

Functional analysis of bovine Nramp1 and production of transgenic cloned embryos *in vitro*

Xiang Cheng², Xiaoli Yu², Yajun Liu², Jie Deng², Xiaoling Ma² and Huayan Wang¹

Department of Animal Biotechnology, College of Veterinary Medicine, Northwest A&F University, Yangling, Shaanxi, China

Date submitted: 09.02.2013. Date accepted: 01.04.2012

Summary

Natural resistance-associated macrophage protein 1 (Nramp1) plays an important role in restraining the growth of intracellular pathogens within macrophages. In this study, *Nramp1* cDNA was cloned from Qinchuan cattle and its anti-bacterial activity was demonstrated as being able to significantly inhibit the growth of *Salmonella abortusovis* and *Brucella abortus* in macrophages. Calf fibroblasts stably transfected with pSP-NRAMP1-HA vector were used to reconstruct bovine embryos by somatic cell nuclear transfer (SCNT). Reconstructed embryos were matured *in vitro* and the blastocyst formation rate (14.0%) was similar to that of control embryos (14.5%). Transgenic blastocysts were transplanted into 43 recipient cattle, of which 14 recipients became pregnant as evidenced by non-return estrus and by rectal palpation. One fetus was aborted after 6½ months of pregnancy and transgene integration was confirmed by semi-quantitative polymerase chain reaction. Together, this study showed that bovine Nramp1 retains biological function against the growth of intracellular bacteria and can be used to reconstruct embryos and produce *Nramp1* transgenic cattle, which may benefit the animal and enhance their ability to prevent attack by intracellular pathogens.

Keywords: Antibacterial activity, Bovine, Nramp1, Reconstructed embryo, Transgenic animal

Introduction

Although antibiotics and vaccines are widely used, infectious diseases caused by intracellular pathogens still result in great economic loss. Previous studies have demonstrated that natural resistance-associated macrophage protein 1 (Nramp1) controls innate resistance and susceptibility to intracellular pathogens such as *Mycobacterium*, *Leishmania*, *Salmonella*, and *Brucella* (Govoni *et al.*, 1999; Barthel *et al.*, 2001). The *Nramp1* gene was first cloned from mouse by the positional cloning method (Vidal *et al.*, 1993). cDNA, genomic DNA and trafficking of the bovine *Nramp1* gene have been characterized subsequently (Feng *et al.*, 1996; Cheng *et al.*, 2011; Cheng & Wang,

2012). The NRAMP1 protein is a hydrophobic integral membrane protein that consists of 12 transmembrane domains (TMD) (Barton *et al.*, 1994). NRAMP1 is expressed in granules of polymorphonuclear leukocytes (Cellier *et al.*, 1997; Canonne-Hergaux *et al.*, 2002), late endosomes and lysosomes in macrophages (Gruenheid *et al.*, 1997; Searle *et al.*, 1998) and in dendritic cells (Stober *et al.*, 2007). NRAMP1 can influence the replication of intracellular pathogens by mediating the activity of macrophages during the early stages of infection (Blackwell *et al.*, 1994), such as by depleting phagolysosomal iron, which is needed for pathogens survival (Forbes & Gros, 2001). NRAMP1 also stimulates the production of nitric oxide, a potent antimicrobial effector, by decreasing intracellular iron availability (Fritsche *et al.*, 2007).

A naturally occurring glycine to aspartic acid substitution at position 169 (G169D) within the mouse *Nramp1* gene results in NRAMP1 losing its ability to inhibit the replication of intracellular pathogens such as *Salmonella* and *Brucella* (Malo *et al.*, 1994; Vidal *et al.*, 1995). In addition, the mouse macrophage cell line RAW264.7, containing a defective NRAMP1 (G169D), has been used widely to determine the *Nramp1*

¹All correspondence to: Huayan Wang, Department of Animal Biotechnology, College of Veterinary Medicine, Northwest A&F University, Yangling, Shaanxi 712100, China. Tel: +86 029 87080069. Fax: +86 029 87080068. E-mail: hhwang101@163.com

²Department of Animal Biotechnology, College of Veterinary Medicine, Northwest A&F University, Yangling, Shaanxi 712100, China.

Table 1 Primers used in this study

Primer	Sequence (5'→3')	Size (bp)
NRAMP1-F	<u>CCGCGGGTCGCCACCATGTCAGGTGACACGGGC</u>	1690 ^a
NRAMP1-R	<u>GGATCCCTTAAGCGTAATCTGGAACATCGTATGGGTATCCCGAGGTCCTCCCCTTCTC</u>	
F1	CATGCTTCAGGGCCTGTTC	1017
R1	CTTAAGCGTAATCTGGAACAT	
F2	CCCACCCTGCCTACTTCAGC	318 or 182
R2	CCGAGGTCCTCCCCTTCT	
GAPDH-F3	CAAGGTCATCCATGACAACCTTTG	496
GAPDH-R3	GTCCACCACCCTGTTGCTGTAG	

^aKozak and HA tag sequences are underlined in NRAMP-F and NRAMP1-R, respectively. *SacII* and *BamHI* sites are shown in bold in NRAMP-F and NRAMP1-R, respectively.

function *in vitro* (Govoni *et al.*, 1999; Barthel *et al.*, 2001). Previous reports have shown that recombinant mouse NRAMP1 protein in RAW264.7 cells abrogated the replication of *Salmonella typhimurium* (Govoni *et al.*, 1999).

Expression of tissue-specific therapeutic genes in transgenic animals is an alternative approach to conventional strategies to avoid the deleterious side effects in the whole body. It has been reported that synthetic promoter (SP) was capable of driving the targeted gene expression especially in macrophages, which provided an ideal tool for driving the *Nramp1* gene to control intracellular bacterial infections in macrophages (He *et al.*, 2006; Wen *et al.*, 2008, 2009). Genetic manipulation combined with somatic cell nuclear transfer (SCNT) is a promising strategy to generate transgenic cloned cattle with a heightened resistance to infections by intracellular pathogens.

The aim of present study was to evaluate the function of bovine *Nramp1* *in vitro* and to produce *Nramp1* transgenic cloned embryos. This study may provide a valuable reference for the production of *Nramp1* transgenic cattle in the future.

Materials and methods

Nramp1 cDNA cloning and vector construction

Primers NRAMP1-F and NRAMP1-R were designed based on GenBank sequence NM_174652.2 (Table 1). The HA tag sequence was fused with the C terminus of *Nramp1* in order to evaluate exogenous *Nramp1* expression. To clone the *Nramp1* gene, total RNA was extracted from the spleen of Qinchuan cattle using Trizol reagent (Invitrogen, USA). Total RNA was reverse transcribed to cDNA with oligo-dT primers and RevertAidTM reverse transcriptase (Fermentas, Canada). Bovine *Nramp1* gene amplified by Triplemaster PCR system (Eppendorf, Germany) was cloned into the pGEM-T-Easy vector (Promega,

USA) and sequenced. The positive clone was named pGEM-T-Easy-NRAMP1-HA.

To construct expression vectors, pEGFP-N1 (Invitrogen, USA) and the macrophage-specific expression vector pSP-EGFP (a gift from Dr Wen Yangan, Chongqing Medical University, China) were digested with *SacII* and *BamHI*, and the *Nramp1* gene from pGEM-T-Easy-NRAMP1-HA was subcloned into plasmids to construct pCMV-NRAMP1-HA-GFP and pSP-NRAMP1-HA-GFP, respectively. Plasmid pCMV-NRAMP1-HA was constructed by deleting the GFP coding sequence and validated by *PstI* digestion. The pSP-NRAMP1-HA vector without GFP was constructed and validated by *XhoI* and *HpaI* enzyme digestion.

Cell culture and transfection

RAW264.7 cells, an immortalized mouse macrophage cell line containing a non-functional *NRAMP1* gene, were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco, USA) high-glucose formulation supplemented with 10% fetal bovine serum (HyClone, USA). In order to evaluate the expression of *Nramp1* constructs, RAW264.7 cells plated on 24-well plates were transfected with pSP-NRAMP1-HA and pCMV-NRAMP1-HA, respectively, using Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's instructions. Twenty-four hours after transfection, total RNA was extracted and subjected to RT-PCR assay using *Nramp1*-specific primers F1 and R1 (Table 1 and Fig. 1A). Primer R1 was within the HA tag sequence. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control.

To determine the subcellular localization of NRAMP1, the Lamp1-RFP plasmid, which was the lysosome marker and purchased from Addgene, was co-transfected with pCMV-NRAMP1-HA into Chinese hamster ovary (CHO) cells using the Lipofectamine 2000. To perform the immunofluorescence assay, cells at 36 h after transfection were washed twice with phosphate-buffered saline (PBS; Gibco,

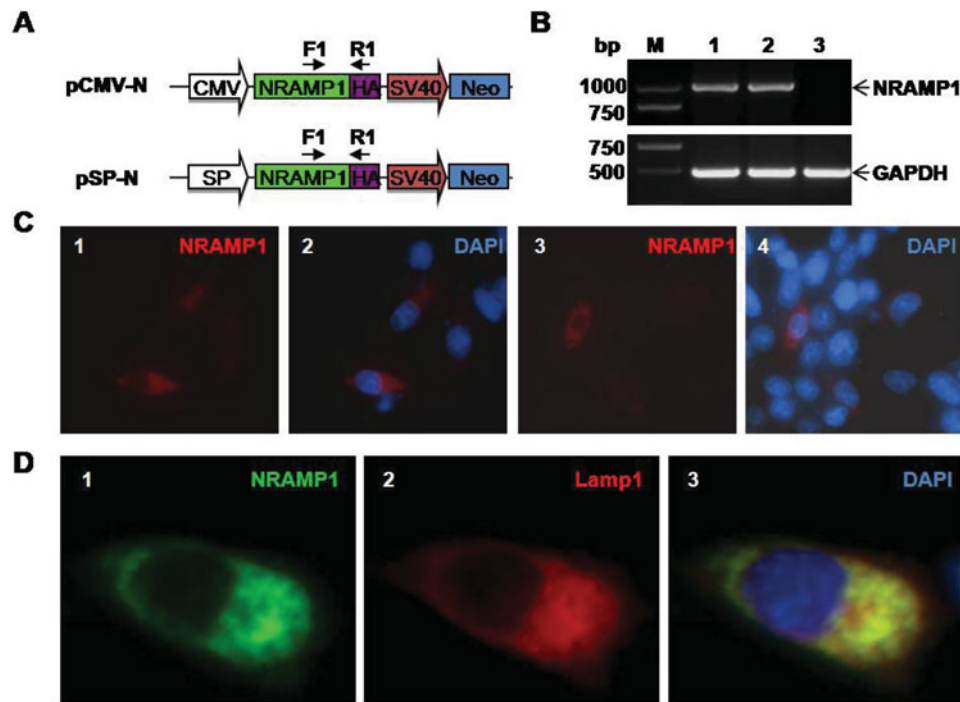


Figure 1 *Nramp1* gene cloning and expression in macrophage RAW264.7 cells. (A) Schematic representation of pCMV-NRAMP1-HA (pCMV-N) plasmid driven by cytomegalovirus (CMV) promoter and pSP-NRAMP1-HA (pSP-N) plasmid driven by macrophage-specific synthetic promoter (SP). The *Nramp1* coding sequence was fused with an HA tag. Primers F1 and R1 were designed to determine exogenous *Nramp1* expression. (B) RT-PCR analysis of *Nramp1* expression in RAW264.7 cells. Lane 1, Cells with pSP-NRAMP1-HA; Lane 2, Cells with pCMV-NRAMP1-HA; Lane 3, RAW264.7 cells. GAPDH was used as internal control. (C) RAW264.7 cells were transfected with pSP-NRAMP1-HA (1 and 2) and pCMV-NRAMP1-HA (3 and 4), respectively. The expression of *Nramp1* was determined by anti-HA antibody followed by secondary antibody conjugated with TRITC. (D) Co-localization of NRAMP1 and Lamp1 in CHO cells. pCMV-NRAMP1-HA was co-transfected using Lamp1-RFP into CHO cells. (1) Immunofluorescence due to NRAMP1 (green) was detected with anti-HA antibody and FITC-conjugated anti-mouse IgG; (2) Lamp1-RFP expression (red); (3) DAPI staining and merged image.

USA) and fixed in 4% paraformaldehyde for 20 min. An immunofluorescence assay was performed using mouse monoclonal anti-HA antibody (GenScript, USA, 1:500) as described previously (Lam-Yuk-Tseung *et al.*, 2006). Cells were then incubated in 100 ng/ml 4',6-diamidino-2-phenylindole (DAPI) for 5 min and visualized under a fluorescence microscope (Nikon, Japan).

In order to determine the antibacterial effect of bovine *Nramp1*, RAW264.7 cells were seeded onto 6-cm plates 1 day before transfection. Plasmids pCMV-NRAMP1-HA and pSP-NRAMP1-HA were transfected into cells using the Lipofectamine 2000 method. At 48 h after transfection, medium containing 400 μ g/ml G418 (Gibco-BRL, USA) was added into the plates. After 10–12 days of incubation, the individual G418-resistant clones were picked and expanded. Stably transfected clones were confirmed by semi-quantitative RT-PCR and F1 and R1 primers.

To obtain reliable transgenic donor cells for SCNT, calf fibroblasts (CF) were generated from the ear skin of newborn female Qinchuan calf by the tissue culture

method described previously (Lipiński *et al.*, 2007). CF cells were transfected with pSP-NRAMP1-HA using Lipofectamine 2000. After 48 h of transfection, cells were transferred into medium that contained 800 μ g/ml of G418 and cultured for 2 weeks. Drug-resistant clones were subcultured and confirmed by RT-PCR. Cytogenetic analysis and the proliferation potential of transgenic fibroblasts were assessed based on a previous report (He *et al.*, 2009).

Antibacterial assay

Assays for bacteria strains *Salmonella abortusovis* and *Brucella abortus* were performed as described previously (Barthel *et al.*, 2001). Briefly, RAW264.7 macrophages (as the control) and *Nramp1* transfected RAW264.7 (RAW264/NRAMP1) cells were grown to 80% confluency. Fifty thousand cells were seeded onto 12-well plates, one plate was used to determine the number of bacteria at the starting point, and the other plates were used to determine the number of bacteria at 24 h after infection. Macrophages from both control

and RAW264/NRAMP1 cells were challenged with 5×10^6 bacteria for 2 h, respectively, to allow for phagocytosis. To inhibit the growth of extracellular bacteria, 25 $\mu\text{g}/\text{ml}$ of G418 was added for 1 h, and cells were washed with PBS three times. Control cells were collected to count the number of colony-forming units at the starting point (CFU_0), and experimental cells were incubated at 37°C for 24 h. Macrophages were lysed osmotically with 0.4 ml 0.01% bovine serum albumin (BSA), and cell lysates were serially diluted and sprayed onto agar plates for the CFU count (CFU_{24}). Replication ability of the bacteria was represented by the ratio of CFU_{24} versus CFU_0 .

Production of transgenic cloned embryos

To produce SCNT embryos, CFs and an *Nramp1* transgenic calf fibroblast clone (SP-CF2) with a normal karyotype were used as donor cells, which were grown in the 6-well plate until 80% confluency, and were then trypsinized. High quality bovine oocytes isolated from ovaries and used to perform SCNT were matured in TCM199 (Gibco, USA) medium supplemented with 2.5 $\mu\text{g}/\text{ml}$ sodium pyruvate, 50 $\mu\text{l}/\text{ml}$ insulin–transferrin–selenium (ITS; Gibco, USA), 0.1 IU/ml human menopausal gonadotropin (HMG; Ningbo animal hormone factory, China), 1 $\mu\text{g}/\text{ml}$ estradiol (Ningbo animal hormone factory, China) and 10 ng/ml epidermal growth factor (EGF; Millipore, USA) for 22 h. One donor cell was inserted into the perivitelline space of an enucleated oocyte. Electrofusion was carried out with two direct current pulses of 140 V/mm for 80 μs , with a 1 s interval. After electrofusion, the reconstructed oocytes were cultured at 38°C in a 5% CO_2 in air atmosphere for 6–7 days. DNA isolated from a single reconstructed embryo at the morula or blastocyst stages was analyzed by PCR using primers F2 and R2 as reported previously (Melo *et al.*, 2005; Zhang *et al.*, 2010). The transgenic hatched blastocysts were incubated in 100 ng/ml DAPI for 5 min, washed three times in PBS, mounted on slides and squashed under a coverslip. Blastocysts were visualized with a fluorescence microscope (Nikon, Japan).

Embryo transfer and pregnancy diagnosis

Animal experiments were performed under animal welfare standards issued and approved by Animal Research Committee of Northwest A&F University (No. 001004150A). To perform embryo transplantation, 86 blastocysts were produced by SCNT using SP-CF2 transgenic fibroblasts. Two reconstructed *Nramp1* transgenic blastocysts were transferred to each recipient bovine ($n = 43$) as described previously (Jang *et al.*, 2006). At 60 days after transplantation, pregnancy was determined by rectal palpation. Thereafter, the

different pregnancy phases were monitored by rectal palpation and ultrasound every 2 months.

Detection of *Nramp1* transgene in the cloned fetus

To evaluate the integration of *Nramp1* transgene from the cloned fetus, genomic DNA was isolated and PCR was performed using primers F1 and R1. In order to confirm the transgene insertion, primers F2 and R2 (Table 1 and Fig. 5B) were designed and used to perform PCR.

Statistical analysis

SCNT experiments were repeated at least three times. Data on development of the SCNT embryos were analyzed using the chi-squared test (SAS, USA). Significant difference was confirmed when the *P*-value was less than 0.05.

Results

Bovine *Nramp1* cDNA cloning and expression in macrophages

The HA tag sequence was fused with the C terminus of cloned *Nramp1* cDNA in order to distinguish the exogenous and endogenous *Nramp1* gene (Table 1 and Fig. 1A). The cDNA sequence was amplified from the spleen of Qinchuan cattle. When compared with the *Nramp1* sequence published in GenBank (NM_174652.2), the cloned sequence displayed 99% identity except for two nucleotide variations. One variation (A to G) was located at the 145th position of the coding region that resulted in a threonine to alanine residue change. The second variation (A to C) located at the 157th position of the coding region did not result in an amino acid change. The *Nramp1* gene was subcloned into pSP-EGFP and pEGFP-N1 plasmids, respectively, which were confirmed by DNA sequencing (Fig. 1A).

RT-PCR and the immunofluorescence assay were used to evaluate the expression of exogenous *Nramp1* gene in macrophages. Exogenous *Nramp1* was identified in the transfected macrophages, but was absent from the untransfected macrophages (Fig. 1B). To verify the expression of Nramp1 protein, immunofluorescence analysis using the anti-HA antibody was conducted and the recombinant protein NRAMP1 fused with HA tag was detected in macrophages (Fig. 1C). The lysosomal localization of NRAMP1 protein was confirmed by co-transfecting the NRAMP1 construct and the LAMP1 (lysosome associated membrane protein 1, lysosome marker) construct into CHO cells and determining protein expression by immunofluorescence assay (Fig. 1D). The result was

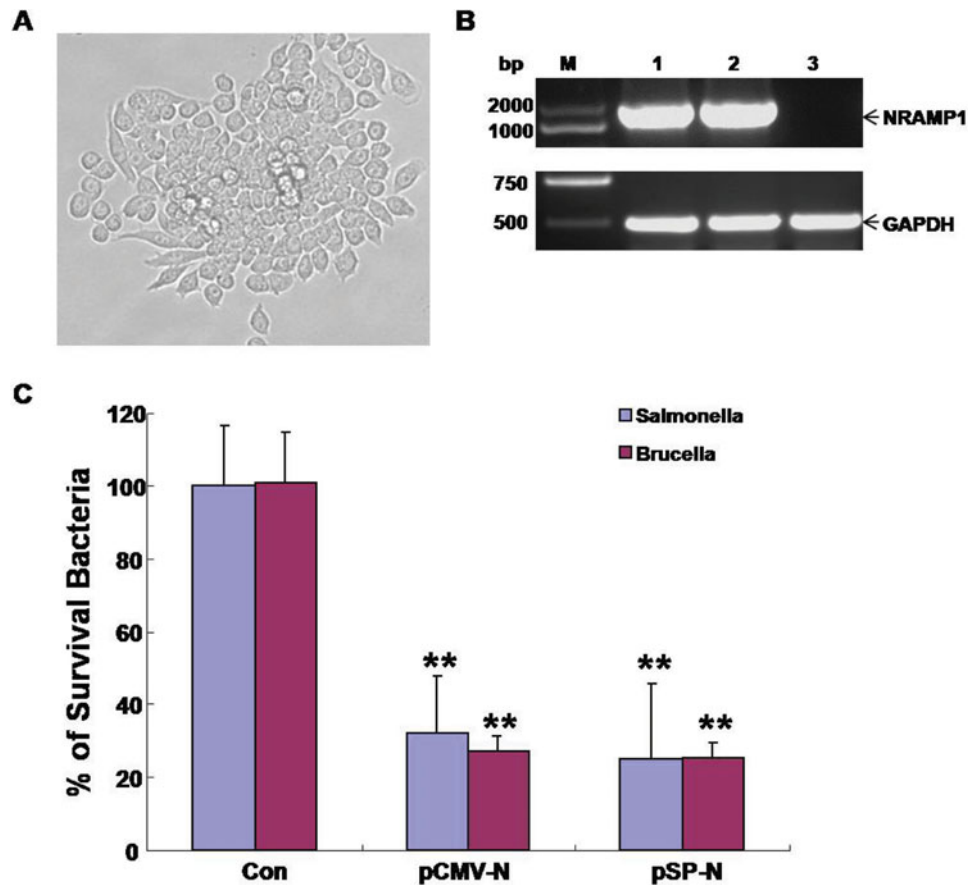


Figure 2 Antimicrobial assay. (A) Morphology of RAW264.7 macrophage clone that was stably transfected with exogenous *Nramp1* cDNA. (B) RT-PCR analysis of stably transfected RAW264.7 clones. Lane 1, RAW264.7 with pCMV-NRAMP1-HA; Lane 2, RAW264.7 with pSP-NRAMP1-HA; Lane 3, RAW264.7 cell as the negative control. GAPDH was used as internal control. (C) Antimicrobial testing *Salmonella abortusovis* and *Brucella abortus* survival after three different treatments. Control (Con), the macrophage RAW264.7 cells infected with *Salmonella* and *Brucella* strains; pCMV-N, RAW264.7 cells with pCMV-NRAMP1-HA plasmid; pSP-N, RAW264.7 cells with pSP-NRAMP1-HA plasmid. The results are presented as a mean \pm SD ($n = 3$) and as the percentage of inhibition versus control cells. ** $P < 0.01$.

consistent with that of previous reports (Lam-Yuk-Tseung *et al.*, 2006; Cheng & Wang, 2012).

NRAMP1 inhibited the growth of *Salmonella* and *Brucella*

Overexpression of mouse NRAMP1 protein has been shown to inhibit the replication of intracellular pathogens such as *Salmonella* and *Brucella* in the macrophage (Govoni *et al.*, 1999). In order to test the function of bovine NRAMP1, two expression plasmids, pCMV-NRAMP1-HA driven by cytomegalovirus (CMV) promoter and pSP-NRAMP1-HA driven by macrophage-specific SP, were constructed and transfected into RAW264.7 macrophages. A representative clone that was stably transfected with pCMV-NRAMP1-HA construct is shown in Fig. 2A. RT-PCR analysis showed the expression of exogenous *Nramp1* in stably transfected RAW264.7 cell lines from both CMV-NRAMP1 and SP-NRAMP1

constructs (Fig. 2B). Anti-*Salmonella abortusovis* and anti-*Brucella abortus* assays were performed based on these stably transfected cell lines. In comparison with untransfected RAW264.7 macrophages (control), both pCMV-NRAMP1-HA and pSP-NRAMP1-HA transfectants significantly inhibited the growth of *Salmonella abortusovis* and *Brucella abortus* (Fig. 2C). Of note, the *Nramp1* macrophage transfectants with a SP promoter displayed similar levels of inhibition as did those with the CMV promoter, further confirming that the SP promoter could be utilized to efficiently direct *Nramp1* expression in macrophages and to produce *Nramp1* transgenic bovines (Fig. 2C).

Reconstruction of transgenic embryos

CFs were isolated from the ear skin of newborn female Qinchuan calf by the tissue culture method described previously (Lipiński *et al.*, 2007) (Fig. 3A). To characterize the stably transfected fibroblasts, 16 clones

Table 2 *In vitro* maturation of bovine embryos reconstructed with calf fibroblast (CF) or SP-CF2 cells

Donor cells	Embryos			
	No. reconstructed	No. fused (%)	No. cleaved (%)	No. blastocysts (%)
CF	130	81 (62.3)	62 (76.5)	9 (14.5)
SP-CF2	138	82 (59.4)	57 (69.5)	8 (14.0)

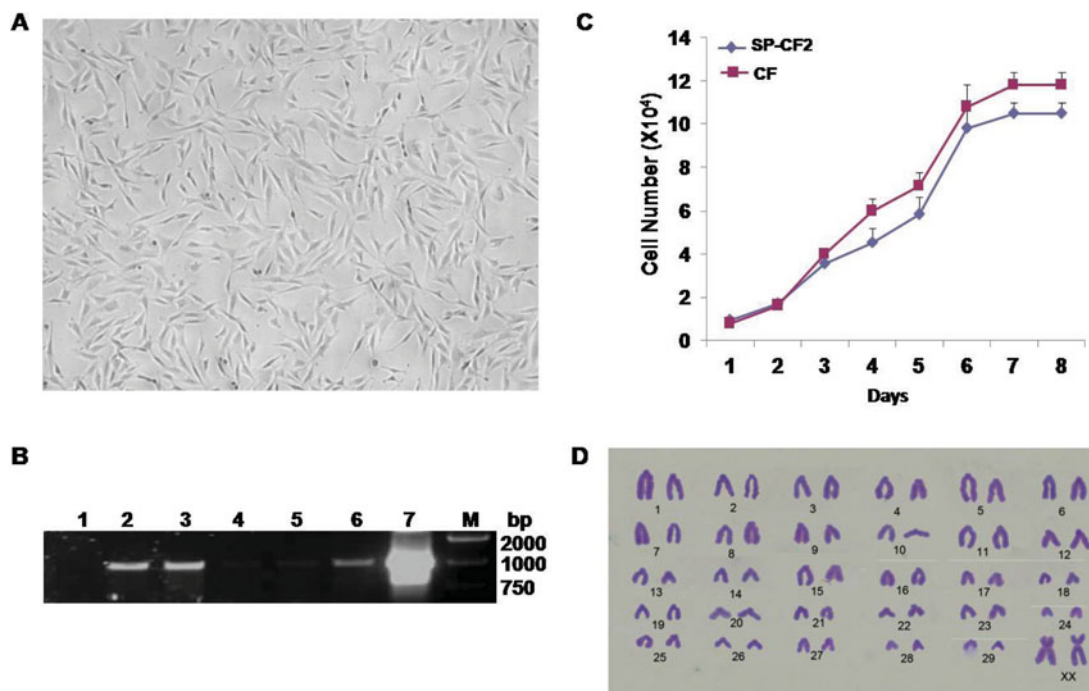


Figure 3 Characterization of stably transfected cell lines. (A) Morphology of isolated newborn calf fibroblasts (CF). (B) Five stably transfected fibroblast clones harbouring pSP-NRAMP1-HA plasmid were analyzed by PCR to detect the insertion of exogenous *Nramp1*. Lane 1, newborn calf fibroblasts; Lanes 2–6, stably transfected clones. Clone SP-CF2 shown in Lane 3; Lane 7, pSP-NRAMP1-HA plasmid is the positive control; M, DNA marker. (C) Growth curve of clone SP-CF2 and the calf fibroblasts (CF). (D) Karyotype of clone SP-CF2.

were picked and cultured in medium that contained G418. Five G418-resistant clones that contained the transgene *Nramp1* were identified (Fig. 3B). One stably transfected clone SP-CF2 showed a normal karyotype containing 30 pairs of chromosomes ($2n = 58 + XX$; Fig. 3D). The proliferation potential of SP-CF2 was measured compared with control CF cells, showing that the doubling time of SP-CF2 is 37.9 h versus 34.8 h for CFs (Fig. 3C).

The results of 138 cloned embryos derived from the SP-CF2 group and 130 cloned embryos derived from the CF group showed that the fusion rate between the SP-CF2 group and CF group did not differ significantly (59.4% versus 62.3%). Moreover, the blastocyst formation rate of SP-CF2 embryos (14.0%) was similar to that of CF embryos (14.5%, $P > 0.05$) (Table 2). The reconstructed embryos could be developed *in vitro* to mature into the hatched blastocyst stage (Fig. 4A). PCR results further

confirmed that the reconstructed embryos retained the *Nramp1* transgene (Fig. 4B).

Transplantation of embryos reconstructed with SP-CF2 cells and characterization of transgenic bovine fetus

The transgenic embryos in the blastocyst stage were transplanted into 43 Qinchuan recipient cattle. At 60 days after transplantation, pregnancy tests were performed by rectal palpation and the recording of a non-return estrus, showing that 14 recipients were pregnant. At 90 days after transplantation, rectal palpation and ultrasonography were then applied to diagnose the 14 pregnant recipients. At this time, three recipients were confirmed to have remained pregnant, while the pregnancy of the remaining animals (11) had been terminated due to the higher percentage of first and second trimester embryonic

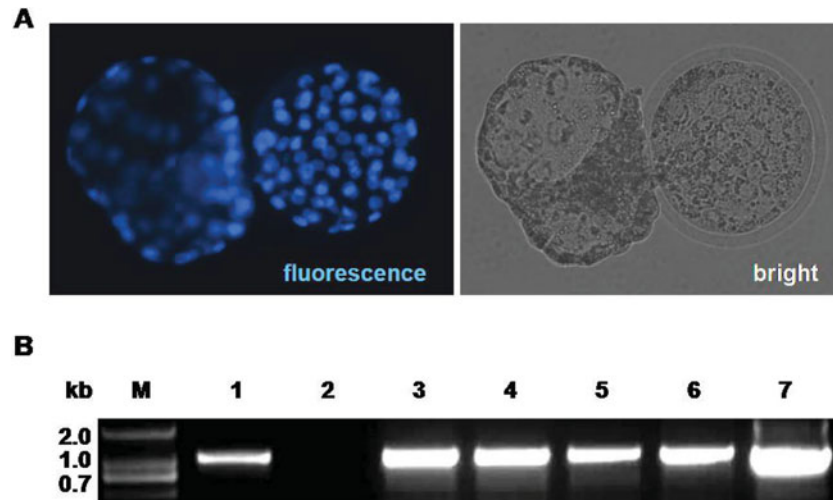


Figure 4 Reconstruction of bovine embryos. SP-CF2 cells and calf fibroblasts (CF) were used as donor cells to perform somatic cell nuclear transfer (SCNT). (A) A hatched blastocyst was derived by SCNT of SP-CF2 cells. (B) PCR analysis of *Nramp1* gene integration in reconstructed embryos with primers F1 and R1. Lane 1, SP-CF2 cells; Lane 2, embryos derived from SCNT of CF; Lanes 3–6, embryos derived from SCNT of SP-CF2; Lane 7, plasmid DNA of pSP-NRAMP1-HA; M: DNA marker.

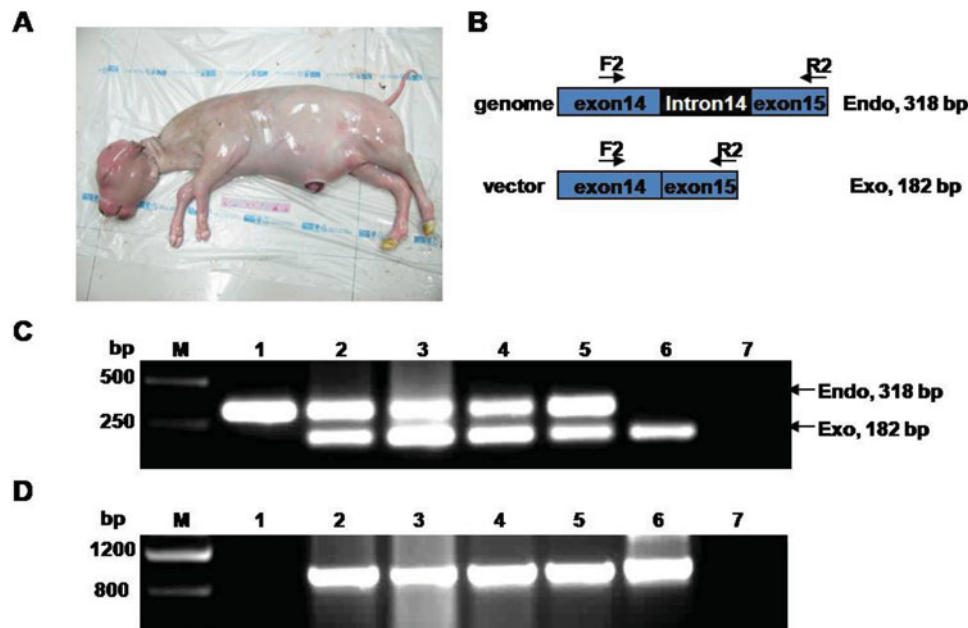


Figure 5 Characterization of transgenic bovine fetus. (A) An aborted fetus from the transplantation of embryos reconstructed with SP-CF2 cells. (B) Schematic representation of the exon 14 to 15 region of bovine *Nramp1* gene and the location of primers. The PCR products of 318 bp and 182 bp fragments are from endogenous and exogenous *Nramp1* gene, respectively. (C, D) PCR analysis of transgene in the transplant fetal tissues with primers F2/R2 (C) and primers F1/R1 (D). Lane 1, calf fibroblasts; Lane 2–4, specimens of brain, skin and umbilical cord from aborted fetus tissues; Lane 5, SP-CF2 cells; Lane 6, pSP-NRAMP1-HA plasmid DNA as positive control; Lane 7, H₂O as negative control. M, DNA marker.

loss or absorption, which has often been reported following the SCNT cloning (Kato *et al.*, 2000). Within the first 6½ months, miscarriages occurred in three pregnant cattle at the different developmental stages because of the abnormal development of fetus or placenta (Farin *et al.*, 2006). One aborted fetus at 6½ months pregnancy is shown in Fig. 5A. To

confirm the integration of the transgene, we designed one specific set of F2 and R2 primers, which were used to differentiate the exogenous and endogenous *Nramp1* gene simultaneously (Fig. 5B). The PCR results of specimens from aborted fetal brain, skin and umbilical cord tissue showed that the aborted fetus had retained the *Nramp1* transgene (Fig. 5C,D). Due to

the decomposition of aborted fetus in recipient bovine uterus over several days, DNA isolated from tissue of muscle, kidney, liver and spleen were degraded heavily and unable to be used for PCR.

Discussion

Intracellular pathogens are challenges in the treatment and control of refractory intracellular infectious diseases as they are ingested and protected in macrophages, thus resistant to the host humoral immunity. There are few effective methods for efficiently controlling intracellular pathogens, which cause great loss in livestock production. Previous reports have shown that *Nramp1* is one candidate gene for controlling the replication of intracellular pathogens (Govoni *et al.*, 1999; Barthel *et al.*, 2001). Here we determined the function of bovine *Nramp1* in macrophages and illustrate the production of a *Nramp1* transgenic cloned embryos *in vitro*.

Although the *Nramp1* gene is conserved in both prokaryotes and eukaryotes (Cellier *et al.*, 1996), there are still polymorphisms among species and even among different breeds. *Nramp1* cDNA was cloned from the spleen of Qinchuan cattle in this study; sequencing results showed that there were two nucleotide differences within the amplified *Nramp1* sequence when compared with the bovine *Nramp1* sequence from GenBank. This finding was confirmed by two further rounds of *Nramp1* gene cloning, suggesting that these differences might be due to polymorphism in the *Nramp1* gene between Qinchuan cattle and other bovine species.

In this study the macrophage cell line RAW264.7 was used to evaluate the functionality of macrophage-specific SP and to examine recombinant bovine *Nramp1* gene expression *in vitro*. The RAW264.7 cell line contains non-functional NRAMP1 with a single glycine to aspartic acid substitution at position 169 (G169D) (Govoni *et al.*, 1999). In addition, overexpression of NRAMP1^{Gly169} protein in RAW macrophages (NRAMP1^{Asp169}) restores these cells to inhibit the replication of *Salmonella typhimurium* (Govoni *et al.*, 1997, 1999; Barthel *et al.*, 2001). Although bovine macrophage cell lines have already been established (Matsumoto *et al.*, 1995; Stabel & Stabel, 1995), previous reports have shown that the RAW264.7 cell line containing a defective NRAMP1 is an optimal cell line to investigate *Nramp1* function *in vitro*. Therefore, in this study, we used the mouse macrophage cell line RAW264.7 to determine *Nramp1* function *in vitro*.

Both RT-PCR and immunofluorescence assay revealed that the *Nramp1* gene could be expressed in the macrophage. Furthermore, as there are two nucleotide differences in the coding sequence of the bovine

Nramp1 gene compared with the published sequence in GenBank, it is important to determine whether the cloned *Nramp1* gene could inhibit the replication of intracellular pathogens in the macrophage. In our study, NRAMP1-HA RAW264.7 transfectants showed strong inhibition of replication of both *Salmonella abortusovis* and *Brucella abortus* in comparison with the untransfected macrophages. This observation confirms a previous report (Barthel *et al.*, 2001). Moreover, macrophage-specific SP exhibited similar levels of antibacterial activity as did the CMV promoter in macrophages, which was consistent with previous reports (He *et al.*, 2006; Wen *et al.*, 2008). This result suggested that the *Nramp1* gene under SP control could be expressed in macrophage *in vivo*.

The genetic background of donor cells used for SCNT is a very important factor that may affect the efficiency of producing transgenic animals and transgene expression in cloned livestock (Powell *et al.*, 2004; Campbell *et al.*, 2007; Zhao *et al.*, 2009). For instance, the chromosome number of the donor cells might be altered during long-term culture under G418 selection. In order to obtain high quality *Nramp1* transgenic fibroblast cells, we performed G418-resistance selection and single transgenic cell cloning selection was confirmed by PCR analysis. The stably transfected cell line SP-CF2 used for SCNT retained the normal karyotype and was propagated for >20 passages. Reconstructed embryos were able to mature *in vitro* and the blastocyst rate was similar to that of the control. This result was consistent with previous reports (Lee *et al.*, 2007; Zhang *et al.*, 2010).

Although live offspring from cattle transgenic for the above cell line have not yet been obtained, the aborted fetus showed transgene insertion in multiple tissues, suggesting that more recipient animals and different cell lines were needed to apply transplantation. Similar observations have reported that recipients were aborted within approximately 90 days (Kato *et al.*, 2000). Embryonic loss or absorption in the SCNT animal could be triggered by: (1) the inappropriate breeding season, or by allantoic malformation, which occurred during the first trimester (Peterson & Lee, 2003); or (2) the incomplete epigenetic modification of recombinant embryos that results in the abnormal development of fetus and placenta of cloned animals (Farin *et al.*, 2006). Because SP is a strong macrophage-specific promoter, we speculate that the *Nramp1* transgene under SP promoter control could be expressed in macrophages in cloned cattle. However, the expression of both RNA and protein from transgene in aborted fetus was unable to be detected due to the decay of specimens. In future, the priority is to demonstrate tissue-specific expression of the *Nramp1* gene in cloned animals and its biological activity against intracellular pathogens.

Acknowledgements

We thank Mrs Wenlin Yang, Wei Li, Hao Dong and Dr Qingmin Wu for the technical support. We are grateful to Dr Renjith Mathew for critically evaluating the manuscript. This work was supported by the National Transgenic Breeding Project (2009ZX08007-008B), China.

References

- Barthel, R., Feng, J., Piedrahita, J.A., McMurray, D.N., Templeton, J.W. & Adams, L.G. (2001). Stable transfection of the bovine *NRAMP1* gene into murine RAW264.7 cells: effect on *Brucella abortus* survival. *Infect. Immun.* **69**, 3110–9.
- Barton, C.H., White, J.K., Roach, T.I. & Blackwell, J.M. (1994). NH₂-terminal sequence of macrophage-expressed natural resistance-associated macrophage protein (Nramp) encodes a proline/serine-rich putative Src homology 3-binding domain. *J. Exp. Med.* **179**, 1683–7.
- Blackwell, J.M., Barton, C.H., White, J.K., Roach, T.I., Shaw, M.A., Whitehead, S.H., Mock, B.A., Searle, S., Williams, H. & Baker, A.M. (1994). Genetic regulation of leishmanial and mycobacterial infections: the *Lsh/Ity/Bcg* gene story continues. *Immunol. Lett.* **43**, 99–107.
- Campbell, K.H., Fisher, P., Chen, W.C., Choi, I., Kelly, R.D., Lee, J.H. & Xhu, J. (2007). Somatic cell nuclear transfer: past, present and future perspectives. *Theriogenology* **68** (Suppl 1), S214–31.
- Canonne-Hergaux, F., Calafat, J., Richer, E., Cellier, M., Grinstein, S., Borregaard, N. & Gros, P. (2002). Expression and subcellular localization of NRAMP1 in human neutrophil granules. *Blood* **100**, 268–75.
- Cellier, M., Belouchi, A. & Gros, P. (1996). Resistance to intracellular infections: comparative genomic analysis of Nramp. *Trends Genet.* **12**, 201–4.
- Cellier, M., Shustik, C., Dalton, W., Rich, E., Hu, J., Malo, D., Schurr, E. & Gros, P. (1997). Expression of the human *NRAMP1* gene in professional primary phagocytes: studies in blood cells and in HL-60 promyelocytic leukemia. *J. Leukoc. Biol.* **61**, 96–105.
- Cheng, X., Deng, J., Meng, S.Y., Lai, W.F. & Wang, H.Y. (2011). The cloning and its expression in the cells and the tissues of the bovine natural resistance-associated macrophage protein 1 gene isoform (NRAMP1-ISO). *Chin. J. Agric. Biotech.* **19**, 685–91.
- Cheng, X. & Wang, H.Y. (2012). Multiple targeting motifs direct NRAMP1 into lysosomes. *Biochem. Biophys. Res. Commun.* **19**, 578–83.
- Farin, P.W., Piedrahita, J.A. & Farin, C.E. (2006). Errors in development of fetuses and placentas from in vitro-produced bovine embryos. *Theriogenology* **65**, 178–91.
- Feng, J., Li, Y., Hashad, M., Schurr, E., Gros, P., Adams, L.G. & Templeton, J.W. (1996). Bovine natural resistance associated macrophage protein 1 (*Nramp1*) gene. *Genome Res.* **6**, 956–64.
- Forbes, J.R. & Gros, P. (2001). Divalent-metal transport by NRAMP proteins at the interface of host-pathogen interactions. *Trends Microbiol.* **9**, 397–403.
- Fritsche, G., Nairz, M., Theurl, I., Mair, S., Bellmann-Weiler, R., Barton, H.C. & Weiss, G. (2007). Modulation of macrophage iron transport by Nramp1 (Slc11a1). *Immunobiology* **212**, 751–7.
- Govoni, G., Gauthier, S., Billia, F., Iscove, N.N. & Gros, P. (1997). Cell-specific and inducible *Nramp1* gene expression in mouse macrophages *in vitro* and *in vivo*. *J. Leukoc. Biol.* **62**, 277–86.
- Govoni, G., Canonne-Hergaux, F., Pfeifer, C.G., Marcus, S.L., Mills, S.D., Hackam, D.J., Grinstein, S., Malo, D., Finlay, B.B. & Gros, P. (1999). Functional expression of Nramp1 *in vitro* in the murine macrophage line RAW264.7. *Infect. Immun.* **67**, 2225–32.
- Gruenheid, S., Pinner, E., Desjardins, M. & Gros, P. (1997). Natural resistance to infection with intracellular pathogens: the Nramp1 protein is recruited to the membrane of the phagosome. *J. Exp. Med.* **185**, 717–30.
- He, W., Qiang, M., Ma, W., Valente, A.J., Quinones, M.P., Wang, W., Reddick, R.L., Xiao, Q., Ahuja, S.S., Clark, R.A., Freeman, G.L. & Li, S. (2006). Development of a synthetic promoter for macrophage gene therapy. *Hum. Gene Ther.* **17**, 949–59.
- He, Y.L., Wu, Y.H., He, X.N., Liu, F.J., He, X.Y. & Zhang, Y. (2009). An immortalized goat mammary epithelial cell line induced with human telomerase reverse transcriptase (hTERT) gene transfer. *Theriogenology* **71**, 1417–24.
- Jang, G., Bhuiyan, M.M., Jeon, H.Y., Ko, K.H., Park, H.J., Kim, M.K., Kim, J.J., Kang, S.K., Lee, B.C. & Hwang, W.S. (2006). An approach for producing transgenic cloned cows by nuclear transfer of cells transfected with human alpha 1-antitrypsin gene. *Theriogenology* **65**, 1800–12.
- Kato, Y., Tani, T. & Tsunoda, Y. (2000). Cloning of calves from various somatic cell types of male and female adult, newborn and fetal cows. *J. Reprod. Fertil.* **120**, 231–7.
- Lam-Yuk-Tseung, S., Picard, V. & Gros, P. (2006). Identification of a tyrosine-based motif (YGSI) in the amino terminus of Nramp1 (Slc11a1) that is important for lysosomal targeting. *J. Biol. Chem.* **281**, 31677–88.
- Lee, S.L., Kumar, B.M., Kim, J.G., Ock, S.A., Jeon, B.G., Balasubramanian, S., Choe, S.Y. & Rho, G.J. (2007). Cellular composition and viability of cloned bovine embryos using exogene-transfected somatic cells. *Reprod. Domest. Anim.* **42**, 44–52.
- Lipiński, D., Duszewska, A.M., Zeyland, J., Mały, E., Gawron, W., Rynkowska, A., Reklewski, Z., & Słomski, R. (2007). Obtaining transgenic bovine skin fibroblasts containing human interferon alpha gene. *Anim. Sci. Pap. Rep.* **25**, 211–20.
- Malo, D., Vogan, K., Vidal, S., Hu, J., Cellier, M., Schurr, E., Fuks, A., Bumstead, N., Morgan, K. & Gros, P. (1994). Haplotype mapping and sequence analysis of the mouse *Nramp* gene predict susceptibility to infection with intracellular parasites. *Genomics* **23**, 51–61.
- Matsumoto, H.N., Tamura, M., Denhardt, D.T., Obinata, M. & Noda, M. (1995). Establishment and characterization of bone marrow stromal cell lines that support osteoclastogenesis. *Endocrinology* **136**, 4084–91.
- Melo, E.O., Sousa, R.V., Iguma, L.T., Franco, M.M., Rech, E.L. & Rumpf, R. (2005). Isolation of transfected fibroblast clones for use in nuclear transfer and transgene detection in cattle embryos. *Genet. Mol. Res.* **4**, 812–21.

- Peterson, A.J. & Lee, R.S. (2003). Improving successful pregnancies after embryo transfer. *Theriogenology* **59**, 687–97.
- Powell, A.M., Talbot, N.C., Wells, K.D., Kerr, D.E., Pursel, V.G. & Wall, R.J. (2004). Cell donor influences success of producing cattle by somatic cell nuclear transfer. *Biol. Reprod.* **71**, 210–6.
- Searle, S., Bright, N.A., Roach, T.I., Atkinson, P.G., Barton, C.H., Meloan, R.H. & Blackwell, J.M. (1998). Localisation of Nramp1 in macrophages: modulation with activation and infection. *J. Cell Sci.* **111** (Pt 19), 2855–66.
- Stabel, J.R. & Stabel, T.J. (1995). Immortalization and characterization of bovine peritoneal macrophages transfected with SV40 plasmid DNA. *Vet. Immunol. Immunopathol.* **45**, 211–20.
- Stober, C.B., White, J.W., Popoff, J-F. & Blackwell, J.M. (2007). Slc11a1, formerly Nramp1, is expressed in dendritic cells and influences major histocompatibility complex class II expression and antigen-presenting cell function *Infect. Immun.* **75**, 5059–67.
- Vidal, S.M., Malo, D., Vogan, K., Skamene, E. & Gros, P. (1993). Natural resistance to infection with intracellular parasites: isolation of a candidate for Bcg. *Cell* **73**, 469–85.
- Vidal, S., Tremblay, M.L., Govoni, G., Gauthier, S., Sebastiani, G., Malo, D., Skamene, E., Olivier, M., Jothy, S. & Gros, P. (1995). The Ity/Lsh/Bcg locus: natural resistance to infection with intracellular parasites is abrogated by disruption of the *Nramp1* gene. *J. Exp. Med.* **182**, 655–66.
- Wen, Y.A., Guo, J.Y., Yu, X.L., Cen, D., Liu, J.B., Chen, Y., Zhang, J. & Tu, Z.G. (2008). Construction and targeting study of eukaryotic expression vector modulated by a macrophage-specific promoter. *Xi Bao Yu Fen Zi Mian Yi Xue Za Zhi* **24**, 441–3.
- Wen, Y.A., Yu, X.L., Xia, Q.F., Cen, D., Liu, J.B. & Tu, Z.G. (2009). Macrophage-specific overexpression of antimicrobial peptide PR-39 inhibits intracellular growth of *Salmonella enterica* serovar *typhimurium* in macrophage cells. *Int. J. Antimicrob. Agents* **34**, 315–21.
- Zhang, Y.L., Wan, Y.J., Wang, Z.Y., Xu, D., Pang, X.S., Meng, L., Wang, L.H., Zhong, B.S. & Wang, F. (2010). Production of dairy goat embryos, by nuclear transfer, transgenic for human acid beta-glucosidase. *Theriogenology* **73**, 681–90.
- Zhao, M.T., Lin, H., Liu, F.J., Quan, F.S., Wang, G.H., Liu, J., Hua, S. & Zhang, Y. (2009). Efficiency of human lactoferrin transgenic donor cell preparation for SCNT. *Theriogenology* **71**, 376–84.