donor cells [15]. Total cell counts in SCNT blastocysts improved with each subsequent experimental series and appear comparable to reports for various grades of human IVF embryos [40]. Blastocysts were also derived from a limited number of parthenogenetically activated oocytes using both of the activation methods employed. DNA fingerprint and mtDNA, of the HVII region, analyses of the parthenogenetic embryos (Coded reference K1 and K6) confirmed they originated solely from Egg Donor 3 (Coded reference F1 and F2). The frequency of blastocyst generation is similar to the recent reported work of Revazova et al. 2007 where a total of 44 donated oocytes resulted in 23 blastocysts after parthenogenetic activation (52%), of which 11 (25%) have a visible ICM and generated 6 human parthenogenetic embryonic stem cell lines [41].

DNA fingerprinting analysis of the adult donor fibroblasts, egg donor cumulus cells and all blastocysts observed following nuclear transfer was conducted to determine if the embryonic pathways in the donor nucleus had been reinitiated. Of the five putative SCNT blastocysts generated, successful DNA fingerprints from three SCNT blastocysts were consistent with those of the somatic cell donor employed with no evidence of contamination from the egg donors, indicating that embryonic development was being controlled by the donor cell genome. Not unexpectedly, the DNA fingerprints comparisons are not identical given the reported incidence of ADO and unvisualized alleles in single cell DNA fingerprinting systems [42, 43].

Further amplification of the samples for forward and reverse HVII region mtDNA sequencing, showed three alterations in sequencing in one cloned blastocyst, T or C at 33bp, G or A at 34bp, and 8C or 9C at 270-1bp and indicates that mtDNA in the cloned blastocyst K8 was from

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egg donor 3 (Coded reference F1 and F2) and not AF2 cell donor (Coded reference C5 and C6). It should be noted that the stutter observed after the polyC stretch is almost certainly due exclusively to the repetitive nature of the sequence; there are no heterozygotes in the K cell series at the T or C, and G or A loci, with only the T allele and G allele from egg donor 3 (Coded reference F1 and F2) clearly visualized. This would indicate that if any mitochondrion from fibroblast donor AF2 (Coded reference C5 and C6) were transferred along with the nuclear material, they are vastly outnumbered by those of egg donor 3 (Coded refernce F1 and F2).

Sequence data in both directions is appropriate until you hit the string of C homopolymer in the forward direction, or G homopolymer in the reverse direction. The presence of repetitive sequence, especially in the case of homopolymer and dinucleotide repeats (less so for higher order repeats), causes slippage by DNA polymerase, and causes stutter in sequence downstream of that sequence. The presence of polyC and polyG is especially problematic, and significant sequence degradation can be seen with less than even 10 repeats (much more so than polyA or T, which can tolerate ~13 to 15 repeats). In the case of the sequences seen here, their overall quality gives the expected result, with significant degradation of sequence quality once the critical repeat number is exceeded for the repetitive sequence in question.

Several modifications to the nuclear transfer process were investigated. Method of enucleation, either by extrusion or aspiration of the metaphase plate, resulted in no discernible differences in terms of embryo lysis or degeneration or in the developmental competence of SCNT embryos. The longer incubation of oocytes in a cell-permeable mycotoxin, cytochalasin B (45 min compared to 15-20 min) prevents the formation of contractile microfilaments and disruption of actin filaments and polymerization. Incubation in cytochalasin B using the time intervals reported in previous studies using failed-to-fertilize and MI oocytes resulted in increased oocyte lysis. Increasing the exposure of oocytes to cytochalasin B (45 min) decreased oocyte lysis and supports the findings of a previous report [16]. It is not known if the use of recombinant human hyaluronidase, instead of the commonly used bovine source, to remove the cumulus matrix also contributed to the efficiency of this procedure, but further studies with this new product are certainly warranted.

Eleven successful enucleation attempts did not remove the first polar body because of untoward location that would not have allowed us to achieve our goal of minimizing the amount of cytoplasm removed during the process. Increasing the amount of cytoplasm removed during the enucleation process impacts on the development potential of nuclear transfer embryos [44, 45]. Anecdotally, removal of the first polar body with either technique appeared to increase the amount of cytoplasm removed.

While mice have been generated from the first polar body following NT, that method of transfer required intracytoplasmic injection of the polar body, primarily as a result of apoptotic events associated with the break down and degeneration of the first polar body in the mouse [46]. The presence of the first polar body is of little concern because, in practice, it is difficult to initiate successful electrical fusion of the polar body with the enucleated oocyte and because the donor cell is easily distinguished from the polar body during the microscopic examinations required as part of the electrical fusion. The presence of two pronuclei during the assessment of remodeling, which would have indicated fusion of the first polar body, was not detected. In addition, DNA fingerprinting analysis did not confirm the presence of maternal DNA in the SCNT blastocysts.

In this study, parthenogenetic activation using either CI in combination with protein synthesis (DMAP) or protein kinase inhibitors (CHX/CYTD), artificially activated fresh oocytes that subsequently underwent pronuclear

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formation and cleavage division in a manner consistent with previous reports [24, 47-49]. Prevention of second polar body extrusion by the inhibition of either protein synthesis pathways (DMAP) or protein kinase pathways (CHX/CYTD) can alter the developmental competence of SCNT embryos [50]. However, in our assessment, there appeared to be no difference between the two activation treatments in terms of pronuclei and blastocyst formation although the limited number of activated human oocytes utilized makes it difficult to draw conclusions. Of note, parthenogentically activated blastocyts observed were of lower embryonic grades due to a slower development rate and lower total cell number.

Further modifications to the activation method, as suggested by a number of recent studies, may improve efficacy. For example, cytosolic extracts from sperm have been shown to cause Ca(2+) oscillations in a range of different mammalian oocytes, including humans [51]. In a further refinement, Saunders et al 2002 [52] isolated the specific isoform of phospholipase C (PLC) [53] thought to be responsible for generating Ca(2+) release and inducing $InsP_3$ production. Microinjection of cRNA encoding human PLC_{zeta} induced a response in aged human oocytes that closely mimicked the repetitive calcium Ca(2+) oscillations stimulus provided by the sperm during human fertilization and induced parthenogenesis and development to the blastocyst stage [54].

The protocols produced high rates of pronuclear formation (65%), early cleavage (43%) and blastocyst development (22%) following incubation in standard IVF culture media. As would be expected, a range of blastocyst grades were observed following both parthenogenetic activation and SCNT; however, all showed the presence of a defined inner cell mass. The observation of morphologically normal SCNT blastocysts is the prelude to obtain embryonic stem cell lines which will now be the focus of this research. This study examined the re-initiation of the embryonic genome in cloned blastocysts using methods of DNA fingerprinting and mtDNA sequencing. However, future studies to support the total cell number findings will examine the quality of the SCNT blastocyst and examine chromosome number, ICM/TE ratio and expression of critical embryonic genes at the time of blastocyst formation (Oct 4, Cdx2) [55, 56]. The process of preparing the samples for DNA fingerprinting however precluded the application of these methods.

Other factors that may have contributed to the success of SCNT procedures include the quality of the oocytes obtained from young egg donors. The best donor candidates are likely to be younger women, without a history of infertility, who respond well to ovarian stimulation. A major impediment to conducting studies of this type is the difficulty of obtaining suitable donor oocytes, something that is influenced by both ethical and legal considerations. Models for donation include a range of potential options, including altruistic oocyte donation, monetary inducement, or compensation in the form of reduced treatment fees. After obtaining the appropriate informed consents from the egg donor and intended parents, this study involved utilizing oocytes that were excess-to-reproductive needs and were donated without compensation. All three intended parents were successful in achieving ongoing pregnancies with the oocytes they retained for reproductive purposes. The average in vitro development rates of ART oocytes following either IVF and/or ICSI was 76% for normal fertilization (2PN) and 92% for cleavage to good quality (grade I and II) embryos determined on Day 2-4 (data not shown). Since a significant percentage of couples undergoing fertility treatments appear willing to participate in this type of research [28, 29], we believe the method described to obtain donated oocytes is a viable and ethically acceptable strategy that can enable optimization of the NTSC method to produce embryonic stem cells [10]. Although it only allows for the acquisition of limited numbers of oocytes and thus small groups of embryos from an individual egg donor, it clearly is a more efficacious

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process than those in previous studies which predominantly obtained oocytes from patients of increasing maternal ages undergoing a variety of IVF treatments [15, 24].

We are currently pursuing the generation of ES lines from SCNT embryos generated using these protocols. The recent findings with mouse [57] and human [58] embryos showing the generation of ESC lines from individual blastomeres, in human from arrested embryos [59] and primate embryonic stem cells following somatic cell nuclear transfer [60] may hasten the attainment of this goal.

In conclusion, it has been demonstrated that heterologous SCNT blastocysts can be successfully and reliably generated from two male adult fibroblast lines using oocytes from young oocyte donors obtained shortly after an oocyte retrieval that followed a standard oocyte donation cycle. Following SCNT, the somatic cell nuclei underwent remodeling, as evidenced by pronuclear formation. Of the five putative cloned blastocyst produced, only one cloned blastocyst was confirmed by DNA and mtDNA fingerprinting analyses, however DNA fingerprinting of two other cloned blastocysts indicated they were generated by SCNT although we were unable to conclusively supported these finding with mitochondrial DNA analyses.

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Figure 1. Figure 1 Established adult male fibroblast cell lines for SCNT
A: Relief Contrast image of established AF1 (Coded reference B3 and B4) fibroblast cell line (Scale Bar = 10 μm).
B: Relief Contrast image of established AF2 (Coded reference C5 and C6) fibroblast cell line (Scale Bar = 10 μm).
C: Chromosome Spread of AF1 (Coded reference B3 and B4) Adult fibroblast cell line (passage 4).
D: Chromosome Spread of AF2 (Coded reference C5 and C6) Adult fibroblast cell line (passage 7).
E: Five chromosome aneuploidy screen (#13 Red, #18 Aqua, #21 Green, #X blue (purple), #Y Gold) of metaphase II AF1 (Coded reference B3 and B4) adult fibroblast line (Multi Vysion PGT Fluorescent Probe Set (Vysis, Abbott Molecular Inc, Des Plaines, IL, USA).
F: Five chromosome aneuploidy screen (#13 Red, #18 Aqua, #21 Green, #X blue (purple), #Y Gold) of interphase AF2 (Coded reference C5 and C6) Adult Fibroblast line.

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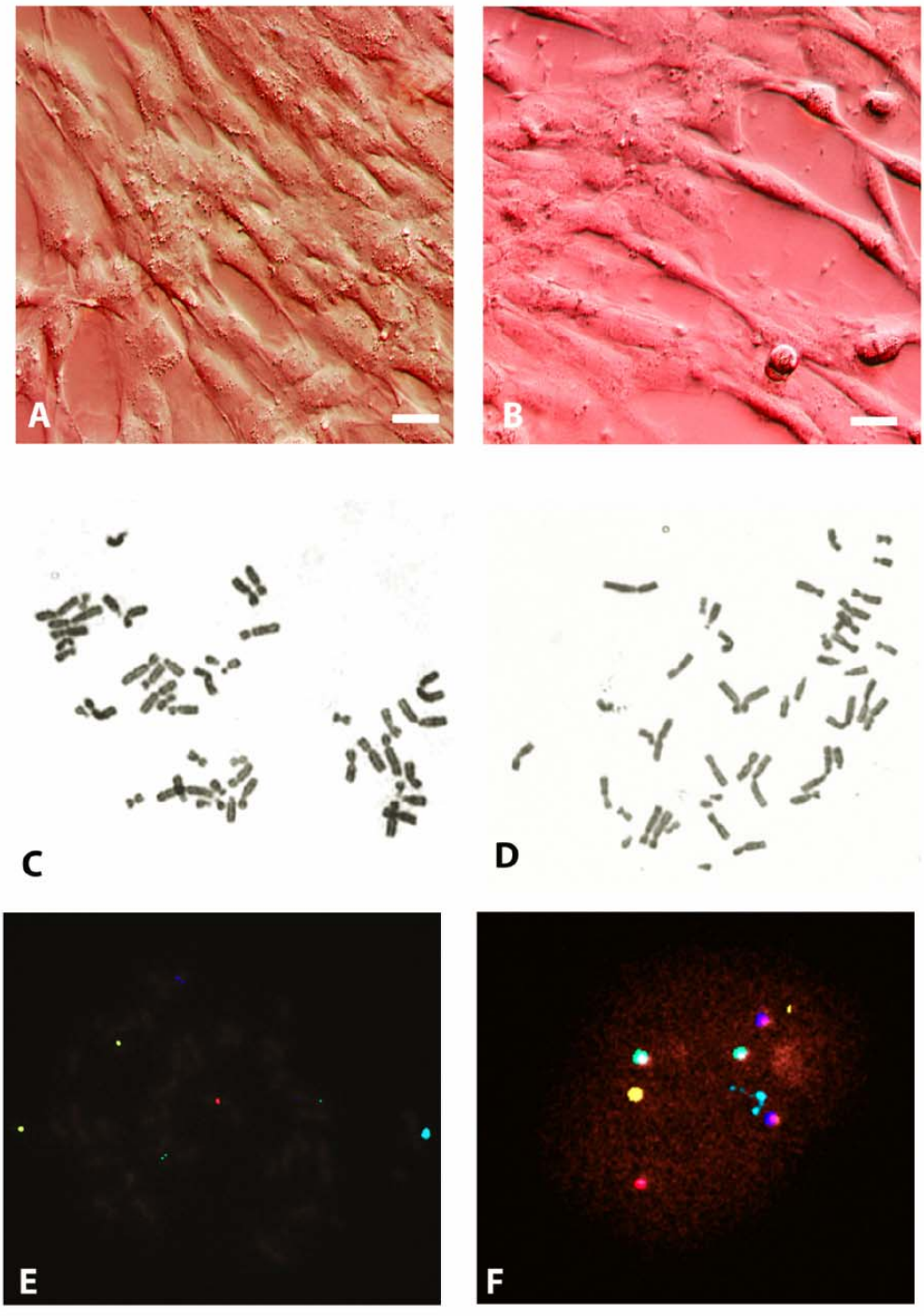

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Figure 2. SCNT and Parthenogenetic Embryo Development
A1: Cumulus matrix-free human metaphase II oocytes.
A2: Enucleation pipette for aspiration of metaphase plate from human oocytes.
A3: UV fluorescence of human metaphase II oocyte stained with Hoechst 33342.
B1: Pronuclear formation (6-7 h post CI activation) in parthenogenetically activated human oocytes.
B2: Embryo cleavage following parthenogenetic activation (Day 3)
B3: Parthenogenetically activated (Coded reference K1) blastocyst (Day 5) from egg donor 3 (Coded reference F1 and F2)
C1: Pronuclear formation (6-7 h post CI activation) following SCNT with an adult fibroblast AF1 (Coded reference B3 and B4).
C2: Pronuclear formation (6-7 h post CI activation) following SCNT with an adult fibroblast AF2 (Coded reference C5 and C6).
C3: Late Day 3 SCNT embryo following nuclear transfer with AF2 (Coded reference C5 and C6) donor cell.
C4: Early Day 3 SCNT embryos following nuclear transfer with AF1 (Coded reference B3 and B4) donor cells.
C5: Late Day 5 SCNT (Coded reference 8E) blastocyst following nuclear transfer with AF1 (Coded reference B3 and B4) donor cell to an oocyte from egg donor 1 (Coded reference A1, A2).
C6: Early Day 6 SCNT (Coded reference K8) blastocyst following nuclear transfer with AF2 (Coded reference C5 and C6) donor cells to oocytes from egg donor 3 (Coded reference F1, F2). Note with attached cleavage arrested blastomere.
Images were captured with a DP70 Digital Camera attached to an Olympus IX71 reflected fluorescence microscope fitted with relief contrast and Differential Interference Contrast optics. (Scale Bar = 20 μm)

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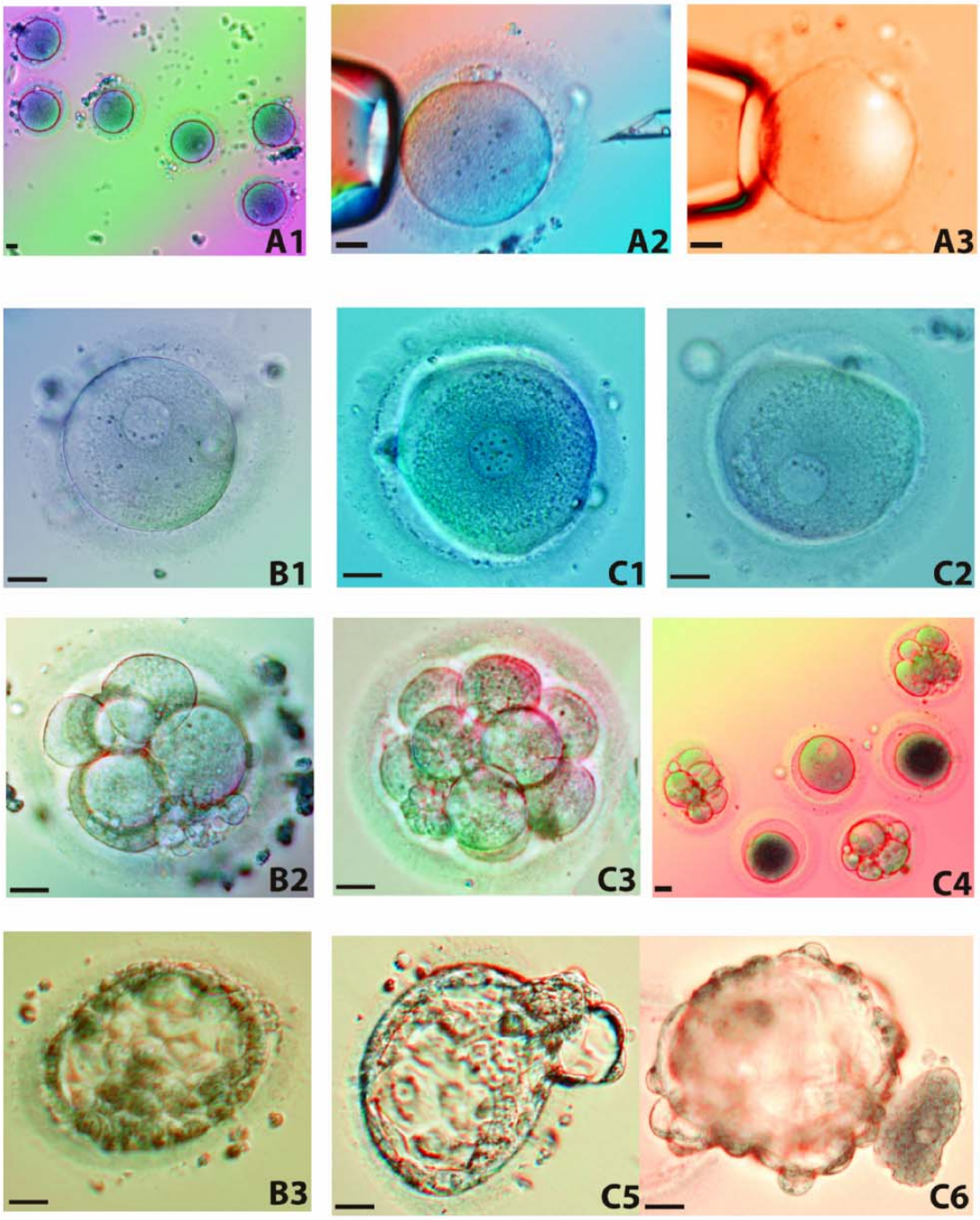

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Figure 3a Microsatellite Analyses of SCNT Blastocysts following nuclear transfer of AF1 (Code reference B3and B4) and AF2 (Coded reference C5 and C6) donor cells using oocytes obtained from Egg Donor 1 (Coded reference A1 and A2)

	Egg Donor A1&2		Fibro donor B3&4		Fibro donor C5&6		SCNT Blastocysts			
	A consensus		B consensus		C consensus		8E		10G	
D3S1358	16	18	15	16	17					
THO1 (11)	6	7	7		-					
D21S11	29	30	31		28	29,30	31		29	30
D18S51	13	20	12	15	14	17			14	
Penta E (15)	8	12	7	13	10	13			10	13
D5S818	11	13	11	13	11		13		11	
D13S317	13	14	11	13	11		11		11	13
D7S820	11		8	9	9	11				
D16S539	12		-		8	13				
CSF1PO (5)	12	13	10	13	11				11	
Penta D (21)	9	13	13	15	10	14			10	
AMELXY	X		X	Y	X	Y				
vWA (12)	17	18	18	19	15	16	19		15	16
D8S1179	13	14	13	15	11	15	13		11	15
TPOX (2)	8		-		-		8			
FGA (4)	18	21	19	21	20	24	21		20	24

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Figure 3b Microsatellite Analyses of SCNT Blastocyst following nuclear transfer of AF2 (Coded reference C5 and C6) donor cell to an oocyte obtained from Egg Donor 3 (Coded reference F1 and F2).

	Egg Donor F1&2		Fibro donor B3&4		Fibro donor C5&6		SCNT Blastocyst	
	F consensus		B consensus		C consensus		K8	
D3S1358	14	17	15	16	17		17	
THO1 (11)	8		7		-		9.3	
D21S11	29	30	31		28	29,30	29	30
D18S51	15	17	12	15	14	17	14	
Penta E (15)	11		7	13	10	13		
D5S818	11	12	11	13	11		11	
D13S317	9	11	11	13	11		13	
D7S820	9		8	9	9	11	11	
D16S539	12	13	-		8	13		
CSF1PO (5)	11	12	10	13	11		11	
Penta D (21)	9	13	13	15	10	14	10	14
AMELXY	X		X	Y	X	Y	X	Y
vWA (12)	16	18	18	19	15	16	15	
D8S1179	11	15	13	15	11	15	11	
TPOX (2)	9		-		-		8	
FGA (4)	23		19	21	20	24	20	

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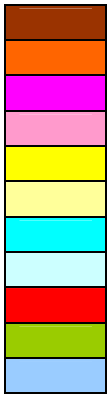
Figure Legend



Only seen in A
consistent with A
Only seen in F
consistent with F
Only seen in B
Consistent with B
Only seen in C
Consistent with C
Allele not seen in F, consistent with ADO of allele in F sample.
Allele not seen in B, consistent with ADO of allele in B sample.
Allele not seen in C, consistent with ADO of allele in C sample.

8E can be explained using alleles from B alone, citing ADO of 8
10G can be explained using alleles from C alone, citing ADO of 13
K8 can be explained using alleles from C alone, and assuming that 9.3, 13, and 8 peaks are due to un-visualized alleles in the C samples.

Notes: The "only seen" designation for B & C changes, in the context of whether the A or F egg donor was used in formation of the SCNT cell line
Also, D21S11 is known to have more than 2 alleles in some individuals; the phase of the three alleles in C is not known.

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Figure 3a and b Microsatellite Analyses of SCNT Blastocysts

Profile of SCNT Blastocysts against Oocyte and Adult Fibroblast cell donors.

Blastocyst 8E can be explained using alleles from adult fibroblast donor AF1 (Coded reference B3 and B4) alone, citing Allele drop-out (ADO) of TPOX (8) allele; Blastocyst 10G can be explained using alleles from adult fibroblast donor AF2 (Coded reference C5 and C6) alone, citing ADO of D13S317 (8) allele; Blastocyst 8K can be explained using alleles from adult fibroblast donor AF2 (Coded reference C5 and C6) alone, assuming that alleles THO1 (9.3), D13S317 (13) and TPOX (8) peaks are due to un-visualized alleles in the AF2 donor cell sample.

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Table 1: SCNT and Parthenogenetic Activation with freshly donated human oocytes

Donor	Oocytes Retrieved	Oocytes Donated	Stage of Development		Treatment		Donor Cells[2]	Parthenogenetic Activation	Embryo Development			
			GV/ MI	MII	PA	SCNT			Oocyte Lysis[3]	PN Formation	Cleavage	Blastocyst
1	21	11	1	10	2			CI/DMAP		2/2	2/2	1
(A1, A2)[1]						8	4 AF1	CI/DMAP	1/4	3/4	3/4	2 (8E, 9F)[3]
							4 AF2	CI/DMAP	1/4	3/4	2/4	1 (10G)[3]
2	27	6	1	5	1			CI/CHX/CYTD		1/1	1/1	1
						4	AF1	CI/CHX/CYTD	2/4	2/4	1/4	0
3	25	12	1	11	2			CI/DMAP	0	2/2	2/2 (K4, K6)	1 (K1)
(F1, F2)[1]						9	AF2	CI/DMAP	3/9	6/9	4/9	2 (K7, K8)
Total	73	29	3	26	5	21						
SCNT Totals									7/21 (33%)	14/21 (66%)	10/21 (47%)	5/21 (23%)

CI: Calcium Ionophore; DMAP: 6-Dimethylaminopurine; CHX: Cycloheximide; CYTD: Cytochalasin D

[1] Coded values for egg donor's and parthenogenetic and SCNT embryos analyzed

[2] Somatic Cells Codes; AF1 (B3, B4) and AF2 (C5, C6) analyzed

[3]Embryo lyses were recorded following enucleation, electrical fusion and at the time of pronuclei formation

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Development of Human cloned Blastocysts Following Somatic Cell Nuclear Transfer (SCNT) with Adult Fibroblasts

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