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Growth performance, reproductive traits and offspring survivability of genetically modified rams overexpressing toll-like receptor 4

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ABSTRACT

Genetic modification provides a means to enhancing disease resistance in animals. Toll-like receptor 4 (*TLR4*), a member of the TLR family, is critical for the recognition of lipopolysaccharide (LPS)/endotoxin from Gram-negative bacteria by host immune cells, which initiates cell activation and subsequently triggers a proinflammatory response to the invading pathogens. In this study, the first generation of genetically modified (GM) sheep overexpressing *TLR4* was produced by microinjection for better disease resistance. Compared with wild-type (WT) rams, the GM rams have similar growth performance, basic semen quality and spermatozoon ultrastructure. The offspring birth rates after cervical artificial insemination were also similar between GM (90.32%) and WT (92.38%) rams. Overall, the presence and expression of the *TLR4* transgene in the genome did not appear to interfere with normal semen production, reproductive traits and the ability of transgene transmission to offspring. The expression levels of *TLR4*, *tumor necrosis factor* and *interferon gamma* genes in monocyte/macrophages from GM sheep were significantly higher than that from WT sheep at early stages after LPS stimulation. The GM offspring born from the founder transgenic ram inseminated ewes had similar survival rate with WT offspring (88.89% vs 84.86%) at weaning. The *TLR4* transgene showed no deleterious effects on growth performance, reproductive traits and offspring survivability of GM rams. Therefore, the GM sheep overexpressing *TLR4* provide a powerful experimental model for analyzing function of *TLR4* in vivo during infection and inflammation.

1. Introduction

A genetically modified (GM) animal is an animal whose genetic material has been altered using genetic engineering techniques. The first successful production of GM animal using microinjection was realized in mouse more than three decades ago. Since that time transgenic techniques have been applied to produce GM animals with varying degrees of success in agriculture [1–4], biomedicine [5–7]and industry [8,9]. In addition to improving farm animal productivity and producing exogenous protein as GM animal bioreactors, these transgenic technologies can be used to improve the health status and disease resistance of the livestock [10–13].

Many Gram-negative bacteria, such as *Brucella*, *Salmonella*, *Escherichia coli* and *Pasteurella* are pathogenic to both animals and humans. Toll-like receptor 4 (*TLR4*), a member of the TLR family, is

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critical for the recognition of lipopolysaccharide (LPS)/endotoxin from Gram-negative bacteria by different host cells initiating cell activation and subsequent triggering of a proinflammatory response to invading pathogens [14–17]. It has been reported that the levels of *TLR4* mRNA expression correlate well with the degree of LPS response in vivo and in vitro [18,19], and overexpression of the *TLR4* gene results in a survival advantage in GM mice during *Salmonella* infection [19,20].

Improved animal health status is an important objective in current animal breeding strategies. However, it is difficult to achieve a significant increase in resistance to disease using traditional breeding. Genetic modification has opened up a potential strategy for enhancing disease resistance in animals. In addition to successful integration of the microinjected gene construct, the stability of transgene transmission to their offspring is also an important factor influencing the efficiency of the production of GM animals [21]. The normal reproductive capability of GM males is important to produce stable lines of GM offspring. A number of studies have been conducted to examine the effects of the transgene on reproductive traits, but yielded limited and contradictory results. Some studies have reported reproductive disorders in GM animals. The decrease in fertility or the infertility of GM males has been related to altered copulatory behavior [22], defective sperm chromatin structure [23] and defective sperm function [24–26]. Other studies, however, found that the presence of transgene in the genome did not influence semen quality [21,27,28].

Because of the scientific importance and potential economic value of GM livestock containing exogenous genes, we successfully produced GM sheep overexpressing *TLR4* through microinjection to improve health status and disease resistance. Then, we evaluated growth performance, sperm characteristics and reproductive capabilities of the founder GM rams, and investigated the survivability of their offspring.

2. Materials and methods

2.1. Ethics statement

All sheep were managed under normal husbandry conditions. All experimental animal protocols were approved and performed in accordance with the requirements of the Animal Care and Use Committee at China Agricultural University (approval ID 2008–017). All surgeries were performed under sodium pentobarbital anesthesia, and all efforts were made to minimize any suffering experience by the animals used in this study.

2.2. Design and construction of the transgenic vector

Total RNA was extracted from sheep (Ovis aries) spleens using an OMEGA kit. According to the *TLR4* mRNA sequence (Genbank Accession No. AM981302), the *TLR4* cDNA sequence was amplified using Reverse Transcript-PCR. To clone into the vector, restriction sites of *Eco*RI and *Sma*I were added to primers. The primers were as follows: forward: ccg gaa ttc ATG GCG CGT GCC CGC CG; reverse: tcc ccc ggg gGG TGG AGG TGG TCG CTT CTT GC. After double enzymatic digestion (*Eco*RI and *Sma*I; NEB, Beverly, MA, USA), PCR products were inserted into the vector to generate a *TLR4* expression vector as described in our previous study [29].

2.3. Production and screening of the founder GM sheep overexpressing TLR4

The estrous periods of the ewes were synchronized with controlled internal drug-releasing insert (CIDR, Pharmacia & Upjohn, New Zealand) for 14 days irrespective of the natural estrous cycles. For superovulation, donors were treated with FSH (Ningbo, Zhejiang, China) over a 4-day period, at 12-h intervals, starting 2.5 days before CIDR removal and continuing within 1 day of CIDR removal. The total dose of FSH for superovulation was 5 IU per kilogram of body weight. A total of 0.1 mg cloprostenol (Ningbo, Zhejiang, China) was administrated at the time of the seventh FSH administration. A total of 100 IU LH (Ningbo, Zhejiang, China) was administrated at 48 h after the CIDR removal to induce ovulation. Eight hours later, laparoscopic insemination was performed. The recipients received the same synchronization treatment and were injected with 330 IU PMSG (Ningbo, Zhejiang, China) when CIDR was removed.

Fertilized eggs were surgically recovered by flushing both oviducts with sterile phosphate-buffered saline 72 h after the CIDR removal. All donors were fasted 36 h prior to surgery. Zygotes were microinjected with 5 pl (5 ng/μL) linear vector in vitro. After examination of the ovaries, oviducts and uterine horns of the recipient, successfully microinjected zygotes were transferred into the ipsilateral oviduct to the ovulated ovary within 1 h. The number of microinjected zygotes transferred per recipient varied from 2 to 5.

Genomic DNA was extracted from ear tissue of the 2 week-old putative GM lambs. Founders and subsequent offspring were screened for the presence of the exogenous gene by PCR and Southern blot analysis as described by our previous study [30]. The following primers were used to identify the potentially GM sheep: cts: ACG GTA AAC TGC CCA CTT G, cta: ACC TGG AGA AGT TAT GGC TG; tsf: GAG CCG TAA GGT GAT TGT CGT G, tsr: GCA TTC ATT TTA TGT TTC AGG TTC A. The GM sheep were finally confirmed by Southern blot analysis (Roche Diagnostics, Mannheim, Germany). Probes were then generated by PCR using the following primers: Ps: ACT GGT AAA GAA CTT GGA GGA GGG, Pr: GTT TCA GGT TCA GGG GGA GGT G.

2.4. Expression of TLR4 in monocyte/macrophages from GM and WT sheep

Peripheral blood monocyte/macrophages from GM and WT sheep were isolated with sheep Lymphocyte Separation Medium (TBD, Tianjin, China). In brief, peripheral blood obtained from jugular vein was separated by centrifugation over Lymphocyte Separation Medium at 400–500 g for 20 min. The layer was collected and incubated with DMEM (TBD, Tianjin, China). The $CD14^{+}$ and CSF1R+ (macrophage-colony stimulating factor receptor) marker (Biostest, Beijing, China), two macrophage-specific proteins, were detected by immunofluorescence. The results validated that the cell population acquired was monocyte/macrophages. According to the manufacturer's protocol, total RNA was extracted using TRIzol method (Invitrogen, Carlsbad, CA, USA) and then used for cDNA synthesis with the Thermo Scientific Revert Aid First Strand cDNA Synthesis Kit #K1611 (Thermo Fisher Scientific Inc., Vilnius, Lithuania). The transcription levels of *TLR4* were examined using quantitative Real-Time PCR. The primer informations for the *TLR4* gene as well as for the reference gene (β-actin) were as follows: TLR4 F: CTG AAT CTC TAC AAA ATC CC, R: CTT AAT TTC GCA TCT GGA TA; β-actin F: AGA TGT GGA TCA GCA AGC AG, R: CCA ATC TCA TCT CGT TTT CTG. The TLR4 protein levels were examined using Elisa (Shanghai, Xinle, China).

2.5. Changes of TLR4 and proinflammatory cytokines in peripheral blood monocyte/macrophages after LPS stimulation

Peripheral blood was collected from 3 month-old GM ($n = 10$) and WT ($n = 10$) sheep. Monocyte/macrophages were isolated as described above, and cultured in RM1640 medium (Gibco, Grand Island, NY, USA) containing 10% FBS for 48 h. Then, monocyte/