Pakistan Veterinary Journal

ISSN: 0253-8318 (PRINT), 2074-7764 (ONLINE)
Accessible at: www.pvj.com.pk

RESEARCH ARTICLE

In Vitro Development Competence of Bovine Nuclear Transfer Embryos Derived from Nanog-Overexpressing Fibroblast Cells

Xi-bang Zheng[§], Yan Yun, Yong-ce Hu, Yong Li[1], Hua-yan Wang, Xiao-ling Ma, Jin-qiang Sui, An-min Lei and Zhong-ying Dou*

Shaanxi Branch of National Stem Cell Engineering and Technology Center, Animal Medicine College, Northwest University of Agriculture and Forestry, Yangling 712100, Shaanxi, P.R. China; [1]College of Life Science, Ningxia University, Yinchuan 750021, Ningxia, P.R. China [§]Also affiliated with Department of Animal Medicine, College of Animal Science and Technology, Guangxi University, Nanning 530005,Guangxi, P.R. China
*Corresponding author: douzhongying@china.com

ARTICLE HISTORY (13-029)

Received: January 18, 2013
Revised: April 27, 2013
Accepted: July 2, 2013

Key words:
Bovine
Development competence
Eukaryotic expression
Molecular cloning
Nanog
Somatic nuclear transfer

ABSTRACT

The purpose of this study was to establish Nanog-expressing cell lines that can be used as donor cells to construct transgenic cloned embryos, and to investigate their *in vitro* development competence. By reverse transcription-polymerase chain reaction (RT-PCR), the cDNA of Nanog gene was cloned from fetal bovine primordial genital ridge tissues. The gene was inserted into PMD18-T vector using recombination techniques and then subcloned into vector pEGFP-C1. After confirmation by restrictive endonuclease digestion and sequencing, the recombinant plasmid pEGFP-Nanog was transfected into skin fibroblast cells. A stable transfected cell line was successfully established after two months of selection with neomycine (G418). Fluorescence microscopy, RT-PCR, and Western Blotting assays indicated that Nanog mRNA and EGFP-Nanog fusion protein were expressed in these cells. The EGFP-Nanog expressing fibroblast cells and the intact fibroblast cells (BEF422) were respectively used to construct cloned embryos. The results showed that the cleavage rate of recombinant embryos in BEF422 cells was significantly ($P<0.05$) higher than in EGFP-Nanog expressing cells (82.14 *vs* 40.38%), but the blastocyst development rate in the latter was slightly higher than in the former (17.30 *vs* 14.29%) ($P<0.05$), indicating that Nanog-overexpressed fibroblasts may be a better candidate of donor cells. To our knowledge, this is the first time that Nanog gene has been introduced into fibroblast cells to produce cloned embryos in bovine.

To Cite This Article: Zheng XB, Y Yun, YC Hu, Y Li, HY Wang, XL Ma, JQ Sui, AM Lei and ZY Dou, 2014. *In vitro* development competence of bovine nuclear transfer embryos derived from Nanog-overexpressing fibroblast cells. Pak Vet J, 34(2): 155-160.

INTRODUCTION

Being transcribed specifically in pluripotent cells such as embryonic stem cells (ESCs), embryonic germ cells (EGCs) and embryonic carcinoma cells (ECs) in the mouse and human, Nanog is considered as an important transcription factor (Yamaguchia *et al.,* 2005; Huang *et al.,* 2007). Since Nanog prevents human ESCs and ECs differentiation into extraembryonic endoderm and trophectoderm lineages, it works as a gatekeeper of pluripotency (Hyslop *et al.,* 2005). Without feeder cells, overexpressed Nanog in human and primate ESCs leads to proliferation of these ES cells while their pluripotency is not compromised (Darr *et al.,* 2006). Nanog not only succeeded in reprogramming somatic cells in serum-free medium supplemented with leukemia inhibitory factor (LIF), but also in reprogramming epiblast-derived stem cells to naive pluripotency in serum-free medium alone (Theunissen *et al*., 2011a). It has been proven that Nanog′s expression in NIH3T3 cells enables their entry in S phase and improves cell propagation (Zhang *et al.,* 2005). Furthermore, Han *et al.* (2012) silenced endogenous NANOG expression in breast cancer cells by small interference RNA (RNAi) technology, and the results showed that silencing of Nanog expression decreases cell proliferation, colony formation and migration ability as well as cell cycle arrests at the G0/G1 phases. In addition, Nanog plays an important role in the propagation and survival of migrating PGCs of wild-type embryos (Yamaguchi *et al.,* 2009). Nanog along with

Oct4, Sox2, and Lin28 were also able to reprogram human skin fibroblast cells to iPS cells (Yu *et al.,* 2007). However, the ecotopic expression of Oct4, Sox2, Klf4, and c-Myc alone was not capable to induce adult fibroblasts to stable iPS cells in bovine, unless the addition of Nanog to the reprogramming cocktail (Sumer *et al.,* 2011). More interestingly, the capacity of Nanog to establish pluripotency is fully conserved in vertebrates (Theunissen *et al.,* 2011b). Taking all factors together, the above studies indicate that Nanog is an essential factor in maintaining pluripotency of ES cells.

It is believed that unfertilized eggs and ES cells contain factors by which pluripotency is given to somatic cells (Takahashi *et al.,* 2006). Somatic nuclear transfer (SNT) is an alternative approach to derivation of stem cells. A recent report showed that, as donor cells, putative ES cells perform better than adult fibroblasts and lymphocytes in cleavage and blastocyst production rate in goat (Dutta *et al.,* 2011). The activity of Nanog is closely linked to an undifferentiated state of cells even in nuclear reprogrammed somatic cells, indicating that Nanog serves a key regulator to maintain pluripotency in a dose-dependent manner (Hatano *et al.,* 2005). It is reasonably speculated that Nanog-overexpressing somatic cells may be a source of better donor cells for SNT. However, there are few reports accessible on the function of ectopic Nanog expression in somatic cells and in SNT embryo development in bovines.

In this study, bovine Nanog gene was cloned from fetal bovine primordial genital ridge tissues by means of RT-PCR. A eukaryotic expression plasmid pEGFP-Nanog was constructed and then transfected into bovine fibroblasts (BEF422) to obtain a stable cell line expressing Nanog. We also constructed SNT embryos originated respectively from control or Nanog-overexpressing fibroblast cells and compared their development potential *in vitro*.

MATERIALS AND METHODS

Experimental animals: The use of all animals in the present study was granted by the Committee of Animal Welfare, Northwest University of Agriculture and Forestry, Shaanxi, China.

Cloning and sequencing of Nanog gene: Primers were designed (Table 1) according to the Nanog mRNA sequence (NM001025344) in GenBank. Extracted from a fetal calf's primordial genital ridges, total RNA was reversely transcribed into the first strand of cDNA. PCR was initiated with pre-denaturation at 94°C for 4 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 65°C for 45 s, extension at 72°C for 1 min, and ended with extension for 10 min at 72°C. After purification, the PCR product was ligated to vector pMD18-T to produce a recombinant cloning plasmid (pMD18-T-Nanog), which was used to transform *E. coli* strain (DH5α). The nucleotide sequence of the target gene was confirmed in positive clones by DNA sequencing.

Construction and identification of expression vector: The sequencing-confirmed plasmid pMD18-T-Nanog was digested by *Bgl*II and *Sac*II, and the Nanog fragment was subcloned to pEGFP-C1 to obtain a recombinant expression plasmid (pEGFP-Nanog). DNA sequencing was performed to make sure that EGFP and Nanog would be in the same open reading frame (ORF).

Cell culture and transfection: An ear skin biopsy was removed from a Holstein cow whose genetic background was well known, and skin fibroblast cells (BEF422, Fig. 2A) were cultured in the DMEM medium (Gibco, USA) supplemented with 10% FBS (Gibco, USA). With 80-90% confluency, the cells on 12 well-plates were cotransfected by the endotoxin-free pEGFP-Nanog and lipofectamine 2000 reagent (Invitrogen, USA) according to the manufacturer's instructions. The medium was changed 24 h after transfection and the cells were trypsinized and split (1:4) before being cultured in fresh DMEM containing 10% FBS, 100 units/100 μg/mL penicillin/streptomycin (Gibco, USA), and 400 μg/mL G418 (Invitrogen, USA). The medium was changed every 3 to 4 days and after 4 to 5 weeks of selection, the positive clones appeared. The locations of these clones were marked on the bottom of culture plate, and negative cells around them were scraped away. Washed by PBS and then trypsinized, the positive clones were individually passed to a 24-well plate, one clone to one well. With a reduction of G418 to 200 μg/mL in the medium, the cells continuously grew until they were 70-80% confluent. In the same way, these cells were passed to 6-well plates and then to 60 mm dishes. Finally, these positive cells were conserved in a freezing medium in liquid nitrogen at -196°C.

Karyotyping: Karyotyping was performed as described in the previous report (Wang *et al.*, 2005). Briefly, EGFP-Nanog expressing cells were incubated in growth media containing 0.08 μg/mL of colcemid for 4-6 h at 37°C. Then, the cells were collected and treated in 0.57% KCl solution for 30 min at 37°C, followed by fixation in acetic methanol (1:3, v/v), and placing drops of cell suspension on clean glass slides to achieve good spreading. The chromosomes were stained with silver staining solution (1 volume of 2% gelatin and 2 volumes of 50% silver nitrate) in the dark at a constant temperature of 37°C for 12 min. The chromosomes were examined under a phase microscope at 1000× magnification.

RT-PCR assay: The pEGFP-Nanog-transfected fibroblast cells were thawed and subcultured at 37°C for subsequent RT-PCR analysis. Untransfected fibroblast cells were used for negative control. For semi-quantification of Nanog expression, the same amount of total RNA (400 ng) and cDNA (2 μL) were respectively used in reverse transcription and PCR. The primers for ß-actin and Nanog are listed in Table 1. The PCR protocol was the same as above except that annealing temperature ranged from 54 to 58°C.

Western Blotting assay: Western blotting was performed as previously reported (Zhang *et al.*, 2005). Briefly, BEF422 and the pEGFP-Nanog-transfected fibroblast cells were harvested, and protein samples were prepared with NP-40 lysis buffer (Beyotime, China), which were subsequently separated by 12% PAGE and transferred to

nitrocellulose (NC) membranes afterward. Then they were blocked in 5% nonfat milk in TBST overnight at 4°C, and incubated with HRP-conjugated anti-GFP antibody (1:10000, Abcam, UK) for 1 h at room temperature, the membrane was washed and incubated with Super Signal West Pico substrate (Pierce, USA) for 5 min, and finally exposed to X-ray film. Beta actin was used as internal control.

Flow cytometry analysis (FCA): After harvest and 3 times of washes in ice-cold PBS, the pEGFP-Nanog-transfected fibroblast cells were re-suspended in PBS supplemented with 0.1% BSA, and then fixed in 70% ethanol overnight. Then they were washed for 3 times and re-suspended at 1×10^6 cells/mL in ice-cold PBS containing 0.1% BSA for FCA.

Collection and maturation of oocytes: Oocyte collection and maturation were performed as previously described with minor modifications (Moore *et al.*, 2007). Briefly, bovine ovaries were collected in a local slaughterhouse, and follicular fluid was aspirated from follicles whose diameter ranged from 5-8 mm, from which cumulus-oocyte complexes (COCs) were recovered. After washing, the COCs in groups of 30-50 were transferred to 4-well dishes containing modified TCM-199 medium (Gibco, USA) supplemented with 2.5 μg/mL sodium pyruvate, 10 mM HEPES, 2 mM glutamine, 50 μL/mL insulin transferrin selenium (ITS) (Gibco, USA), 0.1 IU/mL human menopausal gonadotropin (hMG) (Baoli, China), 1 μg/mL estradiol-17ß, 50 μg/mL uracil, 10 ng/mL EGF and 10 mg/mL BSA. *In vitro* maturation of the COCs was conducted at 38.5°C in a humidified atmosphere of 5% CO_2 in air.

Nuclear transfer: Nuclear transfer was performed as previously described with minor modifications (Moore *et al.*, 2007). Briefly, metaphase II oocytes were enucleated by aspirating the polar body and adjacent cytoplasm. As donor cells, individual EGFP-Nanog-expressing fibroblasts (passage 6-8) and intact fibroblast cells were respectively transferred to the perivitelline space of enucleated oocytes and fused in Cytofusion Medium (Cyto-pulse, Sciences, USA) by using two successive DC pulses of 1.9kV/cm for 15 μs. Activation was carried out 1 h later, and reconstructed embryos were cultured consecutively in sequential medium G-1 and G-2 (Vitrolife, USA) at 38.5°C in a humidified atmosphere of 5% CO_2. On Day 7 to 9, blastocysts were collected for subsequent gene expression analysis.

Statistical analysis: The experiments of nuclear transfer were repeated four times. When each replicate was conducted, we ensured that oocytes would be the ones recovered and cultured on the same day in order to eliminate any effect of different batches. The software package SPSS 18.0 was used to analyze the data. $P<0.05$ was considered statistically significant.

RESULTS

Cloning of bovine Nanog gene: Agarose gel electrophoresis demonstrated that Nanog cDNA fragment was successfully amplified and its length (about 1 kb) was as expected (Fig. 1A). Furthermore, restrictive endonuclase digestion indicated that cDNA fragment inserted in pMD 18-T vector was also nearly 1 kb (Fig. 1B). DNA sequencing showed that this putative Nanog and another bovine Nanog homolog (GI: 70778751) shared 99.9% identity with only one nucleotide substitution-170 (T to C), concomitant with one amino acid substitution-58 (Thr to Ile), convincing that the cDNA fragment inserted in pMD 18-T vector was indeed bovine Nanog. This DNA sequencing-confirmed plasmid was named pMD18-T-Nanog.

Identification of eukaryotic expression vector of bovine Nanog: Restrictive endonuclease digestion and agarose gel electrophoresis illustrated that the Nanog fragment inserted in the pEGFP-C1 vector was a single one (about 1 kb) as shown in Fig. 1C, and DNA sequencing showed that both EGFP and Nanog were in

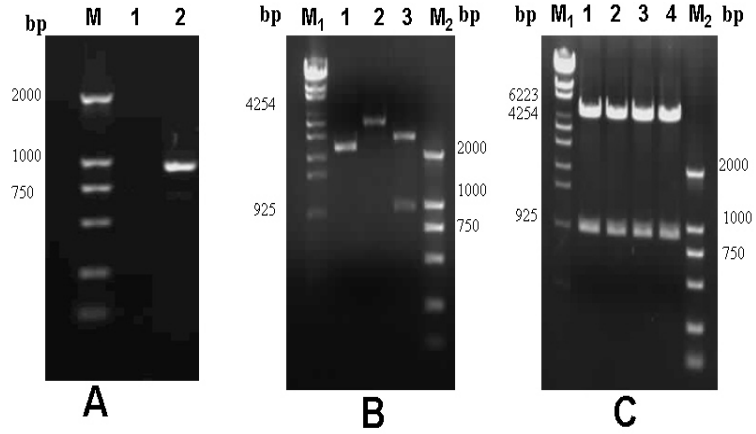

Fig.1: A) Nanog cDNA was cloned by RT-PCR. Lane M, DL2000 DNA marker; lane 1, Negative control using water; lane 2, PCR product of Nanog cDNA (about 1 kb). **B)** Nanog cDNA was ligated to pMD18-T vector by TA cloning. Both single and double digestion of the recombinant plasmid by restriction endonuclease showed that Nanog cDNA was inserted in pMD18-T vector. M_1, λ-*Eco*T14 digest DNA marker; lane 1, Intact pMD18-T-Nanog; lane 2, pMD18-T-Nanog digested by *Bgl*II; lane 3, pMD18-T-Nanog digested by *Bgl*II and *Sac*II; lane M_2, DL 2000 DNA marker. **C)** The eukaryotic expression vector-pEGFP-Nanog was successfully constructed. Lane M_1, λ-*Eco*T14 digest DNA marker; lane 1-4, pEGFP-Nanog digested by *Bgl*II and *Sac*II; lane M_2, DL 2000 DNA marker.

Table 1: Primers for PCR

mRNA	Direction	Primer sequence	Product size (bp)
Nanog(cloning)	Forward	5'-GGAAGATCTATGAGTGTGGGCCCAGCTTGTCCC-3'	900
	Reverse	5'-TCCCCGCGGCAAATCTTCAGGCTGTATGTTGAG-3'	
Nanog(expression)	Forward	5'-ATGAGTGTGGGCCCAGCT-3'	900
	Reverse	5'-TACAAATCTTCAGGCTGTATGTTG-3'	
ß-actin	Forward	5'-CAAGGACCTCTACGCCAACA-3'	444
	Reverse	5'-CTCGATCCAACCGACTGCT-3'	

The primers for Nanog (cloning), the underlined letters represent restrict enzyme sites of *Bgl*II and *Sac*II, respectively.

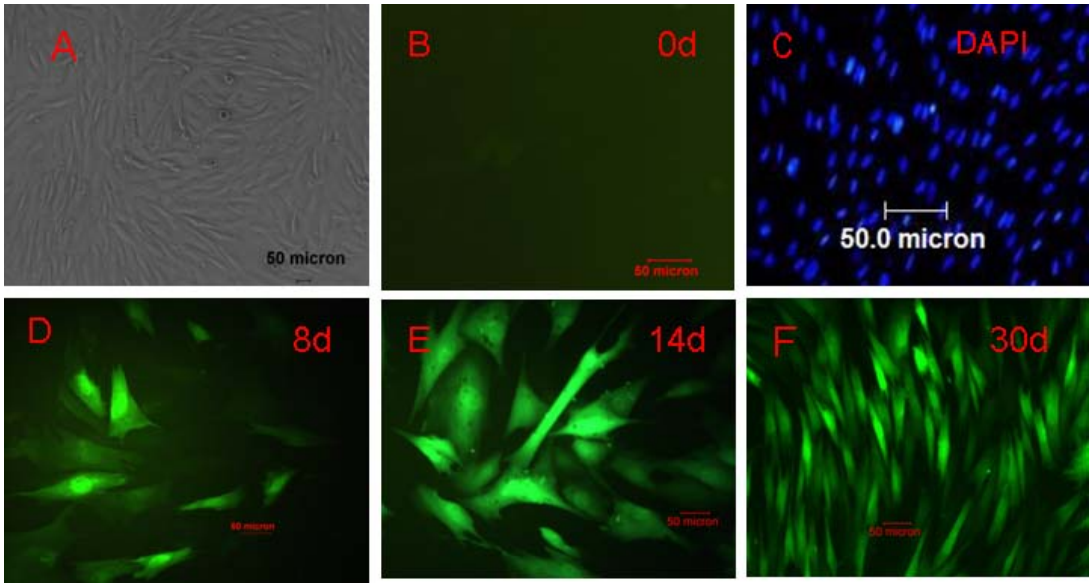

Fig. 2: Nanog gene was expressed in fibroblast cells. Isolated from an ear skin biopsy of a Holstein cow, primary bovine skin fibroblast cells were fusiformed in morphology (A). When 80-90% confluenced, the subcultured fibroblast cells were transfected by the plasmid pEGFP-Nanog with lipofectamine 2000, and G418 (400 µg/ml) was used to select stable transgenic cell clones. There were no fluorescencigenic cells in a negative control (B). The intact fibroblast cells were subsequently counterstained by DAPI (C). After 8 days of transfection, cells with green fluorescence were seen under inverted fluorescence microscope, (D) and the number of them increased after two weeks (E), and positive cell colonies appeared after 4 to 5 weeks (F).

Table 2: *In vitro* development of nuclear transfer embryos with transgentic donor cells and skin fibroblast cells (BEF422)

Donor cell types	Fused %	Cleaved %	Blastocysts%
BEF422	43.75 (56/128)[a]	82.14 (46/56)[a]	14.29 (8/56)[b]
EGFP-Nanog expressing cells	49.84 (156/313)[a]	40.38 (63/156)[b]	17.30 (27/156)[a]

a, b. Percentages with different superscripts within a column are significantly different (P<0.05) in four replications of nuclear transfer. fused, fusion rate of donor cells and enuculated oocytes; cleaved, cleavage rate of nuclear transfer embryos; blastocysts, blastocyst development rate.

the same ORF, which indicated that the eukaryotic expression vector of bovine Nanog has been successfully constructed. This plasmid was named pEGFP-Nanog.

Stable transfection of pEGFP–Nanog into skin fibroblast cells: One week after transfection, cells with green fluorescence were seen under inverted fluorescence microscope (Fig. 2D; Fig. 2E) with positive cell colonies appearing after 4 to 5 weeks (Fig. 2F). Morphologically, most of these cells were fibroblast-like, except with a small number being epithelium-like. The results of FCA showed that the purity of the EGFP-Nanog positive cells accounted for 91.6% of the total isolated cells (data not shown). In addition, the karyotyping of EGFP-Nanog expressing cells showed normal female bovine chromosomes, which was consistent with those of the original skin fibroblast cells (Fig. 3).

Expression of Nanog mRNA and EGFP-Nanog fusion protein in skin fibroblast cells: RT-PCR detection showed that Nanog mRNA expressed in the pEGFP-Nanog-transfected fibroblast cells, but not in the intact BEF422 (Fig. 4A). Western blotting assay also indicated that EGFP-Nanog fusion protein was detectable only in pEGFP-Nanog-transfected fibroblast cells (Fig. 4B).

***In vitro* development competence of transgenic cloned embryos:** The rates for cleavage and blastocyst development of SNT embryos derived from BEF422 and EGFP-Nanog expressing cells were compared (Table 2). The cleavage rate of NT embryos in BEF422 was significantly (P<0.05) higher than in EGFP-Nanog expressing cells (82.14 vs 40.38%), but the blastocyst development rate was slightly higher in the latter than in the former (17.30 *vs* 14.29%) (P<0.05). The EGFP-Nanog expressing embryos were readily seen under fluorescence microscope (Fig. 5).

DISCUSSION

It is well known that Nanog is an important factor to maintain pluripotency of ES cells. Firstly expressed in the compact morula, and then in the inner cell mass (ICM),

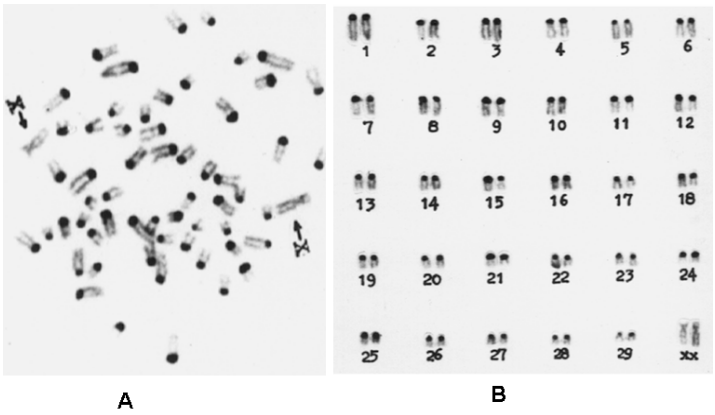

Fig. 3: Metaphase chromosome and karyotype of EGFP-Nanog expressing cells. Metaphase chromosome was prepared from EGFP-Nanog expressing cells (A), and karyotype analysis indicated that these transgenic cells keep the normal 30 XX karyotype (B).

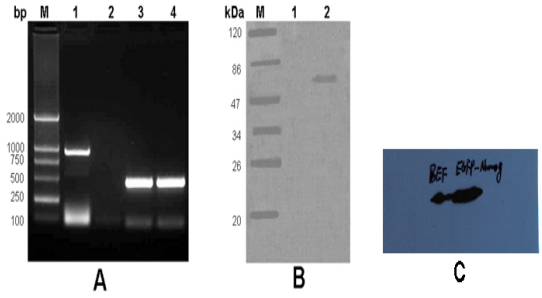

Fig. 4A: Expression of Nanog mRNA in the pEGFP-Nanog transfected fibroblast cells. Total RNA was extracted and RT-PCR analysis was performed with primers to amplify the coding regions of bovine Nanog. Beta actin was used as an internal control (lane3-4). Lane M, DL2000 DNA marker; lane 1, pEGFP-Nanog transfected bovine fibroblast cells; lane 2, Negative control (intact bovine fibroblast cells—BEF422); lane 3, beta actin for EGFP-Nanog expressing fibroblast cells; lane 4, beta actin for intact bovine fibroblast cells.

Fig. 4B: Immunodetection for expression of EGFP-Nanog fusion protein in skin fibroblast cells. 50 μg of protein samples prepared respectively from the pEGFP-Nanog transfected fibroblast cells and BEF422 were separated on SDS-PAGE and analyzed by western blot using anti-GFP antibody. The result showed that EGFP-Nanog fusion protein (about 61 KD) expressed in the pEGFP-Nanog transfected fibroblast cells (lane 2). Intact bovine fibroblast cells—BEF422 was used as negative control (lane 1). Lane M, Protein marker.

Fig. 4C: Internal control—Beta actin detection in intact fibroblast cells (BEF422) and in EGFP-Nanog expressing fibroblast cells.

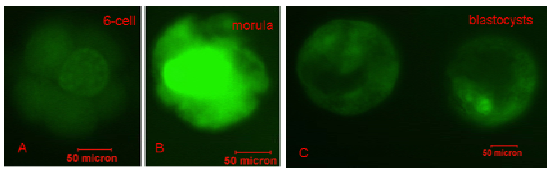

Fig. 5: In vitro development of cloned transgenic embryos. Bovine oocytes were collected from slaughterhouse ovaries, and matured *in vitro*. Oocytes at metaphase II were enucleated, into which single EGFP-Nanog expressing fibroblasts were injected. Fused and activated electrically, the cloned embryos developed to 6-cell stage (A), Morula (B), and blastocysts (C), reflecting green lights under inverted fluorescence microscope.

mouse Nanog expression is down-regulated since implantation, while it can be detected in germ cells of the genital ridges of E11.5 mouse embryos (Chamber *et al.*, 2003). Hambiliki *et al*. (2012) reported that Nanog and another transcript factor-Oct4 were co-expressed in morula and blastocyst of human embryos and in human ES cells. In vitro, Nanog mRNA is highly expressed in ES, EG and EC (embryonic carcinoma) cells, but not in adult tissues (Chamber *et al.*, 2003; Yamaguchia *et al.*, 2005; Huang *et al.*, 2007). Based on this knowledge, we successfully obtained bovine Nanog cDNA by RT-PCR from fetal bovine primordial genital ridges. Afterwards, the eukaryotic expression vector pEGFP-Nanog was constructed and transfected into skin fibroblast cells. After selection with neomycin (G418), stable transfected cells were obtained. As both the reporter gene (EGFP) and the gene of interest (Nanog) occur in the same ORF, Nanog-expressing fibroblast cells are green under fluorescence microscope and so it is possible to trace gene expression in subsequent experiments (such as nuclear transfer). Condition medium (CM) was collected from fibroblast cell cultures during the exponential growth phase and it was found that CM and DMEM (containing G418) at a ratio of 2 to 3 was favorable for the formation and proliferation of positive clones.

By means of SNT technique, animal clones have been successfully produced in different species such as cattle, pigs, goats and sheep etc, but the cloning efficiency has been so low that few of SNT embryos (< 1%) could give rise to live-born offspring. Moreover, a high rate of abortion during early gestation, and increased perinatal death were also linked to SNT (Han *et al.*, 2003). Therefore, insufficient reprogramming of the somatic nucleus by the oocyte may be responsible for the development failure of SNT embryos (Eckardt and McLaughlin, 2004). The fact that ES cells could successfully reprogram a differentiated nucleus implies that some factors expressed in ES cells may transfer the pluripotency to somatic cells. As an impotant factor to maintain ES cell pluripotency, Nanog is the most likely candidate, whose important role has been evidenced in the production of induced pluripotent cells (Okita *et al*., 2007; Yu *et al*., 2007; Takahashi *et al*., 2007). It is suggested that Nanog acts as a molecular switch to initiate the naive pluripo- tency in mammalian cells because it is indispensible for accomplishing somatic cell reprogramming during induction of pluripotency (Theunissen and Silva, 2011c). In other words, cells with pluripotency are better donor cells for nuclear transfer (NT). In the present study, we established EGFP-Nanog

expressing cell lines, which will likely be a source of promising donor cell candidates for NT.

It is reported that NT embryos derived from mesenchymal stem cells (MSCs) had stronger blastocyst development competence, and a lower occurance of apoptosis compared to those from fetal fibroblasts in porcine, suggesting that MSC-NT embryos are of higher quality than fibroblast NT embryos (Kumar *et al.*, 2007). Similarly, NT embryos derived from neural stem cells and amniotic fluid-derived stem cells also had higher developmental potential than those from somatic cells in porcine, implying that the undifferentiated state of the donor cells increases cloning efficiency (Zhao and Zheng, 2010). Similarly, here the blastocyst development rate in EGFP-Nanog expressing cells is slightly higher than in skin fibroblast cells (BEF422) (17.30 *vs* 14.29%) ($P<0.05$). A recent study discovered that Nanog enabled to notably activate the expression of endogenous Nanog, Oct4 and Sox2 both in fibroblasts and embryos, even though its overexpression did not have a significant effect on blastocyst development competence (Zhang *et al.*, 2011). Because of its advantage of easy visual verification of gene expression without damaging embryos, EGFP was used as a selection marker to produce transgenically modified embryos in the present study. It may be promising for these EGFP-Nanog expressing cell-derived embryos to upgrade cloning level and to improve derivation efficiency of bovine ES cells. However, more important work remains to be done to verify our deductions, such as isolating ES cells from these transgenic blastocysts, transferring the embryos to surrogates and investigating the normality of development.

Conclusion: Bovine Nanog was cloned from fetal primordial genital ridges, whose eukaryotic expression vector was introduced to fetal skin fibroblast cells, and a stable cell line expressing EGFP-Nanog was obtained. NT embryos derived from this EGFP-Nanog expressing cells performed better than those ones from intact skin fibroblast cells in blastocysts development.

Acknowledgement: This work was supported by a grant from the Chinese National "863" High-Tech Program (Grant NO.2005AA219050). We thank Dr. Xian-yong Lan, Dr. Jin-lian Hua and associates professor Chun-rong Yang for their kindly help in experiments. We also thank Dr. Simon Walker and Dr. Wan-cun Chang for critical revision of this manuscript.

REFERENCES

Chambers I, D Colby, M Robertson, J Nichols, S Lee, S Tweedie and A Smith, 2003. Functional expression cloning of Nanog, a pluripotency sustaining factor in embryonic stem cells. Cell, 113: 643-655.

Darr H, Y Mayshar and N Benvenisty, 2006. Overexpression of Nanog in human ES cells enables feeder-free growth while inducing primitive ectoderm features. Development, 133: 1193-1201.

Dutta R, D Malakar, K Khate, S Sahu, Y Akshey and M Mukesh, 2011. A comparative study on efficiency of adult fibroblast, putative embryonic stem cell and lymphocyte as donor cells for production of handmade cloned embryos in goat and characterization of putative ntES cells obtained from these embryos. Theriogenology, 76: 851-863.

Eckardt S and KJ McLaughlin, 2004. Interpretation of reprogramming to predict the success of somatic cell cloning. Anim Reprod Sci, 82-83: 97-108.

Hambiliki F, S Stron, P Zhang and A Stavreus-Evers, 2012. Co-locolization of Nanog and Oct4 in human pre-implantation embryos and in human embryonic stem cells. J Asist Reprod Genet, 29: 1021-1028.

Han J, F Zhang, M Yu, P Zhao, W Ji, H Zhang, B Wu, Y Wang and R Niu, 2012. RNA interference-mediated silencing of NANOG reduces cell proliferation and induces G0/G1 cell cycle arrest in breast cancer cells. Cancer Lett, 321: 80-88.

Han, YM, YK Kang, DB Koo and KK Lee, 2003. Nuclear reprogramming of cloned embryos produced in vitro. Theriogenology, 59: 33-44.

Hatano Sy, M Tada, H Kimura, S Yamaguchi, T Kono, T Nakano, H Suemori, N Nakatsuji and T Tada, 2005. Pluripotential competence of cells associated with Nanog activity. Mech Dev, 122: 67-79.

Huang B, TS Xie, DS Shi, T Li, XL Wang, Y Mo, ZQ Wang and MM Li, 2007. Isolation and characterization of EG-like cells from Chinese swamp buffalo (*Bubalus bubalis*). Cell Biol Int, 38: 97-102.

Hyslop L, M Stojkovic, L Armstrong, T Walter, P Stojkovic, S Przyborski, M Herbert and MM Lakoa, 2005. Down regulation of Nanog innduces differentiation of human embryonic stem cells to extraembryonic lineages. Stem Cells, 23: 1035-1043.

Kumar BM, HF Jin, JG Kim, SA Ock, Y Hong, S Balasubramanian, SY Choe and GJ Rho, 2007. Differential gene expression patterns in porcine nuclear transfer embryos reconstructed with fetal fibroblasts and mesenchymal stem cells. Dev Dyn, 236: 435-446.

Moore K, JM Kramer, CJ Rodriguez-Sallaberry, JV Yelich and M Drost, 2007. Insulin-like growth factor (IGF) family genes are aberrantly expressed in bovine conceptuses produced in vitro or by nuclear transfer. Theriogenology, 68: 717-727.

Okita K, T Ichisaka and S Yamanaka, 2007. Generation of germline-competent induced pluripotent stem cells. Nature, 448: 313-317.

Sumer H, J Liu, LF Malaver-Ortega, ML Lim, K Khodadadi and PJ Verma, 2011. Nanog is a key factor for induction of pluripotency in bovine adult fibroblasts. J Anim Sci, 89: 2708-2716.

Takahashi K and S Yamanaka, 2006. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell, 126: 663-676.

Takahashi K, K Tanabe, M Ohnuki, M Narita, T Ichisaka, K Tomoda and S Yamanaka, 2007. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell, 131: 861-872.

Theunissen TW, AL van Oosten, G Castelo-Branco, J Hall, A Smith and JCR Silva, 2011a. Nanog overcomes reprogramming barriers and induces pluripotency in minimal conditions. Curr Biol, 21: 65-71.

Theunissen TW, Y Costa, A Radzisheuskaya, ALV Oosten, F Lavial, B Pain, LFC Castro and JCR Silva, 2011b. Reprogramming capacity of Nanog is functionally conserved in vertebrates and resides in a unique homeodomain. Development, 138: 4853-4865.

Theunissen TW and JCR Silva, 2011c. Switching on pluripotency: a perspective on biological requirement of Nanog. Phil Trans R Soc B, 336: 2222-2229.

Wang L, E Duan, LY Sung, BS Jeong, X Yang and XC Tian, 2005. Generation and characterization of pluripotent stem cells from cloned bovine embryos. Biol Reprod, 73: 149-155.

Yamaguchi S, K Kurimoto, Y Yabuta, H Sasaki, N Nakatsuji, S Mitinori, and T Tada, 2009. Conditional knockdown of Nanog induces apoptotic cell death in mouse migrating primordial germ cells. Development, 136: 4011-4020.

Yamaguchia S, H Kimura, M Tada, N Nakatsujib and T Tada, 2005. Nanog expression in mouse germ cell development. Gene Exp Patterns, 5: 639-646.

Yu J, MA Vodyanik, K Smuga-Otto, J Antosiewicz-Bourget, JL Frane, S Tian, J Nie, GA Jonsdottir, V Ruotti, R Stewart, II Slukvin and JA Thomson, 2007. Induced pluripotent stem cell lines derived from human somatic cells. Science, 318: 1917-1920.

Zhang J, X Wang, B Chen, G Suo, Y Zhao, Z Duan and J Dai, 2005. Expression of Nanog gene promotes NIH3T3 cell proliferation. Biochem Bioph Res Co, 338: 1098-1102.

Zhang L, YB Luo, G Bou, QR Kong, YN Huan, J Zhu, JY Wang, H Li, F Wang, YQ Shi, YC Wei and ZH Liu, 2011. Overexpression Nanog activates pluripotent genes in porcine fetal fibroblasts and nuclear transfer embryos. Anat Record, 294: 1809-1817.

Zhao XU and YM Zheng, 2010. Development of cloned embryos from porcine neural stem cells and amniotic fluid-derived stem cells. Animal, 4: 921-929.