Evaluation of the embryonic preimplantation potential of human adult somatic cells via an embryo interspecies bioassay using bovine oocytes

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Objective: To examine the embryonic preimplantation potential of human adult somatic cells by creating interspecies embryos via somatic cell nuclear transfer (SCNT) using bovine oocytes.
Design: Prospective study.
Setting: Research facility of Reprogen.
Patient(s): Infertile couples.
Intervention(s): Enucleated bovine oocytes were fused via SCNT with either human granulosa (HG) or fibroblast (HF) cells and cultured in vitro. Polymerase chain reaction (PCR) and DNA analysis were performed on the interspecies embryos. Parthenogenetically activated embryos served as controls.
Main Outcome Measure(s): Embryonic preimplantation development after interspecies SCNT.
Result(s): From enucleated bovine oocytes fused with HG cells (n = 48) and HF cells (n = 75), 15 HG- and 22 HF-derived embryos developed, some of which progressed to blastocysts (31.3% vs. 29.3%, respectively). The PCR and DNA analysis showed that the interspecies embryos contained human genomic DNA specific for the individual DNA profile of the HG or HF donor cells used for SCNT. In addition, both bovine- and human-specific mitochondrial DNA was detectable in the interspecies embryos up to the blastocyst stage. Parthenogenetic development was 46.8% and 64.9% for the HG and HF series, respectively. The SCNT efficiency index, defined as the ratio of SCNT and parthenogenetic success rate, was 66.8% for HG cells and 45.5% for HF cells.
Conclusion(s): This interspecies bioassay can be utilized to determine and assess the embryonic preimplantation potential of different types of human adult somatic cells. (Fertil Steril® 2006;85(Suppl 1):1248–60. ©2006 by American Society for Reproductive Medicine.)

Key Words: Human granulosa cell, human fibroblast, bovine oocyte, interspecies SCNT, microPCR, DNA sequencing

In 1981, reproductive cloning in mammals was first reported for rodents by creating adult mice via nuclear transfer from cells of the inner cell mass of blastocysts into enucleated egg cells (1). In 1986, cloned sheep were been obtained from embryonic cell nuclei transfered into enucleated oocytes, thus establishing the cloning procedures for farm animals (2). During the next ten years, cloning procedures have been further developed and extended to pigs (3), goats (4), cows (5), and rhesus monkeys (6), always using embryonic cells as nuclear donors for cloning. In 1997, adult donor cells were employed for successful cloning in sheep by creating "Dolly" from a ewe's mammary gland cell nucleus (7). Subsequently in 1998, eight calves have been cloned from adult cells of a single donor animal (8). In this context, adult bovine granulosa cells have proven to be excellent donor cell types for somatic cell nuclear transfer (SCNT) into enucleated bovine oocytes yielding appreciable success rates in reproductive cloning (9). Bovine oocytes have also been tested for SCNT in combination with various mammalian species using rat, pig, sheep, and rhesus monkey cells as donor cell types. Development up to the blastocyst stage has been observed on these interspecies-specific embryos (10).

In 2000, cloned calves were produced from adult fibroblast cells after long-term culture in vitro (11). Moreover, several cloned calves of female and male genotype were also generated from a variety of different adult cell types (12). Concerning primate cloning with adult somatic donor cells, and particularly relevant for the rhesus monkey model, American researchers have recently reported on the failure to obtain pregnancy from cloned embryos originating from somatic cell nuclear transfer (SCNT) (13).

Several researchers in the cloning field have pointed out that the short period of time for the somatic donor cell nucleus to be properly reprogrammed in the recipient oocytoplasm may not be sufficient to initiate and enable normal embryogenesis. Cloning efficiency in goat and cattle can be significantly improved by exposing donor nuclei from blastomeres of a cloned embryo again to the cytoplasm of recipient oocytes during a second recloning step, thus prolonging the exposure time for genomic remodelling from adult to embryonic gene expression (14, 15). For future applications in reproductive cloning it will be important to further advance our understanding of how to rejuvenate and reprogram human adult cells by modern molecular

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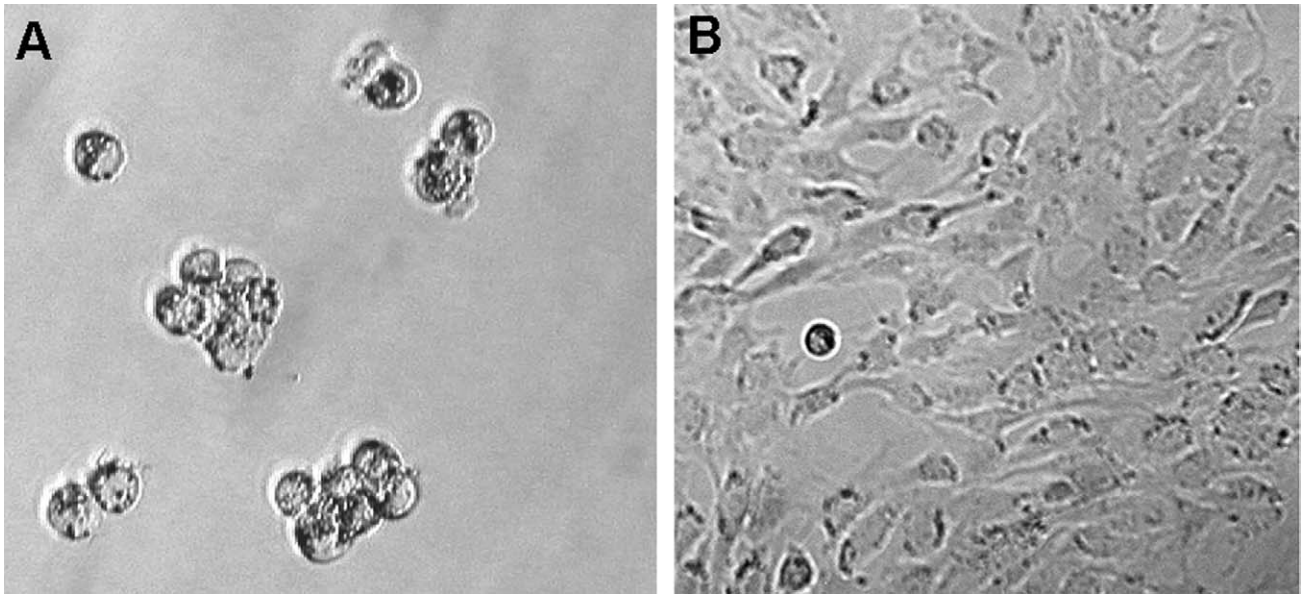
FIGURE 1

Human donor cells used for interspecies somatic cell nuclear transfer. (**A**) Human granulosa (HG) cells derived from the in vitro fertilization cycle. (**B**) Human fibroblast (HF) cells derived from skin tissue in semiconfluent culture.



Illmensee. Embryo bioassay via interspecies SCNT. Fertil Steril 2006.

bioengineering. In this context, the aim of our study was to establish a bioassay to determine the embryonic capacity of human adult somatic cells and explore their developmental potential for embryo cloning via SCNT. We have already presented our preliminary results on interspecies SCNT using enucleated bovine oocytes fused with human granulosa cells from IVF cycles and have shown this to be a very good bioassay model for future studies (16).

MATERIALS AND METHODS

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

Bovine Oocytes and Human Cells

Bovine oocytes were obtained from slaughterhouse ovaries. They were incubated overnight in maturation medium at 5% CO_2 and 38°C. This 15 mL maturation medium contained the following: 13.2 mL Medium 199 with Earle's salts (Invitrogen Corporation, Chicago, IL), 1.5 mL fetal bovine serum (Hyclone, Logan, UT), 75 μL bovine FSH (Sioux Biochemical, Sioux Center, IA), 75 μL bovine LH (Sioux Biochemical), and 150 μL penicillin/streptomycin (Invitrogen).

About 20 h later, they were manually denuded in 300 IU/mL hyaluronidase (Cooper Surgical, Trumbull, CT), washed, and selected for polar body presence. Bovine oocytes were used either for enucleation and SCNT or for parthenogenesis (controls).

Human granulosa (HG) cells were derived from two different donor women (D1 and D2) enrolled in regular IVF/ICSI programs (Fig. 1A). The HG cells were either cultured briefly or maintained in culture for about 3 days at 5% CO_2 and 37°C. Before their use for SCNT, HG cells were micropipetted into microdrops of Ca- and Mg-free phosphate-buffered solution (PBS) (Invitrogen) supplemented with 0.4% bovine serum albumin (BSA; Invitrogen).

Human fibroblast (HF) cells derived from two different donor men (D3 and D4) were established either from frozen skin samples (D3) or from fresh skin biopsy (D4). Such tissue was transfered into a small Nunclon Petri dish (VWR International, West Chester, PA) containing Ca- and Mg-free PBS and minced into very small pieces. These small tissue pieces were then transfered to another small Petri dish containing a preincubated 0.25% trypsin and 1 mmol/L EDTA solution (Invitrogen) and incubated for 20 min at 5% CO_2 and 37°C. After incubation, the contents of the Petri dish were rinsed by repeated pipetting (about 20 times) using a sterile Pasteur pipette and transfered into a 15-mL conical Falcon tube (VWR International) and allowed to sediment. The supernatant was removed and transfered to another Falcon tube containing preincubated Dulbecco's modified eagle medium (DMEM) culture medium (Invitrogen) supplemented with 10% serum substitute supplement (SSS; Irvine Scientific, Santa Ana, CA), centrifuged at 500 rpm for 3 min, and the pellet resuspended in DMEM solution and transfered to two Petri dishes containing DMEM supplemented with 1.25% nonessential amino acid

solution, 1.25% L-glutamine, 1.25% pyruvic acid solution (Invitrogen), 245 μL penicillin/streptomycin solution (Sigma-Aldrich, St. Louis, MO), and 10% SSS and incubated at 5% CO_2 and 37°C. Cultures of primary fibroblast cells were established in vitro (Fig. 1B).

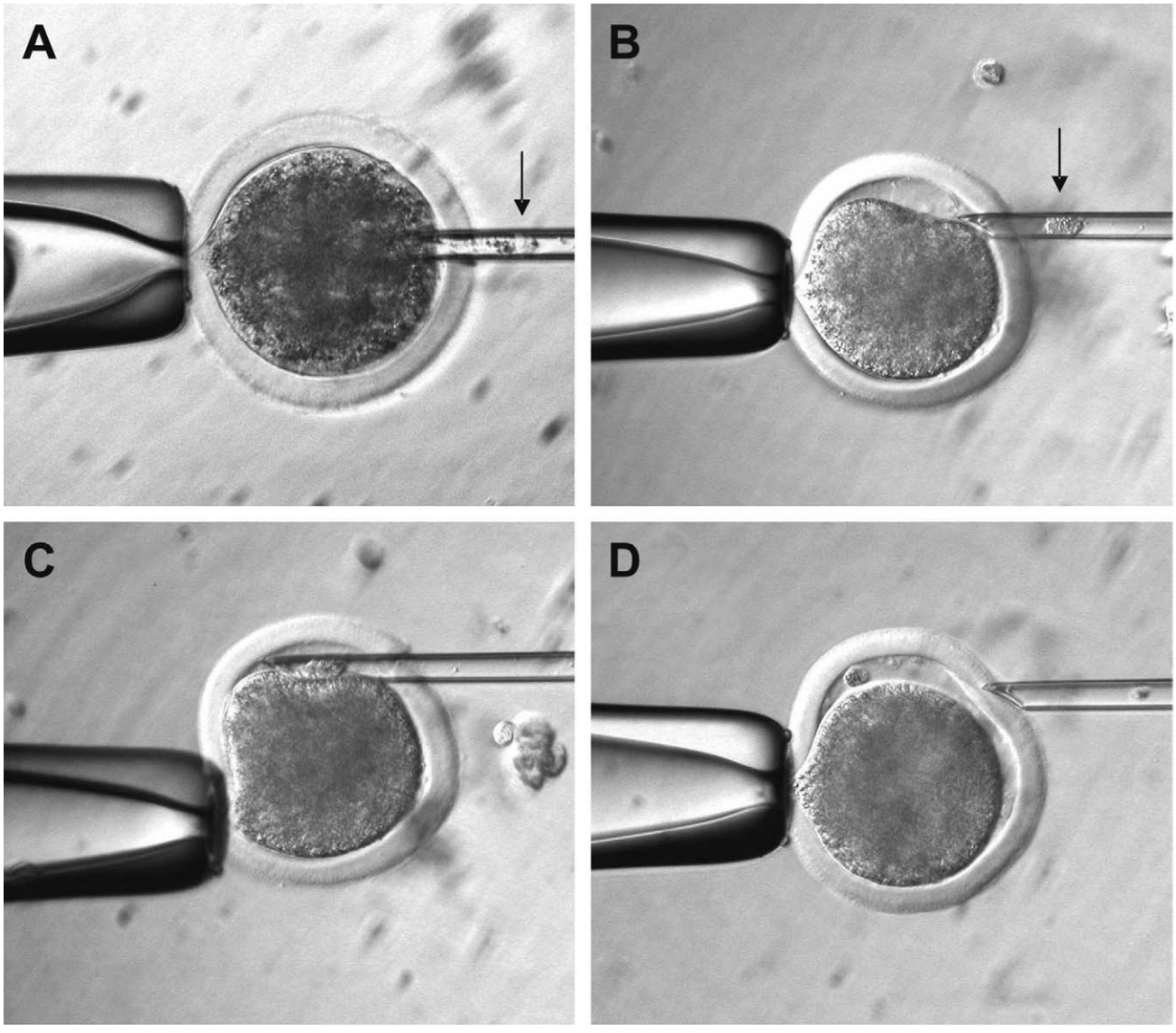
For the cells' further use in SCNT, the culture medium was removed from one Petri dish and replaced initially with Ca- and Mg-free PBS and then with 0.25% Trypsin and 1 mmol/L EDTA solution. After 2 min incubation at 5% CO_2 and 37°C, the cells were detached from the Petri dish, aspirated into a fine glass pipette and transfered through several washing steps in microdrops of human tubal fluid (HTF)+Hepes solution (In Vitro Care, San Diego, CA) supplemented with 5% SSS under properly equilibrated mineral oil (Sigma-Aldrich). These cells served as donor cells for SCNT.

Micromanipulation and SCNT

Enucleation of the bovine oocytes at metaphase II was accomplished via the removal of the polar body and oocyte metaphase II complex which was carried out microsurgically

FIGURE 2

Interspecies somatic cell nuclear transfer. (**A**) Enucleation of bovine oocyte at metaphase II; *arrow* points to the polar body and subsequent metaphase II complex, both being extracted into the pipette. (**B**) Selection of adult human granulosa cell; *arrow* points to the cell to be injected subzonally. (**C**) Injection of the cell into the perivitelline space. (**D**) Human granulosa cell attached to the enucleated bovine oocyte.



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(Fig. 2A), according to previously described procedures (15). Subsequently, the enucleated bovine oocytes were incubated in HTF medium (In Vitro Care) for 1 h at 5% CO_2 and 38°C. Groups of HG or HF cells were transfered into microdrops of Hepes-buffered HTF medium (In Vitro Care) supplemented with 5% BSA and placed in close proximity to the enucleated bovine oocytes.

Single HG cells were selected on the basis of their morphologic appearance (slightly ruffled cell membrane) and transfered microsurgically into the perivitelline space of enucleated bovine oocytes, placed tightly between the zona pellucida and oocyte membrane (Fig. 2B–D).

Single HF cells of medium size with a ruffled pseudopodial cell surface and without a visible nucleus were picked up individually into a micropipette (~20 μm), injected through the zona pellucida of enucleated bovine oocytes, and attached firmly to the oocyte surface. After the injection procedure, the oocytes were incubated for about 20 min at 5% CO_2 and 38°C.

Fusion and Activation

Following careful washing, experimental oocytes were placed individually into a horizontally placed fusion chamber filled with isoosmolar fusion buffer (Eppendorf, Hamburg, Germany) and properly oriented so that the injected cell sticking to the oocyte membrane was placed at either the 12:00 or the 6:00 position. The electrical fusion protocol was 36 V with two pulses of 25 μs, using an Eppendorf multiporator. Oocytes were then removed from the fusion chamber, washed, and incubated as described above. Successful fusion was monitored after 20 min of incubation as evidenced by the incorporation of the somatic donor cell with the oocyte membrane, visualized as a protrusion in the oolemma, thereby indicating that it had fused with the oocyte during this time. Only those oocytes that did not show fusion were again placed into the fusion chamber and pulsed electrically for a second time. The manipulated oocytes were incubated for 1 h as described above before activation. These oocytes were then incubated in culture medium supplemented with 7% ethanol (Sigma-Aldrich) for 5 min, washed carefully, and again incubated in culture medium supplemented with 10 μg/mL cycloheximide (Sigma-Aldrich) and 5 μg/mL cytochalasin B (Sigma-Aldrich) for 5 h (Zarkhartchenko, personal communication). After the activation procedure, oocytes were washed carefully, transfered to IVC-One medium (In Vitro Care) supplemented with 10% SSS, and cultured in vitro at 5% CO_2, 5% O_2, and 38°C.

Nonmanipulated bovine oocytes at metaphase II were preincubated and activated electrically under the same conditions applied to the SCNT-reconstructed oocytes. The parthenogenetically activated oocytes were cultured in vitro and used as controls.

In Vitro Culture

After several careful washings, SCNT-reconstructed and nonmanipulated bovine oocytes (controls) were cultured separately in IVC-One medium supplemented with 10% SSS. Interspecies and parthenogenetic embryonic development was monitored daily up to six days. Embryo quality was assessed using a similar grading system to that employed in IVF programs.

PCR Amplification of Genomic and Mitochondrial DNA

Analysis of Human Genomic Microsatellites. Ten different loci (9 short tandem repeat loci and amelogenin) were generated from Applied Biosystems' AmpFLSTR Profiler Plus Kit for human identification applications. The ten different loci were D3S1358, D5S818, D7S820, D8S1179, D13S317, D18S51, D21S11, FGA, vWA, and amelogenin. Polymerase chain reaction (PCR) was performed according to manufacturers' instructions and electrophoresed for 1 h on an ABI Prism 3100 genetic analyzer, according to the manufacturer's instructions (Applied Biosystems, Foster City, CA).

Analysis of Bovine Genomic Microsatellites. Eleven microsatellite markers were generated from Stock Marks for Cattle Bovine Genotyping Kit (Applied Biosystems). The 11 different loci were SPS115, BM1824, BM2113, TGLA53, INRA 23, ETH10, ETH225, ETH 3, TGLA123, TGLA126, and TGLA 227. The PCR was performed according to manufacturer's instructions and electrophoresed for 1 h on an ABI Prism 3100 genetic analyzer, according to the manufacturer's instructions.

Analysis of Human and Bovine Mitochondrial DNA. A single fragment of either the human or bovine mitochondrial DNA was amplified using the following primer pairs:

Cow: Primer 1 D-loop u: 5′-AAATGTAAAACGACGACG-GCCAGTAATCCCAATAACTCAACAC-3′
Cow: Primer 2 D-loop l: 5′-AAACAGGAAACAGCTAT-GACCACTCATCTAGGCATTTTC-3′
Human: Primer 1 D-loop u: 5′-TTCATGGGGAAGCAGATT-TGG-3′
Human: Primer 2 D-loop l: 5′-TGTGCTATGTACGGTA-AATGGCTT-3′

Amplifications were performed in a final volume of 20 mL in 10× PCR buffer (15 mmol/L $MgCl_2$, pH 8.3) and Q-solution, 100 μmol/L for each deoxynucleotide-triphosphate (dNTP), with 1 U Taq polymerase and 10 pmol each primer. Four microliters of the DNA extract was added to the PCR mix. The amplification was carried out with initial denaturation at 95°C for 10 min, followed by 35 cycles of one denaturation step at 94°C for 40 s, primer annealing at 55°C for 40 s, and primer extension at 72°C for 45 s in a Hybaid thermocycler. The PCR products were purified using the Qiaex II Gel Extraction Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Sequencing was performed using ABI-Prism Dye Kit V3 (Applied Biosystems) in a 10-mL volume containing 2 mL purified PCR product and 5 pmol

primer. Sequencing reactions underwent 27 cycles of 30 s at 94°C, 30 s at 50°C, and 3 min at 60°C in a Techne thermocycler. The dye terminators were removed by Millipore sephadex-G45 column purification. Sequencing reactions were electrophoresed for 2 h on an ABI Prism 3100 genetic analyzer, according to the manufacturer's instructions.

Micro-PCR amplification and DNA sequencing on interspecies SCNT embryos and human granulosa and fibroblast cells were carried out at the Veterinary Institute for Molecular Biology, University of Goettingen, Goettingen, Germany.

RESULTS

In the first experimental series using HG cells for SCNT procedures, these cells were obtained from two different donor women (D1 and D2) enrolled in regular IVF/ICSI programs. In interspecies SCNT, 26 and 22 enucleated bovine oocytes were electrically fused with D1 and D2 donor cells respectively, activated chemically, and were cultured in vitro up to six days. From these SCNT-reconstructed oocytes, eight (for D1) and seven (for D2) embryos developed to various preimplantation stages, and two (for D1) and one (for D2) advanced further into blastocysts (Fig. 3A and B). These developing embryos revealed very similar embryonic capacities and showed quite comparable cellular morphology (Fig. 3A). The SCNT success rate using HG cells was 30.8% for D1 and 31.8% for D2 (Table 1).

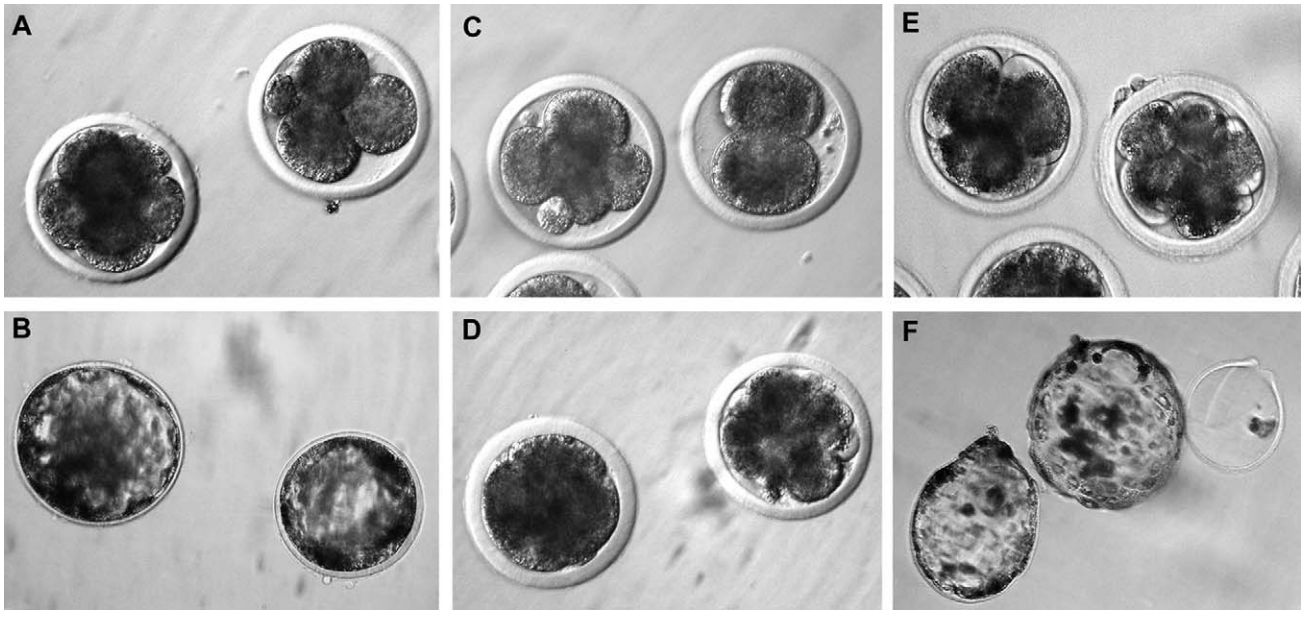
As controls for activation and culture conditions, 36 (for D1) and 41 (for D2) nonmanipulated bovine oocytes at metaphase II were chemically activated under the same conditions as applied to the SCNT-reconstructed oocytes and culture in vitro. From these parthenogenetically activated oocytes, 15 (for D1) and 21 (for D2) developed through various stages of preimplantation up to blastocyst stage. The parthenogenetic success rate for embryo development was 41.6% in the D1 series and 51.2% in the D2 series (Table 1).

Based on these results, we introduced a new parameter for SCNT efficiency which was derived from the overall cloned embryo success rate divided by the parthenogenetic success rate × 100. The overall SCNT efficiency index for HG cells was 66.8%.

In the second experimental series using HF cells for SCNT, such cells were obtained from two different donor men, D3 and D4, from whom fibroblast cultures were established from frozen skin tissue (D3) and fresh skin biopsy (D4). In interspecies SCNT, 62 and 13 enucleated bovine oocytes were electrically fused with D3 and D4 donor cells respectively, activated chemically, and cultured in vitro up to six days. From these SCNT-reconstructed oocytes, 15 (D3) and 7 (D4) embryos developed to various stages of preimplantation up to morula stage (Fig. 3C and D). The developing embryos derived from D3 and D4 donor cells showed comparable cellular and morphologic features, with the exception that SCNT embryos created from HF cells of donor

FIGURE 3

Interspecies somatic cell nuclear transfer preimplantation embryos derived from human granulosa (HG) cells and human fibroblast (HF) cells fused with enucleated bovine oocytes. (**A**) Cleavage embryos and (**B**) blastocysts derived from HG cells. (**C**) Cleavage embryos and (**D**) morulae obtained from HF cells. (**E**) Parthenogenetically developed cleavage embryos and (**F**) blastocysts as controls, showing hatching.



Illmensee. Embryo bioassay via interspecies SCNT. Fertil Steril 2006.

TABLE 1

Interspecies SCNT embryos derived from enucleated bovine oocytes fused with human granulosa (HG) cells and parthenogenetically developed embryos as controls.

Donor cell source	Interspecies SCNT					Parthenotes (controls)				
	Oocytes activated	Embryos developed			Success rate (%)	Oocytes activated	Embryos developed			Success rate (%)	SCNT index[a]
		Morula[b]	Blastocyst	Total			Morula[b]	Blastocyst	Total		
D1	26	6	2	8	30.8	36	9	6	15	41.7	73.8
D2	22	6	1	7	31.8	41	13	8	21	51.2	62.1
Total	48	12	3	15	31.3	77	22	14	36	46.8	66.8

Note: SCNT = somatic cell nuclear transfer.

[a] SCNT efficiency index = Interspecies SCNT success rate/parthenogenetic success rate × 100.

[b] Embryos at various stages of development up to morula.

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D4 progressed more frequently into late preimplantation with an elevated capacity for embryo development compared to SCNT embryos created from D3 donor cells. The SCNT success rate using HF cells was 24.2% for D3 and 53.8% for D4 (Table 2).

As controls for this second SCNT series using HF donor cells, 37 (for D3) and 22 (for D4) nonmanipulated bovine oocytes were chemically activated similarly to the SCNT-reconstructed oocytes and cultured in vitro. From these parthenogenetically activated oocytes, 21 (for D3) and 17 (for D4) developed through various stages of preimplantation up to blastocyst stage (Fig. 3E and F). The parthenogenetic success rate for embryo development was 56.8% in the D3 series and 77.3% in the D4 series (Table 2). By comparing the embryonic developmental rate with the parthenogenetic success rate for HF cells derived from the two different donor cell sources, the SCNT efficiency index was 42.6% for the D3 series and 69.7% for the D4 series. The overall SCNT efficiency index for HF cells was 45.5%.

To summarize our results, from a total of 37 SCNT embryos (15 from HG and 22 from HF), 11 (5 from HG and 6 from HF) reached morula stage (29.5%). For our controls, from 77 parthenogenetically activated embryos (36 for HG and 41 for HF), 39 (16 for HG and 23 for HF) advanced to morula stage (50.9%). Moreover, from these 39 morulae, 19 developed further into well expanded and even hatching blastocysts (24.6%).

In order to reveal and document accurately whether the HG and HF cells were capable of bringing about embryonic development in the enucleated bovine oocytes, we examined the interspecies SCNT embryos at the molecular level, using PCR amplification of species-specific microsatellites and DNA sequencing. We investigated individually the cloned embryos for human genomic and mitochondrial DNA (mtDNA) derived from the HG and HF donor cells as well as bovine genomic and mtDNA originating from the recipient oocytes. Furthermore, using human microsatellites we were able to specify more precisely the genomic DNA profile of somatic donor cells used for SCNT in order to compare these DNA profiles with those obtained from SCNT embryos.

Regarding the SCNT series using HG cells from donors D1 and D2, human microsatellite DNA typing was carried out on 12 interspecies embryos (Table 3). The first set of interspecies embryos (1–5) was derived from HG cells of donor D1. The genotypes generated from these donor cells (see D1 control) were identical to the genotypes revealed for the five interspecies embryos. In a second set of interspecies embryos (6–12) using cells from donor D2, the nuclear genotype of these seven interspecies embryos was identical to the genomic microsatellite DNA from donor cells (see D2 control). Some of the PCR-analyzed and DNA-sequenced samples exhibited an allelic dropout which was most likely due to the minute amounts of DNA extractable from individual interspecies embryos (Table 3).

TABLE 2

Interspecies SCNT embryos derived from enucleated bovine oocytes fused with human fibroblast (HF) cells and parthenogenetically developed embryos as controls.

Donor cell source	Interspecies SCNT					Parthenotes (controls)				
	Oocytes activated	Embryos developed			Success rate (%)	Oocytes activated	Embryos developed			Success rate (%)	SCNT index[a]
		Morula[b]	Blastocyst	Total			Morula[b]	Blastocyst	Total		
D3	62	13	2	15	24.2	37	12	9	21	56.8	42.6
D4	13	5	2	7	53.8	22	10	7	17	77.3	69.6
Total	75	18	4	22	29.3	59	22	16	38	64.4	45.5

Note: SCNT = somatic cell nuclear transfer.

[a] SCNT Efficiency Index = Interspecies SCNT success rate/parthenogenetic success rate × 100.

[b] Embryos at various stages of development up to morula.

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Concerning interspecies SCNT embryos derived from HF cells of donor sources D3 and D4, such detailed human genomic DNA profiles have not been established in this study. However, five interspecies embryos derived from HF-SCNT were analyzed for the presence of human genomic DNA using three different chromosomal microsatellite probes (FGA for chromosome 4, D21S11 for chromosome 21, and D13S17 for chromosome 13) for PCR amplification. From these embryos, human genomic DNA was clearly detectable. More importantly, the genomic DNA sequence profiles of these interspecies embryos were identical to and specific for the ones obtained from the HF donor cells (Fig. 4).

All 37 (15 from HG and 22 from HF) interspecies embryos were analyzed for the presence of bovine-specific genomic DNA, using 11 bovine-specific microsatellite markers for PCR amplification (see "Materials and Methods"). Only in four interspecies embryos, three bovine-specific markers (TGLA 53, BM 2113, and TGLA 227) were clearly amplified and could be detected from the 11 microsatellite markers employed for genomic DNA identification. Three other markers (INRA 23, TGLA 122, and TGLA 126) showed weak amplification, and five other markers (SPS 115, ETH 10, BM 1824, ETH 225, and ETH 3) completely failed to amplify under our PCR conditions. All other interspecies embryos analyzed using bovine-specific DNA markers did not reveal any presence of bovine genomic DNA.

For analyzing the interspecies embryos for the presence of human mtDNA, two human-specific primers were used for PCR amplification (see "Materials and Methods"). From 15 interspecies embryos created from HG cells of donor sources D1 and D2, 14 embryos clearly contained human mtDNA that was extracted from these embryos and could be identified as parts of the D-loop region of human mtDNA (Fig. 5A and B). Only from one of these SCNT embryos could human-specific mtDNA not be detected after PCR amplification and DNA sequencing. Concerning the 22 SCNT embryos derived from HF cells of donor sources D3 and D4, 18 embryos clearly contained human mtDNA as assessed by the presence of human-specific mtD-loop region after PCR analysis. The remaining four SCNT embryos did not show detectable human-specific mtDNA because no such DNA sequencing data could be generated from the extracted embryo DNA.

When tested for bovine mtDNA, all 15 SCNT embryos from the HG series and all 22 SCNT embryos from the HF series exhibited clearly the bovine-specific profiles using two particular primers for mtDNA amplification (see "Material and Methods"). Parts of bovine-specific mtDNA was identified for the D-loop region (Fig. 5C).

DISCUSSION

The current success rates for obtaining mammalian clones from adult cells employed for SCNT remain rather limited.

TABLE 3

DNA sequence analysis of human microsatellites and genes. Seven human microsatellite and three type I markers (AMEL, FGA, vWA) were amplified to determine the presence of human chromosomal (genomic) DNA and the individual genotype of the human granulosa (HG) donor cells in interspecies SCNT embryos.

	Embryo		Microsatellite/gene																			
Source	#	Stage	AMEL		D13S317		D18S51		D21S11		D3S1358		D5S818		D7S820		D8S1179		FGA		vWa	
Interspecies embryos[a]	1	8-cell	X	X	12	12	a.d.	a.d.	28	30	15	16	11	13	a.d.	a.d.	10	15	23	27	14	17
	2	7-cell	X	X	12	12	a.d.	a.d.	28	30	15	16	11	13	8	12	10	15	23	27	14	17
	3	Blast.	X	X	12	12	14	15	28	30	15	16	11	13	8	12	10	15	23	27	14	17
	4	Morula	X	X	12	12	14	15	28	30	15	16	11	13	8	12	10	15	23	27	14	17
	5	6-cell	X	X	a.d.	a.d.	a.d.	a.d.	28	30	15	a.d.	11	13	a.d.	a.d.	10	15	a.d.	a.d.	14	17
D1 cells[c]			X	X	12	12	14	15	28	30	15	16	11	13	8	12	10	15	23	27	14	17
Interspecies embryos[b]	1	5-cell	X	X	11	13	a.d.	a.d.	30	30	15	18	11	12	a.d.	a.d.	8	16	a.d.	a.d.	16	18
	2	8-cell	X	X	11	13	a.d.	a.d.	30	30	15	18	11	12	11	11	8	16	a.d.	a.d.	16	18
	3	4-cell	X	X	a.d.	a.d.	a.d.	a.d.	30	30	15	18	11	12	a.d.	a.d.	8	16	a.d.	a.d.	16	18
	4	Morula	X	X	11	13	a.d.	a.d.	30	30	15	18	11	12	11	11	8	16	a.d.	a.d.	16	18
	5	Blast.	X	X	11	13	a.d.	a.d.	30	30	15	18	11	12	11	11	8	16	20	22	16	18
	6	12-cell	X	X	11	13	a.d.	a.d.	30	30	15	18	11	12	a.d.	a.d.	8	16	20	22	16	18
	7	14-cell	X	X	11	13	a.d.	a.d.	30	30	15	18	11	12	a.d.	a.d.	8	16	20	22	16	18
D2 cells[c]			X	X	a.d.	a.d.	a.d.	a.d.	30	30	15	18	11	12	a.d.	a.d.	8	16	a.d.	a.d.	16	18

Note: SCNT = somatic cell nuclear transfer; a.d. = allelic dropout; X = X chromosome; Blast. = Blastocyst.

[a] Embryos derived from HG cells from D1 female patient.

[b] Embryos derived from HG cells from D2 female patient.

[c] D1 and D2 cells served as controls.

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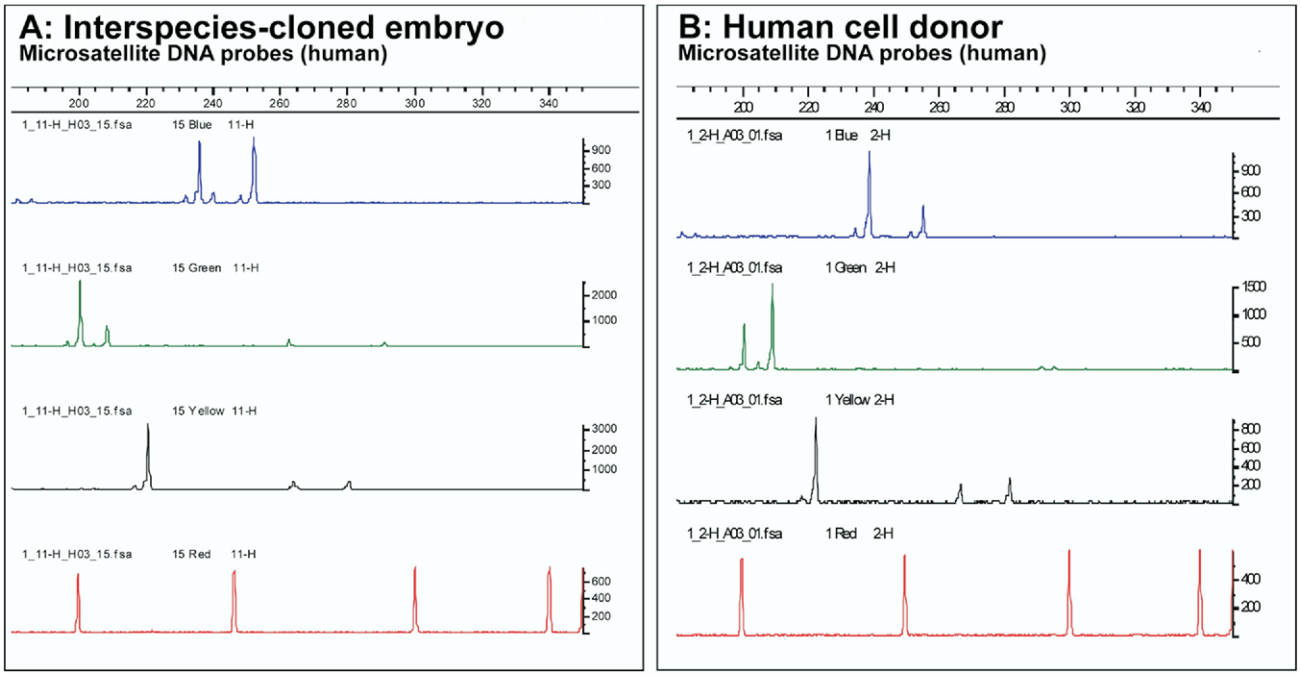
FIGURE 4

Human genomic DNA sequence analysis of interspecies cloned embryo (morula) and human adult fibroblast (HF) donor cells used for somatic cell nuclear transfer. Note the identical DNA sequence profiles concerning peak positions for interspecies embryo (morula) and HF donor cells. Some variations in peak levels result from different sample analysis. Three chromosomal microsatellite probes were used for polymerase chain reaction amplification. Blue = FGA (chromosome 4q28); green = D21S11 (chromosome 21); black = D13S317 (chromosome 13q22–31). A standard marker (ABI Applied Biosystem ROX Reference Dye) served as internal control (for further information, see "Materials and Methods").



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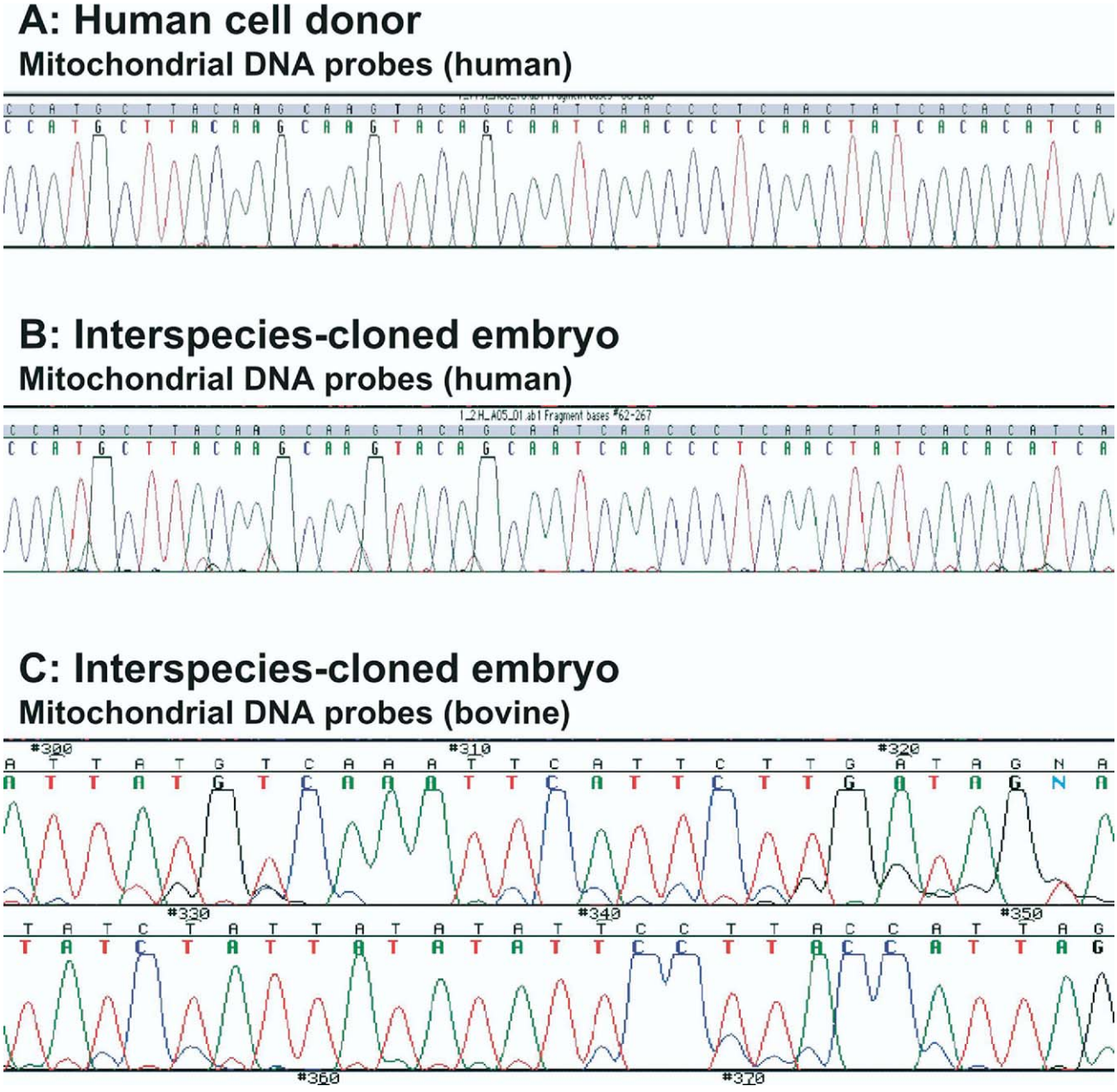
Several important factors may be responsible for and contribute to a low success rate in SCNT cloning. Intensive investigations are focusing on nucleocytoplasmic interactions and reprogramming of transfered somatic cell nuclei by the cytoplasm of recipient oocytes (17), chromatin remodeling of somatic nuclei by oocyte factors (18), rebuilding of telomere length of somatic chromosomes (19–22), and cell-cycle coordination between donor nucleus and cytoplasm of the recipient oocyte during the cloning procedure (23, 24).

It has been argued that epigenetic changes in methylation can result from aberrant or incomplete reprogramming of the donor cell genome within the recipient oocyte cytoplasm, leading to abnormal embryonic and fetal development and resulting in postnatal defects (25, 26). It seems that this epigenetic phenomenon of inferiorly remodeling the somatic chromosomes into a functional embryonic genome is, to some extent, also responsible for a low cloning efficiency (27). Another issue of concern is focusing on nucleocytoplasmic interactions during the performance of SCNT and the karyoplasm-ooplasm volume ratio. It was found that following the removal of different amounts of cytoplasm during enucleation of bovine oocytes, the volume of ooplasm was approximately equivalent to the volume of the somatic donor cells gave the best cloning results (28). It was also shown that embryonic and somatic donor cell nuclei used for SCNT in enucleated bovine oocytes require a different ooplasmic environment for genomic reprogramming, most likely owing to their different cell cycles and differentiation profiles (29). Similarly, different treatments for oocyte activation seem to significantly influence the outcome of SCNT (29, 30).

Bovine oocytes have been successfully employed in interspecies SCNT using adult fibroblast cells from pig, sheep, rat, and rhesus monkey as nuclear donor types (10). Development of cloned interspecies embryos was observed up to the blastocyst stage, but did not lead to pregnancy when transfered in utero. No investigations at the molecular level have been reported to document the embryos' genomic origin from the particular donor cell types. Analogously, enucleated bovine oocytes have been fused in interspecies SCNT with fetal rabbit fibroblasts. Embryonic development to morula stage have been recorded, but no blastocysts so far have been obtained from this interspecies cloning effort (27). In 1999, American researchers reported

FIGURE 5

DNA sequence analysis of human and bovine mitochondrial (mt) DNA extracted from human granulosa (HG) donor cells and interspecies cloned embryo (blastocyst). (**A**) Fluorogram of parts of the human mtD-loop region (position 16191–16239 GeneBank acc. no. AY275537) that were amplified from the human donor cells. (**B**) DNA sequence obtained from the interspecies cloned embryo (blastocyst). Both DNA sequences show identical profiles. (**C**) Fluorogram of parts of a profile of mtDNA sequence from the interspecies embryo (blastocyst) obtained by using bovine mtDNA primers. The region corresponds to position 306–359 of the bovine mtD-loop region (GeneBank acc. no. AF499248).



Illmensee. Embryo bioassay via interspecies SCNT. Fertil Steril 2006.

on interspecies SCNT using bovine oocytes and human lymphocytes or oral mucosal epithelial cells for cloning (31). However, their data were presented briefly in a review on mammalian SCNT without providing specific information about fusion and activation of the bovine oocytes or characteristics of the human somatic donor cells, nor presenting any documentation of the obtained embryos to furnish proof of their origin from the donor cells' genome. In 2003, we

presented our first results on interspecies SCNT using enucleated bovine oocytes fused with human granulosa cells. The embryonic success rate for interspecies SCNT was 32% in comparison to parthenogenetic development of 46% serving as controls (16). Chinese researchers reported on interspecies SCNT using rabbit oocytes and human adult fibroblast cells to create interspecies embryos with an overall success rate for early embryonic development of 36% (32). From interspecies blastocysts, they were successful in establishing embryonic stem (ES) cells in vitro. They also documented very clearly by DNA genotyping and cytogenetic karyotyping that the SCNT embryos and stem cells were derived from the genomic DNA of the human fibroblast donor cells. However, the authors did not present data on mtDNA of the ES cells originating from both rabbit and human cytoplasm (32). This latter molecular analysis of mtDNA was carried out on interspecies embryos derived from SCNT of human cord fibroblasts into enucleated bovine oocytes (33). These authors documented that both human and bovine mtDNA was present in the interspecies embryos up to the 16-cell stage, whereas only bovine mtDNA was detectable beyond the morula stage.

In our current studies, HG cells obtained from IVF/ICSI programs and HF cells taken from fibroblast cultures were chosen as donor cells for fusion with enucleated bovine oocytes. The rationale for selecting these adult cells was based on various cloning reports in different mammalian species that both cell types seem to be currently the most efficient adult donor cells for SCNT purposes (9, 11, 15, 34). Our SCNT series of interspecies cloning with HG and HF cells has resulted in 31.3% and 29.3%, respectively, embryonic development. In this regard, it should be noted that about half of the fused and activated bovine oocytes did not show signs of development. This initial failure may possibly have resulted from intrinsic factors such as: 1) inferior cell-cycle stage of the HG and HF cells during fusion; 2) aberrant reprogramming of the donor cells′ genome after fusion; and 3) nonefficient ooplasmic environment following maturation, enucleation, or activation of the bovine oocytes in interspecies cloning. Some of these physiologic and technical limitations and possible imperfections should be considered and improved in future investigations. At least two possible parameters, i.e., chemical activation and culture conditions, could be ruled out as being responsible for limited developmental capacities of interspecies SCNT embryos. As a matter of fact, for controls, nonmanipulated bovine oocytes were activated chemically and cultured in vitro under the same conditions as the SCNT embryos, and from these parthenogenetically developed embryos, a significant percentage (24.6%) advanced to blastocysts, many of which even hatched under these conditions (Fig. 3F). Our success rate of parthenogenetically developed embryos resulting from chemically activated oocytes compares well with previously published success rates (35, 36). We therefore have to take into consideration other factors such as activation or culture conditions that may be involved in further improving late preimplantation development of SCNT embryos.

From 15 interspecies embryos derived from HG cells, three embryos progressed to blastocyst stage. Blastocysts are currently being employed successfully for isolating ES cells (32, 37, 38). In this respect, Korean researchers have recently obtained patient-specific ES cells from SCNT blastocysts derived from human cord fibroblast cells fused with enucleated bovine oocytes (33). This technology could lead to new possibilities for therapeutic cloning.

In order to properly document that donor nuclei from HG and HF cells were responsible for initiating and promoting the embryonic development noted in our studies, we investigated the interspecies embryos at the molecular level by analyzing species-specific microsatellite DNA and mtDNA. The detection of human microsatellites is a marker for the presence of genomic DNA in SCNT embryos of the corresponding species. In addition, it detects the specific genotype of the individual that provided donor cells and therefore goes to prove the origin and stability of donor nuclei in SCNT embryos. The absence of PCR products originating from bovine microsatellites in almost all developing embryos indicated that the oocytes were completely depleted of the bovine nuclear genome. However, it was also found that in a few instances (4 out of 37), only part of the bovine microsatellites were adequately amplified to signal that some bovine chromosomal DNA seemed to be retained in these SCNT embryos. Currently, there is no experimental proof possible to determine and explain how bovine chromosomal DNA remains associated with these developing embryos. One explanation could be that an adhering granulosa cell that remained attached to the zona pellucida of bovine oocytes after hyaluronidase treatment can lead to bovine chromosomal contamination giving rise to particular amplification of some bovine microsatellite markers. An alternative explanation could be that during enucleation of the bovine oocyte the metaphase II complex can burst, releasing some chromosomal DNA into the ooplasm. Therefore, the limited microsatellite markers will not amplify adequately during early embryogenesis.

Interestingly, in almost all samples of interspecies-cloned embryos, including morulae and blastocysts, human mtDNA was detected, which indicates clearly that mitochondria from the fused human donor cells were carried over to the enucleated bovine oocytes and persisted throughout preimplantation. The resulting interspecies embryos can therefore be considered as heteroplasmic for mtDNA. In our bovine-human interspecies SCNT, human-specific mtDNA was still detectable up to the blastocyst stage, similar to what has been reported for interspecies blastocysts derived from rabbit-monkey SCNT (39), but different from a report on bovine-human SCNT for which human-specific mtDNA could be detected only up to the morula stage (33). Such variability in the persistence of donor cell–specific mtDNA in interspe-

cies SCNT embryos needs further investigations. Viable presence and replicative ability of donor cell–specific mtDNA has been clearly documented in the bovine system by revealing a significant mitochondrial heteroplasmy in cloned adult cattle with 4% to 40% of mtDNA originating from the somatic donor cells used for SCNT (40, 41). Possible mixing and recombination of different mtDNA populations in heteroplasmic cloned animals have been discussed in the context of assisted reproduction and embryo biotechnology (42).

Our interspecies bioassay fusing bovine oocytes with HG and HF cells, respectively, has resulted in appreciable and significant success rates. We introduced a new parameter, defined as SCNT efficiency index, by which the cloning success rate of SCNT embryos can be evaluated in relationship to parthenogenetically induced embryonic development under the same activation and culture conditions. Although the two HG series revealed rather comparable SCNT efficiency indices, there was a significant difference in the SCNT indices observed between the two HF series. One of the plausible explanations could be that HF cells from the D3 donor were established from frozen tissue samples, whereas HF cells from the D4 donor were taken from primary cultures of fresh skin biopsy. Besides these biologic differences, there may be other parameters that could be taken into consideration, such as the genetic background of the individual donors.

The SCNT efficiency index could assist in determining and comparing the embryonic capacity of other human somatic cells using the bovine oocyte model in future interspecies cloning efforts. Such bioassays will enable us to primarily examine, evaluate, and explore the embryonic potential of various human adult somatic cells in their usefulness as nuclear donors for reproductive and therapeutic cloning (43, 44).

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