Journal of Reproduction and Development, Vol. 68, No 1, 2022

Original Article

Transcriptome analysis reveals transforming growth factor-β1 prevents extracellular matrix degradation and cell adhesion during the follicular-luteal transition in cows

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Abstract. Ovarian angiogenesis is an extremely rapid process that occurs during the transition from follicle to corpus luteum (CL) and is crucial for reproduction. It is regulated by numerous factors including transforming growth factor-β1 (TGFB1). However, the regulatory mechanism of TGFB1 in ovarian angiogenesis is not fully understood. To address this, in this study we obtained high-throughput transcriptome analysis (RNA-seq) data from bovine luteinizing follicular cells cultured in a system mimicking angiogenesis and treated with TGFB1, and identified 455 differentially expressed genes (DEGs). Quantitative real-time PCR results confirmed the differential expression patterns of the 12 selected genes. Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis identified that the MAPK and ErbB pathways, cell adhesion molecules (CAMs), and extracellular matrix (ECM)-receptor interactions may play pivotal roles in TGFB1-mediated inhibition of CL angiogenesis. TGFB1 phosphorylated ERK1/2 (MAPK1/3) and Akt, indicating that these pathways may play an important role in the regulation of angiogenesis. Several genes with specific functions in cell adhesion and ECM degradation were identified among the DEGs. In particular, TGFB1-induced upregulation of syndecan-1 (SDC1) and collagen type I alpha 1 chain (*COL1A1*) expression may contribute to the deposition of type I collagen in luteinizing follicular cells. These results indicate that TGFB1 inhibits cell adhesion and ECM degradation processes involving ERK1/2, ErbB, and PI3K/Akt signaling pathways, and leads to inhibition of angiogenesis during the follicular-luteal transition. Our results further reveal the molecular mechanisms underlying the actions of TGFB1 in early luteinization.

Key words: Angiogenesis, Extracellular matrix (ECM), Extracellular signal-regulated kinases 1/2 (ERK1/2), Luteinization, Transforming growth factor-β1 (TGFB1)

(**J. Reprod. Dev. 68:** 12–20, 2022)

The corpus luteum (CL) is formed from the remnants of the ruptured follicle after ovulation, which is accompanied by a highly intense phase of angiogenesis, particularly in the early luteal phase [1]. CL requires an extensive vascular supply to support its rapid growth and steroidogenic function, and inadequate progesterone (P_4) production is associated with poor embryo development and increased pregnancy failure in cows [2].

Angiogenesis plays an indispensable role in CL development and is a highly regulated process that requires coordination between pro- and anti-angiogenic factors, including vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), thrombospondins, and transforming growth factor-β (TGFB1) [3–6].

As a multifunctional growth factor, TGFB1 signals through the cell surface receptor, activin-like kinase (ALK), which then phosphorylates the intracellular signaling molecules (Smad) to regulate gene transcription [7]. TGFB1 has been reported to stimulate or inhibit angiogenesis *in vivo* and *in vitro* in a highly context- and concentration-dependent manner [8]. The stimulatory role of TGFB1 in follicular angiogenesis was indicated by its ability to enhance the secretion of pro-angiogenic factors including VEGFA by rat granulosa cells [9]. In contrast, TGFB1 is involved in the disassembly of capillaries during bovine luteal regression [4, 10].

TGFB1 is expressed in luteal cells in cattle [11, 12] and may contribute to CL formation [13]. However, the exact role and mechanism of TGFB1 in the modulation of angiogenesis during follicle to CL transition has not been fully explored. Previously, we used a serum-free angiogenic culture system [14] that contained many follicular cell types (e.g., steroidogenic, endothelial cells [ECs], and pericytes), stimulated them to undergo luteinization concurrent with angiogenesis, and reported that TGFB1 disrupts the formation of capillary-like structures via TGFB type I receptor (TGFBR1) and Smad2/3 signaling, which may provide critical homeostatic control of angiogenesis during the follicular-luteal transition [6].

In addition to the canonical Smad-dependent pathway [7], TGFB1 activates the MAP kinase, Rho-like GTPase, and phosphatidylinositol-3-kinase (PI3K)-AKT signaling pathways [15, 16]. It also interacts with ErbB, Wnt, Hippo or JAK-STAT signaling pathways [17]. The potential crosstalk and integration of these pathways in CL angiogenesis have not yet been fully understood. A thorough investigation of the molecular events and underlying biochemical mechanisms of TGFB1 in luteal angiogenesis would greatly enhance

Received: June 6, 2021
Accepted: September 29, 2021
Advanced Epub: October 22, 2021

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our knowledge about the critical processes underlying the transition from follicle to CL.

Given the paucity of information on the involvement of TGFB1 signaling in CL angiogenesis, in the present study, we utilized samples from a previously established cell culture system and performed deep RNA sequencing (RNA-Seq) by applying next-generation sequencing (NGS) technology to investigate the potential changes in gene expression. We aimed to elucidate the additional signaling pathways involved in the action of TGFB1 on angiogenesis during the follicular-luteal transition. A network extension of the signaling pathways that mediated by TGFB1 was then created.

Materials and Methods

Preparation of culture medium

Unless stated otherwise, the reagents used for cell culture were purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA). Recombinant human TGFB1 protein was obtained from R&D Systems (Minneapolis, MN, USA). MCDB131 medium (Sigma-Aldrich), a specialized endothelial basal medium, was supplemented with 100 units/ml penicillin, 100 units/ml streptomycin, 18.2 µg/ml hydrocortisone, 1 µg/ml ascorbic acid, 50 ng/ml amphotericin-B, 50 µg/ml gentamicin, 20 ng/ml long arginine 3-insulin like growth factor, 10 IU/ml heparin, 10 µg/ml insulin, 5.5 µg/ml transferrin, 5 ng/ml selenium and 5 ng/ml LH (Prospec-Tany Technogene Ltd., Ness-Ziona, Israel). Fetal bovine serum (FBS, 1%, Thermo Fisher Scientific, Waltham, MA, USA) was added to this medium only for the first 18–24 h of culture, and was then replaced with 1 mg/ml bovine serum albumin (BSA) for the remaining culture period.

Tissues collection and luteinizing follicular angiogenesis

Bovine ovaries were collected from a local slaughterhouse and transported to the laboratory in phosphate-buffered saline (PBS) at 15–20°C. Healthy large antral follicles were selected, and the follicular cells were collected. These cells were then cultured in a validated luteinizing follicular angiogenesis culture system, wherein cells from both the granulosa and theca cell layers (including ECs) were co-cultured in an environment that supported both luteinization and angiogenesis [14]. In brief, ovaries were washed thrice with sterile physiological saline at 37°C, and large follicles (diameter >10 mm) with good vascularization were dissected out, hemisected, and the granulosa cells were dispersed manually into the medium. Clumps of granulosa cells were removed by filtration through a 70 µm mesh filter. The theca shells were dispersed with 1 mg/ml collagenase type IA, 1 mg/ml hyaluronidase type IS, and 0.2 units/ml DNase type IV digestion in a shaking water bath at 37°C. After 30 min, the digestion was stopped by adding FBS, and the cell clumps were removed by filtration (70 µm mesh). The dispersed theca cells were then centrifuged for 5 min at 300 × *g* at 20°C and resuspended in MCDB131 medium. Red blood cells were lysed by adding three volumes of lysis buffer (Solarbio Life Science, Beijing, China) for 5 min. Following a second centrifugation (300 × *g*, 10 min), the theca cells were resuspended in fresh MCDB131 medium. Cell number and viability were determined by trypan blue exclusion and cells were plated at a density of 4×10^5 cells/well (granulosa: theca ratio 3:1) on fibronectin-coated glass cover slips prepared as detailed previously [18] in 12-well plates (Corning Life Sciences, Tewksbury, MA, USA). Cells were incubated in a humidified incubator in 5% CO_2 in air at 39°C, and the medium was replaced with fresh supplemented MDCB131 medium after 24 h of culture. The cells were further treated with 0 or 10 ng/ml TGFB1 for 2 days. At the end of culture, the cells were fixed in acetone: methanol (1:1) at 4°C for 5 min for immunohistochemical analysis or collected for RNA and protein extraction. Each treatment was performed in triplicate per culture, and at least three separate cultures were used.

Immunocytochemistry for von Willebrand factor (VWF)

On day 3 of culture, the endothelial cells were immunostained with VWF antibody as previously validated and described [14, 18]. VWF is an established endothelial cell marker that is used to assess the formation of tubule-like structures over time [19]. In brief, after fixing and blocking with 20% (v/v) normal goat serum (Sigma-Aldrich), the coverslips were incubated with 5 µg/ml rabbit anti-human VWF primary antibody (ab6994; Abcam Ltd., Waltham, MA, USA) overnight in a humidified chamber, followed by incubation with a biotinylated goat anti-rabbit secondary antibody and then detected using the rabbit specific HRP/DAB (ABC) detection IHC Kit (Abcam). All image analyses were performed using Image ProPlus 6.3 (Media Cybernetics, Wokingham, UK), with sections visualized under a × 5 objective. The area of brown (positive) staining was highlighted with only network-like areas included (> 250 μm^2).

RNA extraction and construction of RNA-seq library

Bovine luteinizing follicular cells were stimulated with synthetic TGFB1 (10 ng/ml) for 48 h and RNA-Seq was conducted. Total RNA was extracted using the TRIzol® reagent kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) was used to determine the RNA concentration and RNA integrity number (RIN). Only samples with RIN scores > 8 were used for the sequencing. The mRNA from the samples in each group was enriched using oligo (dT)-attached magnetic beads. Double-stranded cDNA was synthesized and purified prior to ligation of the adaptors to the fragments. These samples were subsequently used to construct a cDNA library using the Illumina HiSeqTM 2500 by Gene Denovo Biotechnology Co. (Guangzhou, China).

Transcriptome data processing

After sequencing, raw reads were filtered using Fastp to remove reads containing adapters, poly-N, and low-quality bases (e.g., N > 5% and quality < 20). The clean reads were then mapped to the reference *Bos taurus* genome by TopHat2, and the unmapped reads (or mapped very poorly) were then re-aligned with Bowtie2. The transcriptome data were deposited at: https://bigd.big.ac.cn/gsa/browse/CRA005656

Identification of differentially expressed genes (DEGs)

Gene abundances were quantified by fragments per kilobase of transcript per million mapped reads (FPKM) using the RSEM software [20]. DEG analysis of the control and TGFB1-stimulated groups was performed using the edgeR package (version 3.12.1). False Discovery Rate (FDR)-corrected p-values were used to screen the DEGs. FDR < 0.05, and log2 (fold change) ≥ 1 was identified as the threshold for significant differential expression.

Gene ontology (GO) enrichment and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis of DEGs

A comprehensive bioinformatics analysis of DEGs was then performed using an online platform (http://www.omicshare.com/tools/), using all the annotated genes in the *Bos taurus* genome as background. GO and KEGG pathway analyses of the DEGs were conducted using GO (http://www.geneontology.org/) and KEGG

(http://www.genome.jp/kegg/) databases, respectively. GO annotation of the DEGs was performed using the cluster-Profiler R package. The calculated p-value was subjected to FDR correction, with FDR ≤ 0.05, as a threshold. GO terms and pathways meeting this condition were defined as significantly enriched by the DEGs.

Quantitative real-time PCR (qRT-PCR) validation

Twelve DEGs were randomly selected for the qRT-PCR analysis. Primer sequences for these genes are listed in Supplementary Table 1. On day 3 of culture, RNA was isolated from the cultured luteinizing follicular cells using the RNAprep Pure Cell Kit (Tiangen Biotech, Beijing, China). One microgram of total RNA was transcribed into cDNA using the PrimeScript™ RT Reagent Kit (Perfect Real Time; TaKaRa, Dalian, China) according to the manufacturer's instructions. qRT-PCR was performed in 20 µl reaction volumes using a SYBR® Premix EX Taq Kit (Takara, Shiga, Japan) according to the manufacturer's instructions. An ABI 7500 system (Applied Biosystems, Foster City, CA, USA) was used to detect the amplification products following: 2 min at 95°C, followed by 40 cycles of 10 sec at 95°C, 30 sec at 60°C, and 30 sec at 72°C. Upon completion of the qRT-PCR, the threshold cycle (Ct) values were calculated using the ABI 7500 software V.2.0.6. The relative expression levels of the selected genes were calculated using the $2^{-\Delta\Delta CT}$ method and normalized to the stably expressed housekeeping gene (*ACTINB*). The stability of *ACTINB* between the control and treatment groups was tested in our previous study [6]. Three separate experiments were performed on cells from different cultures, and each treatment was performed in triplicate.

Western blotting

On day 1 of culture, cells were washed with ice-cold PBS and removed by scraping. After lysis in RIPA lysis buffer containing a protease and phosphatase inhibitor cocktail (Abcam), the extracts were centrifuged at 20 000 × *g* for 20 min at 4°C, and stored at –80°C until analysis. Protein concentrations were quantified using a DC™ Protein Assay Kit (Bio-Rad Laboratories Inc., Hercules, CA, USA). Equal amounts (25 µg) of proteins were separated by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride membranes (Merck Millipore, Burlington, MA, USA). The membranes were then blocked with 5% (w/v) BSA in Tris-buffered saline containing 0.05% Tween (TBST) for 2 h, and then incubated overnight at 4°C with primary antibodies against ERK1/2 (#9102, 1:1000), phospho-ERK1/2 (#9101, 1:1000), phospho-Akt (#4060, 1:1000), Akt (#9272, 1:1000), ACTINB (#4970, 1:1000), COL1A1 (#91144, 1:1000), E-cadherin (#3195, 1:1000), GAPDH (#2118, 1:1000), all from Cell Signaling Technology (Danvers, MA, USA), or antibodies against Syndecan-1 (#10593-1-AP, 1: 600) from Proteintech Group, Inc. (Rosemont, IL, USA). After washing thrice with TBST, the membranes were incubated with the appropriate horseradish peroxidase-conjugated goat anti-rabbit IgG (#sc-2004, Santa Cruz Biotechnology, Inc. Dallas, TX, USA) diluted 1:5000 in 5% (w/v) fat-free dry milk/TBST for 1 h at room temperature. Immunoreactive bands were visualized using SuperSignal™ West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific) with the ImageQuant LAS4000 system (GE, Boston, MA, USA).

Statistical analysis

All data were checked for normality and heterogeneity of variance and are presented as the mean ± standard error of mean (SEM). Differences between the control and TGFB1-stimulated groups were analyzed by Student's *t*-test with SPSS 19.0 (SPSS Inc., Chicago, IL, USA) and PRISM software (GraphPad Software, Inc., San Diego, CA, USA). Statistical significance was set at $P < 0.05$.

Results

Effect of TGFB1 on EC network formation and VWF expression in bovine luteinizing follicular cells

After 3 days of culture, a number of VWF-positive EC clusters were observed in the control wells (Fig. 1A). However, TGFB1 reduced EC network formation; very few clusters were present across the entire well (Fig. 1B). A magnified view of the endothelial cell network formation in the control is shown in Fig. 1C. These observations were further confirmed by the downregulation of *VWF* mRNA expression in luteinizing follicular cells following treatment with 10 ng/ml TGFB1 for 48 h ($P < 0.01$, Fig. 1D).

Analysis of the DEGs

After the clean reads were mapped to the *Bos taurus* reference genome, analysis of the RNA-Seq data for Ensembl (version 92) annotation identified 21,039 genes in all the samples. Using edgeR software, 455 genes were found to be differentially expressed between the control and TGFB1 treatment groups at a false discovery rate FDR < 0.05, and log2 (fold change) ≥ 1, of which 173 genes were upregulated and 282 genes were downregulated in the TGFB1 treatment group compared to the control group. The volcano plot clearly displayed DEGs between the control and TGFB1 groups (Fig. 2A). The ten most upregulated and downregulated genes are listed in Table 1.

GO enrichment analysis of DEGs

To investigate the biological functions of all the DEGs, their functional categories were determined by GO annotation. Of the 455 DEGs, 239 were distributed into three main functional biological categories: biological processes, cellular components, and molecular functions (Fig. 2B). Biological processes (240 genes), including biological regulation, developmental process, metabolic process, reproduction, reproductive process, response to stimulus, signaling, and cell-cell adhesion were involved in the response to TGFB1 and influenced the corresponding biological functions (Fig. 2B). For molecular functions, the binding term was the most abundant category. The 'cellular component' of the GO category encompassed cell junction, membrane and extracellular matrix.

KEGG pathway enrichment analysis

To further understand the biological pathways involved in the regulation of TGFB1 in early CL angiogenesis, the DEGs from both groups were mapped to the KEGG pathway database. The analysis revealed that the DEGs were assigned to multiple reference pathways; the top 20 KEGG pathways are shown as a bubble chart in Fig. 2C. These significantly enriched pathways included cytokine-cytokine receptor interaction, TNF signaling pathway, cell adhesion molecules (CAMs), MAPK signaling pathway, ovarian steroidogenesis, and nitrogen metabolism.

Effect of TGFB1 on activation of MAPK and PI3K-Akt signaling pathways

Phosphorylation levels of ERK1/2 (MAPK1/3) and Akt were assessed to determine the activation of the MAPK and PI3K/Akt signaling pathways. The results of western blotting indicated that treatment with TGFB1 for 30 min induced phosphorylation of ERK1/2, whereas the total ERK1 and ERK2 protein levels remained

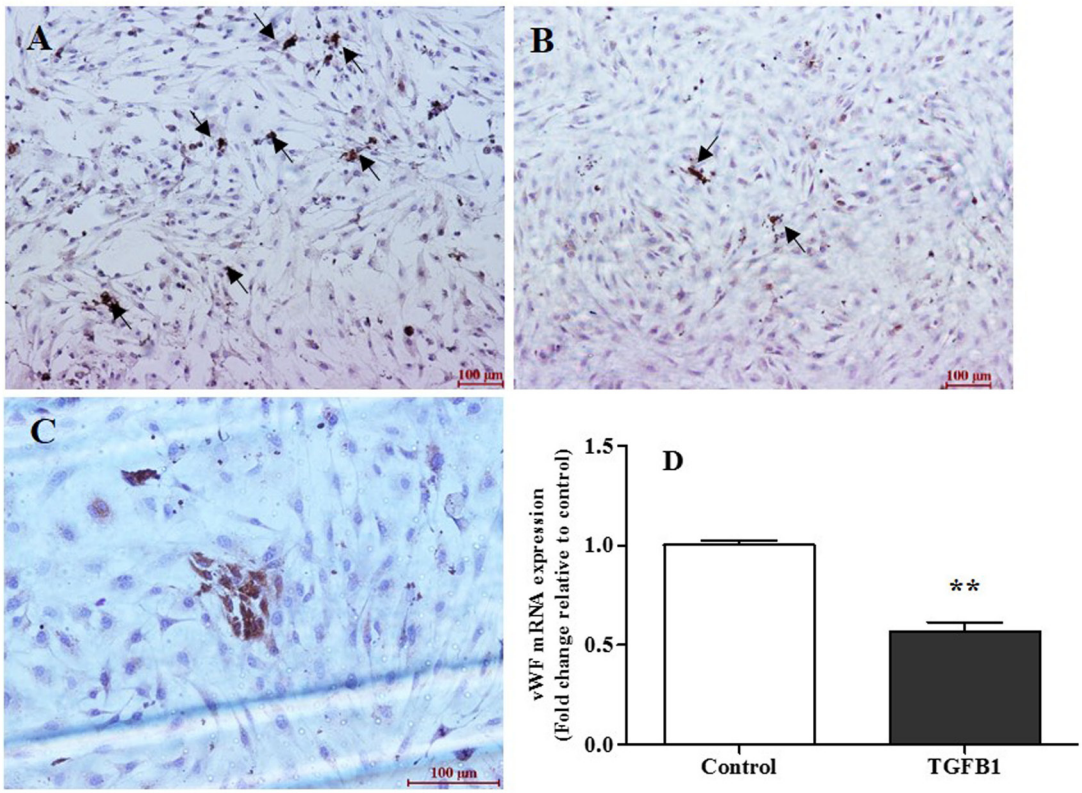

Fig. 1. TGFB1 inhibits the endothelial cell (EC) network formation and von Willebrand factor (VWF) expression in bovine luteinizing follicular cells. Representative images of bovine luteinizing follicular cells (including granulosa, theca and ECs) treated with control medium (A) and 10 ng/ml TGFB1 (B) EC networks were identified by immunohistochemical staining of VWF. Scale bars = 100 μm. (C) Magnified view of the EC network in the control. (D) TGFB1 downregulates *VWF* expression in bovine luteinizing follicular cells. Cells were treated with 10 ng/ml TGFB1 for 48 h, and the mRNA levels of *VWF* were examined using qRT-PCR. Data are presented as mean ± standard error of mean (SEM; n = 3 cultures). * means $P < 0.05$ compared with control; ** means $P < 0.01$ compared with control.

unchanged (Fig. 3A). TGFB1 treatment also induced significant Akt phosphorylation and activated the PI3K-Akt signaling pathway (Fig. 3B).

Selection of key genes and confirmation by qRT-PCR

To confirm the results obtained from RNA sequencing, qRT-PCR was used to verify the relative abundance of the mRNA transcripts of the 12 DEGs. Of the 12 selected genes, mRNA expression of four genes, including ribosomal protein S6 kinase 2 alpha (*RPS6KA2*, $P < 0.01$), cyclic AMP-responsive element-binding protein 5 (*CREB5*, $P < 0.01$), syndecan-1 (*SDC1*, $P < 0.001$), and serpin family B member 5 (*SERPINB5*, $P < 0.01$) were significantly upregulated in the 10 ng/ml TGFB1-stimulated group as compared to the control group (Fig. 4A). However, 10 ng/ml TGFB1 inhibited the other eight genes: epiregulin (*EREG*, $P < 0.001$), amphiregulin (*AREG*, $P < 0.001$), heparin-binding EGF-like growth factor (*HB-EGF*, $P < 0.001$), claudin 1 (*CLDN1*, $P < 0.001$), E-cadherin (*CDH1*, $P < 0.001$), endothelial cell-selective adhesion molecule (ESAM, $P < 0.001$), 3β-hydroxysteroid dehydrogenase 1 (*HSD3B1*, $P < 0.001$), and cytochrome P450 family 11 subfamily A member 1 (*CYP11A1*, $P < 0.001$) mRNA expression (Fig. 4B). The expression profiles of the genes chosen for qRT-PCR verification were consistent with the transcriptome results.

Effect of TGFB1 on the expression of COL1A1, MMP3, SDC1, and CDH1 in bovine luteinizing follicular cells

We next investigated the effect of TGFB1 on the expression of proteins related to ECM degradation in the mammalian ovary, using bovine luteinizing follicular cells as a study model. As shown in Fig. 5A, treatment with 10 ng/ml of TGFB1 for 24 h significantly increased the mRNA expression of *COL1A1 and MMP3* (Fig. 5A). TGFB1 also induced upregulation of COL1A1 and SDC1 protein expression, whereas the protein level of CDH1 was decreased in TGFB1 treated bovine luteinizing follicular cells (Fig. 5B, 5C).

Discussion

In the present study, TGFB1 markedly inhibited EC network formation during the transition from the follicle to the CL. The KEGG pathway analysis predicted that most of the 455 DEGs were mapped to cytokine-cytokine receptor interactions, TNF signaling pathway, CAMs, MAPK signaling pathway, PI3K-Akt signaling pathway, ErbB signaling pathway, and ovarian steroidogenesis. The GO annotations of the ten most upregulated and downregulated genes were cell adhesion, ECM, proteolysis, among others, which are related to angiogenesis. The results of western blotting confirmed that TGFB1 phosphorylated ERK1/2 and Akt in luteinizing follicular cells. In addition, TGFB1 elevated the expression of genes that participate in ECM receptor interaction but downregulated the expression of genes involved in cell adhesion junctions, finally promoting the expression

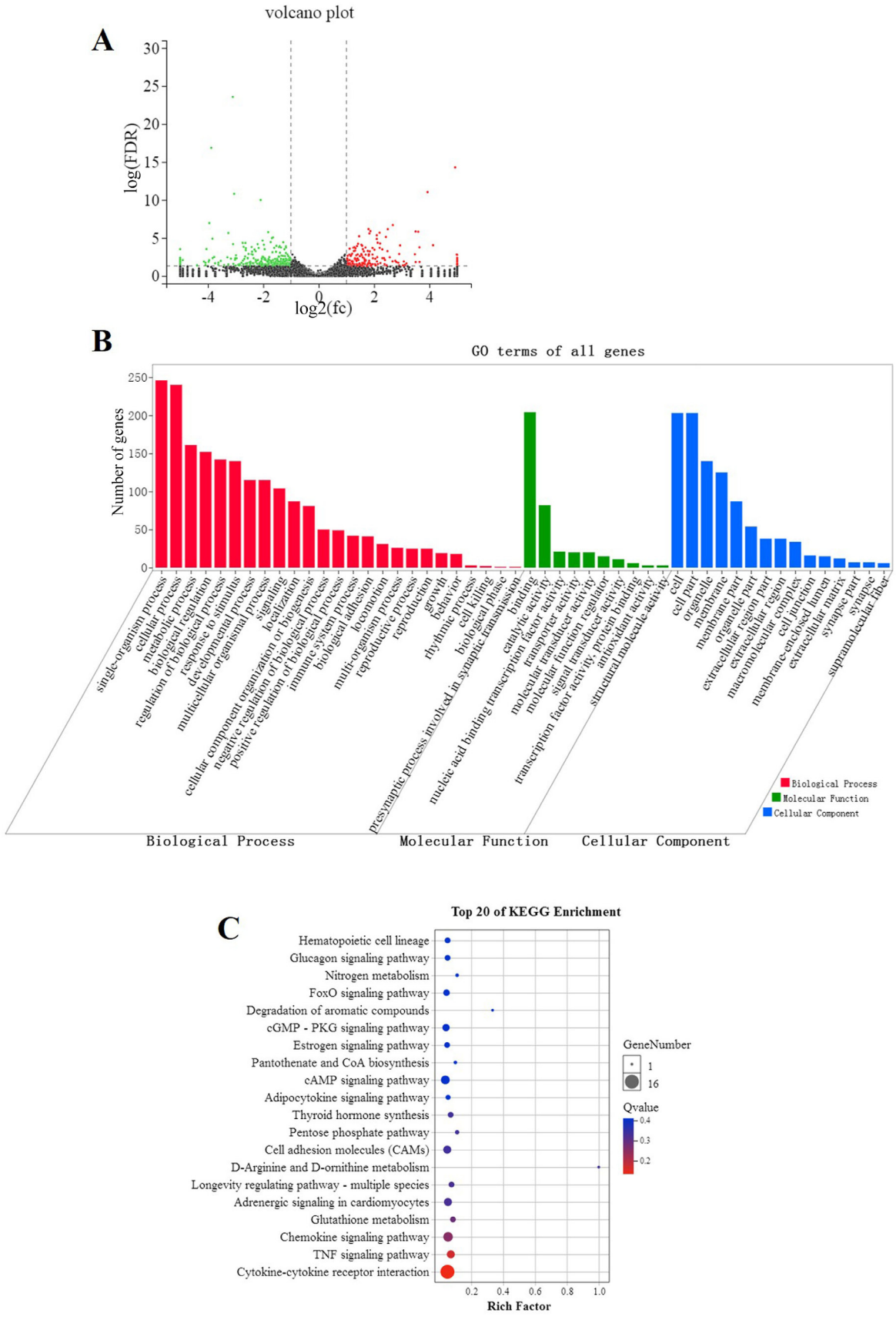

Fig. 2. Analysis of the differentially expressed genes (DEGs) between control and TGFB1 treated bovine luteinizing follicular cells. (A) A volcano plot of the DEGs. The x axis represents the log 2 (fold change, FC) value, and the y axis represents the –log10 (FDR) value. Red points represent upregulated genes, green points represent downregulated genes and black points represent genes that showed no differential expression. (B) GO functional classification of the DEGs. The x axis represents the number of DEGs and the y axis represents the GO terms. (C) Top 20 KEGG pathways of the DEGs. The x axis represents the rich factor, and the y axis represents the pathways. The size and color of each bubble represent the number of DEGs and P-value in each pathway, respectively.

Table 1. Top ten upregulated and downregulated genes in bovine luteinizing follicular cells treated with TGFB1 compared with the untreated controls

Genes	Description	Fold_change	GO annotations
Downregulated			
RAB25	ras-related protein Rab-25	196.66	small molecule binding/cell proliferation
ASGR1	asialoglycoprotein receptor 1	186.66	cell communication/binding
TUBA1D	tubulin alpha-1D	126.67	microtubule cytoskeleton/purine ribonucleoside binding
IGSF5	immunoglobulin superfamily member 5	73.31	cell-cell junction/cell adhesion
FOXB1	forkhead box protein B1	69.55	regulation of biological/nucleic acid binding transcription factor activity
GJC3	gap junction gamma-3	20.99	binding/membrane
AREG	amphiregulin	15.433	branching morphogenesis of an epithelial tube/reproductive process
GSTA1	glutathione S-transferase A1	9.59	cellular process/transferase activity
CLDN1	claudin-1	8.52	cell adhesion/cell-cell junction
EREG	epiregulin	8.28	receptor binding/extracellular region
Upregulated			
EPPIN	eppin precursor	163.33	enzyme inhibitor activity/negative regulation of biological process
AMELX	amelogenin, X isoform	113.33	extracellular matrix/cellular component organization
CYSLTR1	cysteinyl leukotriene receptor 1	60	response to chemical/membrane part
SLC2A5	solute carrier family 2, facilitated glucose transporter member 5	31.66	plasma membrane transmembrane/transporter activity
PAG2	pregnancy-associated glycoprotein 11	30.43	proteolysis/hydrolase activity
IL1RN	Interleukin-1 receptor antagonist protein	15.26	molecular function regulator/ signaling
CRLF1	cytokine receptor-like factor 1	12.46	negative regulation of cellular process/extracellular region
COL1A1	collagen alpha-1(I) chain	5.64	regulation of cell-substrate adhesion/extracellular matrix
SERPINB5	serpin family B member 5	5.38	regulation of proteolysis/ extracellular region
PITX2	pituitary homeobox 2	5.13	angiogenesis/reproduction

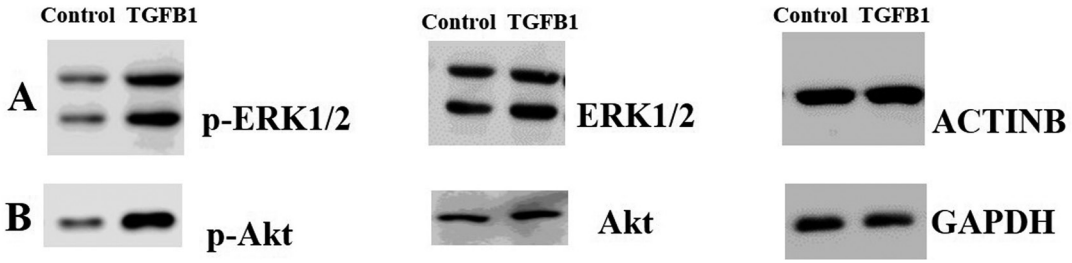

Fig. 3. TGFB1 activated ERK1/2 and PI3K/Akt signaling pathways in bovine luteinizing follicular cells**.** Bovine luteinizing follicular cells were treated with 0 or 10 ng/ml TGFB1 for 30 min. Phosphorylation levels of ERK1/2 (A, left panel) and Akt (B, left panel) were determined by western blotting using specific antibodies for the phosphorylated forms of ERK1/2 (p-ERK1/2) and Akt (p-Akt). The membranes were stripped and re-probed with antibodies to total ERK1/2 (A, middle panel) and Akt (B, middle panel).

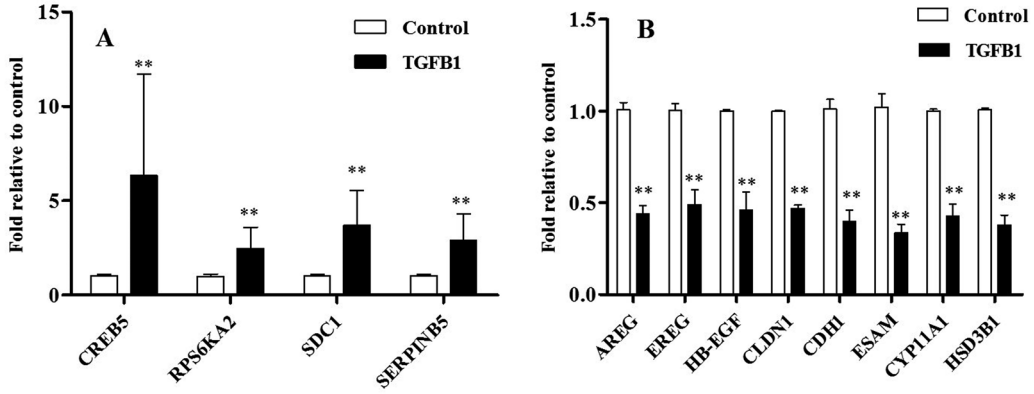

Fig. 4. Validation of the DEGs between the control and TGFB1 treated bovine luteinizing follicular cells by real-time qPCR. Bovine luteinizing follicular cells (including granulosa, theca and endothelial cells) were treated with control medium or 10 ng/ml TGFB1 for 48 h. The mRNA levels of four upregulated genes (A) and eight downregulated genes (B) were quantified using real-time qPCR and normalized by the β-actin housekeeping gene. All data are presented as the mean ± standard error of mean (SEM) of three independent experiments.

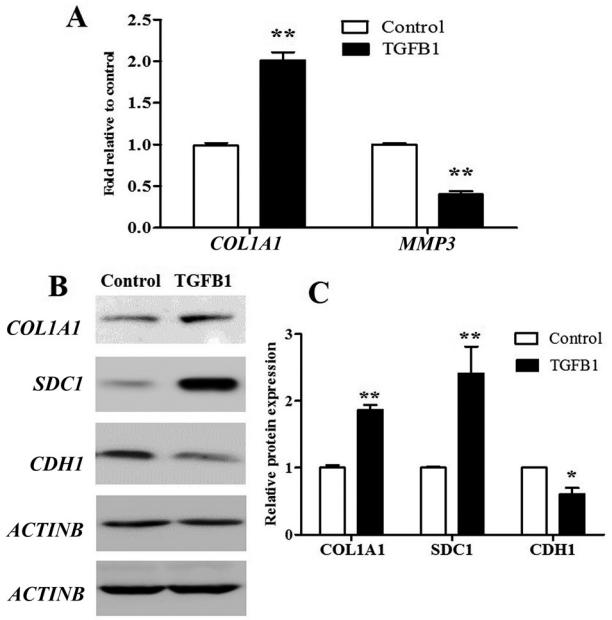

Fig. 5. Effect of TGFB1 on the expression of COL1A1, MMP3, SDC1 and CDH1 in bovine luteinizing follicular cells *in vitro*. Bovine luteinizing follicular cells (including granulosa, theca and endothelial cells) were treated with control or 10 ng/ml TGFB1 for 24 h and the mRNA expressions of *COL1A1* and *MMP3* were analyzed (A), Protein levels of COL1A1, SDC1, and CDH1 were examined using real-time qPCR (B) and western blot analysis (C), respectively.

and deposition of COL1A1, a major ECM protein, in the luteinizing follicular cells. Based on these results, we speculate that TGFB1 inhibits cell adhesion and ECM degradation via the ERK1/2 and PI3K-Akt signaling pathways, which leads to suppressed angiogenesis during the follicular-luteal transition. Ovarian angiogenesis is very intense during the transition of the follicle to CL. This process is intricately controlled by several pro- and anti-angiogenic factors [5, 21]. Although TGFB1 is an important regulator of angiogenesis [22], its exact regulatory mechanism during the follicular-luteal transition period remains unclear.

Ribosomal protein S6 kinase 2 (RPS6KA2), also known as RSK3, is a downstream effector of the Ras/ERK signaling pathway and is activated by ERK [23, 24]. The dual-specificity MAP kinase phosphatases (DUSPs/MKPs) gene is known to encode a phosphatase that dephosphorylates and inactivates MAP kinase isoforms in mammalian cells [25]. In this study, treatment with TGFB1 upregulated *RPS6KA2* and downregulated *DUSP4* mRNA expression in luteinizing follicular cells which demonstrated that the activation of the MAPK pathway during the inhibition of luteal angiogenesis. It is not surprising that the effect of TGFB1 on luteal angiogenesis involves other signaling pathways. Similar activation of signaling pathways by TGFB1 has also been observed in other cells, such as granulosa cells [26] and endothelial cells [27]. These findings suggest that besides the Smad signaling pathway the non-canonical pathway ERK1/2 may be involved in TGFB1-mediated inhibition of angiogenesis in bovine luteinizing follicular cells.

Amphiregulin (AREG), epiregulin (EREG), heparin-binding EGF-like growth factor (HB-EGF), and transforming growth factor alpha (TGFα) belong to the epidermal growth factor (EGF) superfamily and exert their biological functions via the epidermal growth factor receptor (EGFR, a member of the ErbB family of receptor tyrosine kinases). EGF family members have been implicated as regulators of angiogenesis [28]; in addition, HB-EGF- and EGF-induced human umbilical vein endothelial cell (HUVEC) capillary tube formation is dependent on the activation of PI3K and MAPK [29], whereas EREG promotes human corneal epithelial cell proliferation through auto-phosphorylation of EGFR or cross-induction with other EGF family members [30]. In the present study, TGFB1 downregulated *EREG*, *AREG*, *HB-EGF*, and *TGFα* expression in luteinizing follicular cells. A negative interaction between TGFB1 and EGF family members has been reported in previous studies, as TGFB1-induced SMAD3 phosphorylation is selectively diminished by EGF [31] and TGFB1 reduces the phosphorylation of EGFR [32]. Moreover, ablation of Smad4 resulted in the activation of the ErbB2 signaling pathway [33]. Taken together, there may be an antagonistic relationship between TGFB1 and EGF superfamily ligands during early corpus luteal angiogenesis. In the next step, it would be important to evaluate the antagonistic relationship between TGFB1 and EGF superfamily ligands mediated in luteal angiogenesis.

We further examined whether TGFB1 regulates luteal angiogenesis through the PI3K-Akt pathway. Previous studies have reported that TGFB1 can activate the PI3K-Akt pathway, as indicated by the phosphorylation of its downstream effector Akt [34, 35]. Similarly, in TGFB1-treated luteinizing follicular cells, the phosphorylation levels of Akt were increased, suggesting that TGFB1 regulates early luteal angiogenesis through the activation of the PI3K-Akt pathway.

Interactions via the cell contact-dependent pathways are important for maintaining and regulating the integrity of CL and its physiological actions [36]. During luteal angiogenesis, cell adhesion molecules allow endothelial and epithelial cells to form stable contacts with other cells such as the luteal cells and pericytes and/or with the extracellular matrix (ECM). Therefore, cell adhesion is essential for endothelial cell invasion, tissue remodeling, capillary expansion, and steroidogenesis. Our data showed that the CAM pathway is inhibited by TGFB1 during early CL angiogenesis, as the mRNA expression levels of molecules in this pathway, including *E-cadherin*, *ESAM*, and CLDN1 were significantly downregulated. E-cadherin (encoded by the *CDH1* gene) mRNA expression was at its highest level during the early luteal phase in the CL, and its antibody disrupted cell-cell contacts between luteal cells, suggesting that cell-to-cell adhesion by E-cadherin may be necessary for intercellular communication during luteinization [37, 38]. E-cadherin associates with and/or transactivates EGFR [39]. These results prompted us to speculate that the inactivation of EGFR by E-cadherin may be involved in TGFB1 induced anti-angiogenesis. Reduced mRNA levels of *CLDN1* following TGFB1 treatment in the luteinizing follicular cells is also interesting. CLDN1, a cell junctional protein, serves as the backbone of tight junction (TJ) formation and function. It is exclusively expressed in the membrane of luteinizing granulosa cells [40]. Analysis of the RNA present in four bovine cell types revealed CLDN1 as a novel small luteal cell-specific genetic marker [41]. A previous study reported that the abundance of CLDN1 in the luteal phase of cat ovaries was greater than that measured in the follicular and anestrous phases [42]. The results of these studies are now accumulating to support an important function for CLDN1 in regulating ovarian follicular and CL formation, and cell adhesion may be necessary for the establishment of a functional vasculature. In our study, TGFB1 decreased the expression of *CDH1* and *CLDN1* and therefore blocked the adherens junctions (AD) and tight junctions between endothelial and other cells, which subsequently led to the inhibition of angiogenesis and development in early CL.

In preparation for CL formation, the ECM of the ovary undergoes

remodeling that allows cell migration and neovascularization of the newly formed CL. SDC1 acts as a regulator of membrane adhesion-dependent pathways by binding with ECM components and/or soluble ligands [43]. SDC1 null mice show increased corneal angiogenesis [44]. In contrast, SDC1 overexpression leads to delayed wound healing with defective granulation tissue formation and angiogenesis [45]. These results imply that SDC1 may negatively regulate angiogenesis. In this study, we demonstrated that TGFB1 regulates SDC1 protein expression in luteinizing follicular cells. Similarly, TGFB1 induces SDC-1 expression in epithelial cells [46]. Moreover, high levels of SDC1 in human granulosa cells represses cell growth rate, accompanied by reduced progesterone receptor (PGR) and CYP11A1 expression, and steroidogenesis [47]. The results presented in our work brought another mechanism to light: TGFB1 increases *SDC1* expression, resulting in the reduction of matrix degradation and stabilization of the ECM.

Type I collagen as the main component of bovine CL. It accounts for approximately one-sixth of the luteal dry matter [48], and is comprised of two alpha-1 subunits (collagen type I alpha 1 chain, COL1A1) and one alpha-2 subunit (COL1A2). During the post-ovulatory period, the amount of collagen type I first increased and then decreased in the bovine and ovine CL [49]. However, the mRNA levels of *COL1A1* and *COL1A2* were significantly upregulated in the regressing bovine CL [50]. ECM remodeling associated with type I collagen degradation is a major event that triggers CL formation. Our study showed that TGFB1 promoted expression and increased the content of *COL1A1* in bovine luteinizing follicular cells. Similarly, our previous study found that TGFB1 suppressed ECM degradation by increasing SERPINE1 expression [6]. In this regard, TGFB1 may act as a luteotropic inhibitor by modulating the expression of *COL1A1*, *SDC1*, and *SERPINE1*, which results in the failure of the breakdown of vascular basement membranes thereby inhibiting the directional growth of sprouting vessels and disrupting CL angiogenesis over time.

In addition, the ovarian steroidogenesis pathway was one of the downregulated KEGG pathways identified in the analysis of TGFB1 treated bovine luteinizing follicular cells. The primary function of the newly formed CL is to synthesize and secrete progesterone required to establish pregnancy. In the present study, TGFB1 induced repression of CL angiogenesis was accompanied by a decrease in *HSD3B1* and *CYP11A1* expression, which is consistent with previous studies where TGFB1 also decreased progesterone secretion and inhibited HSD3B1 and CYP11A1 expression in bovine luteinizing follicular cells [1]. Downregulation of the steroidogenesis pathway revealed that TGFB1 exerts an adverse effect on CL angiogenesis and leads to the eventual suppression of steroidogenesis.

Collectively, the transcriptomic data obtained in this study revealed that TGFB1 alters the expression of numerous genes associated with cell adhesion junctions and ECM receptors by activating the ERK1/2 and PI3K/Akt signaling pathways. However, the ErbB signaling pathway was inhibited by TGFB1. In addition, TGFB1-induced upregulation of *SDC1* and *COL1A1* expression may contribute to the deposition of type I collagen and ECM remodeling in luteinizing follicular cells. Our findings strongly suggest that TGFB1 inhibits the cell adhesion junction and ECM degradation during follicular-luteal transition and involves the ERK1/2, ErbB, and PI3K/Akt signaling pathways in addition to the canonical Smad signaling pathway, which leads to the suppression of angiogenesis during follicular-luteal transition (Fig. 6).

Conflict of interests: The authors declare no conflicts of interest with regard to the study.

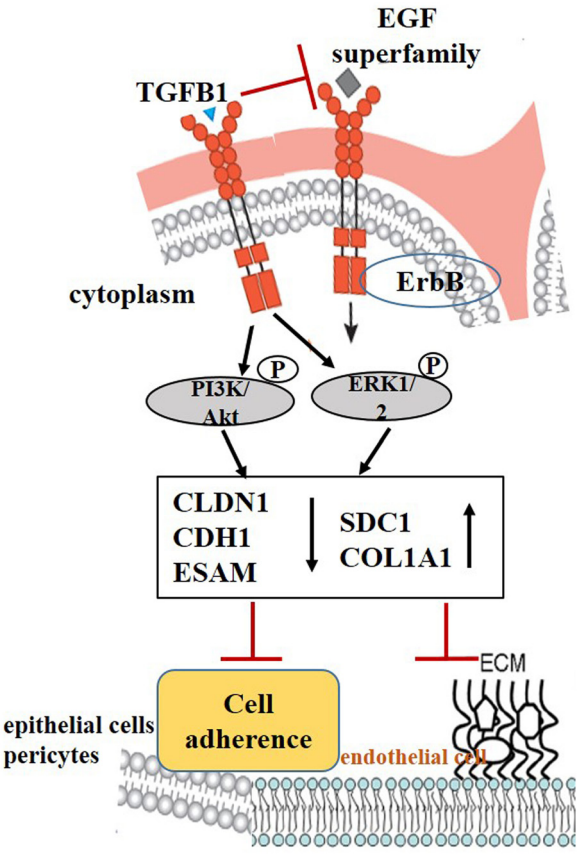

Fig. 6. Hypothetical scheme of the signal pathways mediating TGFB1-induced suppression of angiogenesis during follicular-luteal transition *in vitro*. TGFB1 binds to receptors leading to activation of ERK1/2 and PI3K/Akt signaling pathways and inhibition of ErbB signaling pathway. This suppresses the transcription of *CLDN1, CDH1* and *ESAM* resulting in disruption of the cell adhesion junction, while increasing the expression of *SDC1* and *COL1A1* that inhibit ECM degradation, and promote the deposition of COL1A1. *CLDN1*, claudin 1; *CDH1*, E-cadherin; *ESAM*, endothelial cell-specific adhesion molecule; *SDC1*, syndecan-1; *COL1A1*, collagen type I alpha 1 chain; ECM, extracellular matrix.

Acknowledgments

The present study was supported by grants from the National Natural Science Foundation of China (grant no. 31601943). The authors would like to thank Robert R. Robinson (School of Veterinary Medicine and Science, University of Nottingham) for his suggestions on cell culture and Editage (www.editage.cn) for English language editing.

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