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Somatic Cell Nuclear Transfer Efficiency Associated with Donor Cell Types in the Cashmere Goat

Jianglong Yuan, Yong Jin, Bing Zhu, Wei Wang, Yu Ren, Haiqing Wu, Fei Hao, Mingtu Nuo, Muzi Jin, Zhigang Wang, Ming Chang, Dongjun Liu and Xudong Guo
The Key Laboratory of Mammal Reproductive Biology and Biotechnology, Ministry of Education, College of Life Sciences, Inner Mongolia University, 010021 Huhhot, P.R. China

Abstract: The efficiency of donor cell types in cashmere goat Somatic Cell Nuclear Transfer (SCNT) is undefined. In this study, SCNT was performed using Caprine fetal Fibroblast Cells (CFCs), Caprine Ear Fibroblast Cells (CEFCs) and Transgenic Caprine Fetal Fibroblast Cells (TCFCs) as donor cells to compare the influences of cell type, transgene and sex of nuclear donor fibroblast cells on the SCNT efficiency including fusion and cleavage rates of reconstructed embryos and the birth, postnatal survival and mortality rates of cloned kids. A total of 4,943 reconstructed embryos were obtained. Among them, 3,949 embryos were fused and 3,737 embryos were cultured *in vitro* leading to a total of 3,094 cleavage embryos. Furthermore, embryo transplantation analysis was conducted on 1,873 cloned embryos with relatively normal morphology. A total of 368 recipient goats were transplanted and 48 goats were born of which 35 goats survived.

Key words: Cashmere goat, somatic cell nuclear transfer efficiency, cell type, embryo, China

INTRODUCTION

Since, the cloning of mammals from an adult somatic cell was pioneered in 1996 (1997), the generation of transgenic animals by the combination of Somatic Cell Nuclear Transfer (SCNT) and transgenic technology has become a new means for livestock breeding improvement (Brophy *et al*., 2003; Lai *et al*., 2006; Schnieke *et al*., 1997). Cashmere goat as a major livestock in the west of Inner Mongolia China play a important role in the local agricultural income. Cashmere from the secondary hair follicles of cashmere goat is known as soft gold. So, promoting the yield of cashmere with the transgenic technology is the major purpose.

However, low SCNT efficiency is a major restriction to the development of this approach (Piedrahita and Olby, 2011). Many factors influence SCNT efficiency including somatic cell source, genetic modification and sex with donor cell type being one of the most important factors (Kato and Tsunoda, 2010). Jang *et al*. (2004) showed that there was no difference in the developmental competence of cloned embryos prior to implantation following the use of Bovine Ear Fibroblast Cell (EFCs) or Fetal Fibroblast Cell (FFCs) as nuclear donors although embryo transfer analysis was not conducted. The majority of studies indicate that transgenic SCNT efficiency is lower than non-transgenic (Keefer, 2008; Zakhartchenko *et al*., 2001) and that donor cell sex is a critical factor influencing SCNT efficiency. Chen *et al*. (2003) discovered that the bovine SCNT efficiency of female nuclear donor cells was higher than that of male cells. However, Kato *et al*. (2000) found no significant differences in the SCNT efficiency of male and female cells in cattle. Therefore, the relationship between donor cell type and cloning efficiency varies in different species (Keefer, 2008), although the reason remains to be elucidated.

To date, studies on the relationship of donor cell type with cloning efficiency have focused on model organisms (Sung *et al*., 2006; Wakayama and Yanagimachi, 2001) and investigations in large livestock have been less reported (Kato *et al*., 2000; Powell *et al*., 2004) with no reports of investigations of cashmere goats in particular.

On the basis of earlier established somatic cell nuclear transplantation technology in cashmere goats (Guo *et al*., 2009b), this study used cashmere fibroblasts as an experimental resource to investigate the influences on cloning efficiency of donor cell.

MATERIALS AND METHODS

Reagents: Dulbecco modified Eagle's medium/F12 (DMEM/F12), M199, Dulbecco's Phosphate Buffered saline (D-PBS), G418 and 0.05% trypsin and antibiotics were purchased from Hyclone (Beijing, China). Bovine

Corresponding Author: Dongjun Liu, The Key Laboratory of Mammal Reproductive Biology and Biotechnology, Ministry of Education, College of Life Sciences, Inner Mongolia University, 010021 Huhhot, P.R. China

serum was from TBD (Tianjin, China), inorganic salt and DMSO were from Wako (Oosaka, Japan); Lipofectamine 2000 was from Invitrogen, (Carlsbad, CA, USA); Endotoxin-free plasmid isolation kits were from Qiagen (Valencia, CA, USA), DNA polymerase was from Takara (Dalian, China). All other reagents were purchased from Sigma (St. Louis, MO, USA) except where otherwise stated.

Fibroblast cell isolation and sex determination: Fibroblast cells were isolated from 40 days caprine fetuses and adult ears as described previously (Guo *et al*., 2009a). Sex determination of isolated Caprine fetal Fibroblast Cells (CFCs) and Caprine Ear Fibroblast Cells (CEFCs) was performed by PCR detection of the Sex determining Region Y (*SRY*) gene (Yi *et al*., 2009). All animal experiments were done in accordance with the guidelines on animal care and use established by the Inner Mongolia University Animal Care and Use Committee.

CFC transduction, selection, identification and donor cell preparation: CFCs were transduced with earlier constructed eukaryotic expression vectors (Wang *et al*., 2010) using Lipofectamine™ 2000 according to instructions provided by the manufacturer. Resistant clones (designated TCFC1, TCFC3, TCFC4 and TCFC5) were obtained by G418 selection (Guo *et al*., 2009a). The exogenous gene inserts were identified by PCR as described earlier (Guo *et al*., 2009b). The growth curve and chromosome number were analyzed to verify normal cells for use as donor cells. Donor cells were seeded in 24 well plates (1×10^5/well) and cultured in DMEF/F12 supplemented with 10% FBS at 37°C in 5% CO_2. At 80% confluence, cells were digested by trypsin and harvested by centrifugation at 500×g. The cells were resuspended by DMEM/F12 for use in experiments.

Generation of cloned cashmere goats: Oocytes were matured *in vitro* and nuclear transfer was performed as described earlier (Guo *et al*., 2009b). Reconstructed embryos were cultured for 48 h and the cleavage rate of embryos was calculated. Embryos with suitable morphologies were selected for transplantation into estrus-synchronized recipients. Approximately five embryos were transplanted into each recipient. The pregnancy rate was determined by the absence of estrus after two continuous estrus cycles. Venous blood was drawn from transgenic kids for genomic DNA extraction. Specific primers were designed according to the sequences of exogenous genes to conduct PCR identification (Guo *et al*., 2009a; Wang *et al*., 2010). Ear apex tissues of transgenic somatic cell cloned kids were cultured *in vitro*.

Calculation of cloning efficiency: Rates of fusion, cleavage, pregnancy, birth and postnatal survival among different donor cell type-derived embryos (different individual sources, different fibroblast types, transgenic and non-transgenic, different sexes) and fetal mortality rate were calculated to analyze the influence of cell type on cloning efficiency.

Statistical analysis: All data are expressed as the mean±standard deviation and analyzed with the statistical package SPSS 19 for Windows (SPSS Inc., Chicago, IL, USA). One-way Analysis of Variance (ANOVA) was used to determine statistically significant differences among the groups and χ^2-tests were used for comparison of categorical variables. The $p<0.05$ was considered statistically significant.

RESULTS

Production of transgenic cashmere goat: A total of 4,943 reconstructed embryos were obtained. Among these, 3,949 embryos were fused and 3,737 embryos were cultured *in vitro* resulting in a total of 3,094 cleavage embryos. Morphologies of cloned embryos were relatively normal and a majority of transgenic embryos expressed red fluorescent proteins (Fig. 1). A total of 1,873 cloned embryos were selected on the basis of relatively normal

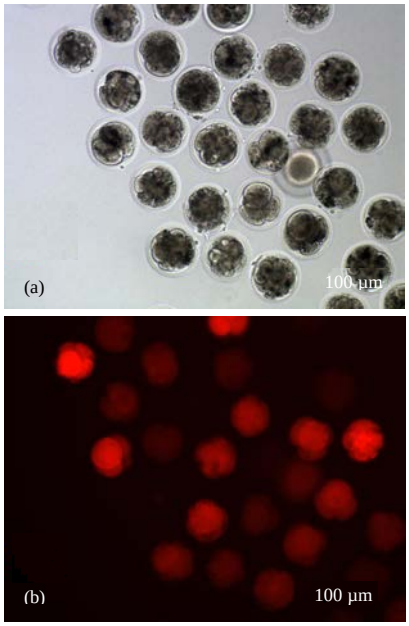

Fig. 1: Transgenic cloned embryos of goats: Transgenic cloned embryos of cashmere goats observed by a) visible light microscopy and b) fluorescence microscopy

Table 1: Influence of cells derived from different individuals on cloning efficiency

Donor cell type	Fusion embryo No. (%)	Cleavage embryo No. (%)	Transplanted embryo No.	Transplanted receptor No.	Pregnancy (%)	Birth No. (%)	Postnatal survival No. (%)
TCFC1 (Male)	729/895 (84.0±2.3)^{z}	654/729 (88.4±4.2)^{zy}	351	68	22 (6.27)	13 (3.70)	11 (3.13)
TCFC5 (Male)	947/1165 (83.2±3.1)^{zy}	647/803 (77.2±3.7)^{yxw}	295	49	13 (4.41)	7 (2.37)	1 (0.34
CFC5 (Male)	171/210 (79.9±3.5)^{zy}	165/171 (96.1±6.5)^{z}	32	8	1 (3.13)	1 (3.13)	1 (3.13)
CEFC1 (Male)	454/650 (70.2±2.5)^{x}	362/395 (92.3±4.6)^{z}	217	39	13 (5.99)	6 (2.76)	5 (2.30)
TCFC3 (Female)	1012/1199 (83.6±1.8)^{z}	842/1003 (83.1±3.4)^{zx}	611	112	28 (4.58)	13 (2.13)	11 (1.80)
TCFC4 (Female)	636/824 (77.2±2.2)^{y}	424/636 (67.2±4.0)^{w}	321	83	9 (2.80)	6 (1.87)	4 (1.25)
CEFC2* (Female)	55/69 (79.71)	46/52 (88.5)	46	9	4 (8.70)	2 (4.35)	2 (4.35)
Total	3949/4943 (79.9)	3094/3737 (82.8)	1873	368	90 (4.80)	48 (2.56)	35 (1.87)

Differences in fusion and cleavage rates were analyzed by One-way Analysis of Variance (ANOVA); $^{w-z}$Values with different superscripts within a column are significantly different ($p<0.05$)

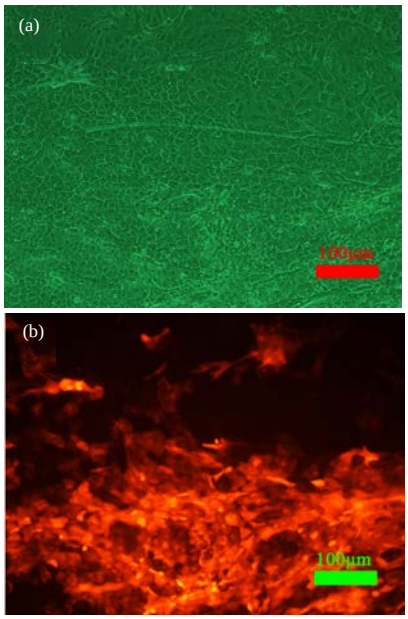

Fig. 2: Identification of transgenic goats: Transgenic primary cells of ear apex of cashmere goats observed by a) visible light microscopy and b) fluorescence microscopy

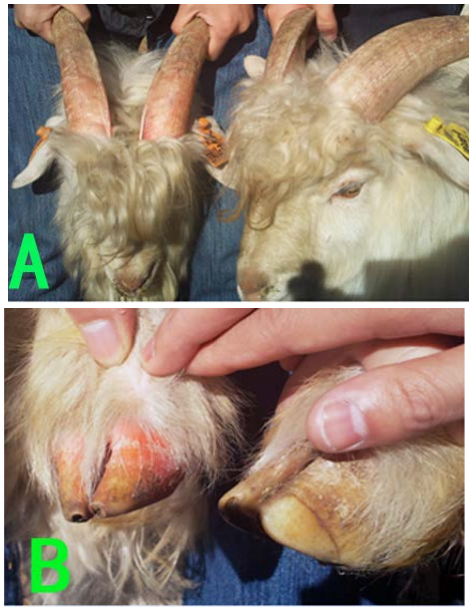

Fig. 3: Transgenic goat expression of red fluorescent protein: Expression of red fluorescent protein in cashmere goat horns A) and goat hoofs; B) (in each case: left, transgenic cashmere goat; right, control)

morphology for embryo transplantation investigations. A total of 368 recipient goats were transplanted and 48 goats were born with 35 survivors remaining after 1 week (Table 1). Sexes of all kids matched the SRY-PCR results. Furthermore, PCR analysis of genomic DNA extracted from transgenic cloned kids showed that exogenous genes (neomycin resistance gene) were integrated into the cashmere goat genome. Ear apex tissues of transgenic cashmere goats were cultured *in vitro* and examined microscopically for the expression of exogenous red fluorescent proteins. Partial transgenic goats highly expressed (Fig. 2a and b) and partial transgenic goats presented exogenous red fluorescent protein expression in appearance to a certain extent (Jang *et al*., 2010) (Fig. 3a and b).

Influences of different individual-derived cells on cloning efficiency: Marked differences in cloning efficiencies of different individual-derived donor cells were observed (Table 1). The cloning efficiency of TCFC1 was found to be higher than that of TCFC5 (both male) with fusion rates of cleavage rates, pregnancy rates of birth rates of and postnatal survival rates of Furthermore, the mortality rate of CFC1T was lower than that of CFC5T. Marked differences were observed in comparisons of the cloning efficiencies of TCFC3 and TCFC4. Compared with TCFC4, TCFC3 exhibited higher rates of fusion (83.6 vs. 77.2%) and cleavage (83.1 vs. 67.2%) pregnancy (4.58 vs. 2.80%), birth (2.13 vs. 1.87%) and postnatal survival (1.80 vs. 1.25%). However, the mortality rate of kids was lower for TCFC3 compared with TCFC4 (15.38 vs. 3.3%). A direct

Table 2: Influence of fibroblast cell type on cloning efficiency

Donor cell type	Fusion embryo No. (%)	Cleavage embryo No. (%)	Transplanted embryo No.	Transplanted receptor No.	Pregnancy No. (%)	Birth No. (%)	Postnatal survival No. (%)
FFCs	171/210 (81.4)^{x}	165/171 (96.5)^{x}	32	8	1 (3.13)	1 (3.13)	1 (3.13)
EFCs	509/719 (70.8)^{y}	408/447 (91.2)^{y}	263	48	17 (6.46)	8 (3.04)	7 (2.66)

Table 3: Influence of non-transgenic and transgenic donor cells on cloning efficiency

Donor cell type	Fusion embryo No. (%)	Cleavage embryo No. (%)	Transplanted embryo No.	Transplanted receptor No.	Pregnancy No. (%)	Birth No. (%)	Postnatal survival No. (%)
Non-transgenic cell	680/929 (73.2)^{x}	576/618 (93.0)^{a}	295	56	18 (6.10)	9 (3.05)	8 (2.71)
Transgenic cell	3324/4083 (81.4)^{y}	2567/3171 (80.9)^{b}	1578	312	72 (4.56)	39 (2.47)	27 (1.71)

Table 4: Influence of donor cell sex on cloning efficiency

Donor cell type	Fusion embryo No. (%)	Cleavage embryo No. (%)	Transplanted embryo No.	Transplanted receptor No.	Pregnancy No. (%)	Birth No. (%)	Postnatal survival No. (%)
Male-derived cell	2301/2920 (78.8)^{x}	1828/2098 (87.1)^{x}	895	164	49 (5.47)	27 (3.02)	18 (2.01)
Female-derived cell	1703/2092 (81.4)^{y}	1312/1691 (77.6)^{y}	978	204	41 (4.19)	21 (2.15)	17 (1.74)

Differences in fusion and cleavage rates were analyzed by χ^2-tests. ^{xy}Values with different superscripts within a column are significantly different ($p<0.05$)

correlation was observed between the early cleavage rate of cloned embryos and the rates of pregnancy, birth and fetal survival (Table 1).

Influence of fibroblast cell type on cloning efficiency: Compared with non-transgenic CEFC-derived cloned embryos, non-transgenic CFC-derived cloned embryos showed significantly higher rates of fusion ($p<0.05$) and cleavage ($p<0.05$) and slightly higher birth and survival rates ($p>0.05$) (Table 2).

Influences of non-transgenic and transgenic donor cells on cloning efficiency: The fusion rate of non-transgenic cell-derived cloned embryos was significantly lower than that of transgenic cell-derived embryos ($p<0.05$) while the cleavage rate of the former was significantly higher than that of the latter ($p<0.05$) (Table 3). The rates of pregnancy ($p>0.05$), birth ($p>0.05$) and postnatal survival ($p>0.05$) of non-transgenic cell-derived cloned embryos were also higher than those of the transgenic cell-derived cloned embryos.

Influence of sex of donor cell type on cloning efficiency: The fusion rate of male cell-derived embryos was significantly lower than that of female cell-derived cloned embryos ($p<0.05$) while cleavage rate of male cloned embryos was significantly higher than that of female cell-derived cloned embryos $p<0.05$) (Table 4).

Subsequent pregnancy, birth and postnatal survival rates were higher in male cell-derived cloned embryos compared with those of female cell-derived cloned embryos ($p>0.05$).

DISCUSSION

To the knowledge, this is the first report describing the generation of cloned transgenic cashmere goats and the investigation of the influence of different donor cell types of on SCNT. In order to minimize experimental variation, SCNT and embryo transplantation investigations were conducted in the autumn of a single year on animals held in the same environment.

Studies of the influences of Ear Fibroblast Cells (EFCs) and Fetal Fibroblast nuclear (FFCs) donors on cloning efficiency have yielded varying results. A bovine study conducted by Srirattana *et al.* (2010) suggested that the fusion rate of EFCs derived embryos was significantly higher than that of FFCs while the cleavage and blastocyst rates of fetal fibroblast-derived embryos were slightly higher than those of FFCs derived embryos. However, further embryo transplantation analyses were not conducted in this study. Wani *et al.* (2010) found no differences in the fusion rates of camel FFCs and EFCs and the cleavage rate of EFCs-derived embryos was slightly higher than that of FFCs-derived embryos. However, the final fetal birth rate was based on a comparison of surviving animals. In the current study, the influence of EFCs and FFCs on cloning efficiency was investigated in cashmere goats. The rates of fusion, cleavage, birth and postnatal survival of CFC cell-derived embryos were higher than those of CEFC-derived embryos. It can be speculated that such variations are species specific and associated with differences in nuclear transplantation operation techniques (Keefer, 2008).

Comparisons of non-transgenic (CFFC5) and Transgenic (TCFFC5) somatic cell donors derived from the same individual showed that the fusion rate of transgenic reconstructed embryos was higher than that of non-transgenic reconstructed embryos. This observation is consistent with a study conducted in goats reported by Zhang *et al.* (2010) and similar to the results of studies conducted in cattle, dogs and pigs (Hong *et al.*, 2011; Kurome *et al.*, 2008; Zakhartchenko *et al.*, 2001). This may be attributable to the generally greater diameter of

transgenic cells compared with non-transgenic cells thus allowing closer contact with the oocyte which facilitates electro-fusion. However, the cleavage rate and corresponding rates of birth and postnatal survival were significantly higher in non-transgenic cloned embryos compared with transgenic cloned embryos. It can be speculated that these effects are associated with the increased culture duration and passage number of transgenic cells compared with non-transgenic cells. Furthermore, earlier studies have suggested that long-term culture of donor cells is associated with cloned blastocyst apoptosis (Jang *et al*., 2004) and that exogenous gene insertion and long-term drug screening in the transfection and culture process of transgenic cells influences donor cell quality and subsequent cleavage rate and cloning efficiency of the embryos (Gao *et al*., 2002; Heidari *et al*., 2010; Schnieke *et al*., 1997; Westhusin *et al*., 2001; Zakhartchenko *et al*., 2001). In the study, it was observed that the logarithmic growth phase and growth rate was reduced in transgenic cells compared with common somatic cells (Guo *et al*., 2009a). Comparisons of non-transgenic cloned fetal goats revealed an apparent increase in the fetal pregnancy duration of transgenic cashmere goats (154.7 vs. 152 days). The development retardation of transgenic cloned embryos may be a causative factor in the low cloning efficiency observed in this study.

Comparison of influence of donor cell sex on the cloning efficiency of cashmere goats showed that the fusion rate of male cell-derived cloned embryos was lower than that of female cell-derived embryos, although the specific reason remains to be elucidated. However, the cleavage, pregnancy, birth and postnatal survival rates of male cell-derived cloned embryos were higher than those of female cell-derived cloned embryos. This suggests the sex of cloned embryos have different developmental abilities. The results of this study in goats are similar to those of a canine study conducted by Kim *et al*. (2009). However, a study by Hosseini *et al*. (2008) in sheep showed that the cleavage rate of female cell-derived cloned embryos was slightly higher than that of male cell-derived cloned embryo, although further embryo transplantation analyses for comparison of birth rates were not conducted. In contrast to the results of the study, Chen *et al*. (2003) showed that the birth rate of bovine female cell-derived cloned embryos was significantly higher than that of male cell-derived cloned embryos although these discrepancies may be caused by species differences and the use of different nuclear transplantation systems. Furthermore, the cloning efficiency of male cell-derived cloned embryos was higher than that of female cell-derived cloned embryos. Female cells carry more genetic material than male cells (XX vs. XY). Therefore, some differences exist in the reprogramming process which may be responsible for the observed differences in cloning efficiency. For example, the female X chromosome is inactivated in early cleavage (Nesterova *et al*., 2001) and mediates a dosage compensation effect although studies suggest that the X-inactive specific transcript (XIST, non-coding RNA that plays a critical role in X chromosome inactivation) is overexpressed in somatic cell cloned embryos (Wrenzycki *et al*., 2002). The abnormal expression of XIST in clones may be involved in the mechanism resulting in the lower cloning efficiency observed in female cloned embryos compared with male cloned embryos. Recent studies have suggested that gene knockout and the interruption of XIST in mice results in increased cloning efficiency (Inoue *et al*., 2010; Matoba *et al*., 2011).

The techniques involved in SCNT are complex and costly. Therefore, preliminary identification of embryos is required prior to the generation of cloned and transgenic animals. The study did not identify a correlation between the fusion and cleavage rates although the cleavage rate of cloned embryos and the cloning efficiency are correlated to some extent (Table 1). For example, the cleavage rate of TCFC4 cell-derived embryos was significantly lower than that of other groups and the subsequent embryo transplantation results also showed that the birth and postnatal survival rates were also lower than those of other donor cells. Furthermore, the cleavage rates and cloning efficiency (both theoretical and actual) of male cell-derived cloned embryos were higher than those of female cell-derived embryos. Therefore, the ability to select cell lines with higher fusion and cleavage rates for preliminary research is advantageous for increasing the cloning efficiency and implementation of subsequent studies.

CONCLUSION

The SCNT efficiency of CFCs cell-derived embryos was higher than that of CEFCs-derived embryos and the cloning efficiency of non-transgenic cloned embryo was higher than that of transgenic cloned embryos. Also, the SCNT efficiency of male cell-derived embryos was higher than that of female cell-derived embryos. These data indicate that donor cashmere goat cell type is an important factor in determining SCNT efficiency.

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