

# Reprogramming of Human Somatic Cells Using Human and Animal Oocytes

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## Abstract

There is renewed interest in using animal oocytes to reprogram human somatic cells. Here we compare the reprogramming of human somatic nuclei using oocytes obtained from animal and human sources. Comparative analysis of gene expression in morula-stage embryos was carried out using single-embryo transcriptome amplification and global gene expression analyses. Genomic DNA fingerprinting and PCR analysis confirmed that the nuclear genome of the cloned embryos originated from the donor somatic cell. Although the human–human, human–bovine, and human–rabbit clones appeared morphologically similar and continued development to the morula stage at approximately the same rate (39, 36, and 36%, respectively), the pattern of reprogramming of the donor genome was dramatically different. In contrast to the interspecies clones, gene expression profiles of the human–human embryos showed that there was extensive reprogramming of the donor nuclei through extensive upregulation, and that the expression pattern was similar in key upregulation in normal control embryos. To account for maternal gene expression, enucleated oocyte transcriptome profiles were subtracted from the corresponding morula-stage embryo profiles. *t*-Test comparisons (median-normalized data @  $fc > 4$ ;  $p < 0.005$ ) between human in vitro fertilization (IVF) embryos and human–bovine or human–rabbit interspecies somatic cell transfer (iSCNT) embryos found between 2400 and 2950 genes that were differentially expressed, the majority (60–70%) of which were downregulated, whereas the same comparison between the bovine and rabbit oocyte profiles found no differences at all. In contrast to the iSCNT embryos, expression profiles of human–human clones compared to the age-matched IVF embryos showed that nearly all of the differentially expressed genes were upregulated in the clones. Importantly, the human oocytes significantly upregulated Oct-4, Sox-2, and nanog (22-fold, 6-fold, and 12-fold, respectively), whereas the bovine and rabbit oocytes either showed no difference or a downregulation of these critical pluripotency-associated genes, effectively silencing them. Without appropriate reprogramming, these data call into question the potential use of these discordant animal oocyte sources to generate patient-specific stem cells.

## Introduction

THE OOCYTE IS THE ONLY CELL IN THE ADULT BODY that can reprogram a terminally differentiated somatic cell back to a totipotent state (Cibelli et al., 2002). This transformation requires a series of molecular events occurring correctly

within a very short period of time, including a global resetting of the gene expression pattern of the donor nucleus. Numerous studies have confirmed the ability of interspecies oocyte cytoplasm to successfully support mitotic cell cycles under the direction of adult somatic cell nuclei to the preimplantation stage (reviewed in Beyhan et al., 2007; Tecirlioglu

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et al., 2006), and in a few closely related bovid species (*Bos-to-Bos* combinations), successful development through to term (Lanza et al., 2000; Li et al., 2006; Meirelles et al., 2001). For this reason, animal oocytes have been considered a potentially important and readily available source for generating patient-specific embryonic stem cells.

However, to date there is no reproducible evidence that human stem cells can be successfully generated using interspecies somatic cell nuclear transfer (iSCNT). It is also unclear whether inter- and intraspecies nuclear transfer techniques share the common ability to reprogram the nucleus of a human somatic cell. There is an abundance of data indicating that iSCNT can generate preimplantation-stage embryos (Tecirlioglu et al., 2006). Although one study carried out in China claimed to have generated stem cells using rabbit oocytes (Chen et al., 2003), this work has not been replicated despite attempts by numerous groups in the last half-decade (Jinguan et al., 2005; News Service, 2006; Vogel, 2006). Additionally, there is strong evidence that DNA methylation/demethylation of the donor nucleus after iSCNT occurs in a species-specific way, and that rabbit and bovine ooplasm might lack the ability to demethylate sequences from discordant species (Chen et al., 2006). Here we compare the genomic-wide reprogramming ability of human somatic cells using oocytes obtained from multiple animal and human sources.

## Materials and Methods

### Oocyte collection

**Human oocytes.** Healthy female donors between the ages of 27 and 34 were treated with luteinizing hormone releasing hormone (LHRH) agonist (Lupron, 0.5 mg, TAP Pharmaceutical, Lake Forest, IL), and then stimulated with recombinant follicular stimulating hormone (FSH) (Follistim) and microdose recombinant LH (Ovidrel, Serono, Geneva, Switzerland) starting from day 1. The FSH usually started with 100–150 units and was adjusted as folliculogenesis proceeded. When two leading follicles reached 18 mm in diameter, a single dose of Ovidrel (250  $\mu$ g) was given to induce oocyte maturation.

Oocytes were retrieved using an ultrasound-guided egg retrieval procedure 36 h post-Ovidrel injection under conscious sedation with Midazolam 5–7.5mg (Versed, Roche, Indianapolis, IN) and Fentanyl 50–75  $\mu$ g (Abbott, Abbott Park, IL). The oocyte–cumulus cell complexes were washed and incubated in IVF medium (Quinn's IVF medium, Cooper Surgical, Trumbull, CT) for 4 h in a high humidified incubator with 5% CO<sub>2</sub> before removing cumulus cells. Follicular fluids containing follicular cells and blood cells from donors were collected and frozen for genotyping. The cumulus cells were removed with a small bore micropipette (100- $\mu$ m diameter) after 2 min of exposure to hyaluronidase (100 IU/mL, Sigma-Aldrich, St. Louis, MO). The denuded oocytes were washed and sorted based on their maturity. Oocytes with GV or missing the first polar body were classified as immature and subjected to *in vitro* maturation. Only oocytes having a first polar body was classified as MII stage and used for SCNT without further maturation. GV or MI stage oocytes were cultured in 500  $\mu$ L of maturation medium (Quinn's IVF medium supplemented with recombinant human FSH (15 ng/mL), LH (1  $\mu$ g/mL), EGF (50 ng/mL), and

E<sub>2</sub> (1  $\mu$ g/mL) in a four-well dish) overnight in a humidified atmosphere at 37°C with 5.5% CO<sub>2</sub> in air. Only oocytes that matured to the MII stage within 24 h after oocyte retrieval were used in this experiment.

**Rabbit oocytes.** All the procedures and animal housing were approved by IACUC of Baylor College of Medicine and complied with the NIH laboratory animal care guide lines. Sexually matured New Zealand white rabbits (6–8 months old) were superovulated by six consecutive intramuscular injections of FSH (0.3, 0.3, 0.4, 0.4, 0.5, 0.5 mg, Follitropin-V, Bioniche, Athens, GA) 12 h apart and intravenous human chorionic gonadotropin (hCG, 200 U, Abraxis, Los Angeles, CA) 12 h after the last FSH injection. The oocytes were collected 12–13 h after the hCG injection. The oviducts and ovaries were removed with the minimum amount of surrounding tissues after the rabbits were sedated by i.v. injection of Propofol (7–10 mg/kg of bodyweight, Abbott). The oocytes were collected by flushing the oviducts with TCM-199 (Medium 199 with 25 mM HEPES, Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) using a 10-mL syringe. The isolated cumulus–oocyte complexes were treated with hyaluronidase (80 U/mL, Cooper Surgical) for 5 min and washed with small bore pipettes (150- $\mu$ m diameter) to remove the cumulus cells. The denuded oocytes were kept until use in KSOM supplemented with 2.5% FBS in an incubator with 5.5% CO<sub>2</sub> in air at 38.5°C. A fraction of rabbit cumulus cells was washed and prepared for genotyping.

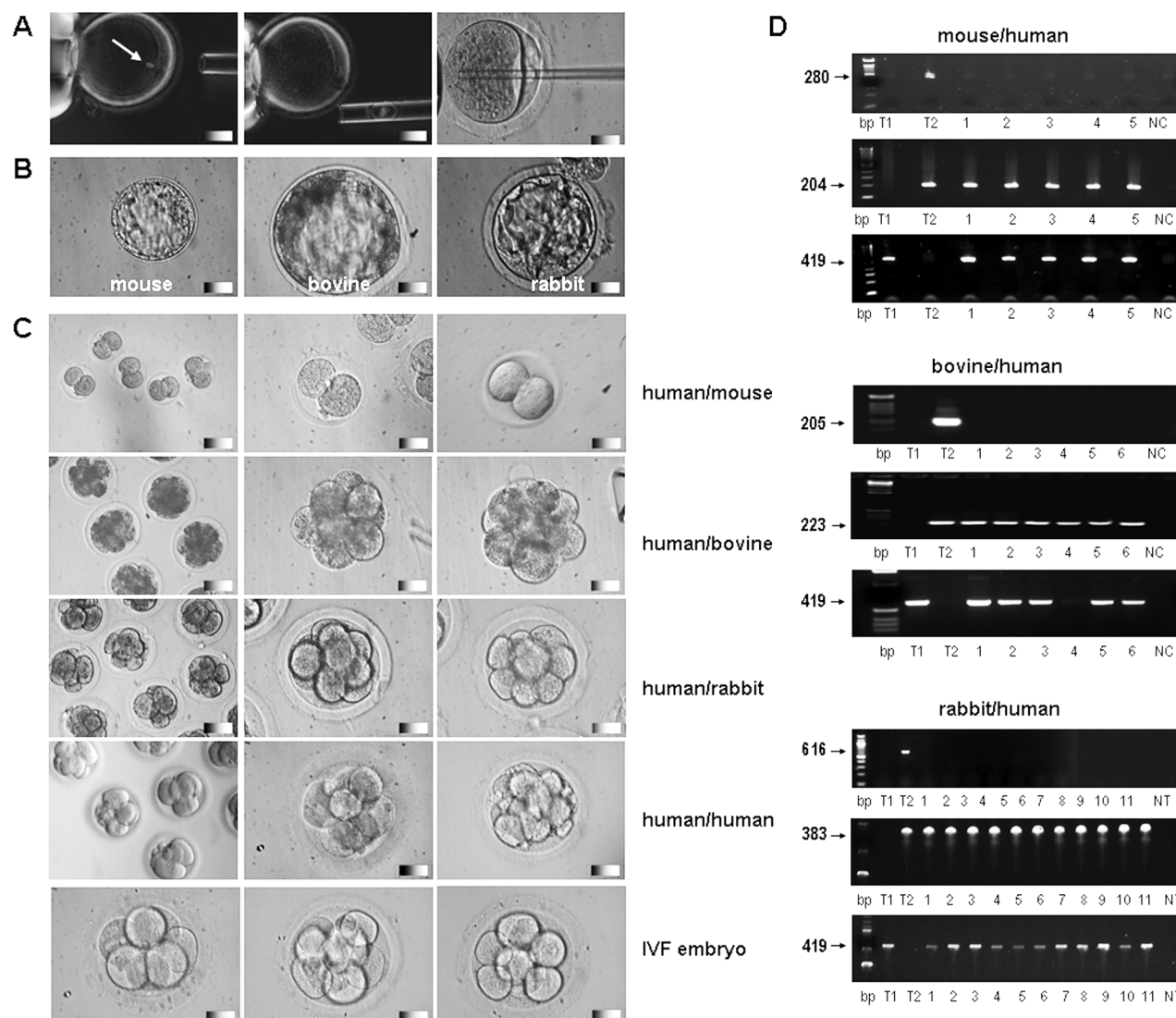
**Bovine oocytes.** Bovine oocytes were obtained from BOMED Inc. (Madison, WI). They were shipped overnight at 39°C in maturation medium composed of TCM-199 supplemented with 15% FBS, bovine FSH and LH, and upon arrival, kept in the same maturation medium in an incubator at 38.5°C up to 20 h after starting the *in vitro* maturation. Cumulus cells were removed as described above. The denuded oocytes were kept in G1.3 medium (VitroLife, Göteborg, Sweden) supplemented with 2.5 mg/mL bovine serum albumin in an incubator with 5.5% CO<sub>2</sub> in air at 38.5°C. A fraction of bovine cumulus cells was washed and prepared for genotyping.

**Mouse oocytes.** Eight- to 10-week-old B6D2 F1 mice were injected with 5 U PMSG (Calbiochem, San Diego, CA) and 5 U hCG (Sigma-Aldrich) at 48 h apart to induce superovulation. The cumulus and oocyte complexes were retrieved by tearing the oviduct in HEPES–CZB. The oocytes were denuded by treating with hyaluronidase as described above, and kept in KSOM medium until use in an incubator with 5.5% CO<sub>2</sub> in air at 37°C. Some mouse cumulus cells were washed and prepared for genotyping.

**Somatic cell nuclear transfer.** MII spindles in human and rabbit oocytes were visualized for enucleation using the Oosight™ spindle imaging system (CRI, Woburn, MA) to avoid Hoechst staining and UV light exposure. Only oocytes with definite metaphase spindles were used in this study. Hoescht staining was used for spindle visualization in bovine oocytes, and the spindles of mouse oocytes were easily detected with Hoffman modulation optics. To facilitate the removal of metaphase spindles, oocytes were preincu-

bated in an egg manipulation medium (Hepes-Quinn's medium, Cooper Surgical) supplemented with cytochalasin B ( $2.5 \mu\text{g}/\text{mL}$ ). Enucleation of oocytes was performed using a Piezo drill (PrimeTech, Japan) with a  $20\text{-}\mu\text{m}$  manipulation pipette. The removal of the metaphase spindle was confirmed by the presence of a bright oval shaped complex in the manipulation pipette (Fig. 1). Fresh cumulus cells from different donors were used as nuclear donors. After denudation of human oocytes, the cumulus cells were washed in manipulation medium (Hepes-Quinn's medium with 5% serum protein substitute, Cooper Surgical) and mixed with 4% Polyvinylpyrrolidone solution (PVP, MW 36,000, Calbiochem). Injection of nuclei with surrounding cytoplasm was performed using a Piezo drill as described previously

(Chung et al., 2006). The reconstructed embryos were washed and kept in embryo culture medium (Quinn's cleavage medium, Cooper Surgical) until activation. All nuclear injections were performed within 15 min after the enucleation. After the nuclear injection, the reconstructed embryos were incubated in culture medium for 1 h before activation. The human reconstruct activation was performed with Ionomycin ( $5 \mu\text{M}$  for 5 min) followed by 6-DMAP (2 mM for 5 h) in an embryo culture medium (Quinn's cleavage medium, Cooper Surgical). For bovine iSCNT embryo activation, Ionomycin ( $10 \mu\text{M}$  for 5 min) followed by 6-DMAP (2 mM for 4 h) in G1.3 (Vitrolife) was used. The rabbit iSCNT reconstructs were activated by applying three direct current pulses of  $3.4 \text{ kV}/\text{cm}$  for a duration of  $20 \mu\text{sec}$  each



**FIG. 1.** Somatic cell nuclear transfer using human and animal oocytes. **(A)** Stages of nuclear transfer. Left panel: visualization of mitotic spindle complex using Oosight™ spindle imaging system, arrow indicates the location of the spindle complex; middle panel: removal of the spindle complex; right panel: injection of somatic cell nucleus. **(B)** Control intraspecies blastocysts generated using mouse, rabbit, and bovine oocytes. **(C)** Development of interspecies cloned embryos and a human IVF embryo. **(D)** genomic DNA and mitochondrial DNA analysis of iSCNT embryos. In each group, top panel: animal genomic DNA, middle panel: animal mtDNA; bottom panel: human mitochondrial DNA. Bp—DNA size ladder; T1—human total DNA; T2—animal total DNA; NC—negative control; numbers correspond to different blastomere samples analyzed.



by BTX 200 Electro Cell Manipulator (BTX Harvard Apparatus, Holliston, MA) in sorbitol-based activation medium (0.3M sorbitol supplemented with 0.1 mM magnesium acetate and 0.1mM CaCl<sub>2</sub>) followed by 1-h incubation in KSOM + 2.5% FBS containing 2.0 mM 6-DMAP and 5 µg/mL cyclohexamide (Sigma-Aldrich). The mouse reconstructs were activated by 6-h incubation in CZB medium containing 10 mM strontium chloride (Sigma-Aldrich). The activated reconstructed embryos were washed, and cultured in 50 µL of culture medium under mineral oil.

**Cloned embryo culture.** The cloned embryos were cultured in different conditions. The human and mouse cloned embryos were cultured in 5.5% CO<sub>2</sub> atmosphere at 37°C in a humidified incubator in Quinn's cleavage medium and KSOM, respectively. The rabbit and bovine cloned embryos were cultured in 5.5% CO<sub>2</sub>/5% O<sub>2</sub>/N<sub>2</sub> balanced atmosphere at 38.5°C in KSOM + 2.5% FBS and G1.3, respectively. The cloned embryo development was assessed daily and culture media was replaced with fresh sequential media: G2.3 for bovine and Quinn's blastocyst medium for human at day 3 after cloning (day 0 = day of cloning). The mouse and rabbit clones were cultured in the same media without change for 4 days.

#### *Preparation of blastomeres for the genotyping and RNA analysis*

**Human embryo biopsy.** Only cloned embryos that developed to the morula stage were biopsied for genotyping and gene expression. In all cases, the zona pellucidae were removed by brief exposure to acidic Tyrode's solution and thorough washing in a HEPES-buffered manipulation medium (HEPES-Quinn's medium, Cooper Surgical). Three to four blastomeres were removed from the denuded embryos for genotyping using a 50-µm manipulation pipette and the remaining parental embryos were processed for gene expression analysis. The parental embryos were washed in RNase-free PCR buffer loaded in a 0.25 mL PCR tube and snap frozen in liquid nitrogen. For genotyping, the biopsied blastomeres were washed and lysed in KCl-based buffer. After 10-min incubation at 37°C the lysates were added to neutralization buffer. The embryonic lysates then were coded, frozen, and shipped on dry ice to RMA of New Jersey (Morristown, NJ) for DNA fingerprinting.

**Interspecies SCNT embryo biopsy.** Interspecies embryos were biopsied at the eight-cell to morula stage, and two to four blastomeres were biopsied and prepared as for genotyping using KCl-based lysis buffer as in the human embryo preparation. The remaining parental embryos were prepared for RNA analysis as described above.

**Embryonic cell WGA and donor total DNA isolation for human cloned embryos.** WGA was performed according to the manufacturer's recommendations using the GenomePlex WGA4 kit (Sigma-Aldrich). WGA DNA was purified using GenElute PCR Purification columns (Sigma-Aldrich). DNA from the oocyte and nuclei donors was isolated from approximately  $1 \times 10^6$  coded cumulus cells using the QIAgen DNeasy Tissue Kit (Qiagen, Chadsworth, CA). Purified WGA and total DNA was quantified using a Nanodrop 8000 spectrophotometer (Nanodrop, Wilmington, DE).

**Genomic single nucleotide polymorphism (SNP) genotyping and mtDNA resequencing of human SCNT embryos.** WGA or total DNA was prepared to perform the NspI 262K SNP microarray analysis according to the manufacturer's instructions (Affymetrix, Kansas City, MO). Embryonic WGA derived DNA profiles were evaluated at a genotyping stringency of 0.01, while profiles of total DNA from donor tissue was evaluated at the default setting of 0.33, using GTYPE 4.1 software (Affymetrix). All 262K SNP genotypes were compared to generate % similarities for all possible comparisons of embryonic WGA with donor total DNA. SNP microarray data was also analyzed and visualized with the Copy Number Analysis Tool (CNAT) 4.1 (Affymetrix) and using a Gaussian smoothing distance of 5 MB to evaluate karyotype. The HV region of the mitochondrial genome was evaluated using the MitoSeqr resequencing system according to the manufacturer's instructions (Applied Biosystems, Medford, MA). SeqScape software was used to observe similarities and differences between all embryonic and donor mitochondrial DNA comparisons. To evaluate the reliability of genomic and mtDNA fingerprinting from single or multiple cells, cell lines were obtained that could serve as controls. Each of three cell lines (Coriell Cell Repository ID#: GM01201, GM11872, GM13121) were subject to both single cell WGA and total DNA processing as described above. The ability to correctly assign match and nonmatch similarities was evaluated and used as a reference to define expected similarities for match and nonmatch genomic and mtDNA relationships between embryonic cells and donor total DNA.

**Karyotype/aneuploidy screening.** Cell lines with known abnormalities (GM02948, trisomy 13 male; GM04610, trisomy 8 female; and GM04435, trisomy 16 and 21 male) were obtained from the Coriell Cell Repository to serve as controls for accurate single-cell aneuploidy screening. SNP microarray data on single cells from these cell lines, putative SCNT, or iSCNT embryonic cells, and single blastomeres from three *in vitro* fertilization derived human embryos, were analyzed and visualized as copy number state graphs with the Copy Number Analysis Tool (CNAT) 4.1 (Affymetrix Inc.).

#### *Genomic SNP genotyping and mtDNA PCR for iSCNT embryos*

**Whole genome amplification.** Because the cell number (two to four cells) available for the genotyping and mtDNA analysis was limited, whole genome DNA amplification was carried out. For the genotyping, two to four blastomeres of each individual iSCNT embryo were biopsied, lysed in alkaline lysis buffer, snap frozen in liquid nitrogen, and sent to RMA of NJ for the genotyping as for the human SCNT. The genomic and mitochondrial DNA of each samples were amplified using the GenomePlex WGA4 kit (Sigma-Aldrich) according to the manufacturer's protocol. Human cumulus cells and mouse, rabbit, and cow cumulus cells also were processed to obtain genomic and mitochondrial DNA using the WGA4 kit. After amplification, all samples yielded approximately 10 µg of DNA.

**Genotyping of iSCNT embryos.** Because the SNP microarray described above is specific for human DNA, the de-

tection of DNA indicates that human DNA is present. SNP genotyping call rates (the percentage of SNP probe sets that a genotype call was made successfully) using GTYPE 4.1 were evaluated from putative iSNCT embryo samples following whole genome amplification and microarray processing as described above. Samples with call rates similar to those obtained from control pure human DNA samples were considered positive for human DNA and demonstrative the human origin of nuclear DNA in putative iSNCT embryonic cells.

**Human mtDNA.** The amplified DNA from the iSCNT cloned embryos was used for evaluating mitochondrial DNA. For the human, the primer set RSA001308206 (Applied Biosystems) was used to amplify a portion of the mitochondrial control region (D-loop). Each reaction mixture (25  $\mu$ L) contains 5  $\mu$ L of DNA samples mixed with 1 $\times$  PCR buffer II (Applied Biosystems) containing 0.5  $\mu$ M primers, 200  $\mu$ M dNTP, and 2 mM  $Mg^{++}$ , plus 2.5 units Gold Taq polymerase. Amplification was carried out in a Gene Amp thermal cycler (Applied Biosystems) programmed to perform a denaturation step of 96°C for 5 min, followed by 40 cycles consisting of 30 sec at 94°C, 45 sec at 60°, and a 45-sec extension at 72°C. The final extension was 10 min. The PCR products (417 bp) were mixed with 2  $\mu$ L of BlueDye gel loading solution (Invitrogen) and electrophoresed on a 2% agarose gel. The resulting DNA fragments were visualized by UV transillumination and analyzed using the Kodak UV gel documentation system-PC (Kodak, Rochester, NY).

**Mouse mtDNA.** For the mouse mtDNA, the mitochondrial Nd3 region, a 204-bp fragment containing the 9461 site, was amplified by PCR with the following primers: forward 5'-TTCCA ATTA GTAGATTCTGAATAAACCCAGAAGAGAGTGAT-3' and reverse 5'-AAATT TT ATT GAGAATGTAGACG-3'. Each reaction mixture (20  $\mu$ L) contained 5 ng template DNA, 200  $\mu$ M of each dNTP, 0.75 U Red Taq polymerase (Sigma-Aldrich), 0.5  $\mu$ M of each primer, and 1 $\times$  Red Taq PCR buffer. Amplification was carried out in a Gene Amp thermal cycler (Applied Biosystems) programmed to perform a denaturation step of 96°C for 2 min, followed by 25 cycles consisting of 45 sec at 95°C, 1 min at 55°, and a 45-sec extension at 72°C. The final extension was 5 min. The resulting PCR products (204 bp) were loaded in a 2% agarose gel and visualized as for the human mtDNA PCR.

**Rabbit mtDNA.** For the rabbit mtDNA, the mitochondrial cytochrome *b* (cytb) gene was amplified by PCR with the following primers: forward 5'-TCTACATACACGTAG GC-CGCGGAA-3' and reverse 5'-GAGGAG AAGAATGGCTACAAGGAAA-3'. Each reaction mixture (10  $\mu$ L) contained a 10-ng template DNA, 200  $\mu$ M of each dNTP, 0.75 U Red Taq polymerase (Sigma-Aldrich), 0.5  $\mu$ M of each primer, and 1 $\times$  Red Taq PCR buffer. Amplification was carried out in a Gene Amp thermal cycler (Applied Biosystems) programmed to perform a denaturing step of 96°C for 5 min, followed by 40 cycles consisting of 30 sec at 94°C, 45 sec at 60°, and a 45-sec extension at 72°C. The final extension was 10 min. The final amplification products of 353 base pairs were loaded in a 2% agarose gel and visualized as for the human mtDNA PCR.

**Bovine mtDNA.** For the bovine mtDNA, the mitochondrial 12S rRNA gene was amplified by PCR using the following primer pair: forward 5'-CTAGAGGAGCC TGTT CTATA-ATCGATAA-3' and reverse 5'-TGGTTTCATAATAACTTTCGCGCT-3'. Each reaction mixture (25  $\mu$ L) contained 5 ng of DNA with 1 $\times$  PCR buffer II (Applied Biosystems) and 0.5  $\mu$ M primers, 200  $\mu$ M dNTPs, and 2 mM  $Mg^{++}$ , plus 2.5 units Gold Taq polymerase. The PCR reaction was run at 95°C for 9 min (1 cycle), 94°C for 45 s, 63°C for 45 sec, and 72°C for 1 min for 40 cycles, with a final extension at 72°C for 2 min. The final amplification products of 223 base pairs were loaded in a 2% agarose gel and visualized as for the human mtDNA PCR.

### Genomic DNA PCR

**Mouse.** The D1Mit46 simple sequence repeat (SSR) polymorphism was used to genotype nuclear DNA from mouse iSCNT by using MapPairs assay B219 forward and reverse unlabeled primers (Invitrogen) as described previously (Tecirlioglu et al., 2006). The reaction parameters included 10 ng of template DNA, 0.5  $\mu$ M of each primer, 200  $\mu$ M each dNTP and 0.75 U Red Taq polymerase, 1 $\times$  Red Taq buffer, with cycling conditions of 96°C for 2 min (1 cycle), 94°C for 45 sec, 55°C for 45 sec, 72°C for 1 min for 30 cycles, and 72°C for 7 min. PCR products (250 bp) were loaded in a 2% agarose gel and visualized as for the human mtDNA PCR.

**Bovine.** Bovine trophoblast protein-1 gene (gene bank access #NM001015511) was amplified by PCR using the following primer pair: forward 5'-ATACAGTGACTGCGCCTGGGAAAT-3' and reverse 5'-TCG GTGGCT GAAGCAGAAATCAGA-3'. Each reaction mixture (25  $\mu$ L) contained 5  $\mu$ L of DNA samples mixed with 1 $\times$  PCR buffer II (Applied Biosystems) containing 0.5  $\mu$ M primers, 200  $\mu$ M dNTP, and 2 mM  $Mg^{++}$ , plus 2.5 units Gold Taq polymerase. PCR reaction was run at 95°C for 9 min (1 cycle), 94°C for 45 sec, 60°C for 45 sec, and 72°C for 1 min for 40 cycles, with a final extension at 72°C for 2 minutes. The 205-bp PCR products were loaded in a 1.5% agarose gel and visualized as for the human mtDNA PCR.

**Rabbit.** The rabbit uteroglobin gene (gene bank access #X01423) was amplified by PCR using the following primer pair: forward 5'-GGGCC AATCAAG CAA AGGATTCGT-3' and reverse 5'-TGATGGCCGAATCAAACCCAAACC-3'. Each reaction mixture (25  $\mu$ L) contains 5  $\mu$ L of DNA samples mixed with 1 $\times$  PCR buffer II (Applied Biosystems) containing 0.5  $\mu$ M primers, 200  $\mu$ M dNTP, and 2 mM  $Mg^{++}$ , plus 2.5 units Gold Taq polymerase. The PCR reaction was run at 95°C for 9 min (1 cycle), 94°C for 45 sec, 60°C for 45 sec, and 72°C for 1 min for 40 cycles, with a final extension at 72°C for 2 min. The 610-bp PCR products were loaded in a 2% agarose gel and visualized as for the human mtDNA PCR.

**Gene expression analysis.** For single-embryo cDNA synthesis and amplification, embryos were placed into thin-wall PCR tubes containing 4  $\mu$ L of ice-cold lysis buffer (50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM,  $MgCl_2$ , 0.5% Triton X, with 50-pM custom synthesized pd(T)19-24 primers, 5 U/mL RNase A-type inhibitor (Qiagen), 344 U/mL RNA-

guard (Roche), and 10  $\mu$ M of dATP, dCTP, dGTP, and dTTP. Embryos were lysed at 65°C for 2 min. First-strand synthesis of cDNA was performed using MMLV (50 U) and AMV (1 U) RTs for 20 min, and the RT terminated by heat inactivation at 65°C for 15 min. A poly(A) tail was added using terminal transferase TdT (New England Biolabs, Ipswich, MA) in NEB4 buffer supplemented with 0.25 mM of  $\text{CoCl}_2$  and 0.2 mM of dATP. The reaction was heat inactivated for 15 min at 65°C. The RT product from each embryo was used as a template for further PCR amplification as follows. The contents of each tube was brought to 100  $\mu$ l by adding buffer D (Epicentre, Halifax, Canada), 5 U of FailSafe polymerase (Epicentre, Madison, WI), and 5  $\mu$ g of PCR primer AL1 5'-ATT GGA TCCAGG CCG CTC TGG ACA AAA TAT GAA TTC (T)24-3' (Tietjen et al., 2003). PCR was performed using two-stage amplification. The first round included 94°C for 45 sec, 42°C for 1 min, 70°C for 6 min, with a 10-sec increase of extension time at each cycle for 25 cycles. An additional 5 U of FailSafe polymerase was added to the reaction for the next 25 cycles of amplification using the same program but no time increase for the extension period. About 2–4  $\mu$ g of cDNA was synthesized from each sample. mRNA levels were identified by PCR with transcript specific primers. We used the following primers: Oct4 (5'-ATGGCGGGACA CCTGGCT-3'; 5'-TCAGTTTGAATGCATGGG-3'), Sox2 (5'-ATGTACAACATGAT GGAGAC-3'; 5'-TCACATGTGTG-AGAGGGG-3'), Nanog (5'-TGCAATGTCTTCT GCTG-AGAT-3'; 5'-GTTCAGGATGTTGGAGAGTTC-3'). Positive products were sequenced to verify their identity.

**Microarray analysis.** SCNT or normal IVF embryos (four to five blastomeres), enucleated oocytes (human,  $n = 4$ ; bovine,  $n = 4$ ; and rabbit,  $n = 3$ ) and ~200 cumulus cells were placed in 10  $\mu$ L of sterile water. Cells were then shipped to Cogenics (Morrisville, NC) for RNA amplification and array hybridization. In brief, total RNA was extracted and amplified using a TargetAmp™ 2-Round Aminoallyl-aRNA Amplification Kit 1.0 (Epicentre, Canada) according to the manufacturer's instructions. All labelings were single color using Cy3 dye. Labeled cRNA (1.3  $\mu$ g) was hybridized to Agilent 44K Whole Human Genome Oligo microarrays and, following washing and scanning, data were extracted from the scanned images using Agilent Technologies' Feature Extraction software version 9.5 (FE9.5). For one-color experiments, gProcessedSignal values from Agilent's Feature Extraction software were generated following background subtraction and include correction for multiplicative surface trends. Normalized microarray data representing gProcessedSignal values for every feature were analyzed with GeneSifter™ data analysis suite. For pairwise compar-

isons, Student's *t*-tests were performed on median-normalized, log-transformed data (fold change  $\geq 4$ ;  $p \leq 0.005$ ). For comparisons of all five groups (IVF embryos, human SCNT, bovine SCNT, rabbit SCNT and cumulus cells), an ANOVA was performed (with Bonferroni correction).

## Results

In order to assess the reprogramming ability of human oocyte cytoplasm, a series of studies were carried out using metaphase II (MII) eggs obtained from either healthy volunteers or germinal vesicle (GV) and metaphase I (MI) oocytes (rejected for IVF) matured *in vitro* to the MII stage. Oocytes and donor cumulus cells were obtained with full informed consent, and used in compliance with Stem Cell Sources' and ACT's ethics advisory (EAB) and institutional review (IRB) boards, and in accord with the standards set by the American Society of Reproductive Medicine.

For SCNT, enucleation of the denuded MII oocytes was performed using a small piezo-driven micropipette. Visualization was carried out with an inverted microscope equipped with the Oosight™ spindle imaging system to avoid Hoescht staining and UV light exposure. The enucleated oocytes were injected with nuclei from fresh human cumulus cells and then activated using ionomycin and 6-dimethylaminopurine (6-DMAP). The majority of the reconstructed embryos cleaved to the two-cell (43 of 49) and four-cell (37 of 49) stage, whereas 19 (39%) of the SCNT embryos developed to the morula (8–16 cell) stage (Table 1, Fig. 1). Six morula-stage embryos were used to assess reprogramming. Genomic DNA SNP genotyping showed with high probability that all of the embryos originated from the respective somatic donor cells (Table 2). Whole-genome amplification (WGA) was also performed since only a portion of each embryo ( $\leq 3$  blastomeres) was used for DNA fingerprinting and mtDNA sequencing. Well-characterized cell lines were used to examine the reliability of genomic and mtDNA fingerprinting from single cells (Supplementary Table 1; see online supplementary material at [www.liebert-online.com](http://www.liebert-online.com)). There was a significant difference between match and no-match similarities ( $p < 0.05$ ; Wilcoxon Rank Sum Test). An IVF embryo and unrelated donor cells were also included as negative controls. There was clear separation of the percent similarities between the cloned embryos and single donor total DNA was observed. This diagnostic technique has been previously shown to distinguish between sibling embryos with 100% accuracy using single blastomeres (Treff et al., 2007a). Because of allelic dropout and preferential amplification during single-cell WGA, there should not be 100% match of SNP genotypes tested. Instead,

TABLE 1. DEVELOPMENT OF SCNT EMBRYOS USING HUMAN AND ANIMAL OOCYTES

Species	Number oocytes	Number reconstructed	Embryo development (%)		
			Two-cell	Four-cell	8–16 cell
Human	60	49	43 (88)	37 (76)	19 (39)
Bovine	88	75	67 (89)	47 (63)	27 (36)
Rabbit	33	33	26 (79)	19 (58)	12 (36)
Mouse	60	57	26 (46)	0 (0)	0 (0)



TABLE 2. PERCENT MATCH OF SCNT EMBRYOS AND DONOR CELLS USING SNP MICROARRAYS

Embryo sample	% Match with donor total DNA								
	14	15	16 <sup>a</sup>	17	18	19	20	21	22
clone 1	60.8	59.2	62.3	61.8	<b>76.4</b>	59.9	60.8	63.1	54.5
clone 2	57.7	56.7	59.6	59.3	<b>69.4</b>	57.9	60.0	60.6	53.5
clone 3	61.8	60.3	62.8	62.2	63.3	60.3	61.9	<b>80.0</b>	55.1
clone 4 <sup>b</sup>	55.8	55.9	56.6	56.5	57.0	56.3	57.6	57.8	<b>77.6</b>
clone 5 <sup>b</sup>	55.4	54.9	55.7	56.2	56.0	56.0	56.1	57.3	<b>73.2</b>
clone 6	55.3	54.7	55.8	55.9	56.5	55.6	56.9	57.4	<b>73.7</b>
clone 7	62.9	60.6	63.4	<b>79.8</b>	63.9	61.4	63.0	65.0	55.6
clone 8	62.7	61.3	63.6	<b>80.2</b>	63.7	61.2	63.2	65.0	56.3
clone 9	73.8	<b>82.1</b>	66.5	66.2	66.6	64.9	66.3	67.5	58.3
clone 10	61.9	<b>79.4</b>	62.6	62.1	62.7	61.8	62.7	63.9	56.3
clone 11 <sup>b</sup>	59.3	58.8	60.2	60.0	59.6	58.8	60.0	<b>72.8</b>	54.7
clone 12 <sup>b</sup>	60.5	59.4	61.5	61.4	61.8	60.1	60.7	<b>77.2</b>	54.5
IVF embryo	63.0	62.6	63.2	63.2	63.6	64.6	65.4	64.6	57.6

<sup>a</sup>Unrelated cell.<sup>b</sup>Different blastomeres tested from the same embryo (clones 4 and 5; and clones 11 and 12).

known matches demonstrated similarities consistent with the expected allele drop-out (ADO) and preferential amplification rates of WGA, and clearly higher than unmatched similarities. The results shown in Table 2 confirm that the clones originated from the respective nuclear donor cells.

The hypervariable (HV) region of the mitochondrial genome of the cloned embryos was evaluated to confirm the maternal origin of the mtDNA. WGA DNA from the cloned embryos and total DNA from oocytes and cumulus cell donors were sequenced and compared with a reference sequence for mitochondrial HV region analysis. The positions where the experimental samples differed from the reference sample are shown in Table 3. The results clearly demonstrated that the mtDNA sequences in the HV1 regions of the cloned embryos matched those of the corresponding oocyte donor.

Twenty-three chromosome karyotyping of the SCNT embryos was carried out using a WGA- and SNP-based microarray paradigm (see Materials and Methods). Copy number analysis settings were utilized as previously described

(Treff et al., 2007b). Each SNP was assigned a value from 0 to 5, and the overall chromosome was assigned a copy number based on the majority of SNP copy number assignments (full karyotypes were based on the copy number of each chromosome). This technique has been previously used to examine the karyotype of stable cell lines with various established karyotypes (Treff et al., 2007b). The cloned human embryos analyzed ranged from normal to various degrees of aneuploidy, whereas all the total DNA samples from oocyte and cumulus cell donors gave the expected normal karyotypes (Supplementary Table 2).

Reprogramming of the donor somatic genome was evaluated using microarray analyses after RNA amplification. Whole genome expression profiling using Agilent Human Genome Arrays (~41,000 unique transcripts) revealed striking differences between the expression patterns of normal and SCNT embryos in comparison to the donor somatic cells (Fig. 2). In *t*-test comparisons between individual groups a large number of genes (6178) were differentially expressed

TABLE 3. SUMMARY OF MITOCHONDRIAL HV1 SEQUENCING

	Mitochondria HV sequences													
	70	88	103	149	160	165	166	170	195	201	269	282	297	340
Index reference	C	T	C	C	C	C	C	T	T	C	T	G	T	T
DOE18	C	T	T	A	C	T	C	T	C	C	T	G	C	T
DOE19	T	T	T	A	C	T	C	T	T	T	T	A	C	C
DOE20	T	T	T	A	C	T	C	T	T	T	T	A	C	C
DOE21	C	T	C	A	C	T	C	T	C	C	T	G	C	T
DOE22	C	C	C	A	C	C	C	T	T	C	T	G	T	T
Clone1	T	T	T	A	C	T	C	T	T	T	T	A	C	C
Clone2	T	T	T	A	C	T	C	T	T	T	T	A	C	C
Clone3	C	T	T	C	T	C	C	C	C	C	T	G	C	T
Clone4 <sup>a</sup>	C	T	C	A	C	T	C	T	C	C	T	G	C	T
Clone5 <sup>a</sup>	C	T	C	A	C	T	C	T	C	C	T	G	C	T
Clone6	C	T	C	A	C	T	C	T	C	C	T	G	C	T

The reference sequence is provided in the SeqScape software and is based on sequence information for a reference genomic DNA sample. The index refers to the position in the mitochondrial sequence of the reference sample.

<sup>a</sup>Different blastomeres tested from the same embryo.

between the somatic cell nuclear donor with the human–human SCNT clones. Over 84% of genes were upregulated (5200) and the rest were downregulated, indicating that SCNT had dramatically changed the expression profile of the donor nucleus. An examination of the expression profiles of Oct-4, Sox-2, nanog, the factors recently used for reprogramming human somatic cells to generate induced pluripotent stem (iPS) cells (Yu et al., 2007), showed that all were significantly upregulated in normal embryos and the human–human SCNT clones. Similarly, genes such as follicle stimulating hormone receptor (FSHR), hyaluronan synthase 2 (HAS2), and steroidogenic acute regulatory protein (STAR), which are normally well expressed in cumulus cells, were no longer expressed in the reprogrammed human–human SCNT clones or in normal embryos. These data indicate that the reprogramming using an enucleated human oocyte was specific, changing the expression profile of the differentiated cumulus cell nucleus to one that had important similarities to normal age-matched human embryos.

To assess the reprogramming ability of animal oocyte cytoplasm, a series of studies were carried out using eggs obtained from rabbits, cows, and mice. All procedures and animal housing were approved by the Baylor College of Medicine's Institutional Animal Care and Use Committee (IACUC), and complied with NIH laboratory animal care guidelines. Sexually matured New Zealand White Rabbits (6–8 months old) and B6D2F1 mice (8–10 weeks old) were superovulated using FSH and human chorionic gonadotrophin (hCG), or PMSG and hCG, respectively. Bovine oocytes were obtained from Bomed Inc. and matured *in vitro* (see Materials and Methods).

Enucleation of the denuded oocytes was performed using a piezo-driven micropipette as described previously (Chung et al., 2006). The enucleated oocytes were injected with nuclei from fresh human cumulus cells and then activated using either ionomycin and 6-DMAP (bovine), direct current pulses using the BTX 200 Electro Cell Manipulator in sorbitol-based activation medium followed by incubation in KSOM/6-DMAP/cyclohexamide (rabbit), or incubation in CZB medium containing strontium chloride (mouse) as described previously (Wakayama et al., 2000). Approximately half (26 of 57) of the human–mouse NT units cleaved to the two-cell stage but then arrested, which is consistent with prior data using a variety of approaches (Table 1, Fig. 1C). In contrast, the majority of the reconstructed embryos generated using bovine and rabbit oocytes cleaved to the two-cell (89% and 79%) and four-cell stage (63 and 58%), respectively, whereas 27 of 75 (36%) of the human–bovine and 12 of 33 (36%) of the human–rabbit embryos developed to the morula (8–16-cell) stage (Table 1, Fig. 1C). Positive intraspecies SCNT controls using cumulus cells confirmed that the mouse, bovine, and rabbit oocytes used in these experiments reliably generated expanded blastocysts [9 of 35 (26%), 7 of 35 (20%), and 5 of 20 (25%), respectively] (Fig. 1B).

Genomic and mitochondrial DNA analysis was carried out on 22 of the iSCNT embryos (6 human–bovine, 11 human–rabbit, and 5 human–mouse). Both PCR and whole genome microarray analysis confirmed that the nuclear genome of the embryos originated from the somatic human donor cells (Supplementary Table 3; Fig. 1D). Amplified total DNA from the iSCNT embryos was digested and hybridized to Affymetrix gene chips containing human specific

probes. The SNP microarray data was analyzed and visualized with the Copy Number Analysis Tool (Affymetrix CNAT 4.1) using a Gaussian smoothing distance of 5 MB to evaluate aneuploidy. Human genomic DNA was confirmed in all of the iSCNT embryos; in fact, 8 of the 11 (73%) human–rabbit embryos, 4 of the 6 (67%) human–bovine, and 3 of the 5 (60%) human–mouse embryos had normal human diploid karyotypes, respectively (Supplementary Table 3). PCR using species-specific primers confirmed that the genomic DNA of the embryos was entirely human (Fig. 1D). The iSCNT embryos were also analyzed for the presence of human and animal mitochondrial DNA (mtDNA) using species-specific PCR primer sets. For humans, the mitochondrial control region (MitoSEQr) was amplified, whereas the Nd3, cytochrome *b*, and 12S rRNA regions were amplified for mouse, rabbit, and bovine, respectively. Species-specific mtDNA was detected in all of the iSCNT embryos (Fig. 1D). There was clear mitochondrial heteroplasmy in the iSCNT embryos.

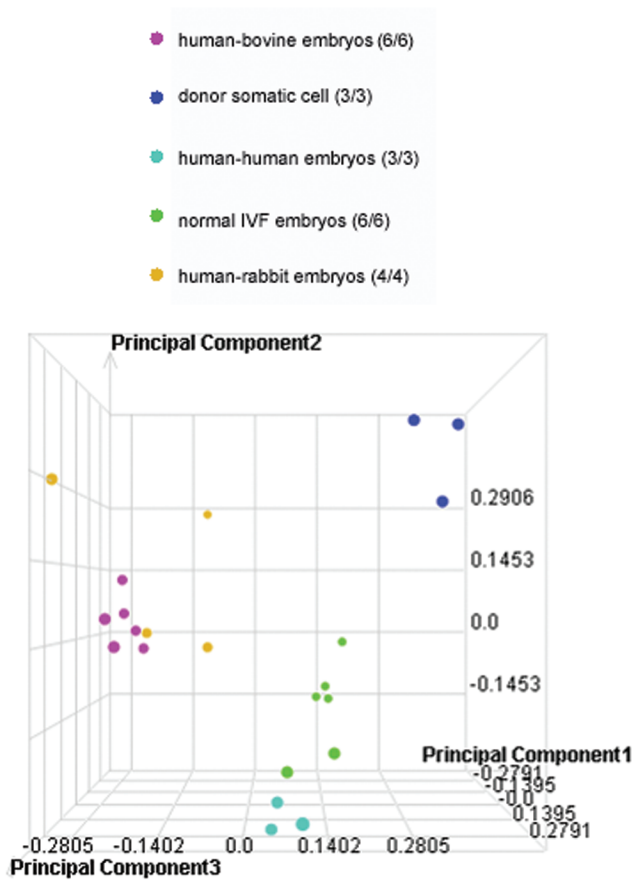
Ten of the morula-stage iSCNT embryos (six human–bovine and four human–rabbit) were used to assess reprogramming of the donor somatic genome. Although the iSCNT embryos generated using bovine and rabbit oocytes appeared morphologically similar to the human–human and normal IVF embryos, their global expression pattern was strikingly different. There were 4629 and 3008 genes that were differentially expressed in human–bovine and human–rabbit clones, respectively, compared to the donor human somatic cells. In both cases, the majority (60–70%) of these genes were downregulated. When the expression profiles between these two crossspecies clones were compared, there were no genes differentially expressed (Fig. 3; lane 4 and 5). When compared to gene expression in normal embryos, the human–rabbit clones showed 2379 differentially expressed genes and the human–bovine had 2950. Again, 60–77% of the genes were downregulated. In addition, in the cross-species clones, none of three critical reprogramming factors examined were upregulated, whereas they were significantly upregulated in both the IVF embryos and the human–human clones. This suggests that although cytoplasmic factors in bovine or rabbit oocytes were capable of supporting limited cell growth and division and changing the expression profile of between 30–50% of the donor nucleus, specific reprogramming toward the normal human embryonic state did not occur in the iSCNT embryos.

## Discussion

Although there have been several previous reports documenting the formation of SCNT embryos using human oocytes (Cibelli et al., 2001; French et al., 2008; Heindryckx et al., 2007; Stojkovic et al., 2005), to date, there has been no data indicating to what extent the donor genome was reprogrammed. Here, we show that, in contrast to animal oocytes, human oocytes have the capacity to extensively reprogram adult human somatic cells. Importantly, microarray analysis confirmed that the expression pattern of the human–human SCNT embryos were highly similar to normal IVF embryos.

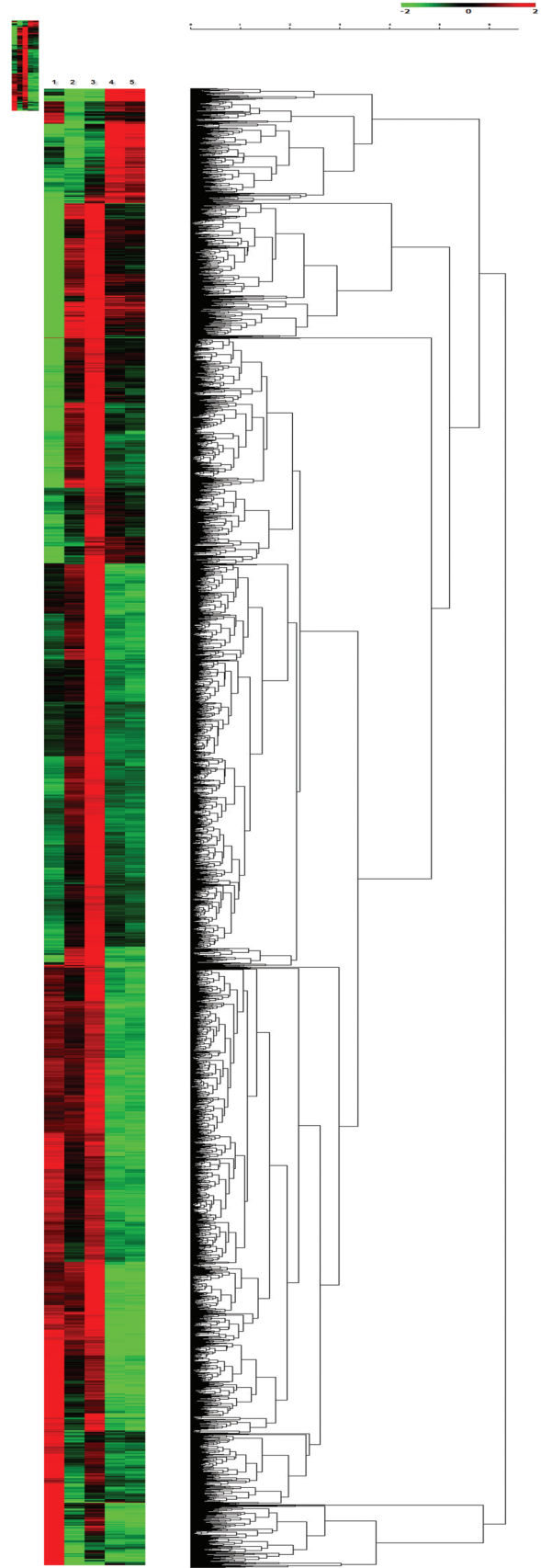
Due to the shortage of human donor eggs, cows, rabbits, and other animals have long been considered attractive surrogate sources of oocyte and egg cytoplasm for SCNT (Lanza





**FIG. 2.** Principal component analysis of the intensity data from 22 samples. A principal component analysis was performed on the intensity data for all noncontrol probes on the Agilent Human Whole Genome Microarray from each hybridization (22 original data files) using Rosetta Resolver. The two-dimensional rendering of this plot was oriented such that principal component (PC) 1 is along the X-axis, while PC2 is depicted along the Y-axis, with the dark blue spots representing donor somatic cell samples, light blue spots representing human–human embryo samples, purple spots representing human–bovine embryo samples, green representing normal IVF embryo samples, and yellow spots representing human–rabbit embryo samples.

**FIG. 3.** Hierarchical clustering of genes that are most differentially expressed in the donor somatic cells compared to the cloned embryos. Following median normalization and log transformation of the microarray data, an ANOVA analysis was performed on all 22 samples, and yielded 4838 differentially expressed genes (fold change threshold >4;  $p < 0.005$ ; with Bonferroni correction). Column 1: averaged global transcription profiles obtained from donor human somatic cells ( $n = 3$ ); column 2: normal human IVF embryos ( $n = 6$ ); column 3: human–human SCNT embryos ( $n = 3$ ); column 4: human–bovine iSCNT embryos ( $n = 6$ ); and column 5: human–rabbit iSCNT embryos ( $n = 4$ ). Red indicates upregulation; green indicates downregulation.



et al., 1999). Clearly, there are different degrees of iSCNT compatibility. Discordant combinations generally arrest at the cleavage stage, although there have been several reports of blastocyst formation (Beyhan et al., 2007; Tecirlioglu et al., 2006). Our group (Lanza et al., 2000; News of the Week, 2003) and others (Li et al., 2006; Meirelles et al., 2001) have successfully used bovine oocytes to clone closely related bovid species, and rabbit oocytes have generated iSCNT embryos using donor cells from cats (Thongphakdee et al., 2006; Wen et al., 2005), panda (Chen et al., 2002), and chickens (Liu et al., 2004), among others (Beyhan et al., 2007). However, it remains unknown whether the donor nuclei in the later combinations were fully reprogrammed. In addition, except for one study, which to date has proven irreproducible, there is no evidence that patient-specific human stem cells can be generated using animal oocytes. This is consistent with studies that indicate that oocyte cytoplasm supports nuclear remodeling, but not reprogramming of discordant iSCNT combinations (Park et al., 2004).

Studies using cow and rabbit oocyte cytoplasm clearly suggest that DNA methylation/demethylation of the donor genome occurs in a species-specific way, and that the ooplasm might lack the ability to demethylate repetitive sequences from other species (Chen et al., 2006). Genome-wide demethylation seems to correlate with the relative timing of zygotic genome activation (Beaujean et al., 2004). Although cleavage division relies on maternal RNA transcripts and proteins, further development requires activation of the embryonic genome to ensure correct progression of the cell cycle. The maternal to zygotic transition starts at different points in different species: as early as the two-cell stage in mouse, and at the 8–16 cell (morula) stage in both bovine and rabbit embryos (Beaujean et al., 2004; Misirlioglu et al., 2006; Nothias et al., 1995). Our results suggest that bovine and rabbit oocytes do not support appropriate embryonic genome reprogramming of human somatic cell nuclei, and call into question the ability of animal ooplasm to generate patient-specific human stem cells.

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## Author Disclosure Statement

V.C., V.M.S., S.B., I.K., R.M.H., S.-J.L., M.M., R.D., and R.L. are (or formerly were) employees of Advanced Cell Technology or Stem Cell Source, companies in the area of stem cell research and regenerative medicine.

## References

Beaujean, N., Taylor, J.E., McGarry, M., et al. (2004). The effect of intraspecific oocytes on demethylation of sperm DNA. *Proc. Natl. Acad. Sci USA* 101, 7636.

Beyhan, Z., Iager, A.E., and Cibelli, J.B. (2007). Interspecies nuclear transfer: implications for embryonic stem cell biology. *Cell Stem Cell* 1, 502.

Chen, D.Y., Wen, D.C., Zhang, Y.P., et al. (2002). Interspecies implantation and mitochondria fate of panda–rabbit cloned embryos. *Biol. Reprod.* 67, 637–642.

Chen, T., Zhang, Y.-L., Jiang, X., et al. (2006). Interspecies nuclear transfer reveals that demethylation of specific repetitive sequences is determined by recipient ooplasm but not by donor intrinsic property in cloned embryos. *Mol. Reprod. Dev.* 73, 313–317.

Chen, Y., He, Z.X., Liu, A., et al. (2003). Embryonic stem cells generated by nuclear transfer of human somatic nuclei into rabbit oocytes. *Cell Res.* 13, 251–263.

Cibelli, J.B., Kiessling, A.A., Cunniff, K., et al. (2001). Somatic cell nuclear transfer in humans: pronuclear and early embryonic development. *E-biomed J. Regen. Med.* 2, 25–31.

Cibelli, J., Lanza, R.P., Campbell, K.H.S., et al., eds. (2002). *Principles of Cloning* (Academic Press, San Diego).

Chung, Y.G., Gao, S., and Latham, K.E. (2006). Optimization of procedures for cloning by somatic cell nuclear transfer in mice. *Methods Mol Biol.* 348, 111–124.

French, A.J., Adams, C.A., Anderson, L.S., et al. (2008). Development of human cloned blastocysts following somatic cell nuclear transfer (SCNT) with adult fibroblasts. *Stem Cells* 26, 494.

Jingjuan, J., Tonghang, G., Xianhong, T., et al. (2005). Experimental cloning of embryos through human–rabbit interspecies nuclear transfer. *Zool. Res.* 26, 416–421.

Heindryckx, B., De Satter, P., Gerris, J., et al. (2007). Embryo development after successful somatic cell nuclear transfer in in vitro matured human germinal vesicle oocytes. *Hum. Reprod.* 22, 1982–1990.

Lanza, R.P., Cibelli, J.B., and West, M.D. (1999). Prospects for the use of nuclear transfer in human transplantation. *Nat. Biotechnol.* 17, 1171–1174.

Lanza, R.P., Cibelli, J.B., Diaz, F., et al. (2000). Cloning of an endangered species (*Bos gaurus*) using interspecies nuclear transfer. *Cloning* 2, 79–90.

Li, Y., Dai, Y., Dol, W., et al. (2006). Cloned endangered species takin (*Budorcas taxicolor*) by inter-species nuclear transfer and comparison of the blastocyst development with yak (*Bos grunniens*) and bovine. *Mol. Reprod. Dev.* 73, 189–195.

Liu, S.Z., Zhou, Z.M., Chen, T., et al. (2004). Blastocysts produced by nuclear transfer between chicken blastodermal cells and rabbit oocytes. *Mol. Reprod. Dev.* 69, 296–302.

Meirelles, F.V., Bordignon, V., Watanabe, Y., et al. (2001). Complete replacement of the mitochondrial genotype in a *Bos indicus* calf reconstructed by nuclear transfer to a *Bos taurus* oocyte. *Genetics* 158, 351.

Misirlioglu, M., Page, G.P., Sagirkaya, H., et al. (2006). Dynamics of global transcriptome in bovine matured oocytes and preimplantation embryos. *Proc. Natl. Acad. Sci USA* 103, 18905–18910.

News of the Week. (2003). Another endangered species cloned. *Science* 300, 421.

News Service. (2006). Stem cell tensions increase. *New Scientist* 2535, 6.

Nothias, J.Y., Majumder, S., Kaneko, K.J., et al. (1995). Regulation of gene expression at the beginning of mammalian development. *J Biol Chem.* 270, 22077–22080.

Park, S.H., M.R. Shin, N.H. Kim (2004). Bovine oocyte cytoplasm supports nuclear remodeling but not reprogramming of murine fibroblast cells. *Mol. Reprod. Dev.* 68, 25.

- Stojkovic, M., Stojkovic, P., Leary, C., et al. (2005). Derivation of a human blastocyst after heterologous nuclear transfer to donated oocytes. *Reprod. Biomed.* 11, 226–231.
- Tecirlioglu, R.T., Jitong, G., and Trounson, A.O. (2006). Interspecies somatic cell nuclear transfer and preliminary data for horse-cow/mouse iSCNT. *Stem Cell Reviews* 2, 277–288.
- Thongphakdee, A., Numchaisri, P., Omsongkram, S., et al. (2006). In vitro development of marbled cat embryos derived from interspecies somatic cell nuclear transfer. *Reprod. Domest. Anim.* 41, 219–226.
- Tietjen, I., Rihel, J.M., Cao, Y., et al. (2003). Single-cell transcriptional analysis of neuronal progenitors. *Neuron* 38, 161–175.
- Treff, N.R., Su, J., Berge, E.P., et al. (2007a). Single-blastomere whole-genome DNA fingerprinting results in unequivocal embryo identification—a powerful new clinical and diagnostic tool. *Fertil. Steril.* 88, S4.
- Treff, N.R., Su, J., Mavrianos, J., et al. (2007b). Accurate 23 chromosome aneuploidy screening in human blastomeres using single nucleotide polymorphism (SNP) microarrays. *Fertil. Steril.* 88, S1.
- Vogel, G. (2006). Stem cells: ethical oocytes, available for a price. *Science* 313, 155.
- Wakayama, T., Shinkai, Y., Tamashiro, K.L., et al. (2000). Cloning of mice to six generations. *Nature* 407, 318–319.
- Wen, D.C., Bi, C.-M., Ying, X., et al. (2005). Hybrid embryos produced by transferring panda or cat somatic nuclei into rabbit MII oocytes can develop to blastocysts in vitro. *J. Exp. Zool. A Comp. Exp. Biol.* 303, 689.
- Yu, J., Vodyanik, M.A., Smago-Otto, K., et al. (2007). Induced pluripotent stem cell lines derived from human somatic cells. *Science* 318, 1917–1920.

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