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Cloning of Asian Yellow Goat (*C. hircus*) by Somatic Cell Nuclear Transfer: Telophase Enucleation Combined With Whole Cell Intracytoplasmic Injection

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ABSTRACT Our and other previous studies have shown that telophase enucleation is an efficient method for preparing recipient cytoplasts in nuclear transfer. Conventional methods of somatic cell nuclear transfer either by electro-fusion or direct nucleus injection have very low efficiency in animal somatic cell cloning. To simplify the manipulation procedure and increase the efficiency of somatic cell nuclear transfer, this study was designed to study in vitro and in vivo development of Asian yellow goat cloned embryos reconstructed by direct whole cell intracytoplasmic injection (WCICI) into in vitro matured oocytes enucleated at telophase II stage. Our results demonstrated that the rates of cleavage and blastocyst development of embryos reconstructed by WCICI were slightly higher than in conventional subzonal injection (SUZI) group, but no statistic difference ($P > 0.05$) existed between these two methods. However, the percentage of successful embryonic reconstruction in WCICI group was significantly higher than that in SUZI group ($P < 0.05$). After embryo transfer at 4-cell stage, the foster in both groups gave birth to offspring. Therefore, the present study suggests that the telophase ooplasm could properly reprogram the genome of somatic cells, produce Asian yellow goat cloned embryos and viable kids, and whole cell intracytoplasmic injection is an efficient protocol for goat somatic cell nuclear transfer. *Mol. Reprod. Dev. 74: 28–34, 2007.* © 2006 Wiley-Liss, Inc.

Key Words: Asian yellow goat (*C. hircus*); telophase ooplasm; whole cell intracytoplasmic injection; subzonal injection; somatic cell nuclear transfer

INTRODUCTION

Somatic nuclear transfer is a method with potential applications in many fields, such as breed selection, production of transgenic animals, wildlife conservation (Chen et al., 1999) and research on the mechanism of cell differentiation and nucleus-cytoplasm interaction. The technique of somatic cell nuclear transfer itself is affected by a variety of factors, including the source of donor somatic cell and its differentiation, the number of passages in donor cell culture, the cell cycle stage of donor cells, recipient oocytes, cytoplast age, activation procedure, and type of culture used for reconstructed embryos (Fulka et al., 1998; Wells et al., 1999; Zakhartchenko et al., 1999). The procedures of enucleation and donor cell injection are two of the key factors that affect somatic cell nuclear transfer.

Enucleation can be done at different stages of the recipient oocyte. In general, the oocyte is enucleated at the second metaphase (MII enucleation), when the first polar body is emitted from the cytoplasm (Kato et al., 1998; Baguisi et al., 1999; Zakhartchenko et al., 1999). Since it is impossible to visualize metaphase chromatin under common light microscopy, MII oocytes are often enucleated blindly and the enucleation is not too accurate because of displacement of the chromatin of MII oocytes from the first polar body (Bordignon and Smith, 1998; Mohamed Nour and Takahashi, 1999). Another choice is to enucleate the oocytes under UV-irradiation, visualizing the chromatin by DNA-specific vital stains. However, this method, although it increases enucleation accuracy, is harmful to embryo development. Some studies showed that the enucleation rate can be increased when the chromatin material of oocytes

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was removed at the activated telophase of the second meiotic division, by aspirating the second polar body and surrounding cytoplasm (TII enucleation) (Bordignon and Smith, 1998; Baguisi et al., 1999; Mohamed Nour and Takahashi, 1999; Liu et al., 2000). In two studies on bovine embryonic nuclear transfer, the enucleation rate was significantly higher at TII stage than that at MII stage (98% vs. 59%, 91.5% vs. 59.9%, respectively). More important, after transferring the donor embryo nucleus to the TII enucleated oocyte, both studies showed very good blastocyst rates (Bordignon and Smith, 1998; Mohamed Nour and Takahashi, 1999). Although the method of TII enucleation was first developed in bovine nuclear transfer, the first mammal cloned using this new procedure was a goat (Baguisi et al., 1999).

Cloned embryos are routinely reconstructed using either subzonal injection (SUZI; Wilmut et al., 1997) method that involves in placing a whole donor cell in the perivitelline space of an enucleated recipient oocyte and fusing the donor and recipient cells with electrical pulses or intracytoplasmic injection (ICI; Wakayama et al., 1998; Onishi et al., 2000) technique in which donor nuclei are isolated and directly injected into enucleated oocytes. However, both methods require prolonged manipulation of either the oocytes fusion or donor cell nucleus isolation, which results in not only intensive labor but also the low overall cloning efficiency due to the low fusion rate or damage to the isolated nucleus. Recently, Lee et al. (2003) reported an efficient and simple method of nuclear transfer in pig cloning that involves direct injection of a whole cell into an enucleated oocyte without both fusion and nucleus isolation processes.

The purpose of this study is to test the feasibility of combining the TII enucleation procedure with whole somatic cell injection in somatic cell nuclear transfer of Asian yellow goat. The Asian yellow goat embryos were constructed by transferring Asian yellow goat somatic cells into the enucleated oocytes and then reconstructed embryos were transferred into the oviducts of foster of another breed (local white mounting goat) with different color.

MATERIALS AND METHODS

Chemicals

All the chemicals used in this experiment were purchased from Sigma Chemical Company (St. Louis, MO) except for those specifically mentioned. Drugs were prepared as stock solutions by dissolving in 0.9% NaCl solution or DMSO, and stored at −20°C. The stock solutions were diluted with culture medium prior to use.

Preparation of Donor Cells

The method of cell culture and assessment has been described previously (Han et al., 2001). Briefly, ear tissue specimens derived from a 6-year-old female adult Asian yellow goat were finely chopped into pieces measuring about 1 mm^2 and enzymatically dissociated using 0.25% trypsin and 1 mM EDTA (Gibco BRL, Grand Island, NY) in PBS (Gibco BRL) for 12 hr at 4°C and then for 30 min at 37°C. The digested cells and tissues were seeded into 75-cm^3 cell culture flasks containing Dulbecco's modified Eagle medium/F-12 (DMEM/F-12; Gibco) plus 10% fetal calf serum (HyClone, Logan, UT) and cultured in a 5% CO_2 incubator at 37°C. After reaching 70%–80% confluency, monolayers of the primary cells with spindle-shaped morphology were disaggregated for further culture. Part of cells at passage 1–3 were frozen and stored in liquid nitrogen for long-term use. Fibroblasts at passage generation 3–8 were used as donors.

Oocyte Collection and In Vitro Maturation

Goat ovaries were transported from a slaughterhouse to the laboratory in saline at 30–35°C. Ovaries were washed in Dulbecco's phosphate-buffered saline (DPBS) containing 4 mg/ml bovine serum albumin (BSA, Sigma, St. Louis, MO) before collection of oocytes. Cumulus-oocyte complexes (COCs) were collected by slicing the surface of ovaries with a blade. The COCs were located and evaluated by using a dissecting microscope. Only COCs surrounded by a minimum of four compact layers of cumulus cells and with homogeneous cytoplasm were considered potentially viable and harvested for maturation.

The maturation medium used was TCM 199 (M199, bicarbonate-buffered Earle's, Gibco, Grand Island, NY) supplemented with 10% (v/v) fetal bovine serum (FBS.), FSH 0.5 μg/ml, LH 5.0 μg/ml (Institute of Zoology, Chinese Academy of Sciences, China) and estradiol-17β 1.0 μg/ml (Sigma). The COCs were washed three times in DPBS and four times in maturation medium and then cultured in pre-incubated 100 μl droplets of maturation medium covered with mineral oil (Sigma). The culture environment was 5% CO_2, and 95% humidified air at 39°C (Zhang et al. 2004).

Oocyte Enucleation

At 24–26 hr after IVM, most goat oocytes (Guo et al., 2002) were spontaneously activated and partially extruded the second polar body (TII stage, Fig. 1A,B).

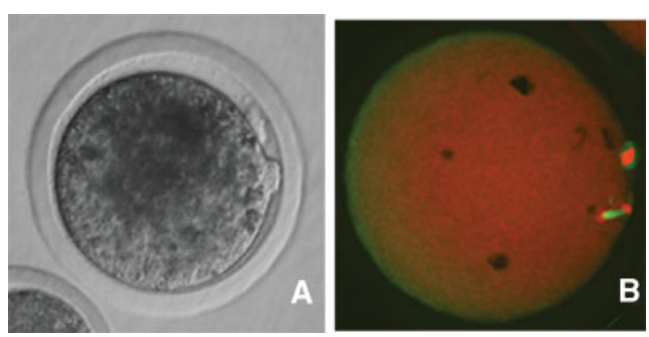

Fig. 1. Spontaneously activated goat oocytes with the first polar body and the partially extruded second polar body after 24 hr of in vitro maturation. **A**: Spontaneously activated goat oocyte under light microscope. **B**: Confocal image of spontaneously activated goat oocyte. [See color version online at www.interscience.wiley.com.]

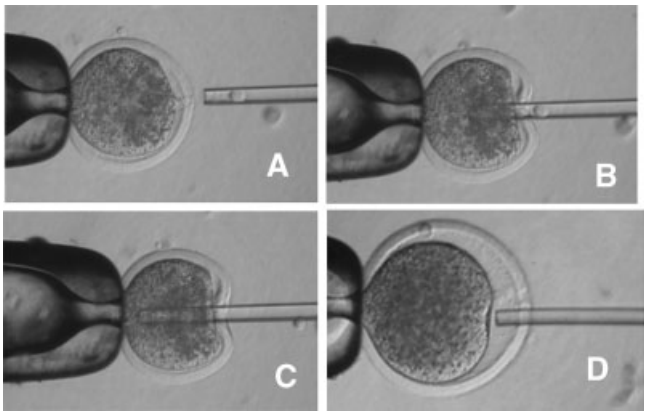

Fig. 2. Whole cell intracytoplasmic injection using piezo-driven manipulator. **A**: The injection needle containing a whole Asian yellow goat somatic cell **B** and **C**. Pushing the whole somatic cell into an enucleated goat oocyte D. Drawing the injection needle out of oocyte.

Activated oocytes were selected to be enucleated. Approximately 10% of the cytoplasm containing the first and second polar body was removed (Bordignon and Smith, 1998).

Whole Somatic Cell Microinjection

Donor cell injection was conducted in the same medium used for oocyte enucleation. Two microinjection protocols were compared. In the SUZI groups, one donor cell was placed between the zona pellucida and the oocyte plasma membrane using the same glass pipette for enucleation. Recipient oocyte-donor cell couplets were equilibrated in fusion medium (0.2 M mannitol, 0.05 mM $MgSO_4$, 0.01 mM HEPES, 0.01 mg/ml BSA) for 5–10 min and then transferred into a fusion chamber containing 100 μl of fusion medium. The couplets were manually aligned so that the contacting membrane of the cytoplast and donor cell was parallel to the electrodes. The couplets were electrically fused with an ECM2001 Elaectrocell Manipulator (BTX, San Diego, CA). Couplets were then washed and incubated in mCR1aa medium for 30 min at 39°C in a humidified air containing 5% CO_2. In the WCICI groups, using pizeo-actuated manipulator with a 10–12 μm glass pipette with a blunt mouth, a whole cell was directly injected into the cytoplasm of an enucleated oocyte (Fig. 2).

Activation and In Vitro Culture of Nuclear Transfer Embryos

Reconstructed embryos were chemically activated by incubation for 5 min in mCR1aa containing 5 μM ionomycin at room temperature, and then incubation for 4 hr in mCR1aa containing 2 mM 6-dimethylaminopurine at 38°C under 5% CO_2. Thereafter, the nuclear transfer embryos were cultured in groups of around 20 embryos in 100 μl droplets of mCR1aa, and 48 hr later, 2∼4-cell embryos were cultured in mCR1aa plus 5% FBS and seeded with mouse fetal fibroblast cells. The droplets were overlaid with mineral oil and incubated at 38°C under 5% CO_2 in air. Half of the medium was renewed every 48 hr. Development from 2-cell stage to morula and blastocyst was evaluated between day 2 and 8.

Embryo Transfer and Microsatellite Analyses

To produce cloned goats, reconstructed embryos were surgically transferred into the oviducts of synchronized foster mothers 48 hr after activation. Genomic DNA was extracted from the blood and tissue collected from the nuclear transfer-derived kids and recipient goats and 10 microsatellite markers (Table 1) were analyzed using methods described elsewhere (Keefer et al., 2001, 2002).

TABLE 1. Ten Microsatellite Markers and Their Primers of Target Sequences

Microsatellite loci	Sequence (5′–3′)	Length (bp)
INRA063	P1 ATTTG CACAA GCTAA ATCTA ACCA	24
	P2 AAACC ACAGA ATGCT TGGAA GAAA	24
INRA023	P1 TAACT ACAGG GTGTT AGATA AACT	24
	P2GAGTA GAGCT ACAAG ATAAA CTTCC	25
INRA011	P1 CGAGT TTCTT TCCTC GTGGT AGGC	24
	P2 GCTCG GCACA TCTTC CTTAG CAACT	25
ILSTS008	P1 TAGCA GTGAG TNAGG TTGG	19
	P2 GAATC ATGGA TTTTCT GGGG	19
LSCV084	P1 CTGGT GGACT ATAGT TCATG	20
	P2 CAGTC ACTCT CACTT GAAAC	20
BM25	P1 GGACA CGTTC TGCAG ATACA ACTAC	25
	P2 GAACT CTCCT TAAGC ATACT TGCTC	25
MM12	P1 CAAGA CAGGT GTTTC AATCT	20
	P2 ATCGA CTCTG GGGAT GATGT	20
SR-SCRP01	P1 TGCAA GAAGT TTTTC CAGAG C	21
	P2 ACCCT GGTTT CACAA AAGG	19
SR-SCRP09	P1 AGAGG ATCTG GAAAT GGAAT C	21
	P2 GCACT CTTTT CAGCC CTAAT G	21
SR-SCRP12	P1 TGACC AGGTG ACTAA CAC	18
	P2 AATCT GATTT CATTT CATG	18

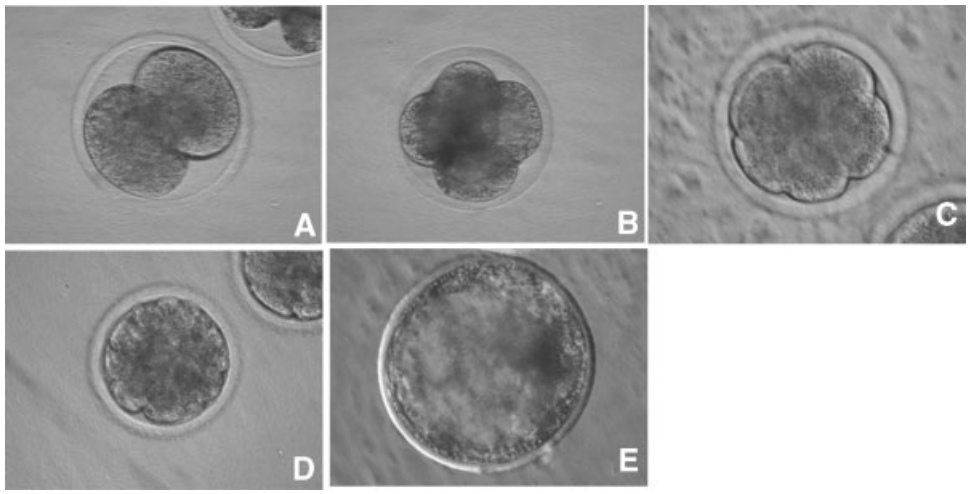

Fig. 3. In vitro development of reconstructed Asian yellow goat embryo by WCICI. **A**: 2-cell reconstructed embryo. **B**: 4-cell reconstructed embryo. **C**: 8-cell reconstructed embryo. **D**: morula. **E**: Blastocyst (magnification 200×).

The resulting microsatellite alleles for the nuclear transfer-derived offspring were compared with those from Asian yellow goat donor cell and contrasted with those from the recipient goats that carried the respective pregnancies.

Statistical Analysis

Reconstruction, cleavage, and development rates were compared by χ^2 analysis. Differences at $P < 0.05$ were considered significant.

RESULTS

In Vitro Development of Asian Yellow Goat Embryos Reconstructed by Suzi and WCICI

Asian yellow goat fibroblast cells were transferred into TII enucleated goat oocytes by SUZI and WCICI, respectively. After in vitro culture in mCR1aa culture medium, the development of Asian yellow goat embryos are shown in Figure 3. Improved development was observed in WCICI group compared with SUZI group. However, there was no significantly statistical difference ($P > 0.05$) in the development rate from the 2-cell to the blastocyst stage between the two groups (Table 2). However, the successful embryo reconstruction rate in WCICI group was significantly higher than that in SUZI group (90.5% vs. 50.0%, $P < 0.05$).

Offspring Birth of Cloned Asian Yellow Goat

A total of 22 similar recipient does received 12–20 embryos/female. The pregnancy rate (defined as the number of pregnant recipients per total number of recipients) at 60 days of gestation was 25% (3/12) and 30% (3/10) for the SUZI group and WCICI group, respectively. As shown in Table 3, 207 and 172 embryos in SUZI and WCICI group were transferred to 12 and 10 recipients, respectively. At 90 days of gestation, one foster aborted in WCICI group. Finally, five recipients gave birth to five viable offspring and one dead neonatal fetus. The overall pregnancy rate was 27.3% (6 of 22 fosters); the percentage of viable cloned kids was 83.3% (5 of 6 cloned kids). The phenotype of all cloned offspring was completely uniform and was significantly different from the foster (Fig. 4). Microsatellite DNA analyses examining 10 loci further confirmed that all the cloned kids were genetically identical to the donor yellow goat and different from the recipient goats (Table 4).

DISCUSSION

In previous studies of embryonic nuclear transfer in cattle, telophase II oocytes were reportedly enucleated effectively with minimal loss of cytoplasm and without exposure to UV light or DNA stain for localization (Bordignon and Smith, 1998; Mohamed Nour and

TABLE 2. In Vitro Development of Asian Yellow Goat Embryos Reconstructed by SUZI and WCICI

	Number of NT oocytes	Fused/injected n (%)	2-cell n (%)	Morula n (%)	Blastocyst n (%)
SUZI	84	42 (50.0)[a]	24 (57.1)[a]	10 (23.8)[a]	3 (7.1)[a]
WCICI	63	57 (90.5)[b]	35 (61.4)[a]	21 (36.8)[a]	5 (8.8)[a]

Values in the same column with different superscripts (a and b) are significantly different ($P < 0.05$).

TABLE 3. Results of Cloned Asian Yellow Goat Offspring Using SUZI and WCICI

	Number of embryos transferred	Transferred foster	Pregnant foster n (%)	Abortive fosters	Viable kids n (%)	Neonatal death	Total pregnant rates n (%)	Total viable kids after birth n (%)
SUZI	207	12	3 (25.0)[a]	—	3 (25.0)[a]	1	6 (27.3)	5 (83.3)
WCICI	172	10	3 (30.0)[a]	1	2 (20.0)[a]	—		

Values within a column with same superscript (a) are not significantly different ($P > 0.05$).

Takahashi, 1999; Liu et al., 2000). In goats, it has been also indicated that the use of an activated telophase II cytoplast may have several practical and biological advantages, for example, easier manipulation and more synchronous recipient cytoplasts (Baguisi et al., 1999; Baguisi and Overström, 2000). Our study confirmed these findings and further demonstrated that goat oocytes enucleated at TII stage, and injected with whole donor somatic cells, had a slight higher rate of embryonic development in vitro than those enucleated at MII stage. Less cytoplasm is removed in TII enucleatlon than in MII enucleation and subsequently there is less disturbance of the micro-environment of the recipient oocyte for coordination with the donor cells.

Fig. 4. Representative cloned Asian yellow goat offspring and their fosters.

Many studies on nuclear transfer have suggested that the activity of maturation M-phase promoting factor (MPF) in recipient oocytes appears important for the reprogramming of donor nuclei. When enucleated MII oocytes are used as recipient cytoplasts, a series of morphological changes are observed in the donor nucleus, including the induction of NEBD and PCC followed by nuclear reformation (Cibelli et al., 1998). Previous studies have suggested that the induction of NEBD and PCC is essential for the reprogramming of gene expression, and that it increases the developmental potential of the reconstructed embryos. In mice cloning, prolonged exposure of transferred nucleus to a cytoplasm rich in MPF showed a relatively higher rate of development (Wakayama et al., 1998). Wells et al. (1999) demonstrated that exposure of the somatic nucleus to enucleated MII ooplasm for 4–6 hr before activation resulted in an increased proportion of fused embryos developing to blastocysts. However, there are also many conflicting observations. Many reports have suggested that MPF is not necessary for nuclear reprogramming. Cloned goats and mice (Baguisi et al., 1999; Gasparrini et al., 2003) have been produced from preactivated or simultaneously activated oocytes in which MPF activity is rather low, even disappears. Cloned cattle using immediate activated oocyte as recipients has also been reported (Akagi et al., 2003).

Lee et al. (2003) reported the production of cloned pigs by whole-cell intracytoplasmic injection (WCICI). They suggested that the method was a simple and efficient NT procedure bypassing electro-fusion of SUZI and the donor nuclei isolation of ICI. Our previous study (Jiang et al., 2004) also used WCICI to reconstruct giant panda embryos reprogrammed by chemically enucleated rabbit oocytes. More than 17.2% of cloned embryos developed to blastocyst stage and the microtubule and nuclear structures of the interspecies cloned giant panda embryos were normal when observed by confocal microscopy.

In the present study, we combined WCICI with TII enucleation in Asian yellow goat cloning. Our results showed that TII enucleation combined WCICI can be used to simplify NT manipulation and to improve the early development of cloned embryos. Furthermore, this study validated the feasibility and effect of this method on the postimplantation development of cloned embryos. Both phenotype observation and microsatellite DNA analysis showed that the cloned kids were genetically identical to the donor Asian yellow goat.

In conclusion, the results of this study show that the method of telophase enucleation combined with WCICI

TABLE 4. Ten Microsatellite Loci Analysis of Tissue Samples From Cloned Asian Yellow Goats and Their Fosters

Samples	Microsatellite loci																			
	INRA023		INRA063		INRA011		ILST008		LSCV084		BM25		MM12		SR-CSRP01		SR-CSRP09		SR-CSRP12	
Donor cell	197	205	160	160	215	215	176	176	144	144	204	204	93	93	136	144	125	125	100	112
Abortive fetus	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Neonatal death (twin)	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Offspring 1 (twin)	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Offspring 2 (single)	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Offspring 3 (single)	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Offspring 4 (single)	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Offspring 5 (single)	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Foster (No. 256)	199	213	162	166	217	—	—	182	142	—	211	211	113	115	140	146	131	135	98	110

"|", denotes the consistency of allele loci between donor cell and offspring; italic Arabic numerals denote the discrepancy between foster and all cloned offspring.

can lead to not only normal in vitro development of the reconstructed embryos but also viable kids in yellow goat somatic nuclear transfer. The telophase II ooplasm can properly reprogram the somatic cells and whole cell intracytoplasmic injection can provide an efficient and less labor-intensive protocol in Asian yellow goat cloning.

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