The development and expression of pluripotency genes in embryos derived from nuclear transfer and *in vitro* fertilization

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Summary

Somatic cell nuclear transfer can be used to produce embryonic stem (ES) cells, cloned animals, and can even increase the population size of endangered animals. However, the application of this technique is limited by the low developmental rate of cloned embryos, a situation that may result from abnormal expression of some zygotic genes. In this study, sheep–sheep intra-species cloned embryos, goat–sheep inter-species cloned embryos, or sheep *in vitro* fertilized embryos were constructed and cultured *in vitro* and the developmental ability and expression of three pluripotency genes, *SSEA-1*, *Nanog* and *Oct4*, were examined. The results showed firstly that the developmental ability of *in vitro* fertilized embryos was significantly higher than that of cloned embryos. In addition, the percentage of intra-species cloned embryos that developed to morula or blastocyst stages was also significantly higher than that of the inter-species cloned embryos. Secondly, all three types of embryos expressed SSEA-1 at the 8-cell and morula stages. At the 8-cell stage, a higher percentage of *in vitro* fertilized embryos expressed SSEA-1 than occurred for cloned embryos. However, at the morula stage, all detected embryos could express SSEA-1. Thirdly, the three types of embryos expressed *Oct4* mRNA at the morula and blastocyst stages, and embryos at the blastocyst stage expressed *Nanog* mRNA. The rate of expression of *Oct4* and *Nanog* mRNA at these developmental stages was higher in *in vitro* fertilized embryos than in cloned embryos. These results indicated that, during early development, the failure to reactivate some pluripotency genes maybe is a reason for the low cloning efficiency found with cloned embryos.

Keywords: Inter-species, Nanog, Oct4, Somatic cell nuclear transfer, SSEA-1

Introduction

The technique of somatic cell nuclear transfer (SCNT), in which a somatic cell nucleus is transferred into an oocyte, and then reprogrammed into a pluripotent state, can be used to produce embryonic stem (ES) cells, even cloned animals (Wilmut *et al.*, 1997; Wakayama *et al.*, 2001). But the application of this technique is limited due to the shortage of oocytes for certain species. Inter-species somatic cell nuclear transfer (iSCNT), an alternative SCNT technique in which a donor somatic cell from one species is transferred into a recipient oocyte of another species, may offer an opportunity to produce ES cells, and increase the population size of endangered mammals whose oocytes are extremely difficult, or sometimes impossible, to obtain (Lanza *et al.*, 2000; Loi *et al.*, 2001; Chen *et al.*, 2003). The overall efficiency of the SCNT procedure is low (Wilmut *et al.*, 2002; Fulka & Fulka, 2007), and is even lower for iSCNT (Lee *et al.*, 2008; Lorthongpanich *et al.*, 2008; Li *et al.*, 2006). It has been proposed that low cloning efficiency may be attributed largely to incomplete epigenetic reprogramming of somatic nuclei (Armstrong *et al.*, 2006; Niemann *et al.*, 2008).

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Ectopic expression of certain types of transcription factors in somatic cells is an alternative technique that can be used to reprogram somatic cells into induced pluripotent stem (iPS) cells (Takahashi & Yamanaka, 2006; Takahashi *et al.*, 2007; Yu *et al.*, 2007). Recently, two studies have shown that the reprogramming of murine fibroblasts into iPS cells follows a defined sequence of molecular events that begins with the downregulation of somatic markers, followed by the upregulation of stage-specific embryonic antigen 1 (SSEA-1). SSEA-1-positive cells then gradually reactivate other markers that are associated with pluripotency, including Oct4, Nanog, Sox2, telomerase, and the silent X chromosome in female fibroblasts (Brambrink *et al.*, 2008; Stadtfeld *et al.*, 2008). These studies lead us consider that the low developmental ability of cloned embryos may be due to incomplete reactivation of pluripotency genes. This idea has been confirmed by recent studies, in which the expression of some pluripotency genes was monitored during the early development of cloned embryos. Most of these studies have shown that pluripotency genes, including *Oct4*, *Sox2*, and *Nanog*, can be reactivated in cloned embryos, although the expression level of certain pluripotency genes is somewhat abnormal (Li *et al.*, 2005; Beyhan *et al.*, 2007; Xing *et al.*, 2009; Aston *et al.*, 2010). Interspecies cloned embryos, even offspring, have been successfully produced by iSCNT (Lanza *et al.*, 2000; Loi *et al.*, 2001; Murakami *et al.*, 2005; Lorthongpanich *et al.*, 2008; Sugawara *et al.*, 2009; Hong *et al.*, 2012; Srirattana *et al.*, 2012), and whether pluripotency genes could be reactivated in iSCNT-derived embryos has been monitored in some studies (Chung *et al.*, 2009; Wang *et al.*, 2011; Hosseini *et al.*, 2012). In our previous studies, goat–sheep or bovine–sheep inter-species cloned embryos were constructed, both of which could develop *in vitro* to the blastocyst stage (Hua *et al.*, 2008, Ma *et al.*, 2008a,b). These results indicated that sheep oocytes could dedifferentiate a foreign somatic cell nucleus from a distant genetic correlated species. However, in these studies, whether pluripotency genes could be reactivated and their relationship with the developmental ability of iSCNT-derived embryos were not evaluated.

In the present study, *in vitro* matured sheep oocytes were used as nuclear recipients and sheep fetal fibroblasts (SFFs) or goat fetal fibroblasts (GFFs) were used as nuclear donors to construct sheep–sheep intra-species or goat–sheep inter-species cloned embryos. Sheep *in vitro* fertilized embryos were also constructed. These three types of embryos were cultured *in vitro*, and their developmental ability and its relationship to the reactivation of certain types of pluripotency genes were evaluated.

Materials and methods

Unless otherwise indicated, all chemicals were purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA).

Donor cell preparation

SFFs or GFFs were isolated from a Mongolian sheep (*ovis aries*) fetus or an Guanzhong diary goat (*Capra hircus*) fetus, respectively, obtained from a slaughterhouse and used as nuclear donors to reconstruct sheep–sheep intra-species or goat–sheep inter-species cloned embryos. Primary cell culture, as well as freeze and thaw, were performed as described previously (Ma *et al.*, 2008a,b). Briefly, after removal of the head and internal organs, the remaining tissues were dissociated mechanically. Explants were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Invitrogen Corporation, Carlsbad, CA, USA) supplemented with 20% (v/v) fetal bovine serum (FBS, HyClone, Logan, UT, USA), 100 IU/ml penicillin and 100 μg/ml streptomycin at 38°C in a humidified atmosphere of 5% CO_2. When the cells from the explants reached 70% confluency, they were removed by treatment with 0.05% (quality/volume percentage concentration; m/v) trypsin–EDTA, counted, and frozen in aliquots in 10% (v/v) dimethyl sulphoxide (DMSO) and 90% (v/v) fetal bovine serum (FBS). Thawed SFFs and GFFs were plated into 96-well plates and cultured in DMEM supplemented with 10% (v/v) FBS at 38°C in a humidified atmosphere of 5% CO_2 until they reached 100% confluency. The medium was then replaced with low serum medium (DMEM supplemented with 0.5% (v/v) FBS) to starve the donor cells for 3–5 days until the day of nuclear transfer (NT). Just prior to NT, the donor cells were collected by trypsinization using 0.05% (m/v) trypsin–EDTA, washed twice, and resuspended in HEPES-buffered synthetic oviduct fluid (H-SOF) supplemented with 10% (v/v) FBS.

Sheep oocyte collection and maturation *in vitro*

The procedure of sheep oocyte collection and maturation has been described previously (Ma *et al.*, 2008a,b). Briefly, slaughterhouse-derived ovaries were collected from mature sheep (*O. aries*), placed in saline (38°C) and transported to the laboratory within 1–2 h. Ovaries were washed three times in fresh phosphate-buffered saline (PBS), sliced using a microblade and the contents released in sterile Petri dishes that contained fresh PBS medium supplemented with 5% (v/v) FBS and 0.054 mg/ml heparin sodium salt. Cumulus–oocyte complexes (COCs) with several intact cumulus cell layers and a homogeneous cytoplasm were

selected for maturation *in vitro*. COCs were washed several times, then cultured in oocyte maturation (OM) medium at 38.5°C in a humidified atmosphere of 5% CO_2. After 22–24 h of culture, cumulus cells were removed from matured oocytes by vortexing the COCs for 3–5 min in Ca^{2+}-/Mg^{2+}-free PBS that contained 0.2% (w/v) hyaluronidase. Denuded oocytes with a polar body were selected and placed in H-SOF supplemented with 10% (v/v) FBS and prepared for enucleation.

Nuclear transfer, electrofusion, activation and embryos culture

Denuded oocytes with a polar body were incubated in H-SOF supplemented with 7.5 µg/ml cytochalasin B, 10 µg/ml Hoechst 33342 stain and 10% (v/v) FBS at 38.5°C for 15 min, then mounted onto a micromanipulator (NT-88NE; Nikon-Narishige, Tokyo, Japan) equipped with epifluorescence. Each oocyte was held with a holding micropipette (20–30 µm inner diameter, 100–150 µm outer diameter), the first polar body and adjacent cytoplasm that contained MII chromosomes were removed using an aspiration micropipette (15–18 µm inner diameter, 20–25 µm outer diameter). The removed cytoplasm was checked for the presence of chromosomes and polar bodies by exposure to UV light. Only oocytes from which all chromosomes were removed were used for NT. One of the SFFs or GFFs was gently aspirated into a micropipette and deposited in the perivitelline space of an enucleated sheep oocyte. The karyoplast–cytoplast couplets were equilibrated in an electrofused medium composed of 0.3 M mannitol, 0.5 mM HEPES, 1% (m/v) fatty acid-free BSA (FAFBSA), 0.05 mM $CaCl_2$ and 0.1 mM $MgCl_2$ for 3 min, then transferred into a cell fusion chamber that contained the same medium used for electrofusion and using a fusion machine (EP-1 Voltain, CryoLogic Pty Ltd, Melbourne, Australia). Karyoplast–cytoplast couplets were aligned manually, then subjected to a double DC fusion pulse of 1.25 kV/cm for 80 µs, as described previously (Beaujean *et al.*, 2004). After electrofusion, the karyoplast–cytoplast couplets were transferred into H-SOF supplemented with 10% (v/v) FBS whilst waiting for fusion to complete. The non-fused couplets were subjected to a second round of fusion 1 h after the first. The fused embryos were activated by culture in H-SOF that contained 5 µM ionomycin and 10% (v/v) FBS for 5 min and were subsequently cultured in SOF that contained 2 mM 6-DMAP and 10% (v/v) FBS for 4 h. The activated embryos were washed twice and cultured in SOF supplemented with 2% (v/v) essential amino acids (Gibco); 1% (v/v) non-essential amino acids (Gibco); 8 mg/ml FAFBSA, 5% (v/v) FBS and 1 mM glutamine as described previously (Loi *et al.*,

2001). The embryos were monitored every 24 h for the progression of development and half the culture medium was replaced with fresh medium every 48 h.

In vitro fertilization and embryos culture

The procedure of *in vitro* fertilization has been described previously and was followed with some modification (Borowczyk *et al.*, 2006). Frozen rams semen was thawed in 2 ml of fertilization medium composed of SOF supplemented with 20% oestrus sheep serum, 5 IU/ml heparin, 5 mM caffine, and were kept in a CO_2 incubator (38.5°C) at an angle of 45°. The sperm were allowed to swim up for 30 min. The matured oocytes were washed 2–3 times and then transferred to 2 ml of fertilization medium. The highly motile spermatozoa from the upper layers were added to the oocytes at a concentration of 1–2 × 10^6/ml approximately, and incubated for 18 h at 38.5°C in a humidified atmosphere of 5% CO_2. Then, the presumptive fertilized embryos were washed 2–3 times and cultured in modified SOF as described above; the progression of embryo development was monitored every 24 h and half the culture medium was replaced every 48 h.

Immunohistochemical labeling of SSEA-1 on the surface of embryo

Embryos derived from nuclear transfer and *in vitro* fertilization were collected at the different developmental stages, 2-cell, 4-cell, 8-cell and morula. Embryos were washed twice in PBS, then placed in PBS supplemented with 0.25% (m/v) pronase for 3–5 min. The zona pellucida was removed completely, then embryos were transferred immediately into PBS. Mouse anti-SSEA-1 monoclonal antibody (Developmental Studies Hybridoma Bank, Iowa City, IA, USA) was used to detect SSEA-1 on the surface of embryos as described previously (Kühholzer *et al.*, 2000; Yan *et al.*, 2008; Park *et al.*, 2009). In brief, embryos were incubated in mouse anti-SSEA-1 monoclonal antibody (1:100) at room temperature for 30 min. Embryos were washed twice in PBS, then incubated in a secondary antibody solution (1.5 µg/ml), either FITC- or TRITC-conjugated rabbit anti-mouse IgG + IgM (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) for 30 min at room temperature in the dark. Subsequently, embryos were washed once in PBS, then placed in a microdroplet of PBS, overlaid with paraffin oil, and observed under an epifluorescence microscope.

Collection of total RNA from embryos

Embryos derived from nuclear transfer and *in vitro* fertilization were collected at different developmental stages, 2-cell, 4-cell, 8-cell, morula and blastocyst, and

Table 1 Information on the primers used for RT-PCR

Species	Type of mRNA	GenBank accession no.	Primer sequences	Location	Product size (bp)
Sheep (*Ovis aries*)	Nanog	FJ970651	5'-CCTCAGTCTCCAGCAAAT-3' 5'-GAGTTCACCAAGACACCC-3'	197–404	208
	Oct4	FJ970649	5'-GTGTTCAGCCAAACGACT-3' 5'-TTCCTCCACCCACTTCT-3'	91–189	99
Goat (*Capra hircus*)	Nanog	AY786437	5'-CCCAGCAACGGCAGAATA-3' 5'-GGTGGCCTCCAGATCACA-3'	489–780	292
	Oct4	HM807284	5'-AGAAAGCGGACGAGTATCGA-3' 5'-GGTGGCCTCCAGATCACA-3'	696–1064	369
pSPTet3 plasmid	Positive control RNA	–	5'-CTGCTCGCTTCGCTACTTGGA-3' 5'-CGGCACCTGTCCTACGAGTTG-3'	–	462

washed three times in PBS. Then, individual embryos were transferred into polymerase chain reaction (PCR) tubes that contained 18 µl ice-cold Cells-to-cDNA II Cell Lysis Buffer (Ambion, Applied Biosystems Inc., Foster City, CA, USA). The mixture was incubated at 75°C for 15 min, then removed from the heat source and placed on ice. Next, 2 µl RNase-free DNase I (2 U/µl, Ambion) was added and the mixture was incubated at 37°C for 15 min to degrade genomic DNA, and then subsequently incubated at 75°C for 5 min to inactivate DNase I, centrifuged and then used as a template for reverse transcription PCR (RT-PCR). In addition, total RNA was extracted from SFFs and GFFs using the same procedure as described above.

RT-PCR and products sequencing

Four pairs of RT-PCR primers were designed based on the sequences of sheep or goat *Oct4* mRNA, or sheep or goat *Nanog* mRNA, using Primer Premier 5.0 software and described in Table 1. In addition, details of positive control RNA from the TaKaRa One Step RNA PCR Kit (TaKaRa, Dalian, Liaoning, China) and its corresponding primers are listed in Table 1. Total RNA from embryos was subjected to RT-PCR using the TaKaRa The One Step RNA PCR Kit. RT-PCR reaction mixture consisted of 5 µl 10× One Step RNA PCR buffer, 10 µl MgCl$_2$ (25 mM), 5 µl dNTP mixture (10 mM each), 1 µl RNase inhibitor (40 U/µl), 1 µl AMV RTase XL (5 U/µl), 1 µl AMV-Optimized *Taq* (5 U/µl); 1 µl forward primer (20 µM), 1 µl reverse primer (20 µM), 1 µl positive control forward primer (20 µM), 1 µl positive control reverse primer (20 µM), 20 µl total RNA, 1 µl positive control RNA and 2 µl RNase-free H$_2$O in a total volume of 50 µl. Moreover, total RNA extracted from SFFs and GFFs was also subjected to RT-PCR in the same reaction system as described above, and was used as a negative control to confirm that the total RNA from embryos and somatic cells was not contaminated by genomic DNA. An RT reaction was carried out at 42°C for 30

min, followed by a step of 94°C for 2 min to inactivate AMV RTase. The RT reaction mixture was used directly for PCR. PCR was performed with the parameters of denaturation at 94°C for 30 s, annealing at a specified temperature (45°C for sheep-specific primers and 50°C for goat-specific primers) for 30 s and an extension step at 72°C for 60 s using a thermocycler (DNA Engine DYAD; MJ Research Inc., Waltham, MA, USA). After 45 cycles, the correct PCR products were identified by electrophoresis and sequencing.

Statistical analyses

The developmental rate of embryos was compared statistically by chi-squared analysis using SPSS software (SPSS Inc., Chicago, IL, USA). Differences at a *P*-value <0.05 were considered to be statistically significant.

Results

Developmental ability of embryos

After culture *in vitro*, all types of embryos were able to develop into blastocysts, even hatched blastocysts (Fig. 1), but the developmental rates were different. As shown in Table 2, at different developmental stages, the developmental rate of *in vitro* fertilized embryos was significantly higher than that of cloned embryos. At the 2-cell to 8-cell stages, the two types of cloned embryos had comparable developmental ability. However, at the morula and blastocyst stages, the developmental rate of intra-species cloned embryos was significantly higher than that of the inter-species cloned embryos.

Expression of SSEA-1 on the surface of embryos

As shown in Fig. 2, SSEA-1 could be detected on the blastomere's outer surface in cloned or in *in vitro* fertilized embryos at the 8-cell and morula

Table 2 The development of embryos derived from *in vitro* fertilization and nuclear transfer*

Type of embryo	No. of cultured embryos	Rate of embryos in different developmental stages (developed embryos/cultured embryos)				
		2-cell stage	4-cell stage	8-cell stage	Morula stage	Blastocyst stage
Sheep *in vitro* fertilized embryos	108	81.5[a] (88/108)	75.0[a] (81/108)	63.0[a] (68/108)	53.7[a] (58/108)	25.9[a] (28/108)
Sheep–sheep intra-species cloned embryos	103	63.1[b] (65/103)	57.3[b] (59/103)	51.5[b] (53/103)	37.9[b] (39/103)	14.6[b] (15/103)
Goat–sheep inter-species cloned embryos	119	60.5[b] (72/119)	53.8[b] (64/119)	47.1[b] (56/119)	18.5[c] (22/119)	6.7[c] (8/119)

Within the same column, values with different superscripts are significantly different ($P < 0.05$).
*Data were collected from 15 series of nuclear transfer and *in vitro* fertilization experiments.
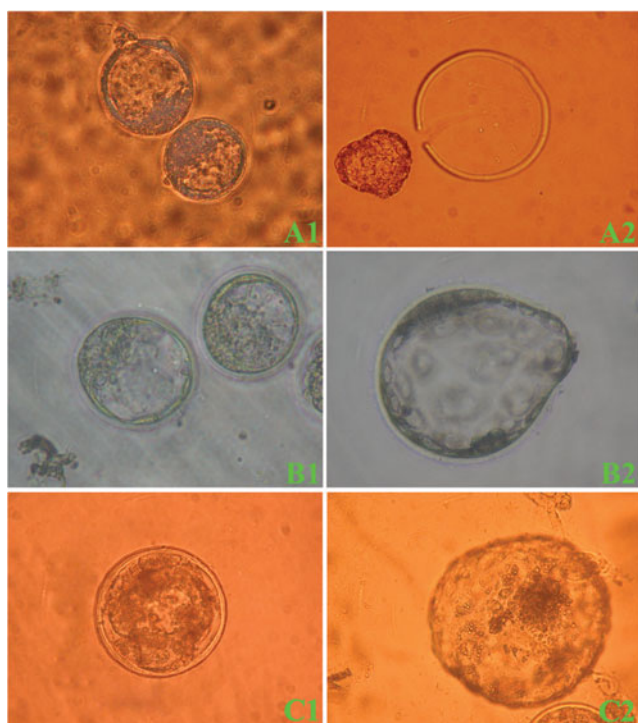

Figure 1 The development of embryos derived from *in vitro* fertilization and nuclear transfer. (*A1*) blastocyst derived from intra-species nuclear transfer. (*A2*) Hatched blastocyst derived from intra-species nuclear transfer. (*B1*) Blastocyst derived from inter-species nuclear transfer. (*B2*) Hatched blastocyst derived from inter-species nuclear transfer. (*C1*) Blastocyst derived from *in vitro* fertilization. (*C2*) Expanded blastocyst derived from *in vitro* fertilization.

developmental stages. Interestingly, as shown in Table 3, not all embryos could express SSEA-1 at the 8-cell stage; only 80.0, 66.7 and 30.0% of *in vitro* fertilized embryos, intra-species and inter-species cloned embryos, respectively, could express SSEA-1. However, at the morula stage, all detected embryos could express SSEA-1.

Expression of *Oct4* and *Nanog* mRNA in embryos

As shown in Fig. 3, all types of embryos expressed *Oct4* mRNA at the morula (M) and blastocyst (B) stages. Moreover, as shown in Table 4, at the morula stage only 80.0, 60.0 and 33.3% of *in vitro* fertilized embryos, intra-species and inter-species cloned embryos, respectively, could express *Oct4* mRNA. At the blastocyst stage, the rate of *Oct4* mRNA-expressing embryos increased to 100, 85.7 and 50.0% respectively for three types of embryos. Also shown in Fig. 3, *Nanog* mRNA was expressed in embryos as late as the blastocyst stage; the rate of *Nanog* mRNA-expressing embryos was 100, 87.5 and 40.0% respectively for three types (Table 4).

When total RNA extracted from SFFs or GFFs was used as a template, *Oct4* and *Nanog* mRNA products could not be amplified by RT-PCR (Fig. 3). This result indicated that RT-PCR products were derived from mRNA, not from genomic DNA. Moreover, products of positive control RNA could be amplified in all RT-PCR reactions (Fig. 3), this indicated that the RT-PCR reaction system was applicable for use. The sizes of the RT-PCR products are shown in Table 1, and were confirmed by electrophoresis (Fig. 3) and sequencing (data not shown).

Discussion

In present study, embryos with the lowest developmental ability were inter-species cloned embryos, as only 6.7% of fused embryos could develop to the blastocyst stage, and this rate was significantly lower than that of *in vitro* fertilized embryos (25.9%) and intra-species cloned embryos (14.6%). This result indicated that sheep oocytes had the capacity comparatively to reprogram homogenous donor nuclei. Similar results have been obtained in other studies, in which bovine and rabbit oocytes were used as nuclear recipients, and in which the

Table 3 The expression of SSEA-1 on the surface of embryos derived from *in vitro* fertilization and nuclear transfer*

Type of embryo	Rate of SSEA-1-expressing embryos in different developmental stages (expressed embryos/detected embryos)			
	2-cell stage	4-cell stage	8-cell stage	Morula stage
Sheep *in vitro* fertilized embryos	0 (0/7)	0 (0/8)	80.0 (4/5)	100.0 (7/7)
Sheep–sheep intra-species cloned embryos	0 (0/7)	0 (0/5)	66.7 (6/9)	100.0 (5/5)
Goat–sheep inter-species cloned embryos	0 (0/5)	0 (0/6)	30.0 (3/10)	100.0 (4/4)

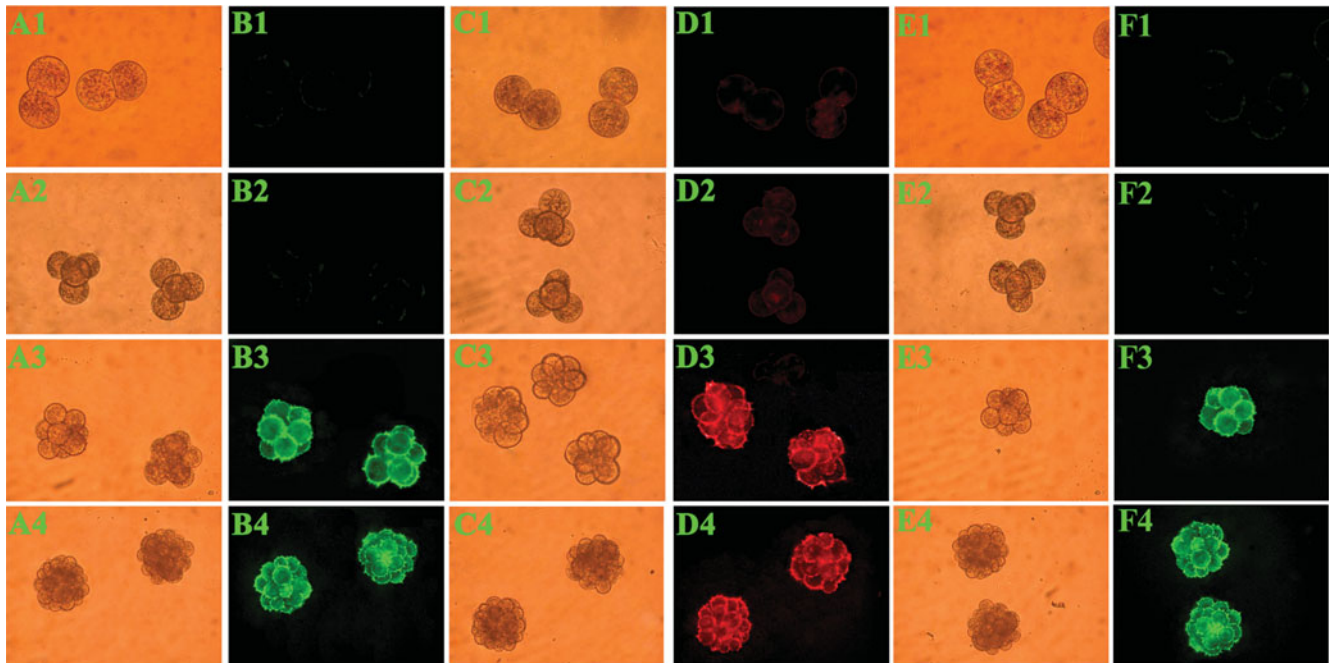*Data were collected from three series of immunohistochemical labeling experiments.

Figure 2 Detection of SSEA-1 on the surface of embryos. (*A, B*) mouse anti-SSEA-1 monoclonal antibody was used as primary antibody, FITC-conjugated rabbit anti-mouse IgG + IgM was used as secondary antibody, SSEA-1 on the surface of intra-species cloned embryos were detected. (*C, D*) mouse anti-SSEA-1 monoclonal antibody was used as primary antibody, TRITC-conjugated rabbit anti-mouse IgG + IgM was used as secondary antibody, SSEA-1 on the surface of goat–sheep inter-species cloned embryos were detected. (*E, F*) Mouse anti-SSEA-1 monoclonal antibody was used as primary antibody, FITC-conjugated rabbit anti-mouse IgG + IgM was used as secondary antibody, SSEA-1 on the surface of *in vitro* fertilized embryos were detected. (*A, C, E*) Observed under visible light; (*B, D, F*) observed under fluorescence. (*A1–F1*): 2-cell embryos; (*A2–F2*): 4-cell embryos; (*A3–F3*): 8-cell embryos; (*A4–F4*) morula.

developmental ability of intra-species cloned embryos was significantly higher than that of inter-species cloned embryos (Murakami *et al.*, 2005; Li *et al.*, 2006; Zhao *et al.*, 2006; Lorthongpanich, *et al.*, 2008).

In the present study, the developmental progression of cloned embryos showed that the difference in blastocyst rate between intra-species and inter-species cloned embryos was mainly due to poor development of inter-species cloned embryos from the 8-cell to the morula stages. Zygotic gene activation (ZGA) is the critical event that governs the transition from maternal to embryonic control of development, and a failure in ZGA can result in the developmental block of embryos. Previous studies have shown that, in both

sheep and goat embryos, ZGA occurs at the 8-cell to 16-cell stages (Ferrer *et al.*, 1995; Pivko *et al.*, 1995). Therefore, in the present study, the low blastocyst rate of inter-species cloned embryos is largely attributed to a failure in ZGA. This conclusion was confirmed as only 30% of inter-species cloned embryos could reactivate gene of *SSEA-1* at the 8-cell stage, a figure that was lower than that of *in vitro* fertilized embryos (80.0%) and intra-species cloned embryos (66.7%).

SSEA-1 is a type of ES (or ES-like) cell surface marker in several species, including mouse, rat, goat, and sheep (Solter & Knowles, 1978; Vassilieva *et al.*, 2000; He *et al.*, 2006; Dattena *et al.*, 2006). During early development of mouse embryos, SSEA-1 is first

Table 4 The expression of *Oct4* and *Nanog* mRNA in embryos derived from *in vitro* fertilization and nuclear transfer[*]

Type of embryo	Rate of *Oct4* mRNA-expressing embryos in different developmental stages (expressed embryos/detected embryos)					Rate of *Nanog* mRNA-expressing embryos in different developmental stages (expressed embryos/detected embryos)				
	2-cell stage	4-cell stage	8-cell stage	Morula stage	Blastocyst stage	2-cell stage	4-cell stage	8-cell stage	Morula stage	Blastocyst stage
Sheep *in vitro* fertilized embryos	0 (0/8)	0 (0/10)	0 (0/6)	80.0 (4/5)	100 (5/5)	0 (0/5)	0 (0/8)	0 (0/9)	0 (0/5)	100 (6/6)
Sheep–sheep intra-species cloned embryos	0 (0/5)	0 (0/6)	0 (0/5)	60.0 (3/5)	85.7 (6/7)	0 (0/5)	0 (0/6)	0 (0/6)	0 (0/5)	87.5 (7/8)
Goat–sheep inter-species cloned embryos	0 (0/7)	0 (0/8)	0 (0/6)	33.3 (2/6)	50.0 (3/6)	0 (0/7)	0 (0/5)	0 (0/5)	0 (0/7)	40.0 (2/5)

[*]Data were collected from 5–10 series of RT-PCR experiments.
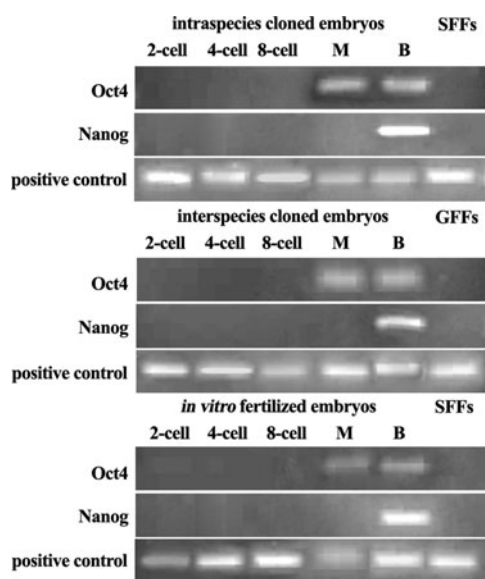
Figure 3 Electrophoresis of Oct4 and Nanog cDNA.

detected on blastomeres at the 8-cell stage (Solter & Knowles, 1978), whereas in other species the early expression of SSEA-1 has not been reported. In the present study, three types of embryos initially expressed SSEA-1 at the 8-cell stage, and SSEA-1 was still expressed at the morula stage. This result indicated that the timing of SSEA-1 expression is identical during early development of mouse, sheep and goat embryos. Moreover, in the present study, a low percentage of intra-species cloned embryos could express SSEA-1 at the 8-cell stage compared with *in vitro* fertilized embryos, this rate was even lower in inter-species cloned embryos. We speculate that this result may be a consequence of only small numbers of cloned embryos able to successfully reactivate zygotic genes, which subsequently resulted in a lower rate of SSEA-1-expressing embryos in cloned embryos. Furthermore, statistical analysis showed that, at the 8-

cell stage, the rate (80.0% for *in vitro* fertilized embryos; 66.7% for intra-species and 30.0% for inter-species cloned embryos; Table 3) of SSEA-1-positive embryos was roughly comparable with the developmental rate (85.3% for *in vitro* fertilized embryos; 73.6% for intra-species and 39.3% for inter-species cloned embryos; Table 2) of 8-cell to morula stages, although the former was somewhat lower than the latter. This difference could be because the detection of SSEA-1 was performed before the occurrence of ZGA in some embryos. Therefore, SSEA-1 could be used as a surface marker to estimate the percentage of embryos that have already successfully reactivated zygotic genes and have the ability to develop into morula.

Oct4 and Nanog are transcription factors that are required to maintain the pluripotency and self-renewal of ES cells (Loh *et al.*, 2006). During the early development of mammal embryos, Oct4 and Nanog can be expressed at certain stages. In mouse embryos, the initial expression of Oct4 and Nanog is at the 8-cell and morula stages respectively, whereas in bovine embryos, these two genes are initially expressed at the 16-cell and blastocyst stages respectively (Chambers *et al.*, 2003; Kurosaka *et al.*, 2004). In the present study, three types of embryos initially expressed *Oct4* and *Nanog* mRNA at the morula and blastocyst stages respectively. These results indicated that the expression pattern of *Oct4* and *Nanog* mRNA is similar in ruminants. Moreover, in the present study, the failure to reactivate *Oct4* and *Nanog* genes was observed in some embryos, although these embryos could develop to the morula stage, even the blastocyst stage, and this phenomenon more frequently occurred in inter-species cloned embryos. In previous studies, the failure to reactivate pluripotency genes and their abnormal expression were also observed in a some intra-species and inter-species cloned embryos (Boiani *et al.*, 2002; Chung *et al.*, 2009; Wang *et al.*, 2011). These studies, together with our study, indicated that the developmental competence of some cloned embryos

is already compromised at the blastocyst stage due to non-activation of certain pluripotency genes. This factor is possibly a reason for the high pregnancy loss of cloned embryos and low survival of cloned offspring.

In conclusion, three types of embryos can develop *in vitro* to the blastocyst stage. *In vitro* fertilized embryos have a higher blastocyst rate than cloned embryos, and ZGA has an important influence on the blastocyst rate of cloned embryos. Moreover, only some cloned embryos can express *SSEA-1*, *Oct4* and *Nanog* genes during early development; the failure in reactivation of some pluripotency genes may be a reason for the low survival rate of cloned embryos in subsequent development.

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References

Armstrong, L., Lako, M., Dean, W. & Stojkovic, M. (2006). Epigenetic modification is central to genome reprogramming in somatic cell nuclear transfer. *Stem Cells* **24**, 805–14.

Aston, K.I., Li, G.P., Hicks, B.A., Sessions, B.R., Davis, A.P., Rickords, L.F., Stevens, J.R. & White, K.L. (2010). Abnormal levels of transcript abundance of developmentally important genes in various stages of preimplantation bovine somatic cell nuclear transfer embryos. *Cell Reprogram* **12**, 23–32.

Beaujean, N., Taylor, J., Gardner, J., Wilmut, I., Meehan, R. & Young, L. (2004). Effect of limited DNA methylation reprogramming in the normal sheep embryo on somatic cell nuclear transfer. *Biol. Reprod.* **71**, 185–93.

Beyhan, Z., Forsberg, E.J., Eilertsen, K.J., Kent-First, M. & First, N.L. (2007). Gene expression in bovine nuclear transfer embryos in relation to donor cell efficiency in producing live offspring. *Mol. Reprod. Dev.* **74**, 18–27.

Boiani, M., Eckardt, S., Schöler, H.R. & McLaughlin, K.J. (2002). Oct4 distribution and level in mouse clones: consequences for pluripotency. *Genes Dev.* **16**, 1209–19.

Borowczyk, E., Caton, J.S., Redmer, D.A., Bilski, J.J., Weigl, R.M., Vonnahme, K.A., Borowicz, P.P., Kirsch, J.D., Kraft, K.C., Reynolds, L.P. & Grazul-Bilska, A.T. (2006). Effects of plane of nutrition on *in vitro* fertilization and early embryonic development in sheep. *J. Anim. Sci.* **84**, 1593–9.

Brambrink, T., Foreman, R., Welstead, G.G., Lengner, C.J., Wernig, M., Suh, H. & Jaenisch, R. (2008). Sequential expression of pluripotency markers during direct reprogramming of mouse somatic cells. *Cell Stem Cell* **2**, 151–9.

Chambers, I., Colby, D., Robertson, M., Nichols, J., Lee, S., Tweedie, S. & Smith, A. (2003). Functional expression cloning of Nanog, a pluripotency sustaining factor in embryonic stem cells. *Cell* **113**, 643–55.

Chen, Y., He, Z.X., Liu, A., Wang, K., Mao, W.W., Chu, J.X., Lu, Y., Fang, Z.F., Shi, Y.T., Yang, Q.Z., Chen, da Y., Wang, M.K., Li, J.S., Huang, S.L., Kong, X.Y., Shi, Y.Z., Wang, Z.Q., Xia, J.H., Long, Z.G., Xue, Z.G., Ding, W.X. & Sheng, H.Z. (2003). Embryonic stem cells generated by nuclear transfer of human somatic nuclei into rabbit oocytes. *Cell Res.* **13**, 251–63.

Chung, Y., Bishop, C.E., Treff, N.R., Walker, S.J., Sandler, V.M., Becker, S., Klimanskaya, I., Wun, W.S., Dunn, R., Hall, R.M., Su, J., Lu, S.J., Maserati, M., Choi, Y.H., Scott, R., Atala, A., Dittman, R. & Lanza, R. (2009). Reprogramming of human somatic cells using human and animal oocytes. *Cloning Stem Cells* **11**, 213–23.

Dattena, M., Chessa, B., Lacerenza, D., Accardo, C., Pilichi, S., Mara, L., Chessa, F., Vincenti, L. & Cappai, P. (2006). Isolation, culture, and characterization of embryonic cell lines from vitrified sheep blastocysts. *Mol. Reprod. Dev.* **73**, 31–9.

Ferrer, F., Garcia, C., Villar, J. & Arias, M. (1995). Ultrastructural study of the early development of the sheep embryo. *Anat. Histol. Embryol.* **24**, 191–6.

Fulka Jr, J. & Fulka, H. (2007). Somatic cell nuclear transfer (SCNT) in mammals: the cytoplast and its reprogramming activities. *Adv. Exp. Med. Biol.* **591**, 93–102.

He, S., Pant, D., Schiffmacher, A., Bischoff, S., Melican, D., Gavin, W. & Keefer, C. (2006). Developmental expression of pluripotency determining factors in caprine embryos: novel pattern of NANOG protein localization in the nucleolus. *Mol. Reprod. Dev.* **73**, 1512–22.

Hong, S.G., Oh, H.J., Park, J.E., Kim, M.J., Kim, G.A., Koo, O.J., Jang, G. & Lee, B.C. (2012). Production of transgenic canine embryos using interspecies somatic cell nuclear transfer. *Zygote* **20**, 67–72.

Hosseini, S.M., Hajian, M., Forouzanfar, M., Moulavi, F., Abedi, P., Asgari, V., Tanhaei, S., Abbasi, H., Jafarpour, F., Ostadhosseini, S., Karamali, F., Karbaliaie, K., Baharvand, H. & Nasr-Esfahani, M.H. (2012). Enucleated ovine oocyte supports human somatic cells reprogramming back to the embryonic stage. *Cell Reprogram* **14**, 155–63.

Hua, S., Zhang, Y., Song, K., Song, J., Zhang, Z., Zhang, L., Zhang, C., Cao, J. & Ma, L. (2008). Development of bovine–ovine interspecies cloned embryos and mitochondria segregation in blastomeres during preimplantation. *Anim. Reprod. Sci.* **105**, 245–57.

Kühholzer, B., Baguisi, A. & Overström, E.W. (2000). Long-term culture and characterization of goat primordial germ cells. *Theriogenology* **53**, 1071–9.

Kurosaka, S., Eckardt, S. & McLaughlin, K.J. (2004). Pluripotent lineage definition in bovine embryos by Oct4 transcript localization. *Biol. Reprod.* **71**, 1578–82.

Lanza, R.P., Cibelli, J.B., Diaz, F., Moraes, C.T., Farin, P.W., Farin, C.E., Hammer, C.J., West, M.D. & Damiani, P. (2000). Cloning of endangered species (*Bos gaurus*) using interspecies nuclear transfer. *Cloning* **2**, 79–90.

Lee, E., Kim, J.H., Park, S.M., Jeong, Y.I., Lee, J.Y., Park, S.W., Choi, J., Kim, H.S., Jeong, Y.W., Kim, S., Hyun, S.H. & Hwang, W.S. (2008). The analysis of chromatin remodeling and the staining for DNA methylation and histone acetylation do not provide definitive indicators of the developmental ability of inter-species cloned embryos. *Anim. Reprod. Sci.* **105**, 438–50.

Li, X., Kato, Y. & Tsunoda, Y. (2005). Comparative analysis of development-related gene expression in mouse preimplantation embryos with different developmental potential. *Mol. Reprod. Dev.* **72**, 152–60.

Li, Y., Dai, Y., Du, W., Zhao, C., Wang, H., Wang, L., Li, R., Liu, Y., Wan, R. & Li, N. (2006). Cloned endangered species takin (*Budorcas taxicolor*) by inter-species nuclear transfer and comparison of the blastocyst development with yak (*Bos grunniens*) and bovine. *Mol. Reprod. Dev.* **73**, 189–95.

Loh, Y.H., Wu, Q., Chew, J.L., Vega, V.B., Zhang, W., Chen, X., Bourque, G., George, J., Leong, B., Liu, J., Wong, K.Y., Sung, K.W., Lee, C.W., Zhao, X.D., Chiu, K.P., Lipovich, L., Kuznetsov, V.A., Robson, P., Stanton, L.W., Wei, C.L., Ruan, Y., Lim, B. & Ng, H.H. (2006). The Oct4 and Nanog transcription network regulates pluripotency in mouse embryonic stem cells. *Nat. Genet.* **38**, 431–40.

Loi, P., Ptak, G., Barboni, B., Fulka Jr, J., Cappai, P. & Clinton, M. (2001). Genetic rescue of an endangered mammal by cross-species nuclear transfer using post-mortem somatic cells. *Nat. Biotechnol.* **19**, 962–4.

Lorthongpanich, C., Laowtammathron, C., Chan, A.W., Ketudat-Cairns, M. & Parnpai, R. (2008). Development of interspecies cloned monkey embryos reconstructed with bovine enucleated oocytes. *J. Reprod. Dev.* **54**, 306–13.

Ma, L.B., Yang, L., Hua, S., Cao, J.W., Li, J.X. & Zhang, Y. (2008a). Development *in vitro* and mitochondrial fate of interspecies cloned embryos. *Reprod. Domest. Anim.* **43**, 279–85.

Ma, L.B., Yang, L., Zhang, Y., Cao, J.W., Hua, S. & Li, J.X. (2008b). Quantitative analysis of mitochondrial RNA in goat–sheep cloned embryos. *Mol. Reprod. Dev.* **75**, 33–9.

Murakami, M., Otoi, T., Wongsrikeao, P., Agung, B., Sambuu, R. & Suzuki, T. (2005). Development of interspecies cloned embryos in yak and dog. *Cloning Stem Cells* **7**, 77–81.

Niemann, H., Tian, X.C., King, W.A. & Lee, R.S. (2008). Epigenetic reprogramming in embryonic and foetal development upon somatic cell nuclear transfer cloning. *Reproduction* **135**, 151–63.

Park, P.J., Colletti, E., Ozturk, F., Wood, J.A., Tellez, J., Almeida-Porada, G. & Porada, C. (2009). Factors determining the risk of inadvertent retroviral transduction of male germ cells after in utero gene transfer in sheep. *Hum. Gene Ther.* **20**, 201–15.

Pivko, J., Grafenau, P. & Kopecný, V. (1995). Nuclear fine structure and transcription in early goat embryos. *Theriogenology* **44**, 661–71.

Solter, D. & Knowles, B.B. (1978). Monoclonal antibody defining a stage-specific mouse embryonic antigen (SSEA-1). *Proc. Natl. Acad. Sci. USA* **75**, 5565–9.

Srirattana, K., Imsoonthornruksa, S., Laowtammathron, C., Sangmalee, A., Tunwattana, W., Thongprapai, T., Chaimongkol, C., Ketudat-Cairns, M. & Parnpai, R. (2012). Full-term development of gaur–bovine interspecies somatic cell nuclear transfer embryos: effect of trichostatin A treatment. *Cell Reprogram* **14**, 248–57.

Stadtfeld, M., Maherali, N., Breault, D.T. & Hochedlinger, K. (2008). Defining molecular cornerstones during fibroblast to iPS cell reprogramming in mouse. *Cell Stem Cell* **2**, 230–40.

Sugawara, A., Sugimura, S., Hoshino, Y. & Sato, E. (2009). Development and spindle formation in rat somatic cell nuclear transfer (SCNT) embryos *in vitro* using porcine recipient oocytes. *Zygote* **17**, 195–202.

Takahashi, K. & Yamanaka, S. (2006). Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* **126**, 663–76.

Takahashi, K., Tanabe, K., Ohnuki, M., Narita, M., Ichisaka, T., Tomoda, K. & Yamanaka, S. (2007). Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* **131**, 861–72.

Vassilieva, S., Guan, K., Pich, U. & Wobus, A.M. (2000). Establishment of SSEA-1- and Oct-4-expressing rat embryonic stem-like cell lines and effects of cytokines of the IL-6 family on clonal growth. *Exp. Cell Res.* **258**, 361–73.

Wakayama, T., Tabar, V., Rodriguez, I., Perry, A.C., Studer, L. & Mombaerts, P. (2001). Differentiation of embryonic stem cell lines generated from adult somatic cells by nuclear transfer. *Science* **292**, 740–3.

Wang, K., Out, H.H., Chen, Y., Lee, Y., Latham, K. & Cibelli, J.B. (2011). Reprogrammed transcriptome in rhesus–bovine interspecies somatic cell nuclear transfer embryos. *PLoS One* **6**, e22197.

Wilmut, I., Schnieke, A.E., McWhir, J., Kind, A.J. & Campbell, K.H. (1997). Viable offspring derived from fetal and adult mammalian cells. *Nature* **385**, 810–3.

Wilmut, I., Beaujean, N., de Sousa, P.A., Dinnyes, A., King, T.J., Paterson, L.A., Wells, D.N. & Young, L.E. (2002). Somatic cell nuclear transfer. *Nature* **419**, 583–6.

Xing, X., Magnani, L., Lee, K., Wang, C., Cabot, R.A. & Machaty, Z. (2009). Gene expression and development of early pig embryos produced by serial nuclear transfer. *Mol. Reprod. Dev.* **76**, 555–63.

Yan, L., Lei, L., Yang, C., Gao, Z., Lei, A., Ma, X. & Dou, Z. (2008). Improved isolation and culture of embryonic germ cells from Guanzhong dairy goat. *Sheng Wu Gong Cheng Xue Bao* **24**, 1670–6.

Yu, J., Vodyanik, M.A., Smuga-Otto, K., Antosiewicz-Bourget, J., Frane, J.L., Tian, S., Nie, J., Jonsdottir, G.A., Ruotti, V., Stewart, R., Slukvin, I.I. & Thomson, J.A. (2007). Induced pluripotent stem cell lines derived from human somatic cells. *Science* **318**, 1917–20.

Zhao, Z.J., Ouyang, Y.C., Nan, C.L., Lei, Z.L., Song, X.F., Sun, Q.Y. & Chen, D.Y. (2006). Rabbit oocyte cytoplasm supports development of nuclear transfer embryos derived from the somatic cells of the camel and tibetan antelope. *J. Reprod. Fertil.* **52**, 449–59.