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Transgenic cloned sheep overexpressing ovine toll-like receptor 4

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ABSTRACT

An ovine fetal fibroblast cell line highly expressing *TLR4* was established by inserting *TLR4* into a reconstructive p3S-LoxP plasmid. Transgenic sheep overexpressing *TLR4* were produced by transferring *TLR4*-transfected fetal fibroblasts into metaphase (M)II-stage enucleated oocytes (using SCNT). Because reconstructed embryos derived from MII-stage enucleated oocytes matured *in vivo* using a delayed-activated method had a higher pregnancy rate (18.52%) than that from MII-stage enucleated oocytes matured *in vitro*, the former procedure was used. Nine *TLR4*-transgenic live births were confirmed using polymerase chain reaction and Southern blot analysis. Increased expression of *TLR4* at mRNA and protein levels in ear tissues of transgenic lambs were verified using reverse transcription polymerase chain reaction and immunohistochemistry, respectively. More toll-like receptor 4 protein was expressed by peripheral blood monocytes and/or macrophages collected from 3-month-old *TLR4*-transgenic than nontransgenic lambs at 0, 1, and 4 hours after lipopolysaccharide stimulation. Furthermore, interferon-γ and tumor necrosis factor α secreted by monocytes and/or macrophages of *TLR4*-transgenic lambs were significantly higher at 1 hour. Therefore, lipopolysaccharide-induced inflammatory responses from monocytes and/or macrophages occurred sooner in *TLR4*-transgenic lambs, consistent with an enhanced host immune response. In conclusion, transgenic sheep overexpressing *TLR4* are a primary model to investigate the role of transgenic animals in disease resistance and have potential for breeding sheep with disease resistance.

1. Introduction

Toll-like receptors (TLRs) have a fundamental role in the innate immune system; they can recognize pathogens and immediately activate immune cells. Toll-like receptor 4 is an important member of the TLR family, which recognizes lipopolysaccharide (LPS) produced by most gram-negative bacteria and subsequently activates innate immunity [1]. After microbial stimulation, TLR4 usually initiates signal transduction to activate nuclear factor-kappa B through the MyD88-independent or MyD88-dependent pathways, which causes release of numerous inflammatory cytokines to enhance neutrophil accumulation at the site of inflammation [2]. In addition, TLR4 can also activate some genes for initiating an adaptive immune response [3]. Furthermore, the TLR4 on dendritic cell cytomembranes can recognize pathogens attacking the organism and induce secretion of cytokines to promote maturation of dendritic cell and differentiation of helper T cells [4]. Dendritic and helper T cells have crucial roles in the adaptive immune system; the former trigger cellular immunity and humoral immunity, whereas the latter are antigen-presenting cells which act as messengers between innate and adaptive immunity.

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It is well-known that some gram-negative bacteria, e.g., *Escherichia coli*, *Proteusbacillus vulgaris*, *Shigella dysenteriae*, and *Brucella melitensis*, are extremely harmful to animal husbandry, especially sheep. Overexpression of *TLR* in transgenic mice enhanced resistance to some microbial infections [5]. In the present study, transgenic sheep overexpressing *TLR4* were produced using SCNT. The protein expression levels of TLR4, interferon (IFN)-γ, and tumor necrosis factor (TNF)-α were compared in peripheral blood monocytes and/or macrophages derived from transgenic and nontransgenic sheep. In addition, an optimized nuclear transplantation method was established. Transgenic cloning technology is broadly used in disease resistance breeding and improvement of animal production performance, but nuclear transplantation has low efficiency and high rates of abortion and abnormal development. Oocyte maturation, valid activation of reconstructed embryos, and embryo culture are crucial factors affecting efficiency of transgenic cloning [6]. Therefore, in this study, the effects of oocyte sources (*in vivo*, *in vitro*) and fusion and/or activation methods (delayed-activated method or simultaneously activated method) on developmental ability of ovine reconstructed embryos were also evaluated.

2. Materials and methods

2.1. Construction of TLR4 expressing vector

The plasmid enhanced green fluorescent Protein (pEGFP)-N1 vector (Addgene, Cambridge, MA, USA) was reconstructed as a p-N1 vector by deleting enhanced green fluorescent protein (EGFP) using restriction enzymes AgeI and NotI (NEB, Beverly, MA, USA) at the respective sites. The internal ribosome entry site (IRES) 2-EGFP fragment amplified from plasmid internal ribosome entry site 2 (pIRES2) -EGFP vector and two LoxP fragments were ligated into p-N1 vector, as a reconstructed vector termed p3S-LoxP.

Extraction of RNA from an ovine spleen was done using an Omega kit and reverse transcribed into cDNA. The *TLR4* was amplified by specific primers containing EcoRI/SmaI sites, and primers of *TLR4* were designed referring to ovine *TLR4* sequence (GenBank accession No. AM_981302) [7], described as follows: *TLR4*-forward (5′- *CCG* **GAA TTC** ATG GCG CGT GCC CGC CG-3′) and *TLR4*-reverse (5′- *TCC* **CCC GGG** TCA GGT GGA GGT GGT CGC TTC TTG-3′). Bases in italic were protective bases, and those in bold were EcoRI/SmaI sites. The *TLR4* amplicon with *EcoRI* and *SmaI* sites and p3S-LoxP vector were ligated and identified to be a *TLR4*-expressing vector termed p3S-LoxP-TLR4.

2.2. Transfection of TLR4 into fetal fibroblasts and TLR4 expression analysis

Fibroblasts were isolated from a 30-day-old Suffolk fetus, and a fetal fibroblast cell line was established using the tissue block method in dulbecco's modified eagle medium (DMEM)-F12 containing 10% fetal bovine serum (FBS) (16000-044; Gibco, Grand Island, NY, USA). The p3S-LoxP-TLR4 vector was transfected into ovine fetal fibroblasts using Lipofectamine TM 2000 (Invitrogen, Carlsbad, CA, USA), which were subjected to screen for 28 days using 0.5 mg/mL G418 (Sigma, Fairfax, VA, USA). Genomic DNA of *TLR4*-transfected fetal fibroblasts was extracted and amplified for the 623-base pair (bp) *TLR4* fragment with the following primers (*TLR4*-p1 forward: 5′-TAC GGT AAA CTG CCC ACT TG-3′; *TLR4*-p1 reverse: 5′-ACC TGG AGA AGT TAT GGC TG-3′). *TLR4*-p1 included a part of cytomegalovirus (CMV) promoter from p3S-LoxP-TLR4; therefore, endogenous *TLR4* was not amplified by this primer.

The amplification reaction was accomplished with a DNA thermal cycler using the step-cycle program set for denaturing at 94 °C for 30 seconds, annealing at 60 °C for 30 seconds, and extension at 72 °C for 45 seconds, for a total of 30 cycles. The mRNA abundance of *TLR4* gene was measured (*β-actin* was the reference) using quantitative (q) polymerase chain reaction (PCR) (Agilent Stratagene Mx3000P), using a SYBR Green kit (Tiangen). The primers for *TLR4* and *β-actin* were: *TLR4*-p2 forward: 5′- CTG AAT CTC TAC AAA ATC CC-3′, TLR4-p2 reverse: 5′- CTT AAT TTC GCA TCT GGA TA-3′; *β-actin*-forward: AGA TGT GGA TCA GCA AGC AG, *β-actin*-reverse: CCA ATC TCA TCT CGT TTT CTG. *TLR4*-p2 included a part of cytomegalovirus (CMV) promoter from p3S-LoxP-TLR4, therefore endogenous *TLR4* was not amplified by this primer. Data from qPCR was analyzed using a Student *t* test. Comparisons were considered significant at $P < 0.05$.

2.3. Collection of in vivo and in vitro matured metaphase II-stage oocytes

Superovulation of the donor was induced by insertion of a progesterone-releasing intravaginal controlled internal drug releasing device (CIDR) and follicle stimulating hormone (FSH; Pharmacia & Upjohn Company, Rydalmere, Australia). *In vivo*-matured oocytes were collected surgically using a fallopian tube flushing method and then placed in the *in vitro* maturation (IVM) medium including 10% FBS TCM-199, 10 ng/mL EGF, 5 μg/mL FSH, 5 μg/mL LH, 1 μg/mL 17β-estradiol, and 2 mM glutamine (Sigma). Oocytes extruding the first polar body were selected for nuclear transplantation. Abattoir-derived ovine ovaries were collected, put in physiological saline and transported to the laboratory (at 30 °C–35 °C) within 2 hours. Cumulus and cumulus-oocyte complexes were selected and cultured in IVM at 38.5 °C in 5% CO_2 for 19 hours. Thereafter, cumulus-oocyte complexes were transferred into TCM-199 medium containing 0.1% hyaluronidase to remove cumulus cells. Oocytes extruding their first polar body were selected for nuclear transfer.

2.4. Collection of donor cells and nuclear transplantation

The fetal fibroblasts passage 10 were used as donor cells for nuclear transfer, which were cultured in 0.5% FBS medium undergoing serum starvation for 5 days until they reached the gap 0 (G0) phase. Only one fetal fibroblast line was used for nuclear transfer. Before nuclear transfer, donor cells were digested as individuals by trypsin. A cumulus-free oocyte and a donor cell were placed in micromanipulation drops, including TCM-199, 5 μg/mL cytochalasin B, 3 mg/mL BSA, and 0.5 mM HEPES (Sigma). Subsequently, the zona pellucida was partially dissected

with a very fine glass needle to make a slit near the first polar body. The first polar body and adjacent cytoplasm containing the chromosomes were extruded by squeezing with the needle using a blind aspiration method. After enucleation, *TLR4*-transfected donor cells were transferred into the perivitelline space from the slit. These couplets were transferred into IVM medium for recovery at 38 °C and 5% CO_2 for 30 minutes.

2.5. Fusion and activation of reconstructed embryos

Nontransgenic fetal fibroblasts and *in vitro* matured oocytes were fused and activated to produce nontransgenic SCNT embryos, which were subjected to two fusion–activation protocols: simultaneously activated and delayed-activated methods. Recovered couplets were placed in a chamber containing two electrodes (electrode weight, 1 mm) that were overlaid with two fusion solutions (fusion solution A and B), respectively. Fusion solution A including 0.3 mol/L mannitol, 0.05 mM Ca^{2+}, 0.1 mM Mg^{2+}, 0.5 mM HEPES, and 0.01% polyvinyl alcohol (Sigma) was used for the simultaneously activated method, and fusion solution B (Ca^{2+}-free fusion solution) including 0.3 M mannitol, 0.1 mM Mg^{2+}, 0.5 mM HEPES, and 0.01% polyvinyl alcohol was used for the delayed-activated method. In the simultaneously activated group, couplets in fusion solution A were electrically fused and then transferred into the *in vitro* culture (IVC) medium including modified synthetic oviduct fluid with amino acids, 0.2 mM glutamine, 6 mg/mL BSA, 3% essential amino acids, 1% non-essential amino acids, and 0.5 mg/mL inositol (Sigma) at 38 °C, 5% CO_2 for 30 minutes, and subsequently activated with 5 μM ionomycin for 5 minutes, followed by 2 mM 6-dimethylaminopyridine for 4 hours. In the delayed-activated group, couplets in fusion solution B were electrically fused and cultured in IVC medium at 38 °C, 5% CO_2 for 2 hours, then activated with 5 μM ionomycin for 5 minutes, followed by 2 mM 6-dimethylaminopyridine for 4 hours. All couplets were fused with two direct current pulses (1.5 kV/cm for 60 μs) using a fusion apparatus (BLS, CF-150/B, Budapest, Hungary). After activation, reconstructed embryos were cultured at 38 °C and 5% CO_2 for development. Cleavage of reconstructed embryos was observed at 48 hours. Then, some were transferred into recipient ewes, whereas others were evaluated for blastocyst quality. Before transplantation, recipient ewes were fasted for 12 hours and treated with controlled internal drug releasing (CIDR) device and pregnant mare serum gonadotropin for estrus synchronization. Transgenic reconstructed embryos after 2 days of culture were loaded in a Tomcat catheter (Sovereign-Sherwood, St. Louis, MO, USA) and transferred into the oviduct of the surrogate after a midventral laparotomy. The number of reconstructed embryos transferred to each recipient varied between five and 10. Pregnancy was detected using a B-mode ultrasound scanner at 60 days after embryo transfer.

2.6. Identification of transgenic animals

To detect the *TLR4* in clone lambs, genomic DNA was extracted from ear tissues and PCR amplification was performed using the primer *TLR4*-p1 and reaction condition, as described already herein. Amplified PCR-positive products were subjected to Southern blot analysis. Briefly, the PCR products were transferred to a nylon membrane and hybridized with a digoxigenin-labeled probe for *TLR4* following the manufacturer's procedure (Roche Molecular Biochemicals, Mannheim, Germany). After washing, membranes were exposed to Hyperfilm ECL (Amersham-Pharmacia Biotech, Little Chalfont, UK). Meanwhile, RNA was also extracted from ear tissues in transgenic lambs, and reverse transcription-PCR amplification was performed with the same primers and reaction condition as qPCR described already herein. The protein expression of TLR4 in ear tissues from transgenic lambs was detected in paraffin-embedded sections with TLR4-fluorescein isothiocyanate antibody (ab59711; Abcam, Cambridge, UK).

2.7. Changes of TLR4 and cytokines of peripheral blood monocytes and/or macrophages after LPS challenge

Peripheral blood was collected from 3-month-old transgenic and nontransgenic lambs. Monocytes and/or macrophages were isolated using sheep lymphocyte separation medium (TBD; Tianjin), and cultured in RM1640 medium (Gibco) containing 10% FBS for 48 hours. Then, monocytes and/or macrophages were stimulated with 1 μg/mL of LPS (Sigma). Cell-free supernatant was collected to measure the concentration of TLR4, IFN-γ, and TNF-α in triplicates using ELISA kits (Xinle, Shanghai, China) at 0, 1, 4, and 48 hours after LPS stimulation according to the instructions. Data from the ELISA was analyzed using a Student *t* test. Comparisons were considered significant at $P < 0.05$.

2.8. Ethics statement

Superovulation, intradermic injection, and blood collection were performed at the experimental station of the China Agricultural University. The entire procedure was carried out in strict accordance with the protocol approved by the Animal Welfare Committee of China Agricultural University (Permit Number: XK662). Sheep spleens were obtained from the Hai Dian Yong Feng abattoir in Beijing, China.

3. Results

3.1. Construction of TLR4-expressing vector

Expression vector, p3S-LoxP-TLR4 (Fig. 1A), was constructed successfully. Amplified cDNA of *TLR4* was confirmed by sequencing a 2598 bp *TLR4* amplicon (Fig. 1B). Expression vector was also correctly digested by restriction enzymes at *EcoRI* and *SmaI* sites (Fig. 1C).

3.2. Transfection of TLR4 into fetal fibroblasts

Positive ovine fetal fibroblasts transfected using p3S-LoxP-TLR4 vector using lipofectamine TM 2000 were identified by detecting the expression of green fluorescent protein (Fig. 2A). The PCR product of *TLR4* amplified from genomic DNA of the positive ovine fetal fibroblasts

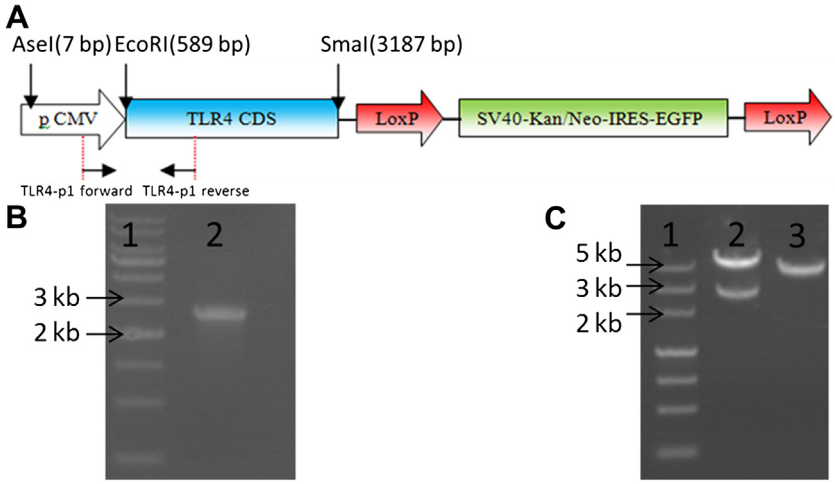

Fig. 1. Expression vector of ovine *TLR4* gene. (A) The expression vector, p3S-LoxP-toll-like receptor (TLR)4 was constructed. Toll-like receptor 4-p1 was for polymerase chain reaction (PCR) and Southern blot analysis, and TLR4-p2 was for quantitative PCR and reverse transcription-PCR. (B) The cDNA of *TLR4* was cloned (length, 2598 bp). Lane 1: marker DNAs; lane 2: amplified cDNA production of *TLR4*. (C) The expression vector, p3S-LoxP-TLR4, was digested by EcoRI and SmaI. Lane 1: marker DNAs; lane 2: two bands after digestion (2531 and 5393 bp, respectively); lane 3: expression vector (p3S-LoxP). Ase1, aquaspirillum serpens restriction enzyme I; bp, base pair; CDS, coding sequence; CMV, cytomegalovirus promoter; EcoRI, E.coli restriction enzyme I; kb, Kilo-base pair; LoxP, locus of X-over P1 site; Neo-IRES-EGFP, neomycin resistance- internal ribosome entry site- enhance green fluorescent protein; p1, primer1; p2, primer2; SmaI, serratia marcescens restriction enzyme I; SV40-Kan, simian vacuolating virus 40-kanamycin resistance.

confirmed chromosomal integration of the *TLR4* in positive ovine fetal fibroblasts (Fig. 2B). The mRNA expression of *TLR4* in the positive ovine fetal fibroblasts was significantly higher than that in nontransfected fetal fibroblasts detected using qPCR (Fig. 2C). Consequently, an overexpressing *TLR4* transgenic cell line was obtained.

3.3. Production of transgenic sheep overexpressing TLR4

The effects of delayed-activated and simultaneously activated methods on developmental competence of nontransgenic SCNT embryos were evaluated. Reconstructed embryos derived from nontransgenic ovine fetal fibroblasts and *in vitro*-matured oocytes occurred with higher frequencies of embryo fusion and blastocyst formation in the delayed-activated group (68.45% and 13.93%, respectively) compared with that observed in the simultaneously activated group (50.79% and 6.17%, respectively, $P < 0.05$; Table 1). In addition, there was no significant difference in frequency of cleavage stage, regardless of fusion–activation methods (Table 1). Therefore, the delayed-activated method was more effective for ovine SCNT than the simultaneously activated method.

Nontransgenic and *TLR4*-transgenic fetal fibroblasts were fused and activated with *in vitro* matured oocytes using the delayed-activated method. Developmental competence between nontransgenic reconstructed embryos and *TLR4*-transgenic reconstructed embryos were

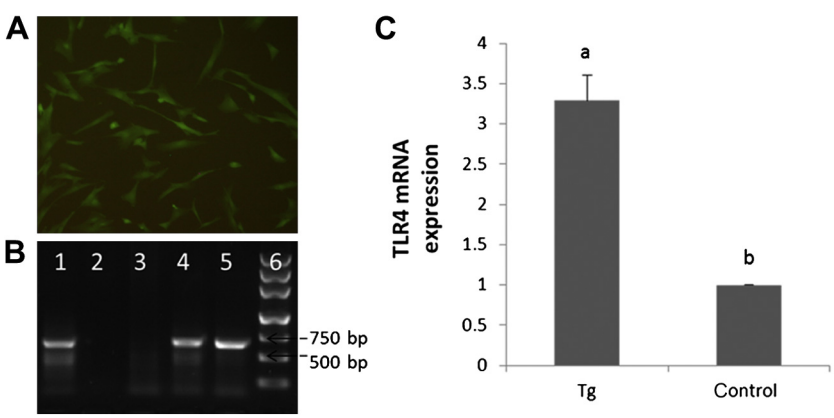

Fig. 2. Identification of *TLR4*-transfected ovine fetal fibroblast line. (A) Green fluorescent protein was expressed in *TLR4*-transfected fetal fibroblasts; (B) positive fetal fibroblasts were detected using polymerase chain reaction. Lane 1: expression vector (p3S-LoxP-TLR4, with primers *TLR4-p1forward* and *TLR4-p1 reverse*); lane 2: negative control without template; lane 3: normal ovine fetal fibroblasts; lane 4 and 5: *TLR4*-transfected ovine fetal fibroblasts; lane 6: marker DNAs. (C) The mRNA expression of *TLR4* was quantified using quantitative polymerase chain reaction. Tg: *TLR4*-transfected ovine fetal fibroblasts, control: normal ovine fetal fibroblasts. Columns without a common letter (a or b) differed ($P < 0.05$). bp, base pair; TLR, toll-like receptor.

Table 1
Development of ovine reconstructed embryos derived from *in vitro* matured enucleated ovine oocytes and nontransgenic fetal fibroblasts in different activated methods.

Activation method	Reconstructed embryos, N	Developmental reconstructed embryos, N	Fusion rate (%)	Cleavage stages, N	Cleavage rate (%)	Blastocysts, N	Blastocyst rate (%)
Simultaneously activated method	33	18	54.54	8	44.44	2	11.11
	52	26	50.00	13	50.00	2	7.69
	87	51	58.62	15	29.41	3	5.88
	15	6	40.00	2	33.33	0	0
Average			50.79 ± 8.0[a]		39.30 ± 9.56		6.17 ± 4.65[a]
Delayed-activated method	35	30	85.71	11	36.67	4	13.33
	45	29	64.44	13	44.83	4	13.79
	58	36	62.07	9	25.00	4	11.11
	30	19	63.33	9	47.37	4	21.05
	87	58	66.67	15	25.86	6	10.34
Average			68.45 ± 9.8[b]		35.95 ± 10.38		13.93 ± 4.24[b]

[a,b] Difference between simultaneously activated and delayed-activated treatments ($P < 0.05$).

compared. Fusion rate of reconstructed embryos derived from nontransgenic fetal fibroblasts was significantly higher (69.71%) than that of reconstructed embryos from *TLR4*-transgenic fetal fibroblasts (55.18%; Table 2). However, there were no significant differences in pregnancy rates of reconstructed embryos from *TLR4*-transgenic and nontransgenic fetal fibroblasts (Table 2). Thus, *TLR4*-transgenic fetal fibroblasts as donor cells were suitable and effective for producing transgenic lambs.

Effects of oocyte source (*in vivo*, *in vitro*) on development ability of *TLR4*-transgenic SCNT embryos were evaluated. Metaphase II-stage enucleated oocytes matured *in vivo* and *in vitro* were fused with *TLR4*-positive fetal fibroblasts and then activated using the delayed activated method. Embryos reconstructed with *in vivo* matured oocytes had better fusion rates and pregnancy rates (78.82% and 18.52%, respectively) than that with *in vitro* matured oocytes (70.82% and 4.16%; Table 3). The 2-day reconstructed embryos (Fig. 3) were transferred into recipients (five to 10 reconstructed embryos for each recipient). Subsequently, six *TLR4*-transgenic live births from *in vivo*-matured oocytes and three *TLR4*-transgenic live births from *in vitro*-matured oocytes were produced (Fig. 4A, Table 3).

3.4. Identification of TLR4-transgenic sheep

The mRNA from ear tissues was extracted and detected using reverse transcription-PCR, which confirmed *TLR4* expression in all *TLR4*-transgenic lambs, but not in nontransgenic lambs (Fig. 4B). Analysis of genomic DNA from ear tissues using PCR-Southern blot assay confirmed integration of *TLR4* in *TLR4*-transgenic cloned lambs. The possibility of cross-contamination was ruled out, because no PCR products were observed and detected in nontransgenic lambs using ethidium bromide staining and Southern blot analysis (Fig. 4C). Toll-like receptor 4 protein expression was detected using immunofluorescence staining, which was increased in ear tissues of transgenic lambs, compared with nontransgenic lambs (Fig. 4D).

3.5. Changes of TLR4 and cytokines in monocytes and/or macrophages after LPS stimulation

Monocytes and/or macroghages isolated from peripheral blood of *TLR4*-transgenic and nontransgenic lambs were stimulated with 1 μg/mL LPS to trigger immune responses. The protein expression level of TLR4 in transgenic lambs was transiently higher than that in nontransgenic lambs at 0, 1, and 4 hours poststimulation ($P < 0.05$). There was no differential expression of TLR4 between transgenic and nontransgenic lambs at 48 hours (Fig. 5A). The IFN-γ secreted from monocytes and/or macroghages in the transgenic lambs group was higher than that in the nontransgenic lambs group at 1 and 4 hours after LPS treatment (Fig. 5B). In addition, TNF-α was also higher ($P < 0.05$) in transgenic lambs compared with nontransgenic lambs at 1 hour (Fig. 5C).

Table 2
Differential SCNT efficiencies between TLR4-transgenic and nontransgenic fetal fibroblasts as donor cells reconstructed with enucleated ovine oocytes.

Fetal fibroblasts	Reconstructed embryos, N	Fusion embryos, N	Fusion rate (%)	SCNT embryos, N	Surrogates, N	Pregnancies, N	Pregnancy rate (%)
TLR4-transgenic fetal fibroblasts	258	138	53.49	130	13	0	0
	639	332	51.96	327	69	6	8.70
	233	140	60.09	136	17	1	5.88
Average			55.18 ± 4.32[a]				4.86 ± 4.44
Nontransgenic fetal fibroblasts	525	363	69.14	359	79	4	5.06
	222	156	70.27	151	31	6	19.35
Average			69.71 ± 0.8[b]				12.21 ± 10.1

[a,b] *TLR4*-transgenic fetal fibroblasts and nontransgenic fetal fibroblasts treatment means differ ($P < 0.05$).

Table 3
In vivo viability of ovine transgenic reconstructed embryos according to oocyte sources (matured *in vivo* and *in vitro*).

Metaphase II-stage oocyte source	Reconstructed embryos, N	Fusion embryos, N	Fusion rate (%)	SCNT embryos, N	Surrogates, N	Pregnancies, N	Pregnancy rate (%)	Births, N
Matured *in vivo*	916	753	82.21	720	53	4	7.55	2
	97	67	69.07	67	10	4	40.00	1
	270	230	85.19	192	50	4	8.00	3
Average			78.82 ± 8.57				18.52 ± 17.8	
Subtotal								6
Matured *in vitro*	635	384	60.47	331	27	1	3.70	0
	887	590	66.52	454	25	1	4.00	0
	273	226	82.78	224	56	5	8.92	3
	151	111	73.51	57	5	0	0	3
Average			70.82 ± 9.59				4.16 ± 3.66	
Subtotal								3

4. Discussion

Transgenic animal technology is a strategy which might have a major role in prevention of animal disease. Host disease resistance can be increased by introducing a resistance-conferring gene into animals or by excluding a susceptibility gene or locus from the animals [8,9]. Candidates for gene transfer experiments included all genes known to modulate nonspecific and specific host defense mechanisms. For example, TLR4, an important member of the pattern recognition receptors family, recognizes LPS, the major exocellular component of gram-negative bacteria, to initiate innate immunity such as acute immune responses [10]. When binding to ligands occurs, TLR4 can trigger MyD88-dependent and MyD88-independent signal transduction pathways to induce cytokine expression [11]. In the present study, TLR4, IFN-γ, and TNF-α in monocytes and/or macrophages from *TLR4*-transgenic lambs were significantly higher than that from nontransgenic lambs at early stages after LPS stimulation. Tumor necrosis factor α, a macrophage-derived cytokine involved in the TLR4 pathway, is an early mediator of the acute-phase response, that can act in an autocrine manner to mediate many LPS-induced effects [12]. Interferon γ is a major product of Th1 cells, and it exerts the immune response toward a Th1 phenotype by promoting innate cell-mediated immunity, specific cytotoxic immunity, and macrophage activation [13]. Moreover, IFN-γ can also be secreted by monocytes and/or macrophages. In the presence of microbial products, a larger amount of IFN-γ was secreted by macrophages rapidly to initiate the early host defense against infection [14,15]. Interferon γ priming of TNF-α responses is responsible for priming a subset of LPS-induced genes [16,17]. Thus, based on higher protein expression levels of TNF-α and IFN-γ in TLR4-transgenic sheep at the early stage after LPS stimulation indicated that transgenic sheep had a higher immune response sensitivity against infection. In addition, IFN-γ and TNF-α secreted by monocytes and/or macrophages had a transient elevation up to 4 hours (rather than lasting for 48 hours), because monocytes and/or macrophages presented acute, not chronic, responses after 1 μg/mL LPS stimulation. Perhaps the inflammatory responses in monocytes and/or macrophages tended toward equilibrium at 48 hours after LPS stimulation.

Approaches to reduce disease susceptibility of animals will benefit animal welfare and agricultural productivity. To date, classical breeding has not yet contributed to a significant increase in resistance to disease [9]. Transgene technology opened up a novel strategy for enhancing disease resistance in animals. Previously, myxovirus Resistance 1 (Mx1) protein was originally characterized in mice, which was able to block the multiplication of some negative-stranded RNA viruses. An attempt was carried out to introduce resistance to influenza A viruses into pigs by transferring mouse *Mx1* constructs into pigs. However, in that study, mouse Mx1 protein was not detected in

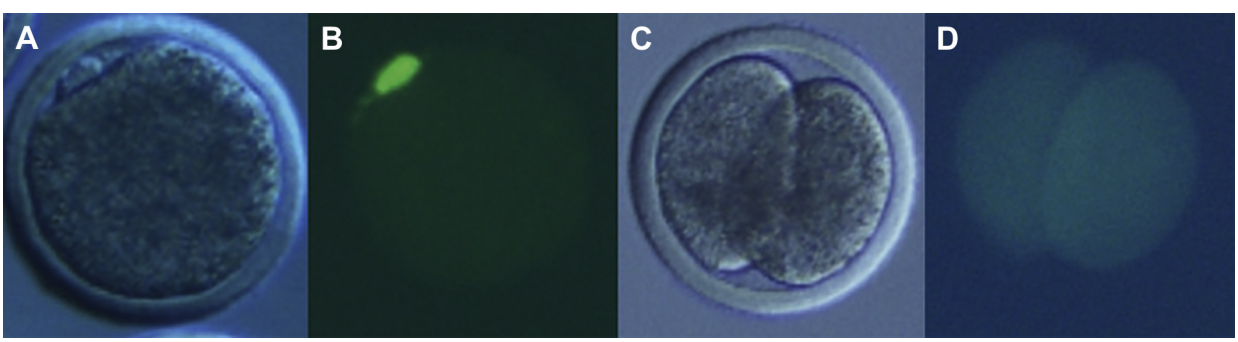

Fig. 3. Production of *TLR4*-transgenic reconstruction embryos. (A) and (B) Reconstructed oocyte (injecting *TLR4* transferred cells into encleated oocyte) before fusion (magnification: ×200). (C) and (D) After fusion, cleavage of reconstructed embryos in the two-cell stage (magnification: ×200).

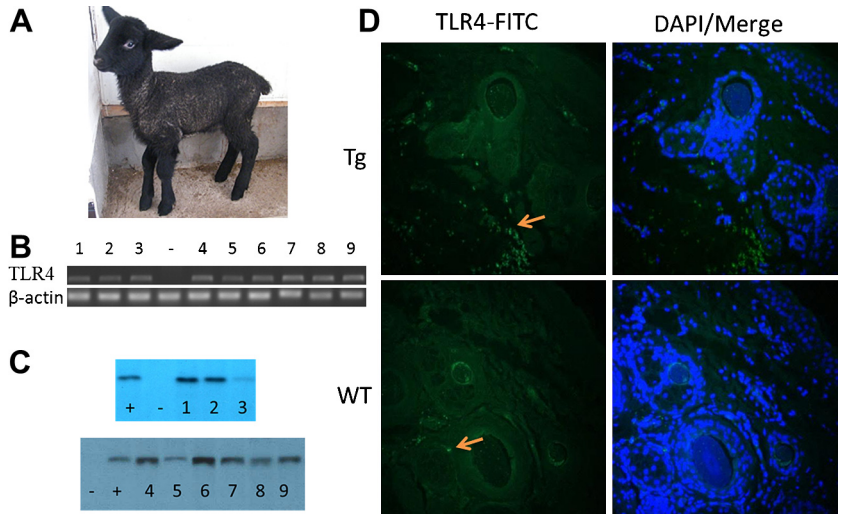

Fig. 4. Production and identification of nuclear transfer-derived lambs using somatic cell nuclear transfer of ovine fetal fibroblasts transfected with *TLR4* into enucleated ovine oocytes. (A) *TLR4*-transgenic lamb. (B) The mRNA expression of *TLR4* in ear tissue from transgenic lambs was detected using reverse transcription polymerase chain reaction. Label "-" indicates RNA of ear tissue from nontransgenic lambs, "1–9" indicates RNA of ear tissue from transgenic lambs, the β-actin gene was used as an endogenous control. (C) Southern blot of *TLR4* insert in transgenic lambs. "-" indicates a nontransgenic lamb, "+" indicates a positive expression vector (p3S-LoxP-TLR4), "1–9" indicates *TLR4*-transgenic lambs. (D) Toll-like receptor 4 protein expression in ear tissue from transgenic lambs was detected using immunohistochemical staining. Toll-like receptor 4 protein was bound with TLR4-FITC antibody specifically in ear tissue (green, left). The arrowhead indicates the TLR4 protein. Paraffin-embedded sections from ear tissue were counter-stained using DAPI (blue), with merged images (right). Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole; FITC, fluorescein isothiocyanate; Tg, *TLR4*-transgenic lambs; TLR, toll-like receptor; WT, nontransgenic lambs.

transgenic pigs [18]. Later on, transgenic cattle carrying a mammary-specific transgene encoding lysostaphin were produced, which were resistant to infection by *S. aureus* [19]. To date, very few successful resistance-enhancing studies with transgene technology have been reported in livestock. However, when the positive biological characters of transgenic animals and theirs offspring was established, the outstanding trait could be spread in the breeding population by traditional breeding methods. In this study, according to the changes of cytokines (TNF-α and IFN-γ) using LPS stimulation, we inferred that transgenic lambs with overexpression of TLR4 produced using SCNT initiated inflammatory reactions more quickly than nontransgenic lambs. Thus, *TLR4*-transgenic sheep provide a promising model to study disease resistance capacity after pathogen challenges.

At present, somatic cell cloning usually has low efficiency, because of several factors, including reprogramming of nucleus, differentiation of donor cells, and situation of oocytes [20]. Somatic nucleus reprogramming is triggered soon after transfer into the cytoplasm of enucleated oocytes and completed in the fusion–activation stage. This reprogramming period is the key stage for optimizing SCNT [21]. In addition, maturation of oocytes is a crucial factor to influence the efficiency of SCNT [22]. Synchronizing maturation of the nucleus and cytoplasm in oocytes is necessary and significant for SCNT. Therefore, various fusion–activation methods and sources of oocytes were evaluated in this study. Although the delayed-activated method has been used in bovine and porcine SCNT [23,24], it is not usually employed in ovine SCNT. In the present study, ovine reconstructed embryos which were

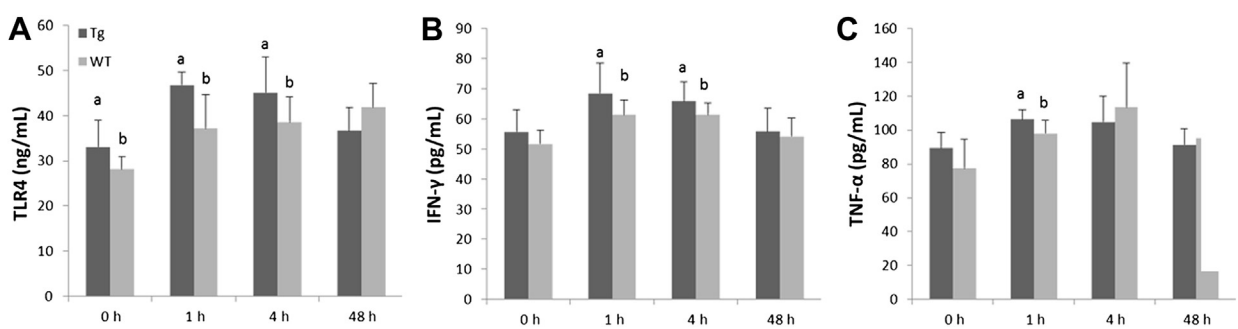

Fig. 5. Mean ± SEM of TLR4 and cytokines (IFN-γ and TNF-α) of peripheral blood monocytes and/or macrophages from *TLR4*-transgenic lambs and nontransgenic lambs at 0, 1, 4, and 48 hours after LPS stimulation. (A), (B), and (C) show the changes of TLR4, IFN-γ, and TNF-α during LPS (1 μg/mL) stimulation, respectively. Columns without a common letter (a or b) differed ($P < 0.05$). IFN, interferon; LPS, lipopolysaccharide; Tg, *TLR4*-transgenic lambs; TLR, toll-like receptor; TNF, tumor necrosis factor; WT, nontransgenic lambs.

delayed-activated at 2 hours after fusion had a significantly higher fusion rate and blastocyst rate than the simultaneously activated group. In addition, cloned transgenic embryos from *in vivo*-matured oocytes had higher SCNT efficiency, compared with that from *in vitro*-matured oocytes, which was in agreement with the results reported by Hyun et al. [25]. Consequently, reconstructing metaphase II-stage enucleated oocytes matured *in vivo* and *TLR4*-positive fetal fibroblast using a delayed-activated method improved the efficiency of SCNT in *TLR4*-transgenic sheep production.

4.1. Conclusions

Transgenic lambs overexpressing *TLR4* were produced successfully by reconstructing transgenic ovine fetal fibroblasts and matured oocytes (*in vivo* and *in vitro*) with a delayed-activated method after fusion. In addition, reconstructing transgenic ovine fetal fibroblasts and *in vivo*-matured oocytes yielded a significantly higher pregnancy rate (18.52%). Transgenic cloned sheep overexpressing TLR4 increased release of inflammatory cytokines, initiated inflammatory responses rapidly, and enhanced innate immunity significantly after LPS stimulation. Collectively, these findings establish the foundation for further studies regarding disease resistance of *TLR4*-transgenic lambs.

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