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# Effects of *Notch2* on proliferation, apoptosis and steroidogenesis in bovine luteinized granulosa cells

Yating Li [a], Jiongjie Jing [a], Wenqing Dang [a], Qi Han [a], Xiangyu Guo [a], Kaiqi Jia [a], Ying Cheng [a], Kai Wang [a], Ermias Kebreab [b], Lihua Lyu [a, *]

[a] College of Animal Science, Shanxi Agricultural University, Taigu, 030801, Shanxi, China
[b] College of Agricultural and Environmental Sciences, University of California, Davis, 95616, CA, USA

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ABSTRACT

Notch signaling pathway plays an important regulatory role in the development of mammalian follicles. This study aimed to explore the effect of *Notch2* on the function of bovine follicles luteinized granulosa cells (LGCs). We detected that the coding sequence (CDS) of bovine *Notch2* gene is 7416 bp, encoding 2471 amino acids (AA). The homology of Notch2 AA sequence between bovine and other species is 86.04%–98.75%, indicating high conservatism. Immunohistochemistry found that Notch2 receptor and its ligand Jagged2 localize in granulosa cells (GCs) and theca cells in bovine antral follicles. And immunofluorescence found that positive signals of Notch2 and Jagged2 overlap in bovine LGCs, speculating that Notch2 receptor may react with Jagged2 ligand to activate Notch signaling pathway and play an important role in bovine LGCs. To further investigate the function of *Notch2*, *Notch2* gene was silenced by short hairpin RNA (shRNA) and CCK-8 analysis showed that the proliferation rate of LGCs was downregulated significantly ($P < 0.01$). Quantitative reverse transcription polymerase chain reaction (qRT-PCR) showed that the mRNA expression of apoptosis related gene *Bcl-2/Bax* decreased ($P < 0.01$) and *Caspase3* increased ($P < 0.05$), cell cycle related gene *CyclinD2/CDK4* complex decreased ($P < 0.01$) and *P21* increased ($P < 0.05$), steroidogenesis gene *STAR* and *3β-HSD* decreased ($P < 0.01$) while *CYP19A1* and *CYP11A1* had no significant difference ($P > 0.05$). In addition, Enzyme-linked immunosorbent assay (ELISA) showed that there was no difference in estradiol ($E_2$) secretion ($P > 0.05$) while the progesterone ($P_4$) secretion decreased ($P < 0.01$). In conclusion, Notch2 plays an important role in regulating bovine LGCs development.



## 1. Introduction

The fecundity of livestock is affected by genetics, external environment, nutritional conditions and other factors. As a monotocous animal, follicular growth and development of bovine directly affect the reproductive cycle and survival rate of the offspring. Exploring the mechanism of follicular development is a key topic in reproduction science. During follicular formation, the primordial germ cells are wrapped by a layer of pregranulosa cells to form primordial follicles, which in turn develop into preantral follicles and antral follicles in the ovary [1,2]. However, most follicles become atretic eventually, and only a few antral follicles are selected as dominant follicles during follicular wave deviation, maintaining high levels of luteinizing hormone-receptor, estradiol ($E_2$), and insulin-like growth factor 1 [3]. The dominant follicle ovulates with the occurrence of peak luteinizing hormone (LH) in each reproductive cycle. The remaining somatic cells in the follicle differentiate and form corpus luteum, which secretes the progesterone necessary to maintain pregnancy [1].

Notch is a widely distributed signaling pathway in organisms, and its dysfunction is associated with several diseases, including cancers [4–6]. In recent years, Notch signaling pathway studies have been highly concerned in regulating cell proliferation and differentiation. Several genes of Notch signaling pathway are expressed in mammalian ovaries, including *Notch1*, *Notch2*, *Notch3*, *Notch4*, *Jagged1*, *Jagged2*, *Hes1*, *Hes5*, *Hey1*, *Hey2*, etc, which strongly

* Corresponding author.
*E-mail address:* lihualvsxau@126.com (L. Lyu).

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suggest that this signaling pathway plays a role in follicular development [7–9]. Notch signaling pathway is activated by intercellular contact of ligands (Delta1, 3, 4 and Jagged1, 2) and receptors (Notch1-4), which initiates the hydrolytic cleavage of Notch receptor protein. First, after the Furin site of Notch is cut in Golgi apparatus, Notch is transferred to the cell membrane as a heterodimer connected by disulfide bonds. The ligand of adjacent cells binds to Notch receptor to induce the S2 site of extracellular region to be cleaved by metalloproteinase TACE, and then the S3 site is also cleaved by γ-secretase, resulting in the intracellular fragment NICD to fracture and enter the nucleus. Finally, NICD and DNA-binding protein CSL form a complex to activate target gene transcription in the nucleus [10]. Blocking Notch signaling pathway led to defective recruitment of primitive follicles, and Notch signaling was involved in the formation of primitive follicles [11]. After activating luteinizing hormone receptor in mouse granulosa cells (GCs), knocking out ligand *Jagged1* of Notch signaling pathway resulted in decreased steroid biosynthesis, enhanced proliferation of GCs, and inhibited differentiation gene regulator *YB-1*, indicating that Notch signaling pathway promotes the differentiation of GCs before ovulation [12].

Notch2 receptor persist in pregranulosa cells, preantral follicular GCs and antral follicular GCs of mice, and expressed in antral follicular oocytes, however, it decreases continuously with the development of follicles and finally disappear in antral follicles [8,11]. Notch2-mediated signaling pathways contribute to signal communication between oocytes and GCs for follicular development, and influence the proliferation of preantral GCs by regulating *c-Myc* expression [8,13]. However, the regulation of Notch signaling pathway is rarely reported in follicular atresia and luteinization of GCs after ovulation, and the regulation of Notch2 signaling pathway in bovine follicular development needs further study.

The LH peak can promote the lutealization of GCs before ovulation, and GCs can also spontaneously lutealize in the serum culture system *in vitro*, which is an effective model for follicular atresia and luteinization [14–16]. In this study, the complete CDS region of bovine *Notch2* gene was amplified by PCR. Then, we explored how *Notch2* regulates luteinized granulosa cells (LGCs), providing theoretical basis for the study of Notch pathway in follicular development in the future.

## 2. Materials and methods

### 2.1. Ethics statement

All animal experiments were approved by the Ethics Committee of Shanxi Agricultural University.

### 2.2. Sample collection

Healthy Holstein cows aged 14 M were slaughtered and fresh ovarian were brought back to the laboratory in 4 °C Dulbecco's phosphate-buffered saline (DPBS) containing 100 IU/mL of penicillin and 100 μg/mL of streptomycin (Solarbio, Beijing, China). After the ovaries were sterilized with 75% alcohol, medium sized follicles with a diameter of 4–8 mm were isolated from ovaries. Some follicles were immersed in 4% paraformaldehyde for 24 h to carry out immunohistochemistry, and the other follicles were cut in half and the GCs in the inner layer of the follicles were gently scraped. Each area was scraped only once to prevent the removal of membrane cells. The mixture of follicular fluid and GCs was collected and filtered through a 100 μm disposable filter. Some GCs were stored at −80 °C for RNA extraction. The others were washed with DPBS 3 times for cell culture.

### 2.3. Cloning the CDS region of Notch2

Total RNA of GCs was extracted using RNAiso Plus (Takara, Dalian, China) following the manufacturer's protocol *in vitro* and then reverse transcribed to cDNA using the PrimeScriptTM 1st strand cDNA Synthesis Kit (Takara, Dalian, China). Segmented primers were designed by NCBI pick primer to amplify CDS of bovine *Notch2* gene. The primer sequences are listed in Table 1. After PCR amplification, the fragments were detected by agarose gel electrophoresis and sent to sequencing. According to the mRNA sequence of *Notch2* CDS region, DNAMAN was used to align the homology of *Notch2* genes between cattle and other 12 species. MEGA7 was used to construct phylogenetic tree with ClustalW and Neighbor-joining algorithms.

### 2.4. Immunohistochemistry

The fixed follicles were dehydrated with gradient ethanol, transparent with xylene and then embedded in paraffin. The embedded follicles were sliced, dewaxed and rehydrated. The sections were boiled in 0.01 M citrate buffer for 15 min for antigenic thermal repair. Then the sections were incubated with endogenous peroxidase for 10 min, goat serum for 10 min, primary antibody (Rabbit Anti-Jagged1 antibody, Bioss, Beijing, China; Rabbit Anti-Jagged2 antibody, Bioss, Beijing, China; Anti-NOTCH2 rabbit polyclonal antibody, Sangon Biotech, Shanghai, China) for 4 °C overnight, Goat anti-Rabbit IgG for 10 min, Streptavidin-HRP for 10 min, and DAB for 1 min according to the instructions of SP Rabbit&Mouse HRP Kit (CWBIO, Jiangsu, China). The follicle sections were re-stained with hematoxylin for 10 s, then dehydrated, sealed and microscopically observed (BX53, Olympus, Japan).

### 2.5. Immunofluorescence

First, appropriate amount of LGCs were seeded on the slides. Next, the LGCs were fixed with 4% paraformaldehyde, permeabilized with 1% TritonX-100 (Solarbio, Beijing, China) and blocked with 1% BSA (Solarbio, Beijing, China), incubated overnight with primary antibodies against FSHR (Bioworld, Minnesota, USA), Notch2 (Cell Signaling Technology, Massachusetts, USA) and Jagged2 (Bioss, Beijing, China) at 4 °C. Then LGCs were incubated with DyLight594 and FITC-conjugated secondary antibodies (BOSTER, Wuhan, China) for 1 h and DAPI solution for 20 min. The slides were observed and photographed under a confocal laser microscope (Leica, Weztlar, Germany).

**Table 1**
Sequences of primers for Notch2 segmented amplification.

| Genes | Primer sequences for PCR | Length (bp) |
|---|---|---|
| *Notch2*-section1 | F1:GTAGGAGGAGGGGAGGAAAA<br>R1:TTCAAAACCTGGAAGGCAAC | 860 |
| *Notch2*-section2 | F2:TCGACTACTCACCCCTGCTT<br>R2:ACGCCATCCATACAGACTCC | 1679 |
| *Notch2*-section3 | F3:GTCTGAATGGGGCAAAGTGT<br>R3:GGCAGAAGGAACCAGTGAAG | 1446 |
| *Notch2*-section4 | F4:CTGTCATACACAGGCAAAAA<br>R4:GCACAGGTAGTCGTTGGTGA | 1404 |
| *Notch2*-section5 | F5:GGCACCTGTATCGACCTTGT<br>R5:TCAGCACCACGATGACCAG | 1060 |
| *Notch2*-section6 | F6:CCGAGTGCCTGTTTGACAAC<br>R6:ACCCTGGTAGACTAGGTCCG | 1139 |
| *Notch2*-section7 | F7:CTGGGGGTTATCATGGCAAAA<br>R7:ACTCTTGGTGTTAGGCCGTCT | 1248 |
| *Notch2*-section8 | F8:ATGCACCATGACATCGTGCGC<br>R8:TCATGCATAGACCTGCATGTT | 1257 |

### 2.6. Package and concentration of lentivirus

ShRNA was designed and synthesized according to Notch2 sequence (GenePharma, Shanghai, China) and linked to lentivirus silencing vector (LV3, GenePharma, Shanghai, China). 293T cells (China Infrastructure of Cell Line Resource, Beijing, China) were cultured to 70% confluence, then the Lip3000 liposome transfection kit (Invitrigen, Carlsbad, USA) was used to transfect the vector plasmid and two package plasmids psPAX2 and pMD2G (Public Protein/Plasmid Library, Jiangsu, China) at the ratio of 1:1:1 for 6 h following its introduction. The fluorescence rate was observed 1 d after transfection and collected lentivirus on days 2 and 3 respectively. The virus was concentrated using a superfast centrifuge at 72000 g/min, 4 °C for 2 h and stored at −80 °C after being packaged separately to avoid repeatedly freezing and thawing.

### 2.7. LGCs culture

First, GCs were added to DME/F12 (HyClone, Shanghai, China) supplemented with 10% FBS (CellMax, Beijing, China), 100 IU/mL penicillin and 100 μg/mL streptomycin (Solarbio, Beijing, China), then cultured in 5% $CO_2$ and 37 °C humidified incubator. The cultured cells were washed with PBS every day for the first two days to remove tissue block impurities surrounded the cells. After 48 h of culture, GCs are luteinized [14]. The LGCs were added to the cell culture plate to make the confluence reach 50%, then an appropriate amount of concentrated virus and 5 μg/mL Polybrene (GenePharma, Shanghai, China) were added. The medium was changed after 24 h of infection. The transfection efficiency was observed by fluorescence microscope and cells were screened by adding 5 μg/mL puromycin (Solarbio, Beijing, China) 2 d later. Finally, the successfully transfected LGCs were collected.

### 2.8. Real-time qRT-PCR

Total RNA was extracted from LGCs using RNAiso Plus (Takara, Dalian, China) according to its instructions. Thereafter, mRNA was reversely transcribed into cDNA using PrimeScriptTM RT reagent Kit with gDNA Eraser (Perfect Real Time) (Takara, Dalian, China) following the manufacturer's protocol. Real-time qRT-PCR was carried out in a 10 μL system with TB Green® Premix Ex Taq™ II (Tli RNaseH Plus) (Takara, Dalian, China). The thermal cycle reaction was set as 95 °C 30 s, 95 °C 5 s, and 60 °C 30 s for 40 cycles in the Applied Biosystems 7500 Real Time PCR System, and the threshold cycle values (Ct) were obtained with *β-actin* as internal reference gene. Primers are shown in Table 2.

### 2.9. Protein extraction and western blotting

Total proteins were extracted from LGCs by adding radioimmunoprecipitation assay (RIPA) buffer (BOSTER, Wuhan, China) containing 1% PMSF (BOSTER, Wuhan, China). Protein concentrations in each well were measured by the BCA protein concentration determination kit (BOSTER, Wuhan, China) and then diluted to a consistent level. Target proteins were separated on sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels (BOSTER, Wuhan, China) and transferred to nitrocellulose membranes (BOSTER, Wuhan, China) which were then soaked in 5% skim milk powder (BOSTER, Wuhan, China) for 1h. The proteins on membranes were incubated in primary antibody of Notch2 (Cell Signaling Technology, Massachusetts, USA) at 4 °C overnight and incubated in fluorescence conjugated secondary antibody (LI-COR, Lincoln, NE, USA) 1h on day 2. Fluorescence images were obtained by LI-COR Odyssey CLx Laser imaging system and analyzed with β-actin as internal reference gene.

**Table 2**
Sequences of primers for qRT-PCR.

| Genes | Primer sequences for qRT-PCR | Length (bp) |
|---|---|---|
| *NICD2* | F:AGACGGCCTAACACCAAGAG<br>R:CTGTTCCCCTTGGCATCCTT | 80 |
| *Hes1* | F:GCCAGTGTCAACACGACACCGGA<br>R:TTCATGCACTCGCTGAAGCCGGC | 302 |
| *Hey2* | F:TCTGAGTTGAGACGACTGGTG<br>R:GCGTGTGCATCAAAGTAGCC | 143 |
| *BCL-2* | F:GTGGATGACCGAGTACCTGAAC<br>R:AGACAGCCAGGAGAAATCAAAC | 124 |
| *BAX* | F:GACATTGGACTTCCTTCGAGA<br>R:AGCACTCCAGCCACAAAGAT | 126 |
| *Caspase3* | F:AGCCATGGTGAAGAAGGAATC<br>R:CTGCAATAGTCCCCTCTGAAG | 89 |
| *Cyclin D2* | F:CACCGATGTGGATTGCCTCA<br>R:TCCAGCTCATCCTCCGACTT | 117 |
| *CDK4* | F:CCTTCATGCCAACTGCATCG<br>R:CCAGAGTGTAACAACCACAGGT | 148 |
| *P21* | F:CGGTGGAACTTCGACTTTGT<br>R:CAAGTGGTCCTCCTGAGACG | 183 |
| *CYP19A1* | F:CACCCATCTTTGCCAGGTAGTC<br>R:ACCCACAGGAGGTAAGCCTATAAA | 78 |
| *CYP11A1* | F:AGGCAGAGGGAGACATAAGCA<br>R:GTGTCTTGGCAGGAATCAGGT | 156 |
| *STAR* | F:CAGAAGGGTGTCATCAGAGCG<br>R:CAAAATCCACCTGGGTCTGC | 169 |
| *3β-HSD* | F:TGCCACAATCTGACCGCATC<br>R:CTCCACCAACAGGCAGATGA | 167 |
| *β-actin* | F:GGATGAGGCTCAGAGCAAGAGA<br>R:TCGTCCCAGTTGGTGACGAT | 78 |

### 2.10. Cell proliferation rate measurement

LGCs treated differently were counted using a blood count board. Each well of the 96-well plate was inoculated with 3000 LGCs. After cells were adherent to the wall, the optical density (OD) value was detected by the CCK-8 kit (Dojindo, Kumamoto, Japan) at 0 h, 24 h, 48 h and 72 h. Eventually, the proliferation rate of the cells was calculated.

### 2.11. Enzyme-linked immunosorbent assay (ELISA)

The concentration of $E_2$ and progesterone ($P_4$) in the cell culture medium was detected by ELISA kit (MEIMIAN, Jiangsu, China). First, cell culture medium was collected. 50 μL medium samples were added to each well of the 96-well enzyme label plate, incubated at 37 °C for 30 min. Then, the medium samples were removed. 50 μL enzyme labeled solution were added and incubated at 37 °C for 30 min. After that enzyme label plate was fully washed. Chromogenic agent A and B were added for 10 min in dark. The reaction was terminated by stop solution and OD value was measured by a microplate reader.

### 2.12. Statistical analysis

Relative gene mRNA expression was calculated with $2^{-\triangle\triangle Ct}$ method. Image J was used to analyze the gray value of the exposed strips of Western blotting for calculating the relative protein expression. The cell proliferation rate was calculated by the formula [OD (Treated) - OD (Blank)]/[OD (Control) − OD (Blank)]. The concentration of $E_2$ and $P_4$ was established according to the standard curve. All experiments were repeated at least three times. The data were analyzed by one-way analysis of variance (one-way ANOVA) in SPSS 22.0 and presented as mean ± standard error of mean (SEM). A $P < 0\ 05$ indicated significant differences while $P < 0\ 01$ were highly significant.

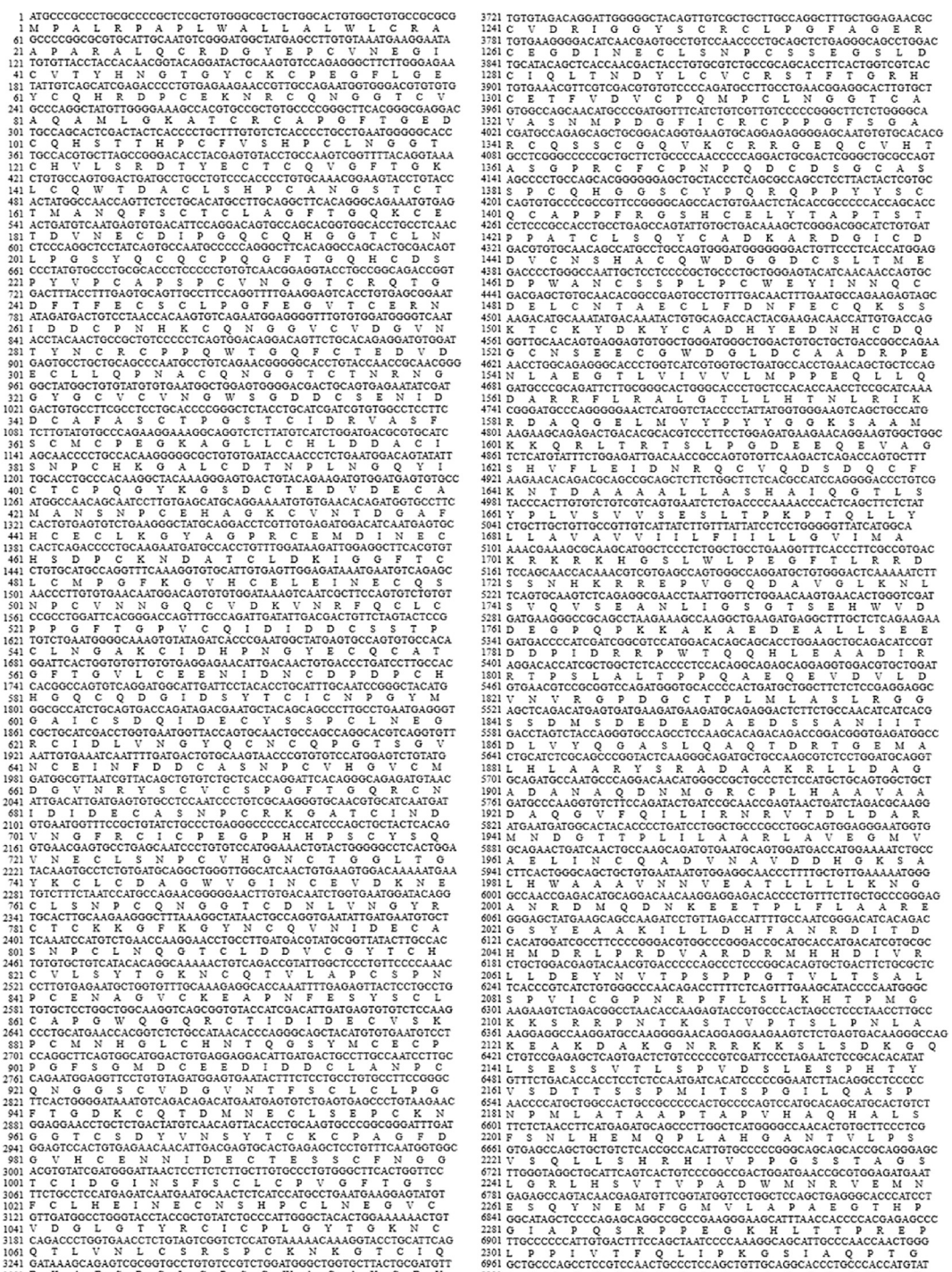

**Fig. 1.** Sequence of bovine *Notch2* gene.

## 3. Results

### 3.1. Homology alignment and phylogenetic tree construction

The mRNA sequence of bovine *Notch2* CDS region was obtained through sequence splicing (Fig. 1) and uploaded to GenBank (accession number: MT951418.1). The length of bovine *Notch2* gene complete CDS region is 7416 bp, encoding 2471 amino acids (AA). N*otch2* nucleotide sequences of different species were retrieved from NCBI database, including *Homo sapiens* (AF315356.1), *Hylobates moloch* (XM_032151593.1), *Macaca nemestrina* (XM_011736856.1), *Pongo abelii* (XM_009245522.2), *Rattus norvegicus* (NM_024358.2), *Mus musculus* (NM_010928.2), *Bubalus bubalis* (XM_006049346.2), *Ovis aries* (KX184272.1), *Capra hircus* (XM_018045986.1), *Sus scrofa* (XM_021090689.1), *Equus asinus* (XM_014831045.1), *Equus caballus* (XM_023642104.1). DNAMAN was used to align the homology of bovine *Notch2* gene with the *Notch2* gene in these 12 species. The sequence similarity was 90.94%, 90.79%, 90.93%, 90.88%, 86.04%, 86.20%, 98.75%, 97.49%, 97.55%, 92.89%, 92.11%, 92.10%, respectively. To represent kinship more significantly, we used MEGA7 to construct the phylogenetic tree with neighbor-joining (Fig. 2).

### 3.2. Localization of Notch2 receptor and its ligands in bovine antral follicles

Immunohistochemical results showed that Notch2 receptor and Jagged2 ligand were expressed in both GCs and follicular theca cells of bovine antral follicles (Fig. 3A,C). However, Jagged1 ligand was not expressed in bovine antral follicles (Fig. 3B).

### 3.3. Immunofluorescence of Notch2 and Jagged2 in LGCs

First, immunofluorescence analysis of the specific receptor FSHR showed that all cells carried green fluorescence, indicating high purity of LGCs (Fig. 4A). Then, the expression of membrane proteins Notch2 and Jagged2 was detected in LGCs. Immunofluorescence confirmed that Notch2 and Jagged2 had strong positive signals on the surface of LGCs, and the red fluorescence of Notch2 overlaps the green fluorescence of Jagged2 (Fig. 4B).

### 3.4. Notch2 silence efficiency

The expression of *Notch2* intracellular fragment *NICD2*, *Notch2* target genes *Hes1* and *Hey2* were detected to determine whether the *Notch2* gene was successfully silenced. The mRNA expression of *NICD2* and *Hey2* down-regulated ($P < 0.01$), mRNA expression of *Hes1* also decreased ($P < 0.05$) after *Notch2* silencing (Fig. 5A). The protein expression of *NICD2* decreased ($P < 0.01$) (Fig. 5B and C).

### 3.5. Effects of Notch2 on the proliferation rate of LGCs

The OD values of bovine follicular LGCs at different time points were detected by CCK-8, and then the cell proliferation rates were calculated. After *Notch2* gene was silenced, the cell proliferation rates at 24 h, 48 h and 72 h were significantly lower than those of the control group (Fig. 6).

### 3.6. Effects of Notch2 on the apoptosis genes and cell cycle genes

The expression of apoptosis related genes *Bax*, *Bcl-2*, *Caspase3* and cell cycle related genes *CyclinD2*, *CDK4*, *P21* were detected. Compared with the control group, the expressions of *Bcl-2* and *CDK4* decreased ($P < 0.01$), and *Caspase3* and *P21* increased ($P < 0.05$). While there was no difference in expressions of *Bax* and *CyclinD2* ($P > 0.05$) (Fig. 7).

### 3.7. Effect of Notch2 on steroid hormone and steroidogenesis genes

The steroid hormone secretion of bovine follicular LGCs was detected by ELISA. First, the standard curve was fitted with $r^2$ of 0.99, then the content of $E_2$ and $P_4$ were calculated by the OD value of the medium. The result showed that silencing *Notch2* had no effect on the $E_2$ secretion ($P > 0.05$) but down-regulated $P_4$ secretion ($P < 0.01$) in bovine follicular LGCs (Fig. 8A). The mRNA expression of *CYP19A1*, *CYP11A1*, *STAR* and *3β-HSD* in bovine follicular LGCs were detected by qRT-PCR, and the results showed that after *Notch2* silencing, expression of *CYP19A1* and *CYP11A1* in bovine follicular LGCs had no difference compared with the control group($P > 0.05$), however expression of *STAR* and *3β-HSD* decreased ($P < 0.01$) (Fig. 8B).

## 4. Discussion

In the present study, we cloned the CDS of bovine *Notch2* gene which is 7416 bp, encoding 2471 AA. Homology analysis showed that bovine *Notch2* gene had the highest homology with buffaloes and the lowest homology with rodents, but the sequence similarity is all higher than 85%, which proved that Notch2 is a highly conserved protein.

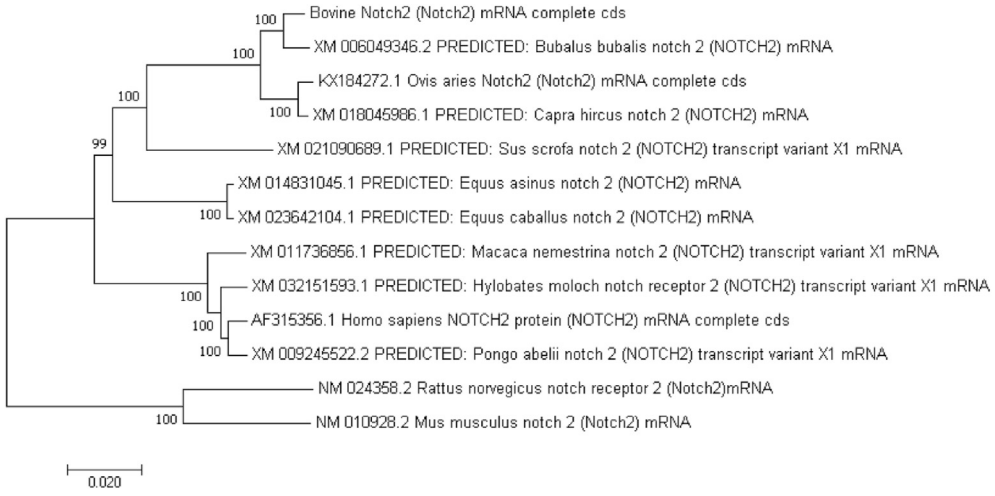


**Fig. 2.** Phylogenetic tree of bovine *Notch2* gene.

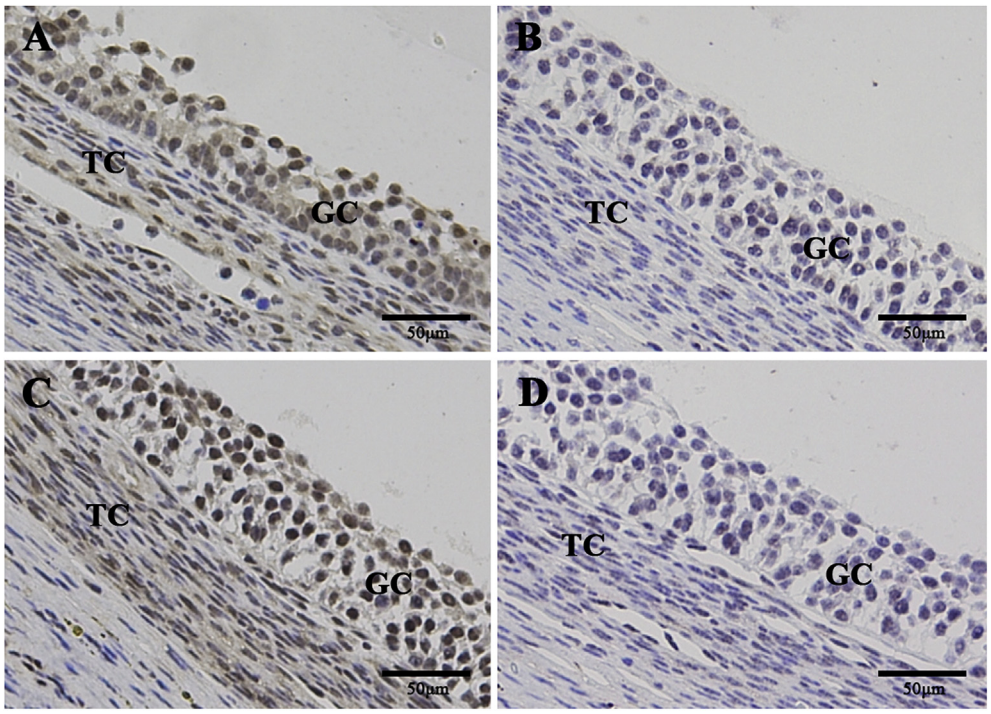


**Fig. 3.** Immunohistochemical localization of Notch2 (**A**), Jagged1 (**B**) and Jagged2 (**C**) in bovine antral follicles. Negative control (**D**). GC: granulosa cell; TC: theca cell. Scale bars correspond to 50 μm.

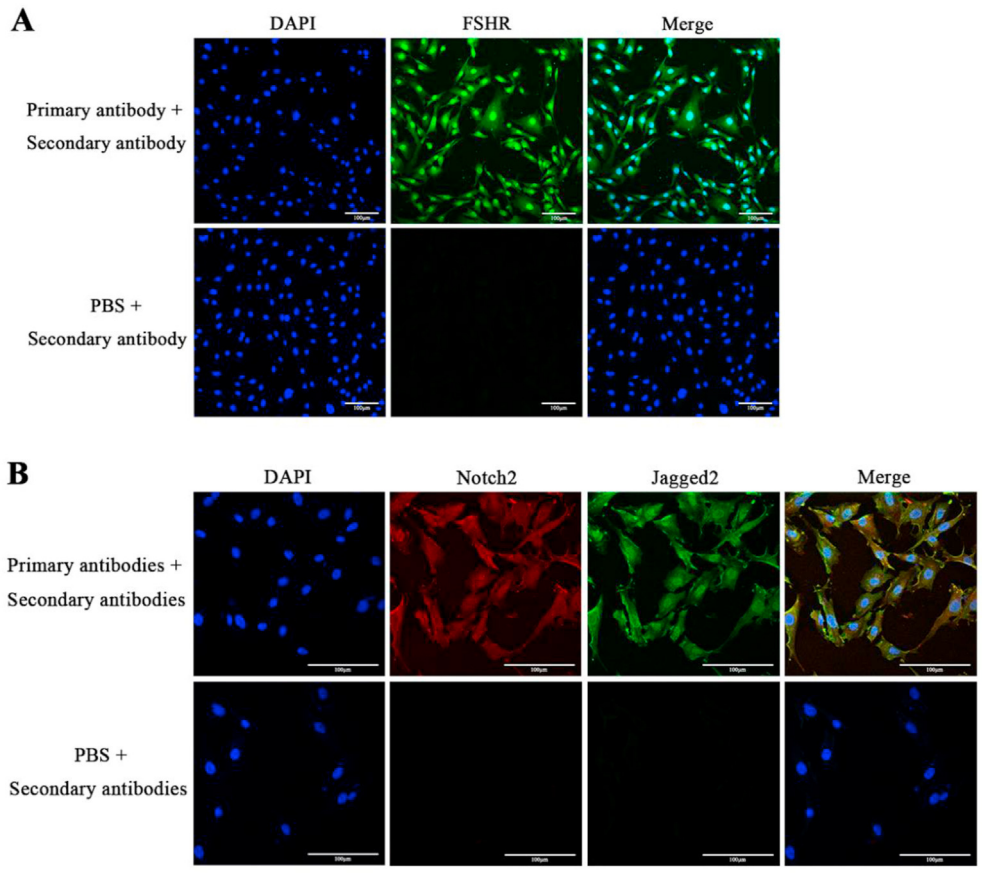


**Fig. 4.** Immunofluorescence analysis of LGCs. Location and expression of FSHR in LGCs (**A**). Location and expression of Notch2 and Jagged2 in LGCs (**B**). Scale bars correspond to 100 μm.

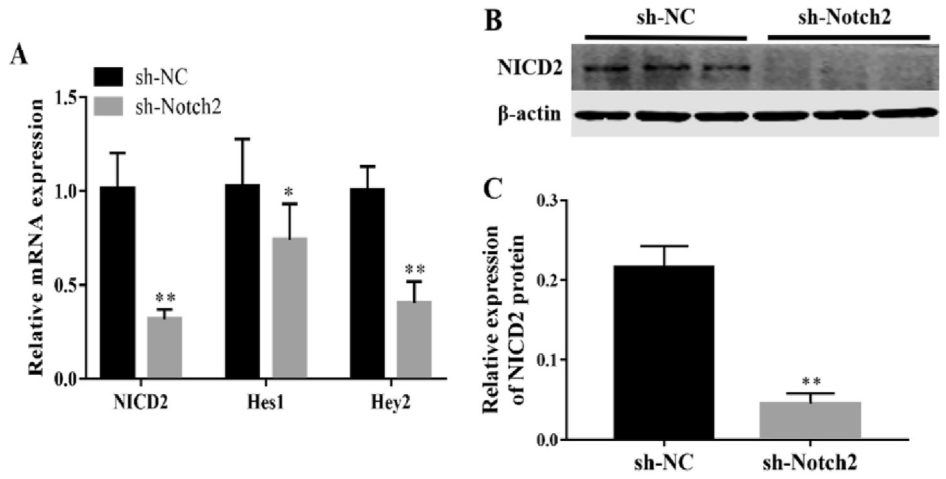


**Fig. 5.** *Notch2* silence effect in bovine follicular LGCs. Relative mRNA expression of *NICD2*, *Hes1* and *Hey2* (**A**). Western blotting bars of *NICD2* in different treatments (**B**). Relative protein expression of *NICD2* (**C**). * indicate $P < 0.05$, ** indicate $P < 0.01$.

Notch signal localization revealed that Notch2 receptor and its ligand Jagged2 were expressed in bovine follicular GCs and theca cells. However, its ligand Jagged1 was not expressed in bovine follicles. A study have found that Notch2 and Jagged2 were expressed in GCs of follicles in mice, and Jagged1 was not expressed in GCs, which is consistent with the results of our study [7]. However, Notch2, Jagged1 and Jagged2 were all expressed in human follicular GCs [9]. These results suggest that the expression of Jagged1 in follicular GCs of different mammals may be different. Then, immunofluorescence detected that the positive signals of membrane proteins Notch2 and Jagged2 overlap completely in LGCs. Both the Notch2 receptor and the Jagged2 ligand were expressed on the membrane of adjacent LGCs. It is speculated that Notch2 may react with Jagged2 to activate Notch signaling pathway between adjacent LGCs and thus play an important role.

To further investigate the effect of *Notch2* gene in bovine LGCs, we designed *Notch2* short hairpin RNA (shRNA) to transfect LGCs according to the amplified bovine *Notch2* gene sequence. *Hes1* and *Hey2* are typical downstream transcription factors of Notch signaling pathway, mediating many Notch signaling activities

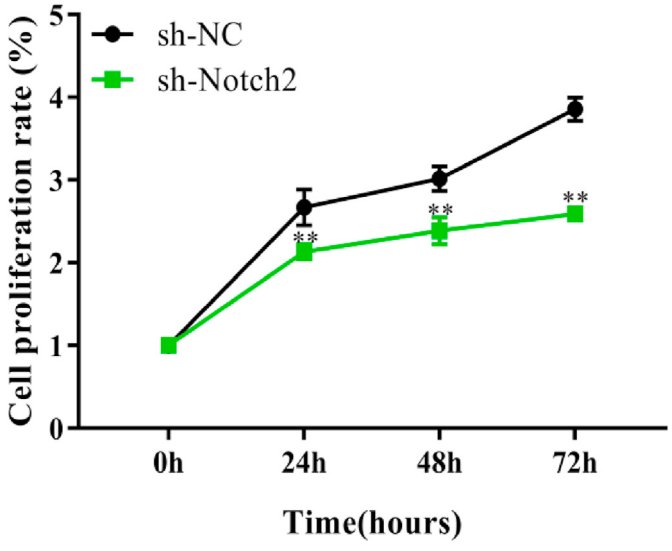


**Fig. 6.** Proliferation rate of bovine follicular LGCs infected lentivirus. ** indicate $P < 0.01$.

[17,18]. The transcription levels of *NICD2*, *Hes1*, *Hey2* mRNA all decreased after *Notch2* silencing. And the protein expression of *NICD2* was also significantly different from control group, indicating that silencing effect of *Notch2* was significant. It has been found that deletion of *Notch2* gene leads to decreased fertility, polyoocyte follicles, reduced number of primitive follicles in mice, and *Notch2* gene plays a role in destruction of germ cells nest and formation of primitive follicles [19]. Decreased proliferation and increased apoptosis occur in follicular GCs of *Notch2* gene knockout mice [20]. Culture *in vitro* of porcine cumulus oocyte complex, inhibition of *Notch2* gene can increase the cleavage rate of oocytes [21]. These indicate that *Notch2* synergistically adjust oocytes and GCs to regulate the occurrence and development of follicles. Cell proliferation rate continued to decrease and the difference was extremely significant at different time points after the *Notch2* gene was silenced by lentivirus in bovine follicular LGCs through CCK-8 test in our study. The *Notch2* gene defection led to the decrease of the proliferation ability of bovine LGCs proving that *Notch2* has a positive regulatory effect on follicular development consistent with previous studies.

Studies have confirmed that dominant follicular selection and follicular atresia are realized by GCs apoptosis [22,23]. The Bcl-2 family and the Caspase family jointly regulate apoptosis in sheep follicular GCs [24]. Bcl-2 family members were divided into anti-apoptotic genes (*Bcl-2*, *Bcl-W*, *Bcl-XL*) and pro-apoptotic genes (*Bax*, *Bad*, *Bim*, *BCL-xS*, *Bod*, *Bok*/*Mtd*) [25]. Bcl-2 family regulates apoptosis by regulating apoptosis induced factors release from mitochondria such as cytochrome C [26]. *Bax* is an antagonist of *Bcl-2*, and may cause the loss of the pro-apoptotic effect of Bcl-2 protein [25]. We detected the expression of apoptosis-related genes at the mRNA level to further explore the effect of *Notch2* silencing on LGCs apoptosis. It was found that the anti-apoptotic gene *Bcl-2* significantly decreased, while the pro-apoptotic gene *Bax* mRNA showed no significant difference before and after *Notch2* silencing. Nevertheless, the ratio of *Bcl-2*/*Bax* significantly decreased indicating that *Bcl-2* and *Bax* jointly regulate the apoptosis of bovine LGCs after *Notch2* gene was down-regulated because of the synergistic effect of *Bcl-2* and *Bax* in the Bcl family. In addition, the Caspase family members are also involved in programmed cell death, which were divided into initiator, effector, executioner, and the lack of certain *Caspases* can cause serious defects in cell apoptosis [27]. *Caspase3* is an executioner gene that induces apoptosis in human ovarian GCs [28]. The expression of *Caspase3* significantly increased after *Notch2* silencing indicating that the apoptosis of LGCs was enhanced,

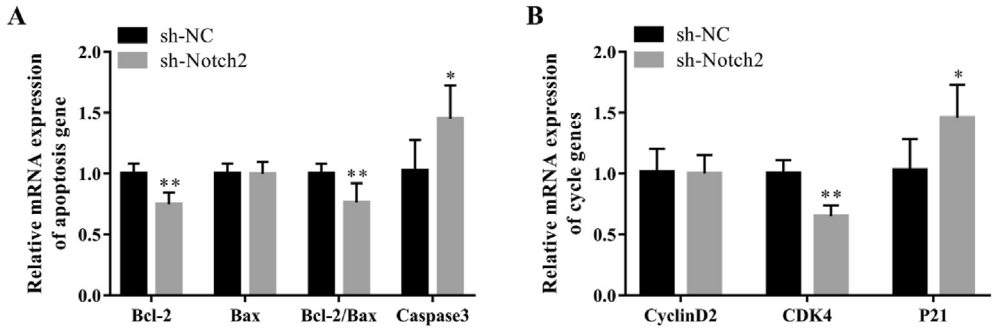


**Fig. 7.** Relative mRNA expression of cell activity related genes in bovine follicular LGCs silenced *Notch2*. Relative mRNA expression of apoptosis related genes *Bcl-2*, *Bax*, *Caspase3* (**A**). Relative mRNA expression of cell cycle related genes *CyclinD2*, *CDK4*, *P21* (**B**). * indicate $P < 0.05$, ** indicate $P < 0.01$.

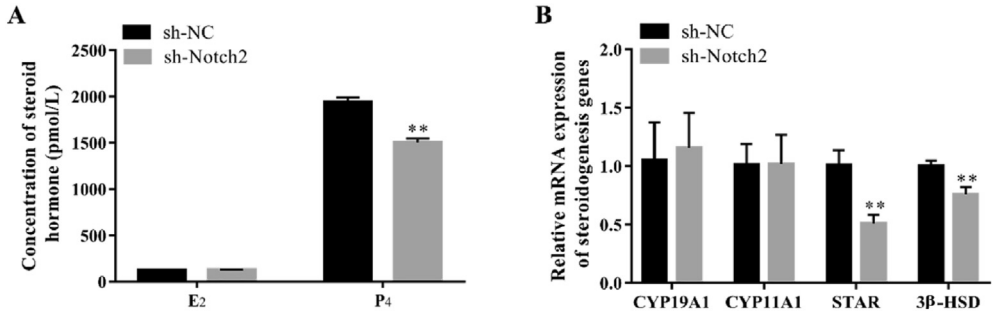


**Fig. 8.** Steroid hormone secretion and steroidogenesis genes expression in bovine follicular LGCs silenced *Notch2*. Concentration of $E_2$ and $P_4$ in cell culture medium (**A**). Relative mRNA expression of *CYP19A1*, *CYP11A1*, *STAR* and *3β-HSD* (**B**). ** indicate $P < 0.01$.

which is consistent with above results in this study. *Caspase3* also participates in the process of apoptosis in bovine LGCs.

Cell proliferation and apoptosis are closely related, so we also examined the expression of some cell cycle related genes. *CyclinD2*/*CDK4* can promote G1 phase cell cycle process and maintain cell mitosis [29]. Inhibition of *CyclinD2* restrains cell proliferation [30], and overexpression of *CyclinD2* and *CDK4* induces cell proliferation [31]. *P21* is a cyclin dependent kinase inhibitor, which binds to cyclins and inhibits their activity, resulting in cell cycle arrest and cell proliferation inhibition. Our results showed that cell cycle related gene *CyclinD2* had no difference, and *CDK4* was significantly decreased after *Notch2* silencing, resulting in the significantly reduced *CyclinD2*/*CDK4* complex. However, the expression of *P21* increased significantly after *Not*ch2 silencing. At least partly maybe the high expression of *P21* inhibits cell cycle genes, thereby reducing cell proliferation. This suggests that the inhibition of *Notch2* gene can lead to cell cycle arrest by regulating cycle-related genes during the development of bovine LGCs.

Steroid hormones play an important role in follicular development. Oocytes of embryonic mouse develop in germline cysts, which are then released and surrounded by GCs to form primitive follicles after mice birth, while $E_2$ can inhibit cyst rupture to inhibite primitive follicle formation [32]. Stimulation of $E_2$ biosynthesis can improve the activity of GCs affecting follicular development in goat [33]. $P_4$ receptor inhibits the apoptosis of rat follicular GCs [34]. $P_4$ inhibits primordial follicle assembly, also inhibits oocyte apoptosis during follicle assembly [35]. We detected the concentration of $E_2$ and $P_4$ in cell culture medium before and after *Notch2* gene silencing. *Notch2* gene did not affect $E_2$ secretion and decreased $P_4$ secretion in LGCs. *CYP19A1* which catalyze androstadienone conversion to $E_2$ was detected and there was no significant difference in *CYP19A1* gene expression. This result consistent with $E_2$ secretion. However, a previous study reported that $E_2$ level of GCs decreases after blocking Notch signal with DAPT [36]. The reason for this difference could be that the LGCs model characterized by low $E_2$ level was adopted in this experiment, and *Notch2* did not participate in the regulation of $E_2$ synthesis in the process of follicular atreia and luteinization. It also could be that other receptors of Notch signal lead to $E_2$ synthesis in bovine GCs. In addition, We detected no significant difference in the expression of $P_4$ synthesis related gene *CYP11A1*, while the *STAR* and *3β-HSD* decreased. STAR delivers free cholesterol from the cytoplasm to the mitochondrial intima, where CYP11A1 catalyzes cholesterol into pregnenolone [37], which is then transported to the endoplasmic reticulum to be converted to $P_4$ by 3β-HSD [38]. This suggested that Notch2 promotes $P_4$ synthesis by affecting cholesterol transport and conversion of pregnenolone to $P_4$ in LGCs. However, there is a study showed that inhibition of Notch signal in preantral follicles can up-regulate the expression of steroid related genes, including *STAR* and *HSD3B2* [39]. It is suggested that Notch signal is a dynamic regulatory process in the development of follicles, and plays a different role in recruitment, selection and dominance of follicles. The regulation mechanism of Notch signal in different stages of follicular development needs further investigation.

## 5. Conclusions

Our study demenstrated that Notch2 and Jagged2 are localized to GCs of bovine antral follicles and their fluorescence signals overlap in bovine LGCs, speculated that Notch2 receptor may activate Notch signaling pathway by reacting with Jagged2 ligand in LGCs. The proliferation rate of LGCs decreased after *Notch2* gene silence. *Notch2* gene silence induced apoptosis by down-regulating *Bcl-2/Bax* and up-regulating *Caspase3*, meanwhile co-regulating the cell cycle of LGCs by down-regulating *CyclinD2/CDK4* and up-regulating cycle suppressor *P21*. In addition, Notch2 gene silence

led to reduced $P_4$ secretion by regulating *STAR* and *3β-HSD* in LGCs. Notch2 determines follicular fate by regulating LGCs proliferation, apoptosis and progesterone production during follicular atresia and luteinization, laying a foundation for further study of Notch signaling pathway.

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## Declaration of competing interest

The authors declare no conflict of interest.

## CRediT authorship contribution statement

**Yating Li:** Conceptualization, Methodology, Investigation. **Jiongjie Jing:** Conceptualization, Methodology. **Wenqing Dang:** Conceptualization, Methodology, Methodology. **Qi Han:** Methodology. **Xiangyu Guo:** Resources. **Kaiqi Jia:** Resources. **Ying Cheng:** Resources. **Kai Wang:** Resources. **Ermias Kebreab:** Writing – review & editing. **Lihua Lyu:** Conceptualization, Writing – review & editing, Funding acquisition.

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