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# Somatic cell bovine cloning: Effect of donor cell and recipients

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Abstr act **Adult somatic cell nuclear transfer was conducted by using cultured ear fibroblast cells obtained from a Holstein female cow (GN) and a Galoway herd bull (GLV). The percentages of reconstructed eggs developed into blastocysts were similar in GN (23.98%, 123 of 513) and in GLV groups (29.55%, 138 of 467). However, the rate of reconstructed female (GN) embryos developed into term was higher than that of male (GLV) (8.02% and 1.82%, respectively). Three kinds of cows, Luxi Yellow cows, Holstein heifers and Holstein cows with normal reproductive records were used as recipients. When the reconstructed embryos from GN were transferred, there was no difference in the pregnancy rate among three kinds of recipients, but the abortion rate of Luxi Yellow cows was significantly higher (85.71%) than in the other two groups (14.29% and 0%, respectively; $P < 0.05$). And the percentages of newborn calves in transferred embryos were significantly different between Luxi Yellow cows and Holstein breed (1.54%, 10.39% and 20.0%, respectively, $P < 0.05$). However, when reconstructed embryos from GLV were transferred, there was no difference among three kinds of recipients in the pregnancy rate, the abortion rate and the delivery rate.**

**Keywords: bovine, somatic cell, nuclear transfer, reconstructed embryo, clone.**

Nuclear transfer (NT) is an efficient technique for assessing the developmental potential of a nucleus and for analyzing the interactions between the donor nucleus and the recipient cytoplasm. In amphibians, thought nuclei of adult kerationocytes support development to the juvenile, tadpole stage, no development to the adult stage was reported[1], leaving open the question of whether a differenttiated adult nucleus can be fully reprogrammed. The first cloned offspring developed from differentiated cells were born after NT from an embryo-derived cell line[2]. Using the same procedure, the live lamb from a cell of adult mammary gland was reported in 1997[3], suggesting that the differentiated somatic cells can dedifferentiate and support development into term. Until now, successful somatic cell cloning has been achieved in mouse[4], goat[5], cattle[6], pig[7], cat[8] and rabbit[9] using adult or fetal cells. The potential applications of cloning in producing copies of elite livestock, transgenic animals, and the propagation of rare animals clearly have great commercial and conservational benefit. An important objective of the present study was to demonstrate the feasibility of adult somatic cell NT in producing copies of highly productive livestock, and the other aim of this study was to evaluate the efficiency of different gender cells and different recipient cows in bovine NT.

## 1 Materials and methods

(ⅰ) Donor cell. The donor cells were from the ears of two elite individuals. One is No. 18 Galoway stock bull (GLV). The other is the highly productive No. 603 Holstein cow (GN). The ear skin was cut into pieces and digested with 0.25% trypsin for 2 h at 4℃ and then for 30 min at 37℃. The digested cells were cultured and passaged in Dulbecco's Modified Eagle Medium : Nutrient Mixture F-12 (Ham) = 1∶1 (D-MEM/F-12, GIBCO BRL, Life Technologies) supplemented with 20% FBS (GIBCO) according to previous report[10].

(ⅱ) *In vitro* maturation of oocytes. Slaughterhouse ovaries were collected from mature Luxi Yellow cows, placed in saline plus 1% penicillin/streptomycin (30℃), and transported to the laboratory within 4 h. Cumulus-oocyte complexes (COCs) were recovered by aspiration of 2—10 mm follicles using an 18-gauge needle. COCs were collected into DPBS. The COCs surrounded by at least three layers of cumulus cells were selected for *in vitro* maturation (IVM). They were washed twice in medium 199+10% FBS before being washed once in maturation medium. Ten COCs were cultured in a 100 μL drop of maturation medium in a 10-cm Petri dish overlaid with paraffin oil. The maturation medium comprised tissue culture medium 199 supplemented with 10% FBS, 0.5 μg/mL FSH, 0.01IU/mL LH, 1.0 μg/mL estradiol. Microdrop dishes were cultured at 38.5℃ in a humidified 5% $CO_2$ in air atmosphere for 19 h. After maturation culture, the COCs were placed in DPBS supplemented with 0.5% hyaluronidase for 3—5 min and cumulus cells were removed by repeated gentle pipetting through a fine bore pipette. Oocytes with the first polar body were selected for NT.

(ⅲ) Prepartion of Donor Cells. Donor cells passed for 1—5 generations were used for NT. The cells were induced to enter G0 stage by culturing in the medium containing only 0.5% FBS for 2—7 days. To obtain donor cells from the adherent cell layer, the fibroblasts from ears were digested with 0.25% trypsin at 39℃ for 2—3

min and neutralized by D-MEM/F-12 once the majority of the cells withdrew their pseudopodia and became round in shape.

(iv) Nuclear transfer. The NT was conducted as previously reported[11]. Briefly, oocytes were preincubated for 15 min in M2 medium with 7.5 μg/mL cytochalasin B (CB, Sigma Co.). The oocyte which just extruded the first polar body was hold by a holding pipette under an inverted microscope. The injector pipette pushed the zona pellucida and the first polar body with approximately the adjacent cytoplasm containing metaphase Ⅱ spindle was expelled. After injection, the couplets were immediately placed into the electrical fusion solution (EFS) comprising 0.28 mol/L mannitol, 0.5 mmol/L Hepes, 0.05 mmol/L calcium and 0.1 mmol/L magnesium for 1—2 min. Then they were transferred into a fusion chamber overlaid with EFS. Cell fusion was induced with two direct current (DC) pulses (2.0 kv/cm, 10 μs each, 1 s apart) and examined after the couplets were cultured in M199 with 10% FBS for 0.5 h. Unfused oocytes were treated with electrical pulses an hour later.

(v) Chemical activation. All fused oocytes were activated 24 h after maturation. Oocyte activation was induced by incubation in 5 μmol/L A23187 in CR1-aa (NaCl 114.7 mmol/L, KCl 3.1 mmol/L, $NaHCO_3$ 26.2 mmol/L, L-Glutamine 1.0 mmol/L, Sodium pyrurate 0.4 mmol/L Hemicalcium lactate 5.0 mmol/L BSA 3 mg/mL Phenol Red 10 μg/mL, Essential amono acids 2%, Non-Essential amono acids 1%) for 4 min at 39℃. Oocytes were then extensively washed in CR1-aa for 5 min before culture for 1 h in 10 μg/mL cycloheximide + 3μg/mL cytochalasin D (CD) in CR1-aa supplemented with 10% FBS, and then cultured in the same medium without CD for 4 h.

(vi) *In vitro* culture of NT embryos. Embryos culture was performed in 100-μL drops of CR1-aa supplemented with 10% FBS overlaid with paraffin oil. Embryos were cultured in a humidified modular incubator chamber at 39℃ in 5% $CO_2$ in air for 48 h. From day 3 (day 1: day of NT), the embryos were co-cultured with mouse fetal fibroblast cells in the same medium, which was changed half every 48 h. On day 6, glucose was added to the medium at a final concentration of 1 mg/mL without changing the medium. On days 7, 8 and 9 of *in vitro* culture, development into morulae and blastocysts was recorded. Difference between experimental groups was verified using chi-square analysis.

(vii) Embryo transfer. One to three morphologically normal blastocysts were transferred into naturally cycling recipient cows 7 days after estrus. A total of 230 cloned embryos were transferred into 112 recipient cows. Sixty of these embryos were derived from GLV and 170 from GN. We used three kinds of recipient cows, Luxi Yellow cows, Holstein heifers, and Holstein cows with normal reproductive record.

(viii) Microsatellite DNA analysis. DNA samples were compared using microsatellite DNA analysis to determine whether the cloned calves have the same genotype as the donor. Genomic DNA samples were extracted from the donor fibroblasts from a cow (GN) and a bull (GLV), the ear skin of 5 alive cloned calves, the muscle of 9 dead cloned calves and the ear skin of 11 recipients by 10 mmol/L Tris (pH 8.0), 100 mmol/L NaCl, 1 mmol/L EDTA, 1% SDS and 0.5 mg/mL protein K. The multiplex set contains 12 different PCR primer pairs (ETH3, ETH225, BM1824, BM2113, TGLA126, TGLA53, ETH10, MGTG4B, TGLA227, TGLA122, INRA23, SPS113) which were labeled with FAM or HEX and amplified in three PCR reactions. The denatured PCR products were loaded into ABI 310 Genetic for electrophoretic separation (Applied Biosystems).

## 2 Results

(i) Developmental potential of NT Embryos *in vitro*. A total of 5278 COCs were collected for IVM. At 19 h, 2897 (54.89%) were judged as matured by the presence of the first polar body in the pervitelline space. A total of 2016 of MII oocytes were used for NT. The results of NT and embryos development are shown in Table 1. The fusion of somatic cells of GLV to cytoplasts was not different with the fusion of somatic cells of GN. Electro-fusion with somatic cells was not affected by either the length of serum starvation (2—7 days) or cell passage number (1—5). There was no difference in the blastocyst development between fused embryos derived from GLV and those from GN.

(ii) Developmental potential of NT embryos in different recipients. A total of 55 blastocysts from GLV were transferred to 26 recipients. Six recipients were pregnant at day 60 (23.08%, Table 2), 5 of which aborted and 1 delivered a cloned calf (Fig. 1(d)). The rate of reconstructed male embryos developing into term was 1.82%. There was no difference among three kinds of recipients in the pregnancy rate, the abortion rate and the delivery rate when reconstructed embryos from GLV were transferred. A total of 162 blastocysts from GN were transferred to 80 recipients, and 17 (21.25%) were pregnant at day 60. Then 7 recipients aborted (41.18%) and 10 delivered 13 calves (Fig. 1(b), (c)) from GN, which was 8.02% on the basis of the number of embryos. When the reconstructed embryos from GN were transferred, there was no difference among three kinds of recipients in their pregnancy rates, but the abortion rate of Luxi Yellow cows was significantly higher (85.71%) than that of the other two groups (14.29%, 0%; $P < 0.05$). On the basis of the number of embryos transferred to recipient cows, the percentages of newborn calves were significantly different between Luxi Yellow cows and Holstein group

Table 1 Development in vitro of cloned embryos from donor cells of GLV and GN

| Type of donor cells | No. NT | No. fused | No. cultured embryos | No. 2-cell embryos (%) | No. 4—8 cell embryos (%) | No. blastocyst (%) |
|---|---|---|---|---|---|---|
| GLV | 982 | 548 | 467 | 365 (78.16%) | 322 (68.95%) | 138 (29.55%) |
| GN | 1034 | 623 | 513 | 424 (82.65%) | 349 (68.03%) | 123 (23.98%) |
| Total | 2016 | 1171 | 980 | 789 (80.5%) | 671 (68.47%) | 261 (26.63%) |

Table 2 Development of different reconstructed embryos in different recipients

| Type of embryos | Type of recipients | No. of embryos | No. of recipients | No. (%) of pregnant[A] | No. (%) of aborted[B] | No. (%) of calves[C] |
|---|---|---|---|---|---|---|
| GLV | Yellow cow | 29 | 14 | 4 (28.57)[a] | 3 (75.0)[a] | 1 (3.45)[a] |
| | Holstein heifer | 20 | 9 | 1 (11.1)[a] | 1 (100.0)[a] | 0 (0)[a] |
| | Holstein cow | 6 | 3 | 1 (33.33)[a] | 1 (100.0)[a] | 0 (0)[a] |
| | | 55 | 26 | 6 (23.08) | 5 (83.33) | 1 (1.82) |
| GN | Yellow cow | 65 | 29 | 7 (24.14)[a] | 6 (85.71)[a] | 1 (1.54)[a] |
| | Holstein heifer | 77 | 40 | 7 (17.5)[a] | 1 (14.29)[b] | 8 (10.39)[b] |
| | Holstein cow | 20 | 11 | 3 (27.27)[a] | 0 (0)[b] | 4 (20.0)[b] |
| | | 162 | 80 | 17 (21.25) | 7 (41.18) | 13 (8.02) |
| GLV+GN | Yellow cow | 6 | 3 | 3 (100.0)[a] | 2 (66.67) | 0*(0) |
| | Holstein heifer | 5 | 2 | 0 (0)[b] | 0 (0) | 0 (0) |
| | Holstein cow | 2 | 1 | 0 (0)[b] | 0 (0) | 0 (0) |
| | | 13 | 6 | 3 (50.0) | 2 (66.67) | 0 (0) |
| Total | | 230 | 112 | 26 (23.21) | 14 (53.85) | 14 (6.09) |

A, Pregnant detected by rectal palpation day 60 after embryo transfer; B, percentage based on the number of pregnant cows; C, percentage based on the number of embryos; * mummy; a, b, c: Within the same column, values with different superscripts were significantly different ($P < 0.05$).

(1.54%, 10.39% and 20.0%, $P < 0.05$). When 13 blastocysts from both GLV and GN were transferred to 6 recipients, 3 (50%) of them were pregnant, but 2 aborted and the other one delivered a mummy.

Fourteen calves were delivered from 12 recipients by natural parturition, assisted parturition or cesarean section between days 276 and 295. One was from the donor cell of GLV (male), and the others were from GN (female). Nine of them died shortly before or after birth, and five are still alive.

(iii) Analysis of Cloned Calves. Except one Yellow cow recipient delivered a mummy, total 14 clones were born from the other 11 recipients. Among the 14 clones, one was from GLV and died of acute gastro-bleeding one month after birth. The other 13 were from GN, 8 of which died owing to rupture of fetal membrane (one twin claves), abnormal position of fetus (one twin clones), underdevelopment (died 1.5 h after birth), improper assistance during parturition, acute pneumonia (after 3 days), intestine twist (after 7 days) shortly before or after birth. The five surviving clones including twins are now 9 months old.

Except two clones were delivered by cesarean section from two Yellow cow recipients before the expected birth date, the gestation periods of the others were 5—9 days longer than the average gestation period of the breed (286 days). The average birth weights of 7 clones from GN (except three twin clones) were $54.14 \pm 5.65$ kg, heavier than the average birth weight of female calves of this breed ($43.46 \pm 3.13$ kg).

(iv) Phenotype of cloned calves. The phenotype of all clones from GN are similar to the donor bovine, especially in the visual coat color pattern on the head and the neck (Fig. 1(a), (b)). However, there was obvious difference in coat color markings on the body.

(v) Microsatellite DNA analysis of cloned calves. Microsatellite DNA analysis confirmed that 13 cloned female calves originated from the donor fibroblast of GN and one cloned male calf originated from the donor fibroblast of GLV, and there was no genetic relationship between clones and recipients.

## 3 Discussion

We have proved that the cultured fibroblasts from bovine ears can be reprogrammed in enucleated MII oocytes and result in the production of viable cloned claves. Many types of somatic cells[12-15] were used as donor cells to produce clone claves. However, the rates of bovine cloning are different due to many factors, one of which is the different cell types. It has not been determined which cell type from the adult is most efficient for somatic cell NT. Here, we used the fibroblasts from bovine ear of male and female individuals. Though cloned animals were produced from cells synchronized at G0 stage or unsychronized, the blastocyst rate of bovine cloning from unsynchronized fetal[16] cells is only 10%—12% while the rate of cloned blastocyst from both quies

Fig. 1. (a) The donor Holstein 603 cow; (b) seven dead cloned calves from GN; (c) five surviving cloned calves from GN; (d) the cloned calf from GLV.

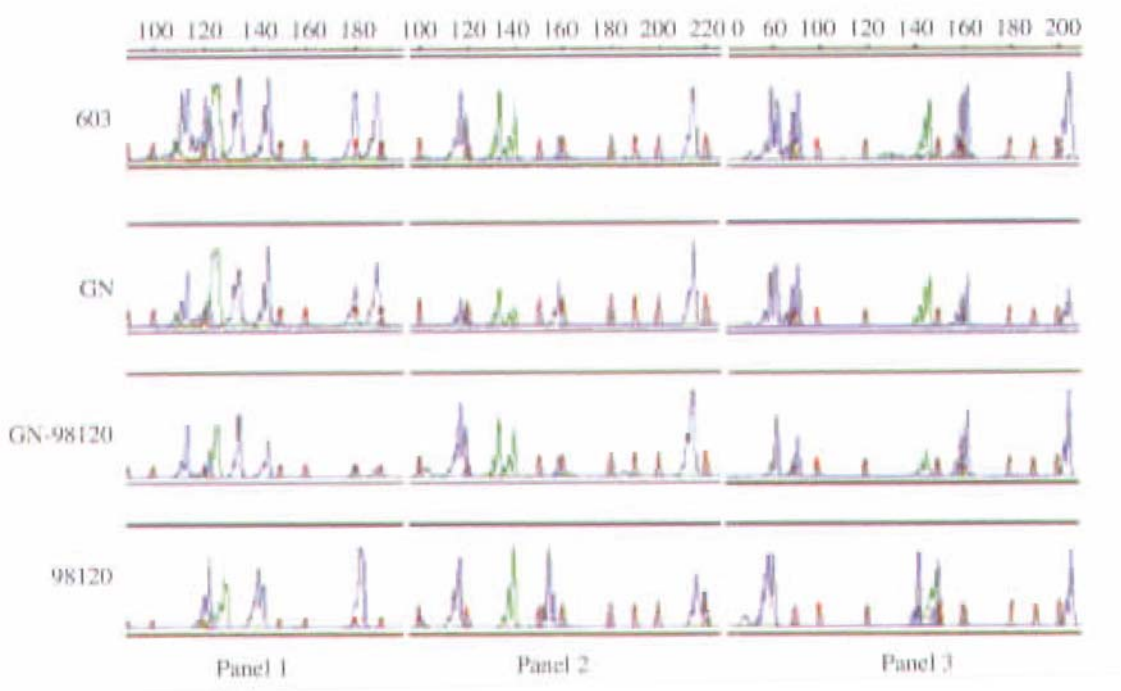


Fig. 2. Microsatellite DNA analyses of one of the cloned calf (GN-98120); donor cow tissue (603); donor cell line (GN); recipient mother (98120). Red curve is standard molecular weight; blue (B) and green (G) are amplified products. Panel 1: Primers ETH3 (B), BM2113 (G), ETH225 (B), BM1824 (B); Panel 2: Primers TGLA126 (B), MGTG4B (G), TGLA53 (B), ETH10 (B); Panel 3: Primers TGLA227 (B), SPS113 (G), TGLA122 (B), INRA23 (B). The same result was obtained in the other cloned calves.

cent adult[12] and fetal[16] cells were 51%—52%. Here, the blastocyst rate was 26.63% when adult G0 cells were used.

Though there was no difference in the blastocyst rate and the pregnancy rate between GLV and GN reconstructed embryos, the rate of reconstructed female embryos developing into term was higher than that of male embryos (8.02% and 1.82%). As we know, some types of somatic cells from female and male have been successfully used in nuclear transfer. However, there is not any report about the cloned efficiency of different gender. Here we used the same kind of somatic cell from female (GN) and male (GLV) in the same experiment. The result indicated that the early development of the reconstructed embryos from female's and male's somatic cells was similar, and the rate of reconstructed embryos developing into term was significantly higher when female cells were used as donors. This is comparable to the latest report about the cloned cat[8], in which, 84 cloned embryos from fibroblast cells of an adult male cat's oral mucosa resulted in one failed pregnancy; however, 3 cloned embryos derived from cumulus cells led to one live clone.

As we know, the location of the spindle relative to the polar body varies among oocytes, which may reduce the efficiency of current enucleation methods using the polar body as an indicator. In order to improve the efficiency of enucleation, we reduced the period of IVM from 22—24 h to 19 h in the present study. After IVM for 19 h, most of the matured oocytes just extruded the first polar body, which could be used as an enucleation indicator. To our knowledge, this is the new enucleated method we put forward for the first time in this study.

Oocyte activation is one of the key steps in NT. Wells et al.[12] reported that negliable proportion of reconstructed embryos would have been activated (around 1%) by the electrical stimulation employed to achieve fusion at 22— 24 hpm. In addition, Wells et al. demonstrated that embryo development is significantly increased by fusing quiescent donor cells with MII cytoplasts before activation (fusion before activation treatment, FBA), in comparison to simultaneous fusion and activation at either 24 or 30 hpm with bovine fetal fibroblasts[16] or adult mural granulose cells[18]. It is suggested that the prolonged exposure of transferred nuclei to oocyte cytoplasmic factors possibly facilitates nuclear remodeling and reprogramming. Here, reconstructed embryos were fused at 21—22 hpm, and activated at 24 hpm. There were 2—3 h for somatic nucleus exposing to MII cytoplasm, which could be beneficial to the development of embryos. The blastocyst rate was 26.63%, which was higher than some other reports[6,17].

We used three kinds of recipients, Luxi Yellow cow, Holstein heifers and Holstein cows with normal reproductive record. When reconstructed embryos from GLV were transferred, there was no difference among three kinds of recipients in the pregnant rate, the abortion rate and the delivery rate. Though there was no difference in their pregnancy rates among three kinds of recipients when the reconstructed embryos from GN were transferred, the abortion rate of Luxi Yellow cows was significantly higher (85.71%) than that of the other two groups (14.29%, 0%; $P < 0.05$). On the basis of the number of transferred embryos, the percentages of newborn calves were significantly different between Luxi Yellow cows and Holstein groups (1.54%, 10.39% and 20.0%, $P < 0.05$). Collectively, these data suggest that Luxi Yellow cows are not suitable to be used as recipient cows, and the Holstein heifers or Holstein cows with normal reproductive record are ideal recipients in bovine NT. To our knowledge, this is the first report about the efficiency using different recipients in bovine NT.

To date, the overall cloning efficiency using somatic cells is low, ranging from 0 to nearly 10%[14]. This could be explained by the high abortion rate and the high neonatal mortality. Here, the abortion rate was 53.84% and the neonatal mortality was 64.3%, which are consistent with previous reports[6,13,14,18]. The exact mechanisms of early or late embryonic loss and neonatal death of clones are still not clear. Recent reports have shown that fetal loss is associated with placental abnormalities[19,20]. It is possible that the excessive accumulation of allantoic fluid and increased fetal or birth weight (i.e. large offspring syndrome) are key factors leading to the abortion or death of the clones[12,19]. In this study, twin claves died in uterus shortly before parturition due to the rupture of foetal membrane, and another twin claves aborted because of excessive accumulation of allantoic fluid. The average birth weight of the clones from GN was 25% heavier than that of normal female calves of this breed. In this study, we do not expel the possibilities that some clones may be died of improper nursing except some unclear mechanism which exist in the methods of cloning. Our results, together with previous reports in bovine, sheep[3,21] and mouse[22] cloning, demonstrate that many problems still exist in nuclear transfer, such as high abortion rate, high neonatal death rate and large offspring syndrome. However, in cloned goats[23—25], unlike in cloned bovine, mouse and sheep, prenatal losses and high birth weights are not observed.

Microsatellite DNA analysis has been used for detecting the genotype of clones[7,14,26]. While microsatellite DNA analysis showed that clones were genetically identical to the donor cows and donor cells, there was difference in the black and white coat color patterns on bodies among the cloned calves and donor 603 cow. The difference is also observed in other reports[12,18]. These differences presumably reflect the environmental influences in utero affecting the multiplication and migration of melanoblasts in the developing fetal skin[27].

In conclusion, the present findings suggest female cloned bovines are easier to generate than males and different kinds of recipient cows are one of the important factors influencing the efficiency of reconstructed embryos developed into term in bovine NT. Here, we found that Holstein heifer or Holstein cows with normal reproductive record are ideal recipient and Luxi Yellow cows are not suitable to be used as recipient in bovine NT.

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