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Effect of potassium simplex optimization medium (KSOM) and embryo screening on the production of human lactoferrin transgenic cloned dairy goats

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In this study, we produced cloned transgenic dairy goat based on dairy goat ear skin fibroblast as donor cells for nuclear transfer (NT), which were modified by human lactoferrin (*hLF*) gene. The developmental competence of NT embryos was compared with either between different embryo culture medium, potassium simplex optimization medium (KSOM) and tissue culture medium (TCM 199), or different classification of NT embryos (48 h after fusion). First we cultured NT embryos to cleavage stage (48 h after fusion) by TCM 199 supplemented with 1 mg/ml bovine serum albumin BSA and KSOM, then used TCM 199 supplemented with 10% FBS to culture them to blastula stage. The results show that the NT embryos in KSOM (19.5%) were superior to TCM 199 (10.6%) in blastulation. In the second experiment, we found that the growth rate of NT embryos (48 h after fusion) was different, then we divided them into four groups: 2-cell, 3- to 4-cell, 5- to 8-cell and >8-cell in stereo microscope and cultured them *in vitro* respectively. The results show day-2 embryos at 3-4cell and 5-8cell stage (31.9 and 28.2%, $P < 0.05$) had higher blastocyst formation rates than those at both 2-cell (9.1%) and >8-cell (8.3%) stage, and finally three healthy cloned transgenic goat were successfully produced using 3-8 cell embryos at Day-2 (82%). Using Hoechst 33342 staining, we also found that the >8 cells embryos at Day-2 demonstrated higher frequency of fragmentation, which may be the one cause of the low blastocyst formation rate. This study therefore demonstrates that KSOM medium could be selected as the early embryo culture medium, and 3-8 cell embryos at day-2 (48 h after fusion) may be the suitable embryos for transplantation, which could reduce the nuclei fragmentation and result in good quality blastocysts that may also enhance the efficiency of transgenic cloned dairy goats production, as well as decrease the economic loss due to embryonic mortality when embryos are transferred to synchronized recipients.

Key words: Nuclear transfer, KSOM, transgenic, human lactoferrin, dairy goat.

INTRODUCTION

Since the first report of a live mammal produced by nuclear transfer (NT) of a cultured cell line in 1996 (Campbell et al., 1996), cloned mammals have been produced successfully in sheep (Wilmut et al., 1997), cattle (Kato et al., 1998), goat (Baguisi et al., 1999) and pig (Polejaeva et al., 2000). An effective system for genetic modification and somatic cell nuclear transfer (SCNT) to produce transgenic animals would find application in the fields of agriculture, biotechnology and human medicine. Although, there are reports of live transgenic cloned offspring produced by SCNT in goats, the efficiency of cloned animal production is still less than

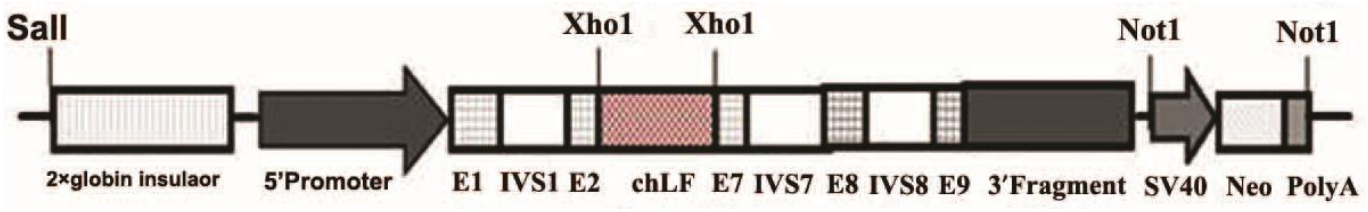

Figure 1. Schematic diagram of the pBC1-*hLF*-Neo mammary gland-specific vector.

satisfactory. The main barriers for transgenic animal production are culture conditions and nuclear transfer protocols.

Numerous studies have been performed to improve *in vitro* developmental competence of goat SCNT embryos using different fusion/activation protocols or culture medium by adding a variety of serum factors or different donor cells (Apimeteetumrong et al., 2004; Melican et al., 2005; Lan et al., 2006; Guo et al., 2009, 2010). However, the development rate of transgenic NT embryo currently remains low, indicating that embryo culture medium cannot provide the necessary ingredients for the development of the *in vitro* embryo. Potassium simplex optimization medium (KSOM) is an optimized medium (Lawitts and Biggers, 1993) that has achieved good results in mouse, rat and rabbit embryos. However, there has been no report of the production of transgenic goat embryos in livestock using KSOM medium.

Human Lactoferrin (*hLF*) is an iron-binding protein of the transferring family (Hyvonen et al., 2006). The molecular mass of LF is approximately 80 kDa. The N- and C-terminus of LF fold into two globular lobes, and each lobe contains one iron-binding site which can bind two ferric ions reversible (Anderson et al., 1989). This protein has been found to have a number of biological functions, including antimicrobial, anticancer, antioxidant and immunomodulatory effects (Yen et al., 2011). Several studies indicate that *hLF* binds to DNA (He and Furmanski, 1995), and Baumrucker et al. (2006) found that exogenous application of *hLF* stimulates the expression of a retinoic acid DR5 luciferase reporter construct in a mammary cell line. *hLF* is produced by various exocrine glands in the human body and is abundantly present in milk and colostrums (Amini and Nair, 2011). However, there is a potential safety problem and limitations on human lactoferrin (*hLF*) production by purification from human body. Hence, it is much more practical and effective to produce *hLF* from the mammary gland of transgenic animals.

The objective of the present study was to establish a system to produce transgenic goats harboring the *hLF* gene by using NT. We also evaluated the effect of the KSOM and tissue culture medium (TCM199) on the developmental ability of transgenic goat embryo by NT.

MATERIALS AND METHODS

Chemicals

Unless otherwise indicated, all chemicals used in this study were obtained from Sigma-Aldrich Company (St. Louis, MO, USA) and the media from Gibco Invitrogen Corporation (Grand Island, NY, USA).

Transgene construct and transfection

Human *hLF* genomic cDNA was cloned using the goat beta-casein promoter as a regulatory controller. The neomycin gene was isolated from the pcDNA 3.1 vector and cloned into the goat b-casein promoter/*hLF* vector (Figure 1). The goat β-casein promoter/*hLF* vector linearized with Lipofectamine LTX (Invitrogen, Grand Island, NY, USA) was used for *hLF* gene transfection. The dairy goat-derived fetal fibroblasts were seeded in a 6-well dish at a concentration of 3×10^3 cells per well and incubated in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) for 48 h. The cells were transfected with linearized plasmid DNA according to the protocol described by the manufacturer. The transfected cells were split into100-mm plates. After 48 h of culture, colonies were selected with 500 mg/ml of G418 (Invitrogen) for 14 days. Well-proliferated colonies were isolated and screened for the presence of the transgene. The colonies were then frozen until use for nuclear transfer.

Collection and *in vitro* maturation of goat oocytes

Goat ovaries were collected at a local abattoir and transported to the laboratory within 3 h in sterilized saline containing 100 IU/ml gentamycin at 30 to35°C. Cumulus-oocyte complexes (COCs) were obtained by slicing the ovarian surface with a razor blade. COCs with more than three cumulus layers and a finely granulated homogeneous ooplasm were selected and cultured in TCM 199 medium supplemented with 10% FBS, 10 µg/ml FSH, 10 µg/ml LH, 1 µg/ml E_2 and 100 IU/ml penicillin/streptomycin for 19 to 21 h at 38.5°C under 5% CO_2 in humidified air. Then cumulus cells were removed from the oocytes by manual pipetting in the presence of 1

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Abbreviation: NT, Nuclear transfer; **hlF,** human lactoferrin; **KSOM,** potassium simplex optimization medium; **TCM,** tissue culture medium; **BSA,** bovine serum albumin.

Table 1. Effect of culture medium on the embryo development competence.

Treatment	Number of embryo cultured	Number (%) of embryo developed to	
		Cleaved (2 day)	Blastocyst (7 day)
TCM 199	168	124 (73.6 ± 4.2)[a]	18 (10.6 ± 2.1)[a]
KSOM -TCM 199	122	91 (74.0 ± 3.9)[a]	24 (19.5 ± 1.4)[b]

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

mg/ml of hyaluronidase, and oocytes with extruded first polar body were selected for enucleation.

Preparation of recipient oocytes

Denuded oocytes with a polar body were incubated in TCM 199 supplemented with 7.5 µg/ml cytochalasin B, 10 µg/ml Hoechst 33342 and 10% FBS at 38.5°C for 10 min. First, the polar body was placed at the 2 o'clock position, and the location of the chromosomes was determined after a brief exposure of the cytoplasm to UV light, then the polar body and nucleus were removed by aspiration using a pipette placed at the 3 o'clock position.

Nuclear transfer

A cell was slipped into the perivitelline space of the enucleated oocyte. Nuclear transfer couplets were electrically fused in medium comprising of 0.25 M D-Sorbitol, 0.5 mM $(CH_3COO)_2$ $Mg.4H_2O$, 0.1 mM $(CH3COO)_2$ Ca, 0.5 mM Hepes and 1 mg/ml bovine serum albumin (BSA), by applying a single electric pulse (20 µs each, 1.2 kv /cm). Activation of fused embryos was achieved chemically by incubation in 5 µM ionomycin for 5 min at room temperature, and were then incubated at 38.5°C under 5% CO_2 in humidified air in TCM 199 containing 2 mM 6-DMAP for 4 h.

Embryo culture and transfer

After activation treatments, the reconstructed embryos were transferred to equilibrated KSOM media (Millipore, USA), and cultured undisturbed for 36 to 40 h at 38.5°C under 5% CO_2 in humidified air. The NT embryos that had been cultured for 40 to 44 h after fusion were transferred into oviducts of the recipients at 40 to 48 h after estrus, respectively.

PCR analysis

The goat ear cells, donor cells and surrogate skin cells were resuspended in lysis buffer (40 mM Tris, pH 8.9, 0.9% TritonX-100, 0.9% NonidetP-40 and 0.4mg/mL proteinase K), incubated at 55°C for 5 h, and heated to 95°C for 10 min in order to inactivate proteinase K. The genomic DNA was amplified using a PCR machine (PE Applied Biosystems, Foster City, CA, USA) in a 20 µL reaction volume with the following variables: 30 cycles of 1 min at 95°C, 1 min at 62°C and 1min at 72°C and a final 10 min extension period. The PCR primers set were used to amplify 750 bp *hLF* gene. The forward primer and reverse primer are as follows: 5′-GAATGGCTGGCAGTGAAACA-3′ and 5′-CTCAATGGGCTCAGGTGGAC-3′.

Experimental designs

Experiment 1: After activation treatments, the reconstructed embryos were cultured in groups of 10 in 35 µL drops of KSOM or TCM 199 under mineral oil in 5% CO_2 in humidified air. Reconstructed embryos were allocated to two culture medium treatments: (1) TCM 199 + 0.1% BSA for 2 days, then TCM 199 + 10% FBS until Day 7; (2) KSOM for 2 days, then TCM 199+10% FBS until day 7.

Experiment 2: We divided Day-2 (48 h after fusion) reconstructed embryos into four groups based on number of blastomeres: 2-cell; 3- to 4-cell; 5- to 8-cell; and >8-cell. They were cultured in the same conditions, and formation rates of blastocyst were counted after 7 days. Besides, each was stained with Hoechst 33342 to observe the situation of blastomere nuclei.

Statistical analysis

For each treatment, at least three replicates were run. Statistical analyses were carried out by ANOVA (multiple comparisons are made with Bonferroni test). Data are expressed as mean ± SE, and $P < 0.05$ is considered significant.

RESULTS

Effect of culture medium on the embryo development competence

The efficiency of TCM 199 and KSOM-TCM 199 medium on the transgenic embryos developmental capability were examined (Table 1). The cleavage rate in culture system of TCM 199 and KSOM-TCM 199 were 73.6 and 74%, respectively ($P > 0.05$). Meanwhile, the blastocyst rate in KSOM-TCM 199 medium was greater than that in TCM199 medium (19.5 and 10.6%, respectively, $P < 0.05$), thus indicating that the KSOM-TCM 199 culture system might be more suitable in culturing transgenic NT embryos *in vitro*.

The formation rates of blastocysts from *hLF* transgenic cloned embryos (48 h after fusion)

The cleavage of *in vitro* cultured *hLF* transgenic cloned embryos was not fully synchronized, and their development capacity was also different (Table 2). The blastocyst formation rate of NT embryos, which were at 3-4 cell (31.9%) and 5-8 cell (28.2%) stage 48 h after fusion were greater than those at 2-cell (9.1%) and > 8-cell (8.3%) stages ($P < 0.05$). This result may indicate that the number of Day-2 embryos at 3-8 cell stage (79.46%) was more than those at 2-cell (8.90%) and > 8-

Table 2. The formation rate of blastocysts from *hLF* transgenic cloned embryos (48 h after fusion).

Stage of embryos at day 2	Number of embryo	Proportion of stage of embryo at day 2 (%)	Number (%) of blastocysts at day 7
2-cell	26	8.90	3 (9.1±4.1)[a]
3-4 cell	91	31.17	28 (31.9±2.7)[b]
5-8 cell	141	48.29	38 (28.2±2.8)[b]
>8-cell	34	11.64	4 (8.3±3.9)[a]

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

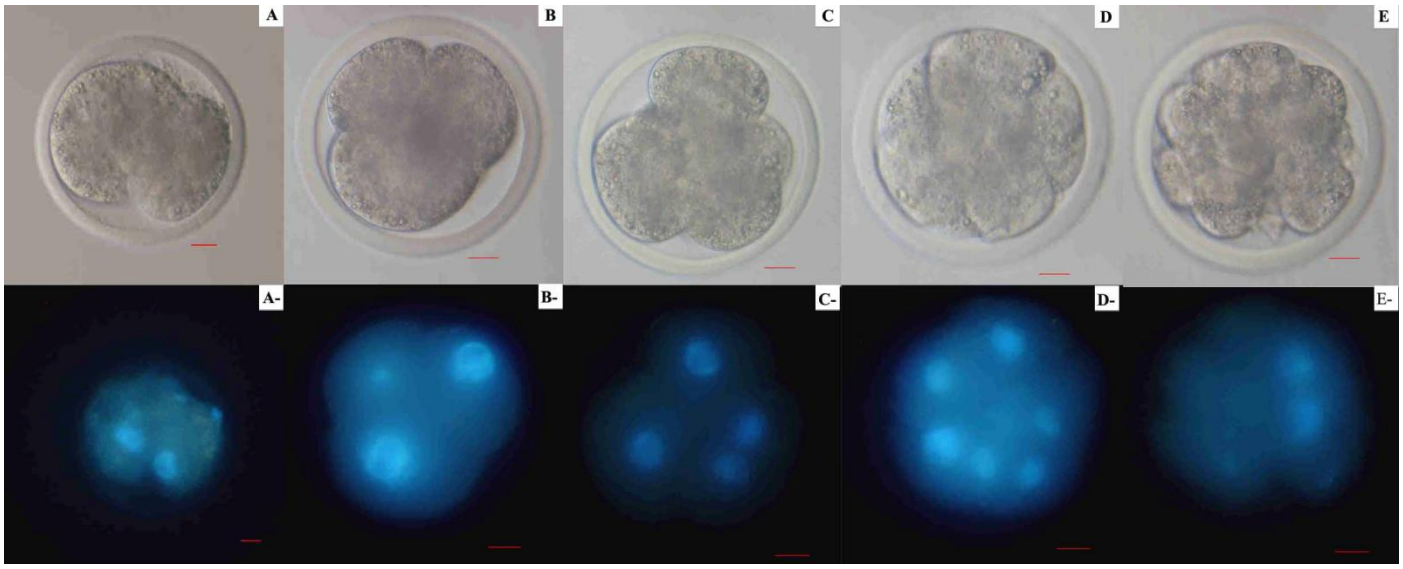

Figure 2. Transgenic cloned embryos developed *in vitro* (48 h after fusion). A, B, C, D, E are 2-cell, 3-cell, 4-cell, 8-cell and >8-cell under bright view, A-, B-, C-, D-, E-, are 2-cell, 3-cell, 4-cell, 8-cell and >8cell under UV. Scale bar = 20 µM.

cell stages (11.64%), whose blastocyst development capacity was negatively affected by some factors.

We could find that the number of Day-2 embryo cell nucleus at 2-cell stage (Figure 2A-) and 3-8 cell stage (3-cell Figure 2B-, 4-cell 2C- and 8-cell 2D-) was 2, 3, 4 and 7, respectively by Hoechst 33342 staining in accordance with the number of their blastomeres (Figure 2A to D). However, the Day-2 embryos at > 8-cell stage (Figure 2E-) mostly reflected a high frequency of fragmentation (> 50%), which appeared blastomere-like without nucleus existed.

Nuclear transfer to produce transgenic goat

The NT embryos were cultured in KSOM medium for 2 days and surgically transferred to surrogate goats. In total, we had 536 reconstructed embryos before activation with Ionomycin and 6-DMAP, and 371 (69%) embryos cleavaged *in vitro* culture in the present study. The Day-2 embryos at 2-cell and > 8-cell stages were abandoned and a total of 304 (82%) SCNT embryos were selected and transferred into 43 recipient females (Table 3). Finally, 9 pregnancies were detected by non-return estrus and B ultrasound scanner, while 5 healthy female lambs was delivered (Figure 3A to C). However, 2 NT dairy goats had perinatal death, 1 fetus was aborted at Day 118 (Figure 3D), and 3 of the pregnancies failed to maintain to term. The presence of the *hLF* gene in the 5 lambs was confirmed by PCR using DNA from ear cells of each lamb (Figure 4).

DISCUSSION

The combination of somatic cell nuclear transfer (SCNT) and transgenic technology led to the production of transgenic cloned animals. During the process, the *in vitro* culture system was a pivotal step. Although, there were a variety of mediums such as TCM 199 (Baguisi et al., 1999), Charles Rosenkrans medium **(**CR1) (Lan et al., 2006), and synthetic oviductal fluid (SOF) (Melican et

Table 3. Results of *hLF* transgenic cloned offspring by using KSOM medium

Number fused Couplets	Number (%) embryos (cleavage stage)	Number (%) of embryo transferred	Transferred recipient	Pregnant recipient	Abortive recipient	Viable offspring
536	371 (69)	304 (82)	43	9	4	5

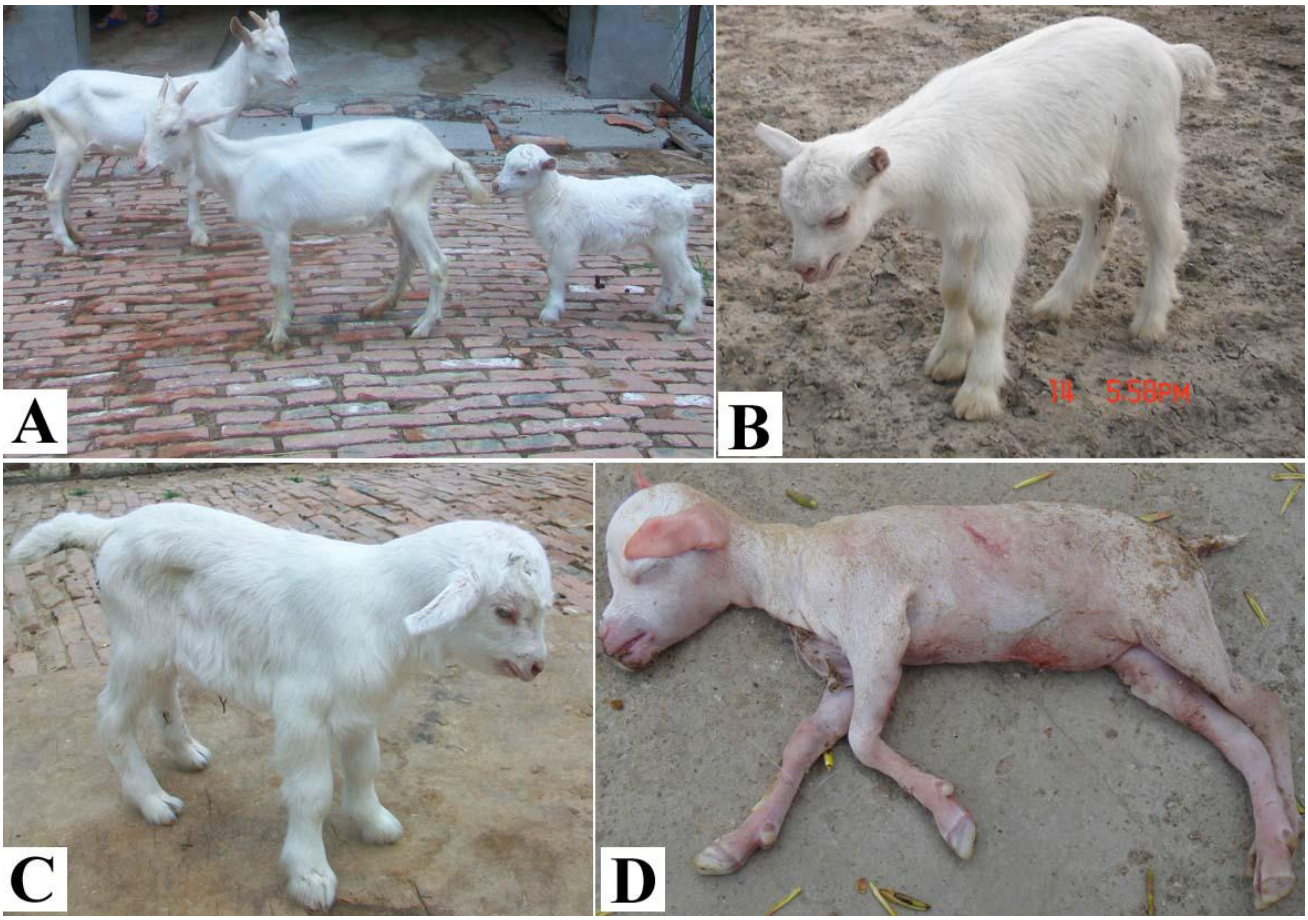

Figure 3. Cloned kids derived from the *hLF* gene transgenic fibroblast cells. (A) Three survived NT dairy goats; (B, C) two NT dairy goats that died perinatally; (D) aborted fetus at Day 118.

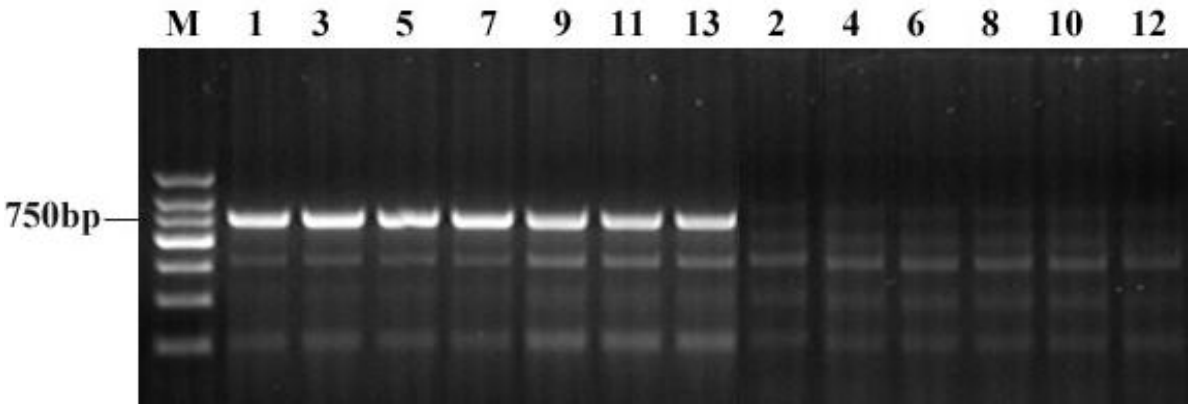

Figure 4. Identification of *hLF* gene in the cloned kid by PCR amplification. Lane 1. Donor cell; lanes 3, 5, 7, 9 and 11, the cloned kid; lane 13, aborted fetus; lanes 2, 4, 6, 8, 10 and 12, the recipient goat as negative control.

al., 2005) shown to culture the early goat embryos *in vitro*, none transgenic offspring was produced by KSOM medium. In the present study, we successfully produced *hLF* transgenic cloned dairy goat and confirmed the KSOM medium as an ideal culture medium for transgenic cloned embryos during the cleavage stage. *In vitro* culture medium, KSOM was superior to TCM 199 in culturing the transgenic cloned embryo at cleavage stage, and the embryos cultured by the KSOM were transferred into the synchronized recipient goats. At last, 4 healthy transgenic cloned dairy goats were produced.

The metabolism of embryos cultured *in vitro* was maintained by energy substances such as glucose, lactate and pyruvate. Glucose had been shown to be very important in the process of blastocyst formation. Nevertheless, high concentration of glucose would block the development of embryo during the cleavage stage (Schini and Bavister, 1988; Chatot et al., 1989; Lawitts and Biggers, 1991; Thompson et al., 1991). These contradictory results were explained by a biphasic effect of glucose: lower concentrations (< 3 mM) stimulated early cleavage stage embryo development, whereas high concentrations (3 - 5 mM) inhibited early embryo development (Lim et al., 2007). Our study also confirmed that high concentrations inhibited early embryo development. In the present study, the glucose concentration in KSOM medium was 0.2 mM, whereas the glucose concentration in TCM 199 medium was 5.6 mM. Therefore, our result demonstrated that the KSOM medium was more appropriate than TCM 199 medium to culture the SCNT embryos from the pronuclear to the cleavage stage.

Currently, most of early goat NT embryos at the cleavage stage (48 h after fusion) were used for embryo transfer (ET) (Baguisi et al., 1999; Reggio et al., 2001; Melican et al., 2005; Chen et al., 2007). It was essential to screen the NT embryos quality before ET. Since *in vitro* culture embryos were influenced by several factors, the development of pre-implantation embryo was not synchronized. In the Day-2 embryos in the present study, the blastocyst formation rate of 3-8 cell stage embryos was greater than that of other stage embryos. Furthermore, similar studies have also shown that 3-8 cell stage embryos had lower chromosome abnormalities (Ulloa Ullo et al., 2008a, b). Therefore, it is reasonable to believe that chromosome abnormality may be related to the development capability of cleavage-stage embryos, which lead to a lower blastocyst formation rate (Magli et al., 2007; Ulloa Ullo et al., 2008b). In addition, we also found that most transgenic cloned embryos were in 3-8 cell stage embryos at Day 2 (79.46%). Meanwhile the > 8-cell stage embryos showed higher frequency of fragmentation (> 50%), which might be the debris due to the abnormal embryonic development, and this might also be the reason of the low formation rate of blastocyst. Therefore, it was necessary to eliminate transgenic cloned embryos with abnormal morphology and select the 3-8 cell stage embryos before ET.

In conclusion, KSOM could be selected as the early embryo culture medium. In order to enhance the efficiency of transgenic cloned dairy goat production and decrease the economic cost, selecting the 3-8 cell stage transgenic cloned embryos (48 h after fusion) with good morphology was necessary when the embryos were transferred to synchronized recipients.

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REFERENCES

Amini AA, Nair LS (2011). Lactoferrin: a biologically active molecule for bone regeneration. Curr. Med. Chem. 18(8):1220-1229.

Anderson BF, Baker HM, Norris GE, Rice DW, Baker EN (1989). Structure of human lactoferrin: crystallographic structure analysis and refinement at 2.8 A resolution. J. Mol. Biol. 209(4):711-734.

Apimeteetumrong M, Thuangsanthia A, Leingcharoen N, Yiengvisavakul V, Harintharanon A, Kunavongkrit A, Sumretprasong J, Vignon X, Techakumphu M (2004). The effect of activation protocols on the development of cloned goat embryos. J. Vet. Med. Sci. 66(12):1529-1534.

Baguisi A, Behboodi E, Melican DT, Pollock JS, Destrempes MM, Cammuso C, Williams JL, Nims SD, Porter CA, Midura P, Palacios MJ, Ayres SL, Denniston RS, Hayes ML, Ziomek CA, Meade HM, Godke RA, Gavin WG, Overstrom EW, Echelard Y (1999). Production of goats by somatic cell nuclear transfer. Nat. Biotechnol. 17(5):456-461.

Baumrucker CR, Schanbacher F, Shang Y, Green MH (2006). Lactoferrin interaction with retinoid signaling: cell growth and apoptosis in mammary cells. Domest. Anim. Endocrinol. 30(4):289-303.

Campbell KH, McWhir J, Ritchie WA, Wilmut I (1996). Sheep cloned by nuclear transfer from a cultured cell line. Nature 380(6569):64-66.

Chatot CL, Ziomek CA, Bavister BD, Lewis JL, Torres I (1989). An improved culture medium supports development of random-bred 1-cell mouse embryos *in vitro*. J. Reprod. Fertil. 86(2):679-688.

Chen DY, Jiang MX, Zhao ZJ, Wang HL, Sun QY, Zhang LS, Li RC, Cao HH, Zhang QJ, Ma DL (2007). Cloning of Asian yellow goat (C. hircus) by somatic cell nuclear transfer: telophase enucleation combined with whole cell intracytoplasmic injection. Mol. Reprod. Dev. 74(1): 28-34.

Guo J, Liu F, Guo Z, Li Y, An Z, Li X, Zhang Y (2010). *In vitro* development of goat parthenogenetic and somatic cell nuclear transfer embryos derived from different activation protocols. Zygote 18(1):51-59.

Guo X, Yang D, Ao X, Wu X, Li G, Wang L, Bao MT, Xue L, Bou S (2009). Production of transgenic cashmere goat embryos expressing red fluorescent protein and containing IGF1 hair-follicle-cell specific expression cassette by somatic cell nuclear transfer. Sci. China C. Life. Sci. 52(4):390-397.

He J, Furmanski P (1995). Sequence specificity and transcriptional activation in the binding of lactoferrin to DNA. Nature 373(6516):721-724.

Hyvonen P, Suojala L, Orro T, Haaranen J, Simola O, Rontved C, Pyorala S (2006). Transgenic cows that produce recombinant human lactoferrin in milk are not protected from experimental Escherichia coli intramammary infection. Infect. Immun. 74(11):6206-6212.

Kato Y, Tani T, Sotomaru Y, Kurokawa K, Kato J, Doguchi H, Yasue H,

Tsunoda Y (1998). Eight calves cloned from somatic cells of a single adult. Science 282(5396):2095-2098.
Lan GC, Chang ZL, Luo MJ, Jiang YL, Han D, Wu YG, Han ZB, Ma SF, Tan JH (2006). Production of cloned goats by nuclear transfer of cumulus cells and long-term cultured fetal fibroblast cells into abattoir-derived oocytes. Mol. Reprod. Dev. 73(7):834-840.
Lawitts JA, Biggers JD (1991). Overcoming the 2-cell block by modifying standard components in a mouse embryo culture medium. Biol. Reprod. 45(2):245-251.
Lawitts JA, Biggers JD (1993). Culture of preimplantation embryos. Methods Enzymol. 225(153-164.
Lim KT, Jang G, Ko KH, Lee WW, Park HJ, Kim JJ, Lee SH, Hwang WS, Lee BC, Kang SK (2007). Improved *in vitro* bovine embryo development and increased efficiency in producing viable calves using defined media. Theriogenology 67(2):293-302.
Magli MC, Gianaroli L, Ferraretti AP, Lappi M, Ruberti A, Farfalli V (2007). Embryo morphology and development are dependent on the chromosomal complement. Fertil. Steril. 87(3):534-541.
Melican D, Butler R, Hawkins N, Chen LH, Hayden E, Destrempes M, Williams J, Lewis T, Behboodi E, Ziomek C, Meade H, Echelard Y, Gavin W (2005). Effect of serum concentration, method of trypsinization and fusion/activation utilizing transfected fetal cells to generate transgenic dairy goats by somatic cell nuclear transfer. Theriogenology 63(6):1549-1563.
Polejaeva IA, Chen SH, Vaught TD, Page RL, Mullins J, Ball S, Dai Y, Boone J, Walker S, Ayares DL, Colman A, Campbell KH (2000). Cloned pigs produced by nuclear transfer from adult somatic cells. Nature 407(6800):86-90.
Reggio BC, James AN, Green HL, Gavin WG, Behboodi E, Echelard Y, Godke RA (2001). Cloned transgenic offspring resulting from somatic cell nuclear transfer in the goat: oocytes derived from both follicle-stimulating hormone-stimulated and nonstimulated abattoir-derived ovaries. Biol. Reprod. 65(5):1528-1533.
Schini SA, Bavister BD (1988). Two-cell block to development of cultured hamster embryos is caused by phosphate and glucose. Biol. Reprod. 39(5):1183-1192.
Thompson JG, Simpson AC, Pugh PA, Wright RW, Jr., Tervit HR (1991). Glucose utilization by sheep embryos derived in vivo and *in vitro*. Reprod. Fertil. Dev. 3(5):571-576.
Ulloa Ullo CM, Yoshizawa M, Komoriya E, Mitsui A, Nagai T, Kikuchi K (2008a). The blastocyst production rate and incidence of chromosomal abnormalities by developmental stage in *in vitro* produced porcine embryos. J. Reprod. Dev. 54(1):22-29.
Ulloa Ullo CM, Yoshizawa M, Yamashita A, Hama S, Mitsui A, Hashi C, Abe H, Hoshi H, Fukui E, Matsumoto H (2008b). Blastocyst Production from *In Vitro*-produced Day-2 Bovine Embryos Classified by Cleavage Stage, and Cytogenetical Evaluation of the Resultant Day-8 Blastocysts. J. Reprod. Dev. 54(6):465-472.
Wilmut I, Schnieke AE, McWhir J, Kind AJ, Campbell KH (1997). Viable offspring derived from fetal and adult mammalian cells. Nature 385(6619):810-813.
Yen CC, Shen CJ, Hsu WH, Chang YH, Lin HT, Chen HL, Chen CM (2011). Lactoferrin: an iron-binding antimicrobial protein against Escherichia coli infection. Biometals 24(4): 585-594.