# Interferon-τ regulates the expression and function of bovine leukocyte antigen by downregulating bta-miR-204

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**Abstract.** IFN-τ is a pregnancy recognition factor that regulates embryo implantation in ruminants. IFN-τ has been suggested to be involved in the expression of microRNA (miRNA/miR) and bovine leukocyte antigen (BoLA), which is an analog of the human major histocompatibility complex class I. However, little is known about whether the miRNAs are involved in the expression of BoLA in ruminants. The present study firstly verified that bta-miR-204 was downregulated and that BoLA was upregulated in the uterine tissues of dairy cows during early pregnancy. Subsequently, luciferase reporter assays, reverse transcription-quantitative PCR and western blot analysis were used to validate BoLA as the target gene of bta-miR-204. Moreover, BoLA was markedly upregulated and bta-miR-204 was downregulated in bovine endometrial epithelial cells (bEECs) treated with IFN-τ. In addition, the results indicated that when the expression level of BoLA was increased by IFN-τ, the expression level of programmed death-ligand 1 (PD-L1) and programmed death-ligand 2 (PD-L2) was also increased. Furthermore, when BoLA was silenced in bEECs by small interfering RNA, the expression of PD-L1 and PD-L2 was not affected by IFN-τ. The expression level of PD-L1 and PD-L2 was also increased in the uterine tissues of pregnant dairy cattle. In conclusion, IFN-τ may function by suppressing the expression of bta-miR-204 to increase the expression of BoLA during the embryo implantation period in cattle. IFN-τ may induce PD-L1 and PD-L2 transcription by regulating BoLA, which may influence the T cell immune response, thereby regulating pregnant cattle immunization.

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*Key words:* dairy cattle, pregnancy, immunity, interferon-τ, bta-miR-204, bovine leukocyte antigen, programmed death-ligand 1, programmed death-ligand 2

## Introduction

Successful embryo implantation is a key step in pregnancy and is a complicated physiological process that consists of various steps, including blastocyst hatching, invasion, migration, attachment and placentation (1,2). During this period, the uterus undergoes several alterations to establish an optimal environment for embryonic growth. A complex regulatory network of molecular interactions exists at the fetomaternal interface (3). IFN-τ is produced during the early embryo implantation, especially in ruminants, such as cattle, sheep and deer, and serves a key role in the maternal recognition of pregnancy (4-6). Previous studies have indicated that IFN-τ upregulated the expression of bovine leukocyte antigen (BoLA)-I, which is the equivalent of the major histocompatibility complex class I (MHC-I) antigen in bovines, and may modulate immune responses and contribute to fetomaternal tolerance in dairy cattle (7-10). Our previous has revealed that IFN-τ stimulation activated a wide variety of microRNAs (miRNAs/miRs) in BEECs (11). Whether the upregulation of BoLA-I expression is associated with miRNAs is still unknown. Moreover, the underlying mechanisms of the contributions of IFN-τ to embryo implantation also remain unclear.

miRNAs are a class of small, noncoding, single-stranded RNAs comprising 22-25 nucleotides. miRNAs have been suggested to negatively regulate gene expression by targeting mRNAs to interfere with post-transcriptional protein translation (12). Because of their ability to silence genes, miRNAs can modulate a variety of physiological and pathological processes, including cellular proliferation, apoptosis and immune responses (13-15). A previous study has indicated that miRNAs regulated the molecules involved in peri-implantation and pregnancy, such as let-7a and miR-320 (16). The functional study of miRNAs may be helpful in revealing the molecular pathways associated with the embryo implantation process.

Preliminary deep sequencing data indicated that bta-miRNA-204 was downregulated in bovine endometrial epithelial cells (bEECs) following IFN-τ treatment (11). Gene Ontology and Kyoto Encyclopedia of Genes and Genomes

pathway analyses indicated the target genes of bta-miRNA-204, whose function analysis found that those predicted genes were enriched in graft-vs.-host disease and allograft rejection, including BoLA-I (17,18) In this research, we will reveal the inner association between bta-miRNA-204 and BoLA-IIn the present study, bioinformatics algorithms were used to predict which genes may be regulated by bta-miRNA-204. Of note, BoLA was indicated to be a target gene of bta-miRNA-204. BoLA gene, which is also known as BoLA-A, belongs to the classic BoLA-I family, and encodes the heavy chain of BoLA class I molecules (19). As BoLA-I serves a crucial role in the regulation of immunosuppression and implantation during early embryonic development (20), the present study investigated whether IFN-τ-mediated regulation of bta-miRNA-204 contributes to effective embryo implantation.

Programmed cell death receptor 1 (PD-1) binds to the programmed death-ligand 1 or 2 (PD-L1/PD-L2) to form a costimulatory signal that negatively regulates T cell immunity. PD-L1 and PD-L2 are expressed by antigen presenting cells (APCs) (21). On the other hand, APCs also express MHC molecules that can activate T cells by interacting with the T cell receptor (TCR) (22,23). Tumor cells frequently upregulate the expression of PD-L1 or PD-L2 to facilitate their escape from the immune system. Although there have been numerous studies about the PD-1/PD-L signaling pathway in tumor immune escape, this pathway has not been well described in pregnant immune tolerance (24). As the expression of PD-L1 and PD-L2 is regulated by several factors and IFN-τ can stimulate bEECs to produce MHC, whether it can also stimulate bEECs to express PD-L1 and PD-L2 is still unknown (10,25,26). Moreover, there may be a connection between MHC and PD1 ligands based on the costimulatory signaling pathway of T cells (27), but few reports exist on this domain, therefore their relationship should be further examined. The main purpose of the present study was to preliminary explore whether IFN-τ could stimulate bEECs to produce both MHC and PD-L1 and PD-L2.

## Materials and methods

*Reagents*. Recombinant ovine IFN-τ was purchased from Creative Bioarray. FBS was purchased from SAFC Biosciences Pty Ltd. Bovine leukocyte antigen (HLA) class I (Thermo Fisher Scientific, Inc.; cat. no. MA5-28477), Actin Monoclonal Antibody (Thermo Fisher Scientific, Inc.; cat. no. MA1-744) HRP-conjugated goat anti-rabbit antibody and the primary antibody anti-cytokeratin-18 (CK-18; cat. no. MA5-12104) were provided by Abcam. Cell Counting Kit-8 (CCK-8) was purchased from Dojindo Molecular Technologies, Inc. LightCycler® FastStart DNA Master PLUS SYBR Green kit was purchased from Roche Applied Science. The microRNA and U6 small nuclear RNA normalization reverse transcription-quantitative PCR (RT-qPCR) kit was purchased from Shanghai GenePharma Co., Ltd. The bta-miR-204 mimic (bta-miR-204 agomir) and an inhibitor (bta-miR-204 antagomir), as well as were three BoLA small interfering (si) RNAs and a negative control (NC) siRNA were synthesized by Shanghai GenePharma Co., Ltd. The FastDigest *Xho*I and *Not*I, Lipofectamine® 2000 and Lipofectamine RNAiMAX kits were obtained from Thermo Fisher Scientific, Inc. Dual-Luciferase Reporter Assay System and psi-CHECK™-2 plasmid were obtained from Promega Corporation. The sequences of all primers were synthesized by Shanghai GenePharma Co., Ltd. and are listed in Table I. The sequences of the agomirs and antagomirs are presented in Table II. All other chemicals were reagent grade.

*Animals and experimental groups*. All experimental procedures involving animals and their care conformed to the Guide for the Care and Use of Laboratory Animals of National Veterinary Research of China. The present study was approved by the Huazhong Agricultural University Animal Care and Use Committee (Wuhan, China; approval no. 20171354CA).

The pregnant cow (n=3) and nonpregnant female dairy cattle (n=5) were obtained from the Animal Experimental Center of Huazhong Agricultural University. All cattle were between 16 and 24 months old, and weighed between 350 and 390 kg. They were acclimatized for one week (24±1˚C, relative humidity of 60-65%, 12 h light/dark cycle with *ad libitum* supply of food and water. The body temperature and food intake of each cow was recorded every day. The dairy cattle were anesthetized with an intravenous injection of 40 mg/kg sodium pentobarbital to minimize suffering (28). The endometrial epithelium (for cell culture) and endometrial tissues of nonpregnant cows (n=5) were collected before ovulation. Pregnancy was induced by artificial insemination, and the day of insemination was designated day 0 of pregnancy (10,29). The endometrial tissues of cattle in early pregnancy were collected during implantation (day 9-25; n=3). An intravenous injection of ≥100 mg/kg sodium pentobarbital was used for euthanasia. All samples were obtained within 30 min after exsanguination and immediately transported to the laboratory on ice.

*Primary bovine endometrial epithelial cell (bEEC) culture and identification*. The collected caruncular endometrial epithelium was mixed with adequate 1% collagenase I (10-15 ml), cut into 1x1 mm pieces and incubated for 1 h in a sealed container in a thermostatic shaker at 37˚C and 88 revolutions/minute. Collagenase I was neutralized with FBS (1-1.5 ml) after the incubation period, and the tissue pieces were placed in a culture dish (35 mm) in 0.5-1 cm intervals. The culture dish was incubated in 5% $CO_2$ at 37˚C for 3 h. After the cells adhered to the dish, the bEECs were cultured in DMEM/F12 (Thermo Fisher Scientific, Inc.; cat. no. 21041033) supplemented with 15% FBS, 2 mM L-glutamine, 50 U/ml penicillin, 50 U/ml streptomycin, 100 U/ml gentamicin and 10 ng/ml EGF, and maintained in a 5% $CO_2$ humidified incubator at 37˚C. The nutrient solution was replaced after 8 h, and thereafter it was replaced every 12 h. Following 48 h, the tissue block was removed, and the medium was replaced every 48 h. The cells were transferred (via trypsinization at room temperature for 20 sec) to 6-well plates on coverslips and analyzed for the expression of the epithelial-specific marker CK-18. Cells were grown to approximately 70% confluence, fixed with 4% paraformaldehyde for 15 min at room temperature and washed three times with PBS. The cells were blocked with 10% normal goat serum at room temperature for 30 min and incubated with CK-18 primary antibody (diluted 1:100) overnight at 4˚C. The

Table I. Primer sequences.

A, Primers for 3'-UTR cloning

| Name | Sequence (5'-3') |
|---|---|
| BoLA 3'-UTR-F | ATCTCGAGATGACATCGAGTGGCCAGAG |
| BoLA 3'-UTR-R | GAGCGGCCGCAGGCGATTGGATTTGTCGGC |
| Mut-BoLA 3'-UTR-F | ATACTCGAGCGAAAGCATGCGTCGTACCT |
| Mut-BoLA 3'-UTR-R | ATGCGGCCGGCCGAAAGTTCCTTTGTGGG |

B, Primers for reverse transcription-quantitative PCR

| Name | Sequence (5'-3') |
|---|---|
| β-actin-F | TGGACTTCGAGCAGGAGAT |
| β-actin-R | CGTCACACTTCATGATGGAA |
| IFN-τ-F | TGAACAGACTCTCTCCTCATCCC |
| IFN-τ-R | TGGTTGATGAAGAGAGGGCTCT |
| BoLA-F | CTCACACCGTCCAAGAGATG |
| BoLA-R | CTCGTTCAGGGCGATGTAAT |
| RT-bta-miR-204 | CTCAACTGGTGTCGTGGAGTCGG<br>CAATTCAGTTGAGAGGCATAG |
| bta-miR-204-F | CGTGGACTTCCCTTTGTCA |
| bta-miR-204-R | CTCAACTGGTGTCGTGGA |
| PD-L1-F | TTGGTCATCCCAGAACCATATC |
| PD-L1-R | CCTTCCAGGGTACCTTTATTCC |
| PD-L2-F | CTACAAGTACCTGACGCTGAAA |
| PD-L2-R | CAACGATGAGGGAGAGAATGAA |
| U6-F | CTCGCTTCGGCAGCACATATACT |
| U6-R | ACGCTTCACGAATTTGCGTGTC |

F, forward; R, reverse; miR, microRNA; Mut, mutant; UTR, untranslated region; BoLA, bovine leukocyte antigen; PD-L, programmed death-ligand.

Table II. Sequences of agomirs and antagomirs.

| Name | Sequence (5'-3') |
|---|---|
| bta-miR-204 agomir | UUCCCUUUGUCAUCCUAUGCCU<br>GCAUAGGAUGACAAAGGGAAUU |
| bta-miR-204 agomir NC | UUCUCCGAACGUGUCACGUTT<br>ACGUGACACGUUCGGAGAATT |
| bta-miR-204 antagomir | AGGCAUAGGAUGACAAAGGGAA |
| bta-miR-204 antagomir NC | CAGUACUUUUGUGUAGUACAA |

NC, negative control; miR, microRNA.

fluorescently labeled DyLight 594 (diluted 1:1,000) secondary antibody was incubated for 45 min at room temperature. DAPI (300 nM) was used to stain the cell nuclei for 1-5 min at room temperature. Fluorescent images were captured using laser scanning confocal microscopy (magnification, x50 and x100) and analyzed using ImageJ (https://imagej.nih.gov/ij/, National Institutes of Health; ImageJ bundled with 64-bit Java 1.8.0_172). The sixth or seventh generation of bEECs was used for subsequent experiments.

*bta-miR-204 target analysis*. The bioinformatics database TargetScan 7.2 (http://www.targetscan.org/) and RNAhybrid (https://bibiserv.cebitec.uni-bielefeld.de/rnahybrid/) were used to search for the target genes of bta-miR-204. The duplexes

and the minimum free energy (mFE) between bta-miR-204 and the 3'-untraslated regions (3'-UTRs) of the potential targets were analyzed by RNA hybridization (30).

*Plasmid construction*. To construct the wild-type and mutant BoLA 3'-UTR luciferase reporter plasmids, the full length of the BoLA 3'-UTR or fragments covering the putative bta-miR-204 binding site were amplified by RT-qPCR using cDNA resulting from extractions from dairy cattle uterine tissues. The amplified products were subcloned into the *Xho*I and *Not*I sites of the psi-CHECK-2 vector. Mutagenesis of the seed region (the bta-miR-204 target site) was achieved by PCR (Shanghai GenePharma Co., Ltd.), using the 3'-UTR plasmid as the template. The following thermocycling conditions were used: 94˚C for 2 min; followed by 35 cycles of 94˚C for 20 sec; 56˚C for 20 sec and 72˚C for 150 sec; followed by 72˚C for 5 min and 15˚C for 1 min. The amplified products were digested using the *Dpn*I restriction enzyme. *E. coli* DH5α cells were transformed with the plasmids, and colonies were grown on Luria-Bertani plates containing ampicillin at 37˚C for 16-18 h. The wild-type and mutant sequences were confirmed by enzyme digestion and sequencing. The recombinant wild-type and mutant plasmids were named Luc-BoLA (3'UTR) and Luc-BoLA (3'UTR)-Mut, respectively.

*Dual-luciferase reporter assay*. The dual-luciferase reporter assay included two reporters. One was *Renilla* luciferase, and the other was firefly luciferase in pmirGLO vector (Promega Corporation), which contained the examined 3'-UTR sequence. For the luciferase reporter assay, bEECs were plated in a 6-well plate to a density of 20-30%, 1 day before transfection. On the second day, 200 ng luciferase reporter plasmid and 10 pmol bta-miR-204 agomir, agomir NC, antagomir and antagomir NC were also transfected into bEECs using Lipofectamine 2000. Luc-BoLA (3'UTR) was transfected into bEECs without the indicated agomirs/antagomirs as the control. The cells were collected at 24 h post transfection, and dual-luciferase activity assays were performed using Dual-Luciferase Reporter Assay System according to the manufacturer's instructions. The luciferase activity was detected using a Lumat LB 9507 Ultra-Sensitive Tube Luminometer (Titertek-Berthold). The firefly luciferase activity of each sample was normalized to the *Renilla* luciferase activity. At least three independent repeats were performed for all the aforementioned transfection experiments.

*siRNA design and cell transfection*. The selection of siRNAs was based on the characterization of siRNAs in a previous study (31). According to the sequence characteristics of siRNA, the general design principle and previous design experience, several siRNAs were designed using siRNA online design tools. Three siRNA sequences were established to target cattle BoLA mRNA (BoLA siRNA1-3; Table III). To identify the most effective siRNA, 100 pmol (the amount recommended in the transfection protocol) of each of these siRNAs were used for transfection into primary bEECs using Lipofectamine RNAiMAX. RT-qPCR was used to evaluate the efficacy of the siRNAs in downregulating BoLA expression in the cells. The BoLA siRNA that exhibited the best inhibitory effect on BoLA mRNA expression was used in subsequent experiments.

Table III. Sequences of siRNA oligonucleotides.

| Name | Sequence (5'-3') |
|---|---|
| BoLA siRNA1 | GCUCAAGUCACCAAGCACATT |
| | UGUGCUUGGUGACUUGAGCTT |
| BoLA siRNA2 | GCAUCAUUGUUGGACUGGUTT |
| | ACCAGUCCAACAAUGAUGCTT |
| BoLA siRNA3 | GUGUCUCUCAUGGUUCCUATT |
| | UAGGAACCAUGAGAGACACTT |
| NC siRNA | UUCUCCGAACGUGUCACGUTT |
| | ACGUGACACGUUCGGAGAATT |

NC, negative control; si, small interfering; BoLA, bovine leukocyte antigen.

Primary bEECs were seeded into a 6-well plate with 30-40% cell density. The bEECs were treated with IFN-τ (200 ng/ml dissolved in DMSO). The blank group was treated with the same amount of DMSO. In addition, the other experimental groups were transfected with 100 pmol BoLA siRNA or NC siRNA at 37˚C for 6 h and subsequently treated with 200 ng/ml IFN-τ at 37˚C for another 12 h immediately after removal of transfection media.

*Cell treatment and cell proliferation assays*. Primary bEECs were seeded into a 6-well plate with 30-40% cell density. The bEECs were treated with 200 ng/ml IFN-τ (11). The blank group was treated with the same amount of DMSO. Furthermore, the other experimental groups were transfected with 100 pmol (in accordance with the transfection protocol) bta-miR-204 agomir, agomir NC, antagomir or antagomir NC using Lipofectamine 2000 at 37˚C for 6 h, as aforementioned. The siRNA groups were transfected with 100 pmol BoLA siRNA or NC siRNA. CCK-8 was used to examine cell proliferation according to the manufacturer's protocol at 6, 12, 24 and 48 h post-treatment. The cells were treated with 1 ml DMEM/F-12 with CCK-8 reagent (1:10 v/v) and incubated for 1 h. The absorbance of each well was measured at 450 nm using a microplate reader (Bio-Rad Laboratories, Inc.) to estimate the cell number.

*RT-qPCR*. Total RNA from primary bEECs was isolated using TRIzol® Reagent (cat. no. 15596018; Thermo Fisher Scientific, Inc.) and converted into cDNA (30˚C for 10 min followed by 42˚C for 30 min) with the PrimeScript 1st strand cDNA Synthesis Kit according to the manufacturer's instructions (Takara Bio, Inc.). Primer Premier 5 software (Premier Biosoft International) was used to design the specific primers (Table I). qPCR was performed on a StepOne Real-time PCR System (Thermo Fisher Scientific, Inc.) with the LightCycler® FastStart DNA Master PLUS SYBR Green mix in a 25-$\mu$l reaction. U6 was used as the housekeeping gene. The PCR producer for the bta-miR-204 reverse transcription reaction was 95˚C for 5 min; 40 cycles of 95˚C for 10 sec and 60˚C for 30 sec; 95˚C for 15 sec, 60˚C for 60 sec, 95˚C for 15 sec. The producer for the PCR reaction was 95˚C 3 min; followed by 95˚C for 12 sec and 62˚C for 40 sec. Each sample was assayed

in triplicate. The results (fold changes) were quantified using the $2^{-\Delta\Delta Cq}$ method (32).

*Western blot analysis*. Total protein from primary bECCs and tissues was extracted by RIPA Lysis and Extraction Buffer (cat. no. 8990, Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The protein concentration was determined using a BCA Protein Assay kit. Samples with equal amounts of protein (50 $\mu$g) were separated using 10% SDS-PAGE and transferred to a PVDF membrane, which was blocked in 5% skimmed milk in 0.4% TBS-Tween-20 (TBST) at room temperature for 2 h. The membrane was incubated with the primary antibody (1:500 dilution) at 4˚C overnight. Following washing with TBST, the membrane was incubated with the secondary antibody (1:1,500 dilution) at room temperature for 2 h. Protein expression was detected using the ECL Plus Western Blotting Detection system (Hangzhou Bioer Co., Ltd) and analyzed using ImageQuant LAS 4000 mini software (Cytiva) according to the ImageQuant LAS 4000 User Manual 28-9607-42 AC. β-actin was used as a loading control.

*Statistical analysis*. Data are presented as the mean ± SEM (n=3). Statistical analyses were performed using Microsoft Excel 2016 (Microsoft Corporation) and GraphPad Prism 6 (GraphPad Software, Inc.). Comparisons among all groups were performed with one-way ANOVA followed by Tukey's multiple comparisons test. P<0.05 was considered to indicate a statistically significant difference.

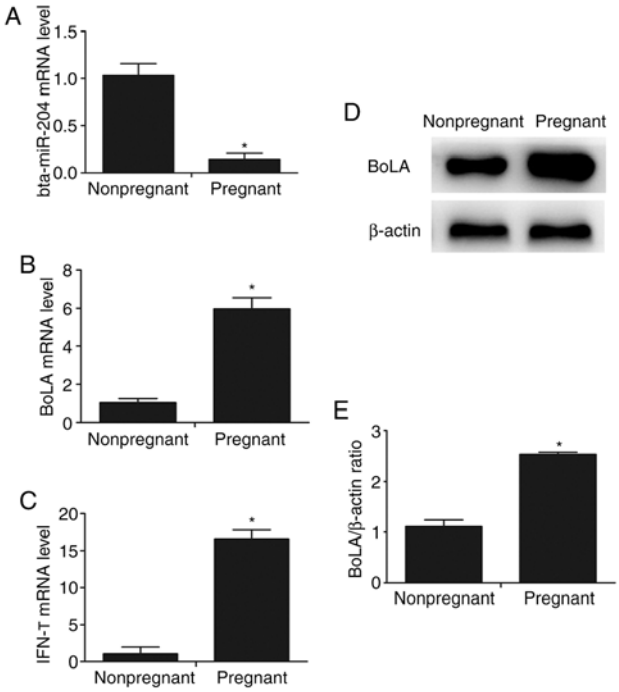


Figure 1. Expression of bta-miR-204, IFN-τ and BoLA in the endometrial tissues of dairy cattle. (A) bta-miR-204, (B) BoLA and (C) IFN-τ mRNA expression level the in endometrial tissues of pregnant and nonpregnant cattle was measured by reverse transcription-quantitative PCR. (D and E) Western blotting was performed to detect the BoLA protein levels in the endometrial tissues of pregnant and nonpregnant cattle. The data are presented as the mean ± SEM (n=3). *P<0.05 vs. the nonpregnant group. BoLA, bovine leukocyte antigen; miR, microRNA.

## Results

*Expression of bta-miR-204, IFN-τ and BoLA in the endometrial tissues of dairy cattle*. A previous study using deep sequencing indicated that IFN-τ (200 ng/ml) decreased the expression of bta-miRNA-204 in bEECs (10). To evaluate whether pregnancy affects the expression of IFN-τ, bta-miR-204 and BoLA, their levels in pregnant cattle were compared with the respective levels in nonpregnant cattle. The results revealed that the mRNA expression level of bta-miR-204 was significantly decreased in cattle during early pregnancy, but the expression level of BoLA in early pregnant cattle was increased compared with that in nonpregnant cattle (Fig. 1A and B). Moreover, the mRNA level of IFN-τ was significantly higher in the endometrium of early pregnant cattle compared with that in nonpregnant cattle (Fig. 1C). Moreover, the protein expression level of BoLA was indicated to be increased in cattle during early pregnancy using western blot analysis (Fig. 1D and E).

*Identification of primary bEECs*. Immunofluorescent studies were performed according to the aforementioned procedure. Primary bEECs were positive for the epithelial-specific marker CK-18. The proportion of CK-18$^+$ cells was >95%, as determined using confocal microscopy (Fig. 2).

*Prediction of the target gene of bta-miR-204*. As miRNAs are well documented to exert their function by affecting the expression of their target gene(s) (33), the present study attempted to identify the direct target of bta-miR-204 associated with IFN-τ treatment. Data collection and analysis revealed that BoLA was a potential target gene of bta-miR-204. The predicted target site is a 1437-1462 base sequence of BoLA mRNA, and the seed region of bta-miR-204 is a 23-30 base sequence (Fig. 3A). The mFE between the BoLA 3'-UTR and bta-miR-204 was calculated with RNAhybrid software (https://bibiserv.cebitec.uni-bielefeld.de/rnahybrid/). The mFE was ~16.9 kcal/mol, which indicated high stability of the duplex.

*BoLA is a direct target of bta-miR-204*. Luciferase reporter assays were performed to further investigate whether bta-miR-204 directly targets the 3'-UTR of BoLA in bEECs. The target sequences of the wild-type and mutant 3'-UTR of BoLA were cloned separately into a luciferase reporter vector to generate Luc-BoLA (3'UTR) and Luc-BoLA (3'UTR)-Mut, respectively (Fig. 3A). The luciferase activity was inhibited by the binding of bta-miR-204 to the 3'-UTR of BoLA. Co-transfection of Luc-BoLA (3'UTR) with bta-miR-204 agomir and bta-miR-204 antagomir in bEECs resulted in a significant alteration in the luciferase activity compared with that in the control groups, which were transfected with Luc-BoLA (3'UTR) only or co-transfected with Luc-BoLA (3'UTR) and bta-miR-204 agomir NC or Luc-BoLA (3'UTR) and bta-miR-204 antagomir NC. BoLA 3'-UTR luciferase activity was inhibited by bta-miR-204 agomir, which mimics bta-miR-204, while it was increased following transfection with bta-miR-204 antagomir, which is an inhibitor of bta-miR-204. Furthermore, the luciferase activity of BoLA (3'UTR)-Mut was unaffected following transfection with bta-miR-204 agomir compared with the transfection with Luc-BoLA (3'UTR) only or the co-transfection of Luc-BoLA

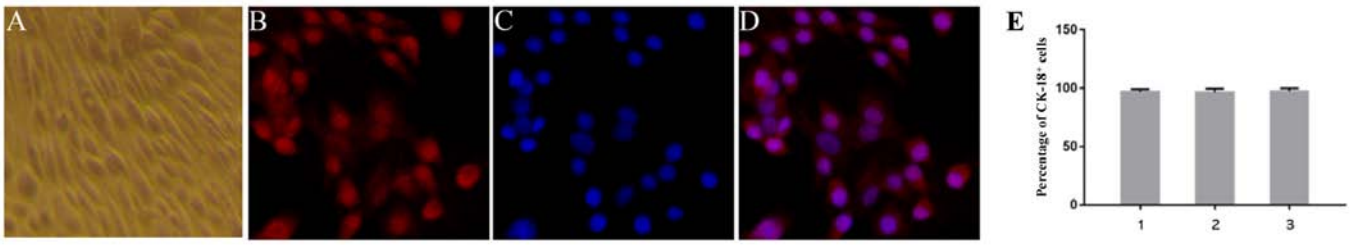


Figure 2. Primary bEEC culture and identification. (A) Morphological observation of bEECs by ordinary optical microscopy (magnification, x200). (B) Primary bEECs were positive for the epithelial-specific marker CK-18 (red). (C) Nuclei were stained with DAPI (blue). (D) Merge of CK-18 and DAPI (magnification, x400). (E) The CK-18$^+$ cells were counted using ImageJ. bEEC, bovine endometrial epithelial cell; CK-18, cytokeratin-18; IF, immunofluorescence.

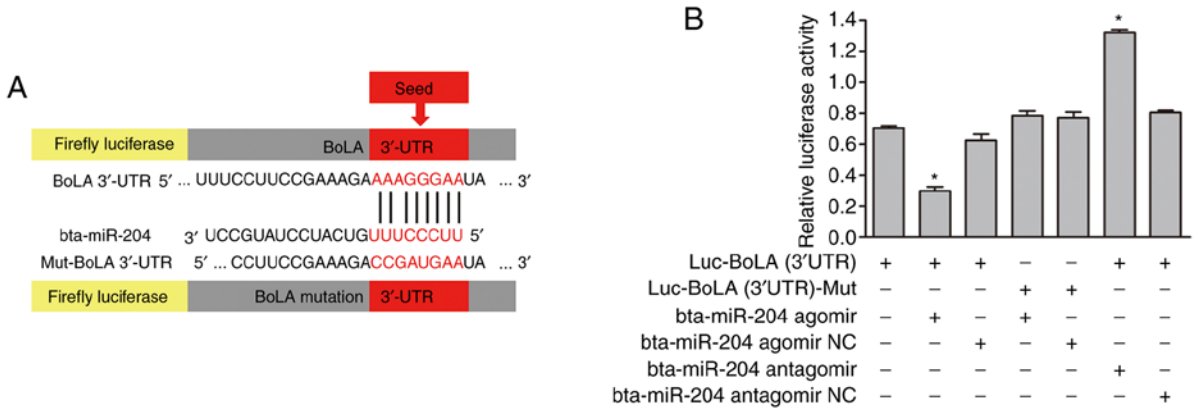


Figure 3. BoLA is a direct target of bta-miR-204. (A) Luciferase reporter vector containing the 3'-UTR (wild-type or mutant) of BoLA. (B) bEECs were co-transfected with Luc-BoLA (3'UTR) and Luc-BoLA (3'UTR)-Mut and the indicated agomirs/antagomirs. At 24 h post transfection, the cells were assayed for both firefly and *Renilla* luciferase activity using a dual-luciferase reporter assay. The data are presented as the mean ± SEM (n=3). $^*$P<0.05 vs. Luc-BoLA (3'UTR). BoLA, bovine leukocyte antigen; miR, microRNA; UTR, untranslated region, Mut, mutant; Luc, luciferase; NC, negative control.

(3'UTR)-Mut and bta-miR-204 agomir NC. This result indicated that the mutated seed sequence of BoLA (3'-UTR) and bta-miR-204 agomir did not bind to each other to affect the luciferase activity (Fig. 3B). The results demonstrated that bta-miR-204 negatively regulated BoLA expression by directly binding to its complementary sequence in the 3'-UTR of BoLA in a sequence-specific manner.

*Effects of IFN-τ, bta-miR-204 and BoLA siRNAs on cell proliferation*. CCK-8 cell proliferation assays were performed to investigate whether the proliferative capacity of bEEC was affected by IFN-τ, bta-miR-204 agomir/NC, bta-miR-204 antagomir/NC and BoLA siRNAs/NC. The results indicated that little difference existed between the IFN-τ-treated group and the blank group. Similarly, little difference was observed between the transfection groups and the blank group, respectively (Fig. 4). Therefore, IFN-τ, bta-miR-204 agomir, and bta-miR-204 antagomir exhibited little effect on the proliferation of primary bEECs compared with the control groups.

*IFN-τ positively regulates BoLA expression*. To determine the roles of IFN-τ and bta-miR-204 in the regulation of BoLA expression, treatment of bEECs with IFN-τ and suppression or overexpression of bta-miR-204 was performed. After transient transfection with bta-miR-204 agomir, bta-miR-204 overexpression was detected in bEECs by qPCR (Fig. 5A). Furthermore, bta-miR-204 agomir NC, bta-miR-204 antagomir and bta-miR-204 antagomir NC were

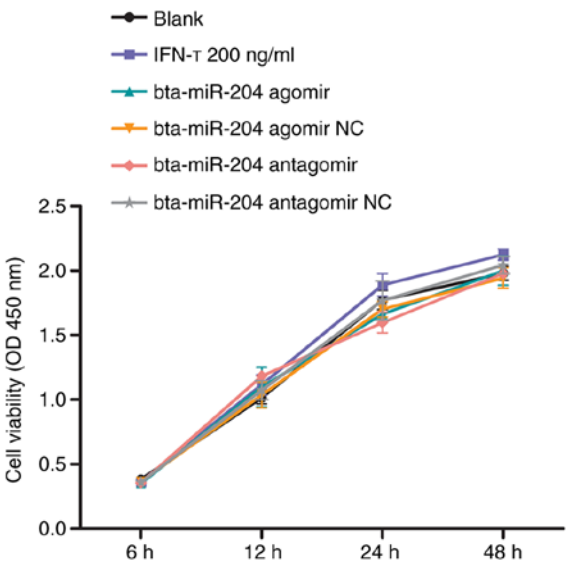


Figure 4. Proliferation of primary bEECs. bEECs treated with IFN-τ (200 ng/ml) or transfected with bta-miR-204 agomir, bta-miR-204 agomir NC, bta-miR-204 antagomir and bta-miR-204 antagomir NC were the experimental groups and the blank group (untreated) was used as the control. The data are presented as the mean ± SEM (n=3). NC, negative control; bEECs, bovine endometrial epithelial cells; BoLA, bovine leukocyte antigen; miR, microRNA; OD, optical density.

also overexpressed in bEECs via transient transfection. The statistical analysis demonstrated that compared with the blank

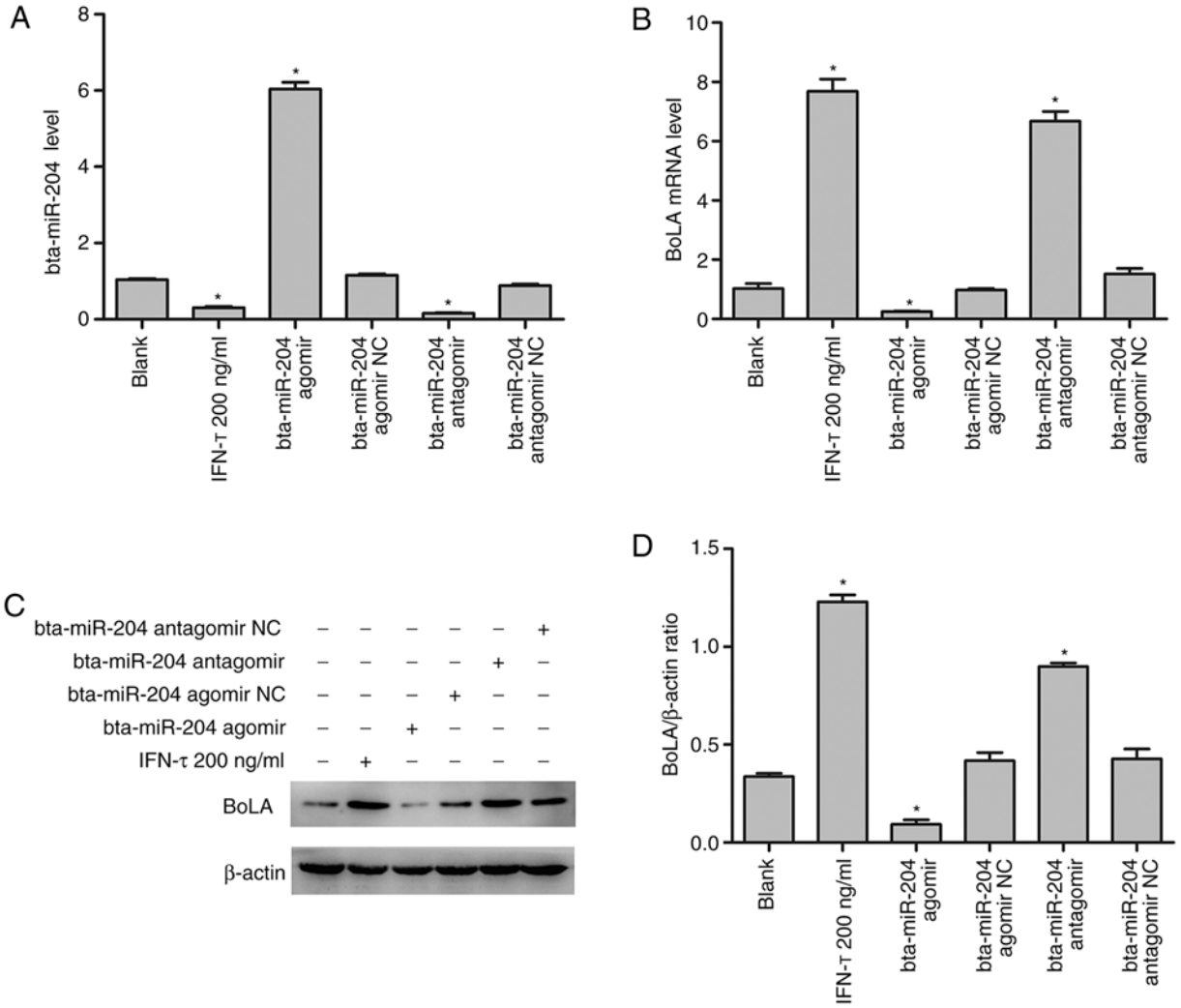


Figure 5. IFN-τ regulates BoLA expression in bEECs. (A) bta-miR-204 and (B) BoLA expression after IFN-τ (200 ng/ml) treatment or bta-miR-204 agomir, agomir NC, antagomir and antagomir NC transfection in bEECs was measured by reverse transcription-quantitative PCR. (C) BoLA protein levels in these groups were detected by western blotting. (D) Gray values of the indicated protein were measured by Fusion software. The data are presented as the mean ± SEM (n=3). *P<0.05 vs. the control group (blank). NC, negative control; bEECs, bovine endometrial epithelial cells; BoLA, bovine leukocyte antigen; miR, microRNA.

group, the expression level of bta-miR-204 was downregulated both after IFN-τ treatment and in the bta-miR-204 antagomir group (Fig. 5A). In addition, compared with the NC group the mRNA (Fig. 5B) and protein (Fig. 5C) expression of BoLA were upregulated as a consequence of the decreased expression of bta-miR-204 While, mRNA and protein expression levels of BoLA were downregulated in the bta-miR-204 groups. Collectively, these results indicated that IFN-τ upregulated BoLA expression by negatively regulating bta-miR-204 expression in bEECs.

*Expression of PD-L1 and PD-L2 in IFN-τ-treated bEECs and endometrial tissues of dairy cattle*. A previous study has reported that the expression level of MHC-I was positively associated with that of PD-L1 and PD-L2 (34). Uterine tissues of pregnant or nonpregnant cattle and BoLA siRNA-transfected bEECs were used in this experiment to evaluate the effect of IFN-τ and BoLA on the PD-L1 and PD-L2 expression level. The results revealed that the mRNA expression of PD-L1 and PD-L2 in cattle during early pregnancy was significantly increased compared with that in nonpregnant cattle (Fig. 6A). RT-qPCR was used to evaluate the efficacy of the siRNAs in downregulating BoLA expression in bEECs. The results indicated that BoLA siRNA 1 exhibited the best inhibitory effect on BoLA mRNA expression, therefore it was used in subsequent experiments (Fig. 6B). In accordance with the *in vivo* results, the mRNA expression level of PD-L1 and PD-L2 in the bEECs was increased following IFN-τ treatment compared with that in the blank group. However, the expression level of PD-L1 and PD-L2 in the BoLA siRNA-transfected groups decreased compared with that in the IFN-τ treatment group (Fig. 6C). These results indicated that IFN-τ may induce PD-L1 and PD-L2 transcription via regulating BoLA expression.

## Discussion

Embryo implantation is an important step for the establishment of normal pregnancy. In ruminants and humans, the interaction between the trophoblast cells and the cells of the apical surface of the luminal epithelium indicates implantation (35).

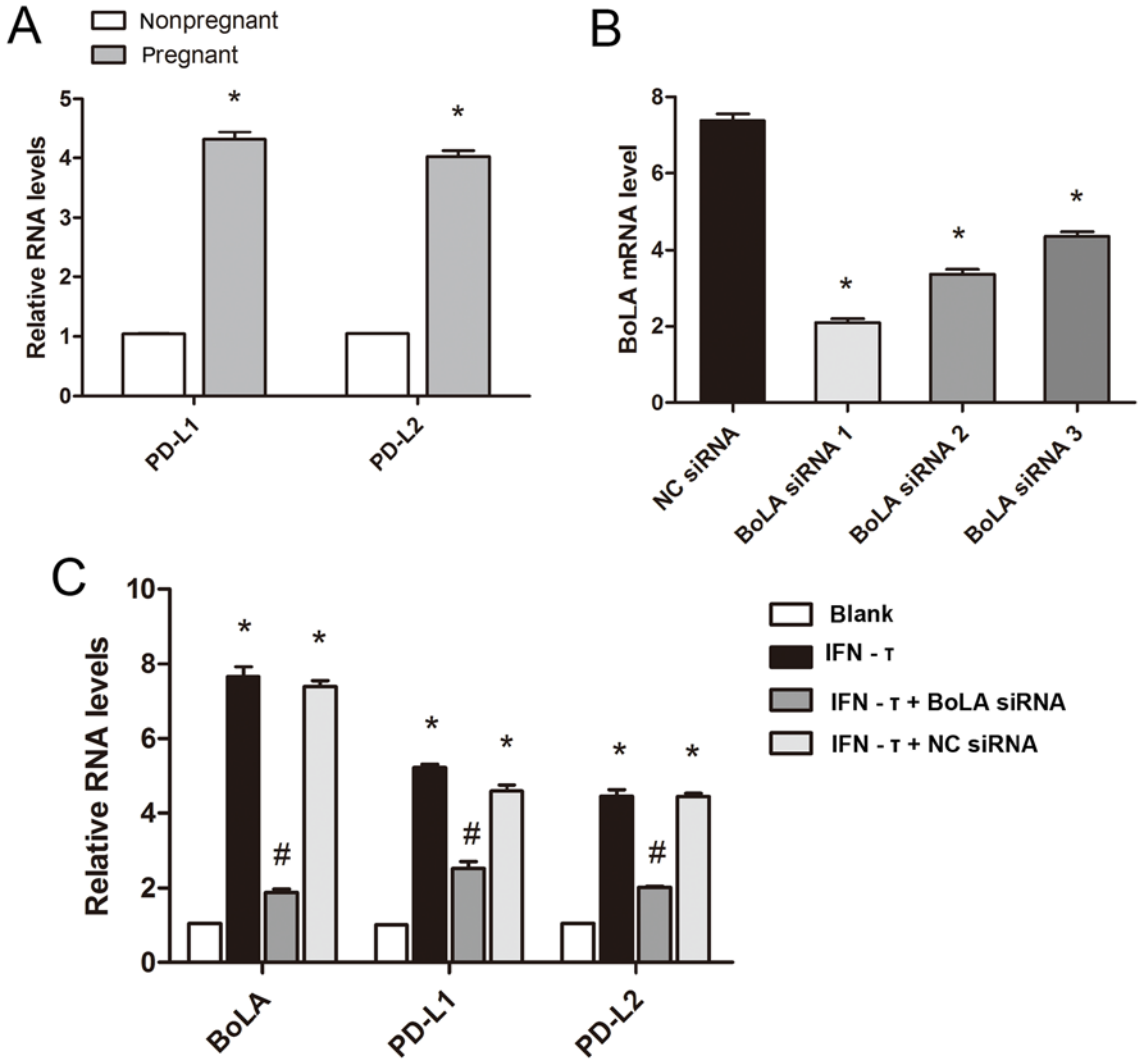


Figure 6. IFN-τ regulates PD-L1 and PD-L2 expression. (A) PD-L1 and PD-L2 expression in the endometrial tissues of pregnant and nonpregnant dairy cattle was measured by RT-qPCR. (B) BoLA expression in bEECs transfected with different siRNAs was measured by RT-qPCR. (C) PD-L1 and PD-L2 expression following IFN-τ (200 ng/ml) treatment and transfection with BoLA siRNA or NC siRNA in bEECs was measured by RT-qPCR. The data are presented as the mean ± SEM (n=3). *P<0.05 vs. nonpregnant, NC siRNA or blank. #P<0.05 vs. IFN-τ group. RT-qPCR, reverse transcription-quantitative PCR; si, small interfering; NC, negative control; bEECs, bovine endometrial epithelial cells; BoLA, bovine leukocyte antigen; PD-L, programmed death-ligand.

The difference between ruminants and humans in controlling embryo implantation is that, in addition to estrogen and progesterone, ruminants also secrete IFN-τ to regulate the expression of various cytokines and transcription factors (36,37).

Previous studies have identified an important function of miRNAs in regulating potential gene expression during implantation in a range of species, such as humans, mice and swine (38-42). For instance, miR-200a was reported to regulate progesterone-progesterone receptor signaling via negatively regulating the expression of progesterone receptor and 20-hydroxysteroid dehydrogenase to influence endometrial receptivity and embryo implantation (43). miR-29a was indicated to regulate the expression of proapoptotic and antiapoptotic factors, thereby serving an important role during embryo implantation (44). Moreover, miR-148a and miR-152 were revealed to regulate the expression of HLA-G to affect the acceptance of the fetus (45,46).

In the present study, IFN-τ was indicated to reduce the expression of bta-miR-204 in bEECs. miR-204 is highly conserved in humans, rabbits, rats, mice and other vertebrates, and it has been revealed to be one of the most commonly altered miRNA in tumors (47-49). Previous studies have indicated that the expression of miR-204 was downregulated in tumor tissues and cells (50-52). This finding suggested that low-level miR-204 via the negative regulation of its target genes may aid the tumor to escape the immune system or proliferate, migrate and invade the tissues.

Previous studies have demonstrated that there were a number of similarities between embryo implantation and tumor invasion and metastasis, such as pathophysiological processes, gene expression, angiogenesis and immune escape (53-55). Based on these similarities, an analogy may be drawn between pregnancy and cancer in terms of immune tolerance (35,56). To further verify whether bta-miR-204 serves an important role in regulating embryo implantation as in regulating tumors, it was firstly observed that the mRNA and protein expression of BoLA were significantly increased in the endometrial tissues of pregnant dairy cattle compared

with nonpregnant cattle and in bEECs treated with IFN-τ compared with control cells. These results were in accordance with the results of previous studies, which demonstrated the important and beneficial role of MHC-I in the establishment of pregnancy (57-59). Subsequently, using dual-luciferase reporter assay, BoLA was verified to be the direct target gene of the bta-miR-204.

Although previous research has revealed MHC-I as a key factor in regulating embryonic development, the specific molecular mechanisms and genes involved are not well understood (60). Several embryonic development-associated genes were analyzed to explore the possible mechanisms in embryo development (61). PD-L1 and PD-L2 are two ligands known to bind to PD-1 and have been associated with the regulation of tolerance and autoimmunity (62,63). PD-L1 and PD-L2 mRNAs have been detected in a variety of tissues, including the heart, lungs, placenta and tumor tissues (64,65). In the present study, the mRNA levels of PD-L1, PD-L2, BoLA and IFN-τ were indicated to be significantly increased in the endometrial tissues of pregnant dairy cattle compared with those in nonpregnant cattle. Aust *et al* (34) reported that a large number of MHC-I and MHC-II genes were positively associated with PD-L1 expression levels in tumor cells. To further elucidate the relationship between BoLA and PD-L1 or PD-L2, experiments were conducted using bEECs. The results revealed that PD-L1 and PD-L2 mRNA expression level increased following IFN-τ treatment, whereas it decreased after transfection with BoLA siRNA. These results indicated that IFN-τ upregulated PD-L1 and PD-L2 transcription, potentially via regulating BoLA expression. These data may provide a basis for explaining the diversity in immune escape mechanisms in pregnant dairy cattle during embryo implantation. A common immune escape mechanism of tumor cells is the downregulation of HLA-I (66). Another common immune escape mechanism in tumors and transplants is the activation of the PD/PD-L negative costimulatory pathway, which alters the balance between pathogenic and regulatory T cells (21,67,68). In particular, PD-L1 expression is crucial for the maintenance of tolerance at the utero-placental interface (69).

In the present study, the results revealed that IFN-τ may induce BoLA expression due to its negative regulation of bta-miR-204. In addition, as the expression level of BoLA was induced by IFN-τ, the mRNA level of PD-L1 and PD-L2 was also increased, and it was positively associated with the expression level of BoLA. These results further indicated the immune escape mechanism of IFN-τ in regulating implantation during early pregnancy in dairy cattle. However, the use of a single animal model is a limitation to the present study, and although the method presented was accurate, different species of animals should be tested *in vivo* or *in vitro*.

To conclude, on the basis of the experimental results, the current study indicated that IFN-τ increased the expression of BoLA by reducing the expression of bta-miR-204, and IFN-τ may induce the transcription of PD-L1 and PD-L2 by inhibiting bta-miR-204 to upregulate the expression of BoLA, thereby affecting the immune microenvironment of the maternal-fetal interface and promoting fetal immune escape.

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## Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

## Authors' contributions

XW and GD designed the study and participated in the data analysis and interpretation, manuscript drafting and critical revision. XW, NY and XM performed the experiments and contributed to data acquisition. TY, YY, JY and AS provided reagents and contributed to data analysis and interpretation and to critical revision of the manuscript. All authors read and approved the final manuscript.

## Ethics approval and consent to participate

The present study was approved by the Huazhong Agricultural University Animal Care and Use Committee (Wuhan, China; approval no. 20171354CA).

## Patient consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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