



## OPEN Structural analysis and core promoter prediction of STAR gene and its regulatory mechanism of progesterone synthesis in bovine luteal cells

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The steroidogenic acute regulatory protein (STAR) plays a crucial role in facilitating cholesterol transfer across the inner mitochondrial membrane during the process of steroidogenesis. However, the transcriptional regulation of the bovine *STAR* gene and its function of progesterone synthesis in luteal cells remain poorly understood. The objective of this study was to analyze the bovine *STAR* gene structure, identify its active promoter region, and explore its potential roles in progesterone synthesis. Bioinformatics analysis revealed that the bovine *STAR* gene encodes an 858-bp mRNA transcript, which translates into a protein consisting of 285 amino acids. The phylogenetic tree analysis showed that its genetic distance was closest to that of sheep. Notably, the promoter region of bovine *STAR* lacks CpG islands, and the core promoter is located within the -1990/-1 region, which contains potential binding sites for transcription factors such as NF- $\kappa$ B, Sp1, NF-1, and LyF-1. Dual-luciferase reporter assays confirmed the core promoter activity within this region, aligning with the prediction results. Overexpression of the *STAR* gene in bovine luteal cells significantly enhanced progesterone production and upregulated the expression of steroidogenic enzymes, particularly 3 $\beta$ HSD and CYP11A1. In conclusion, this study identifies the core promoter region of the bovine *STAR* gene is positioned at -1990/-1. By regulating key steroidogenic enzymes, particularly 3 $\beta$ HSD and CYP11A1, *STAR* is involved in the synthesis of progesterone in corpus luteum cells.

**Keywords** Bovine, *STAR* gene, Bioinformatics, Core promoter, Luteal cell, Progesterone

The corpus luteum (CL) is a temporary endocrine tissue formed post-ovulation in mammals, persisting throughout the entire reproductive cycle. Progesterone synthesis in luteal cells has been shown to directly correlate with the normal reproductive functions and embryo viability in female animals<sup>1</sup>. Recent research has revealed that the steroidogenic acute regulatory protein (STAR) is a key regulator in the rapid synthesis of progesterone<sup>2</sup>. STAR, a transport protein encoded by specific genes<sup>3</sup>, exhibits high tissue specificity and facilitates the transfer of free cholesterol across the outer mitochondrial membrane to the inner mitochondrial membrane—a critical rate-limiting step in progesterone synthesis<sup>4,5</sup>. Within the inner mitochondrial membrane, cholesterol is converted to pregnenolone via cytochrome P450 family (specifically Cytochrome P450 family 11A1, CYP11A1), followed by its conversion to progesterone (P<sub>4</sub>) in the smooth endoplasmic reticulum through catalysis by 3 $\beta$  hydroxysteroid dehydrogenase (3 $\beta$ HSD)<sup>6</sup>. Progesterone regulates a series of physiological processes, including ovulation, follicular growth and atresia, and the differentiation of cells within mature follicles in the ovary<sup>7</sup>. Following ovulation and during the luteal phase, the enzymatic activities of CYP11A1 and 3 $\beta$ HSD are elevated, promoting accelerated progesterone synthesis, with spatiotemporal coordination of STAR expression<sup>8</sup>. It is noteworthy that interference with Hand2os1 in mouse corpus luteum cells resulted in decreased progesterone synthesis, which could be rescued by overexpressing STAR<sup>9</sup>, highlighting STAR protein is a key factor in regulating steroid hormone synthesis in mammals.

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Gene name	Primer sequence(5'-3')	Annealing Temperature/°C	Product Length/bp	Primer Usage
STAR-F	<i>atttgcggccgc</i> GCCACCATGCTTCTCGGACGTTTAAGCT	63	858	CDS region amplification
STAR-R	<i>ceggatttc</i> TCAACACCTAGCTTCAAGAGCAGG	63		
STAR-1 F	<i>gaagatct</i> CAGGTAGACAGCAAAGGGACTCAGT	64	472	Different deletion fragments Amplification
STAR-2 F	<i>gaagatct</i> AGAATGGTCTGGAGTGAGTGAGGTTG	64	1003	
STAR-3 F	<i>gaagatct</i> AATGTGCGCTTGAGTGTCCGGTAC	64	1499	
STAR-4 F	<i>gaagatct</i> TAGAGTGCTCAGGGCATCTGCTG	64	1990	
STAR-R	<i>ctagctagc</i> TACAGGTGGCTCAGTGGCAAAGAA	66		

**Table 1.** Primers used in vector construction. Note: The Italicized characters indicate the cleavage site and protection base; The Underscores indicate cleavage sites, The Bold letters are the protective bases of restriction enzymes.

Gene name	Primer sequence(5'-3')	Annealing Temperature/°C	Product Length/bp	NCBI Accession NO.
STAR	F: GACGTTTAAGCTGTGTGCCG R: TTGGTCGCTGTAGAGAGGGT	60	202	NM_174189.3
3βHSD	F: GAGGAAATGGGGGTGCTAGA R: AACAGCAGCTGGGTACCTTTC	60	239	NM_174343.3
CYP11A1	F: AGAGAATCCACTTTGCGCCACT R: GTAGGATCCCTCGAACTTGGA	60	197	XM_010809903.3
GAPDH	F: GTACATGGTCTACATGTTCCAG R: GGTGTGGATGGTAGTGAAGA	60	200	NM_001034034.2

**Table 2.** Primers used in RT-qPCR. Note: F. Forward primer; R.Reverse primer.

The promoter is a complex region containing numerous transcription factor binding sites, crucial for regulating gene expression. It directly determines the transcription initiation site and the efficiency of gene transcription, making it essential for understanding gene function<sup>10</sup>. For instance, Li et al. mapped the core promoter of porcine *ATG7* and predicted transcription factors potentially regulating ovarian development<sup>11</sup>. Similarly, Song et al. characterized the miR-200b promoter in sheep, revealing its role in granulosa cell senescence via MYBL2 and CDK1 targeting<sup>12</sup>. Given its pivotal role in regulating gene expression, the promoter has garnered attention in studies aimed at improving reproductive traits in livestock production. This study aims to dissect the core promoter sequence of the *STAR* gene using advanced online prediction tools, identify potential transcription factors, and elucidates the molecular mechanisms underlying *STAR* transcriptional regulation. These findings may provide novel insights for enhancing the reproductive performance of livestock such as bovine.

## Materials and methods

### Materials

Bovine corpus luteum cell line (preserved in the laboratory, which was constructed by transduction of SV40T antigen in bovine luteal cells in our laboratory); T4 ligase, endonuclease, DH5α competent cells, pscheck-2 vector (Takara Bio, Japan); Animal cell RNA extraction kit, Animal genomic DNA extraction kit (Tiangen Biotech, China); Reverse transcription kit, Fluorescence quantification kit (Vazyme Biotech, China); Fetal bovine serum, DMEM high glucose medium (Gibco, USA); Dual-Luciferase<sup>®</sup> reporter gene test kit (Promega, USA); Lipofectamine 2000 transfection reagent (Invitrogen, USA); Rabbit polyclonal antibody to *STAR* (1:10,000, Cat# ab180804), rabbit monoclonal antibody to *3βHSD* (1:1,000, Cat# ab167417), rabbit monoclonal antibody to *CYP11A1* (1:1,000, Cat# ab272494) and rabbit monoclonal antibody to *GAPDH* (1:5,000, Cat# ab8245) (Abcam, USA). Equipment included a refrigerated centrifuge (4 °C), cell culture incubator, microplate reader, and real-time PCR system (Light Cycler 96, Roche, Switzerland) and so on.

### Primer design

The gene sequences were determined according to the NCBI database, and Premier 5.0 software was used for primer design, and the primer information was as follows (Tables 1 and 2).

### Bioinformatics analysis

Bioinformatics software and online websites were used to analyze the physicochemical properties of *STAR* genes, protein structure prediction, etc. The software information and specific functions are as follows (Table 3).

### Construction and characterization of different promoter truncations of bovine *STAR* gene

Referring to the instructions of the animal genomic DNA extraction kit, genomic DNA of bovine was extracted, and using DNA as template, *STAR*-R was amplified with *STAR*-1 F, *STAR*-2 F, *STAR*-3 F, and *STAR*-4 F respectively by PCR to obtain four truncated promoter fragments of the *STAR* gene. After recovery and purification by agarose gel electrophoresis, the fragments were double-digested with restriction enzymes *NheI* and *BglII* T4 ligase was

Software name	Website	Purpose
BDGP	<a href="https://fruitfly.org/seq_tools/promoter.html">https://fruitfly.org/seq_tools/promoter.html</a>	Promoter prediction
MEGA	MEGA-X software NJ method to construct phylogenetic tree	Construct evolutionary tree
Gene Cards	<a href="https://www.genecards.org/">https://www.genecards.org/</a>	Subcellular localization
ExpASY	<a href="https://web.expasy.org/protparam/">https://web.expasy.org/protparam/</a>	Physical and chemical property analysis
TMHMM-2.0	<a href="https://services.healthtech.dtu.dk/services/TMHMM-2.0/">https://services.healthtech.dtu.dk/services/TMHMM-2.0/</a>	Transmembrane domain prediction
SignalP-6.0	<a href="https://services.healthtech.dtu.dk/services/SignalP-6.0/">https://services.healthtech.dtu.dk/services/SignalP-6.0/</a>	Signal peptide prediction
NetSurfP-2.0	<a href="https://services.healthtech.dtu.dk/services/NetSurfP-2.0/">https://services.healthtech.dtu.dk/services/NetSurfP-2.0/</a>	Protein secondary structure prediction
SWISS-MODEL	<a href="https://swissmodel.expasy.org/interactive">https://swissmodel.expasy.org/interactive</a>	Protein tertiary structure prediction
STRING	<a href="https://string-db.org/">https://string-db.org/</a>	Protein Interaction
AliBaba2.1	<a href="http://generegulation.com/pub/programs/alibaba2/index.html">http://generegulation.com/pub/programs/alibaba2/index.html</a>	Transcription factor binding site prediction
MethPrimer	<a href="http://www.urogene.org/methprimer2/">http://www.urogene.org/methprimer2/</a>	CPG island prediction

**Table 3.** Website for bioinformatics analysis.

used to connect the target fragment with psicheck-2 vector according to the instructions. The recombinant plasmids psicheck-2-STAR-1 (-472/-1), psicheck-2-STAR-2 (-1003/-1), psicheck-2-STAR-3 (-1499/-1), and psicheck-2-STAR-4 (-1990/-1) were constructed.

### Bovine STAR gene promoter region activity detection

293T cells were revived and seeded into a 24-well plate at a density of  $1 \times 10^5$  cells per well. At 70% confluency, 293T cells were transfected with the four recombinant plasmids or the psicheck-2 empty vector (control) using Lipofectamine 2000 (three biological replicates per group). After 48 h, cell lysates were collected, firefly (*Photinus pyralis*) and Renilla (*Renilla reniformis*) luciferase activities were measured using a dual-luciferase assay kit. Relative luciferase activity was calculated as firefly/Renilla ratio.

### Bovine STAR gene CDS full-length amplification and overexpression vector construction

Resuscitate the bovine luteal cells, after the cell density grows to 90%, the total RNA was extracted using an RNA isolation kit, cDNA was synthesized from qualified RNA samples ( $OD_{260/280} = 1.8\sim 2.0$ ). The full-length coding sequence (CDS) of STAR was amplified by PCR, purified, and cloned into the pLV-CMV vector using NotI and EcoRI restriction sites. The recombinant plasmid (pLV-CMV-STAR) was constructed.

### Transfection of luteal cells with STAR overexpression vector

Luteal cells were passaged into 6-well plates and when the cells reached at 70~ 80% confluency, the pLV-CMV empty vector (control group) and pLV-CMV-STAR recombinant plasmid (experimental group) were transfected into the bovine luteal cells using Lipofectamine 3000 at a concentration of 2.5  $\mu\text{g}$  per well. Each group had three biological replicates.

### Determination of $P_4$ level in luteal cells

After 48 h of transfection with the pLV-CMV-STAR recombinant plasmid, the cell culture supernatant was collected, appropriately labeled, and stored at  $-20\text{ }^\circ\text{C}$  for subsequent analyses. The concentration of progesterone ( $P_4$ ) in the supernatant was quantified using an enzyme-linked immunosorbent assay (ELISA) kit. Analyze the differential secretion levels of  $P_4$  between the STAR overexpression group and the control group.

### Real-time fluorescence quantitative PCR detection

Total RNA was extracted from transfected luteal cells 48 h post-transfection and immediately reverse-transcribed into cDNA, and real-time fluorescence quantitative PCR was used to detect the relative expression of STAR gene, progesterone synthesis-related genes *3 $\beta$ HSD* and *CYP11A1* with *GAPDH* as the internal reference gene. RT-qPCR was performed using SYBR Green Idye method and Light Cycler 96.

### Western-Blot

Total protein was extracted from each group of luteal cells 48 h post-transfection using RIPA lysis buffer, the protein concentration was detected by BCA Protein Quantification Kit. After normalizing protein concentrations, the SDS-PAGE operation was performed, and the protein was transferred to PVDF membrane. After blocking with 5% BSA, incubate the membrane with the primary antibody at  $4\text{ }^\circ\text{C}$  for 12 h, then add the secondary antibody corresponding to the species of primary antibody and incubate it at room temperature for 2 h. Between each step, wash the membrane with TBST. Utilize ECL chemiluminescence to observe the corresponding bands, and quantify the intensity of bands with Image-J software.

### Data statistics and analysis

Relative gene expression was calculated using the Livak method  $2^{-\Delta\Delta C_t}$  with *GAPDH* as the internal reference gene. Relative protein expression was calculated with *GAPDH* as the internal reference protein. Data were

statistically analyzed and graphically plotted using GraphPad Prism software. One-way ANOVA was used to compare gene expression, protein expression and promoter luciferase relative activity, and  $P < 0.05$  was considered statistically significant.

## Results

### Genetic analysis of STAR gene in different species

In order to explore the genetic characteristics of STAR, species evolutionary trees were constructed using the NCBI website and molecular evolutionary genetics software (Mega-X) based on the STAR amino acid sequences (Table 4) from 17 species (Fig. 1). The bovine STAR genes clustered with that of sheep, indicating a high level of homology and close evolutionary affinity. However, the homology between bovine STAR and those of avian, amphibian, and insect species was low.

### Structure prediction analysis of bovine STAR proteins

The subcellular localization of bovine STAR protein was analyzed using the GeneCards online tool, which revealed its primary localization in the mitochondria (Fig. 2-A). Physicochemical properties of the bovine STAR protein were assessed using the ExPASy online tool, which indicated a molecular formula of  $C_{1398}H_{2290}N_{408}O_{409}S_{16}$ , comprising 285 amino acids, with a molecular weight of 31.87 kDa and an isoelectric point of 9, suggesting that the bovine STAR protein is acidic. The lipophilicity index of the STAR protein was found to be 94.42, and its instability index is 42.81 ( $> 40$ ), while the hydropathy index ranges from 2 to -3 (Fig. 2-B), indicating that this protein is an unstable lipophilic protein. Secondary structure prediction of STAR using the NetSurfP-2.0 online software showed that  $\alpha$ -helices account for 38.25%,  $\beta$ -sheets for 5.26%, and random coils for 38.25% (Fig. 2-C). Prediction of the tertiary structure of the STAR protein indicated that the bovine STAR protein is predominantly composed of  $\alpha$ -helices and random coils (Fig. 2-D), which was consistent with the predicted secondary structure results. Further analysis of the transmembrane structure (TMHMM) and signal peptide analysis (SignalP 5.0) using online software revealed the absence of signal peptide regions in bovine STAR (Fig. 2-E) and no transmembrane structures (Fig. 2-F). Additionally, potential interacting proteins of STAR were predicted using the STRING online database, identifying regulatory relationships with key progesterone synthesis enzymes CYP11A1 and  $3\beta$ HSD (Fig. 2-G).

### Analysis of cattle STAR gene promoter region

The sequence of the cattle STAR promoter was analyzed using BDGP online software, which showed the presence of multiple transcription start sites (TSS) in the STAR gene promoter (Table 5). MethPrimer software prediction found that there were no CpG island in the STAR promoter, but multiple CpG regions were identified (Fig. 3).

### Transcription factor binding site prediction

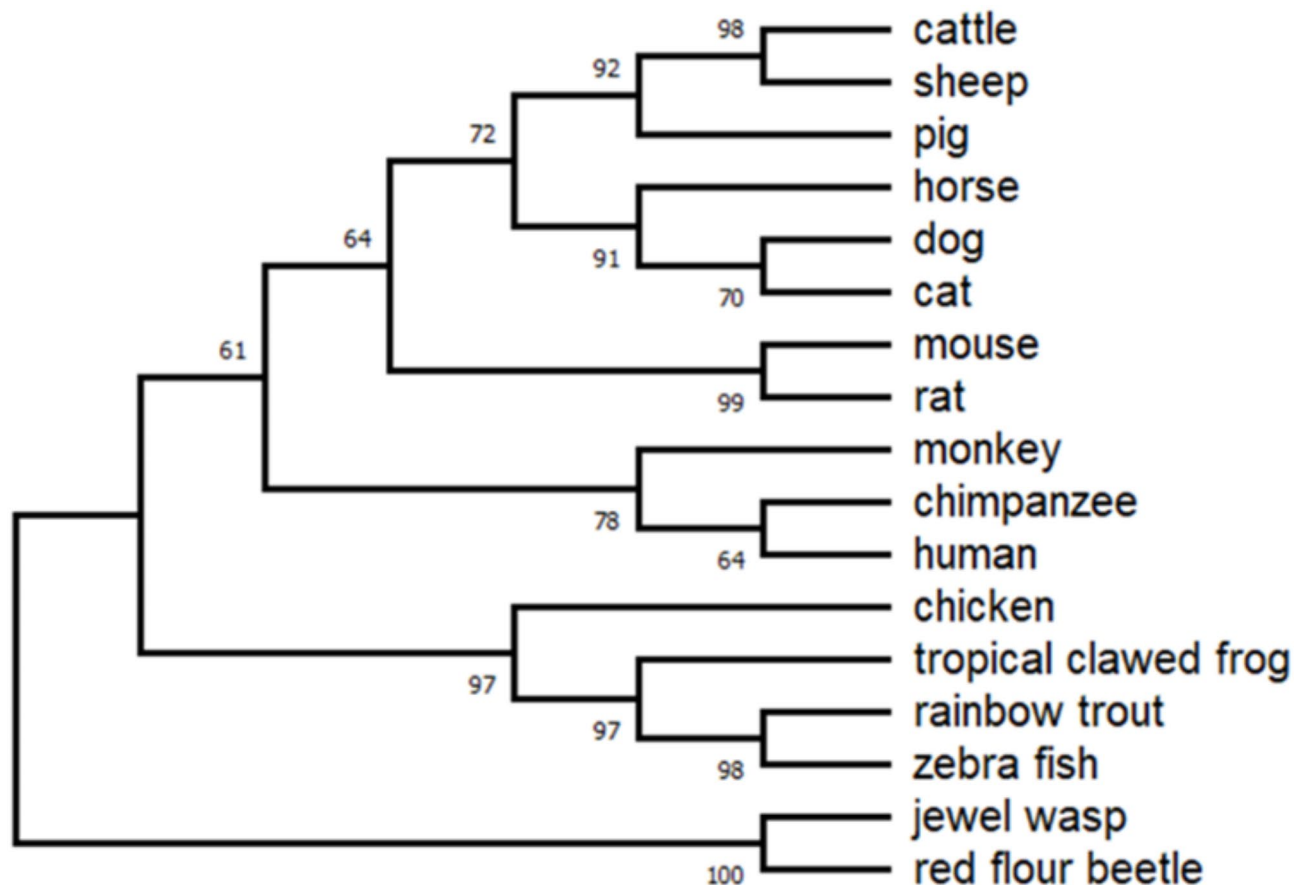
Transcription factors binding sites in the STAR promoter region were predicted using AliBaba2.1 online tool, which identified binding sites for NF- $\kappa$ B, Sp1, NF-1, and LyF-1 transcription factors (Fig. 4).

### Construction of promoter deletion fragments and dual luciferase assay analysis

Different truncated fragments of the 5' flanking promoter of bovine STAR gene were amplified by PCR using bovine genomic DNA as template (Table 1), and the amplified PCR products were purified and recovered, cloned into the dual luciferase pscheck-2 plasmid vector to construct the recombinant plasmid pscheck-2-STAR-1 (-472/-1), pscheck-2-STAR-2 (-1003/-1), pscheck-2-STAR-3 (-1499/-1), and pscheck-2-STAR-4 (-1990/-1).

Species	GenBank No.	CDS length/bp
Homo sapiens (human)	NP_000340.2	858
Mus musculus (house mouse)	NP_035615.2	855
Rattus norvegicus (Norway rat)	NP_113746.1	855
Danio rerio (zebrafish)	NP_571738.1	858
Nasonia vitripennis (jewel wasp)	NP_001234901.1	1029
Oncorhynchus mykiss (rainbow trout)	NP_001117674.1	864
Equus caballus (horse)	NP_001075269.2	858
Macaca mulatta (Rhesus monkey)	NP_001252698.1	858
Bos taurus (cattle)	NP_776614.2	858
Sus scrofa (pig)	NP_998920.2	858
Ovis aries (sheep)	NP_001009243.1	858
Gallus gallus (chicken)	NP_990017.1	846
Canis lupus familiaris (dog)	NP_001091011.1	858
Felis catus (domestic cat)	NP_001233125.1	858
Pan troglodytes (chimpanzee)	XP_001170375.1	858
tropical clawed frog	XP_002932770.1	858
Tribolium castaneum (red flour beetle)	NP_001164123.1	967

**Table 4.** Amino acid sequences and lengths of different species.



**Fig. 1.** Phylogenetic tree of STAR. Phylogenetic tree of the above STAR amino acid sequence based on Neighbor-Joining (NJ) method. The phylogeny is presented as an unrooted transformed cladogram for best presentation.

Double-enzyme digestion was performed to verify that the recombinant plasmid fragments were correct in size (Fig. 5-A). The control plasmid pscheck-2 and the recombinant plasmid were transfected into 293T cell lines, respectively, and the luciferase activity was detected by the dual luciferase reporter gene kit 24 h post-transfection. The results showed that all four STAR promoter reporter vectors constructed were active in cells, with pscheck-2-STAR-4 (-1990/-1) exhibiting the highest relative dual luciferase activity ( $P < 0.01$ ) (Fig. 5-B).

#### Amplification and full-length cloning of CDS region of bovine STAR gene

The complete coding region (CDS) of bovine STAR gene was amplified by PCR, and the amplified PCR product was purified and recovered. After sequencing, the full-length CDS of bovine STAR gene was 858 bp, encoding a total of 285 amino acids, which was consistent with the sequence published by GenBank. The target gene was cloned into pLV-CMV plasmid vector, and double enzyme digestion verified that the recombinant plasmid pLV-CMV-STAR fragment size was correct (Fig. 6).

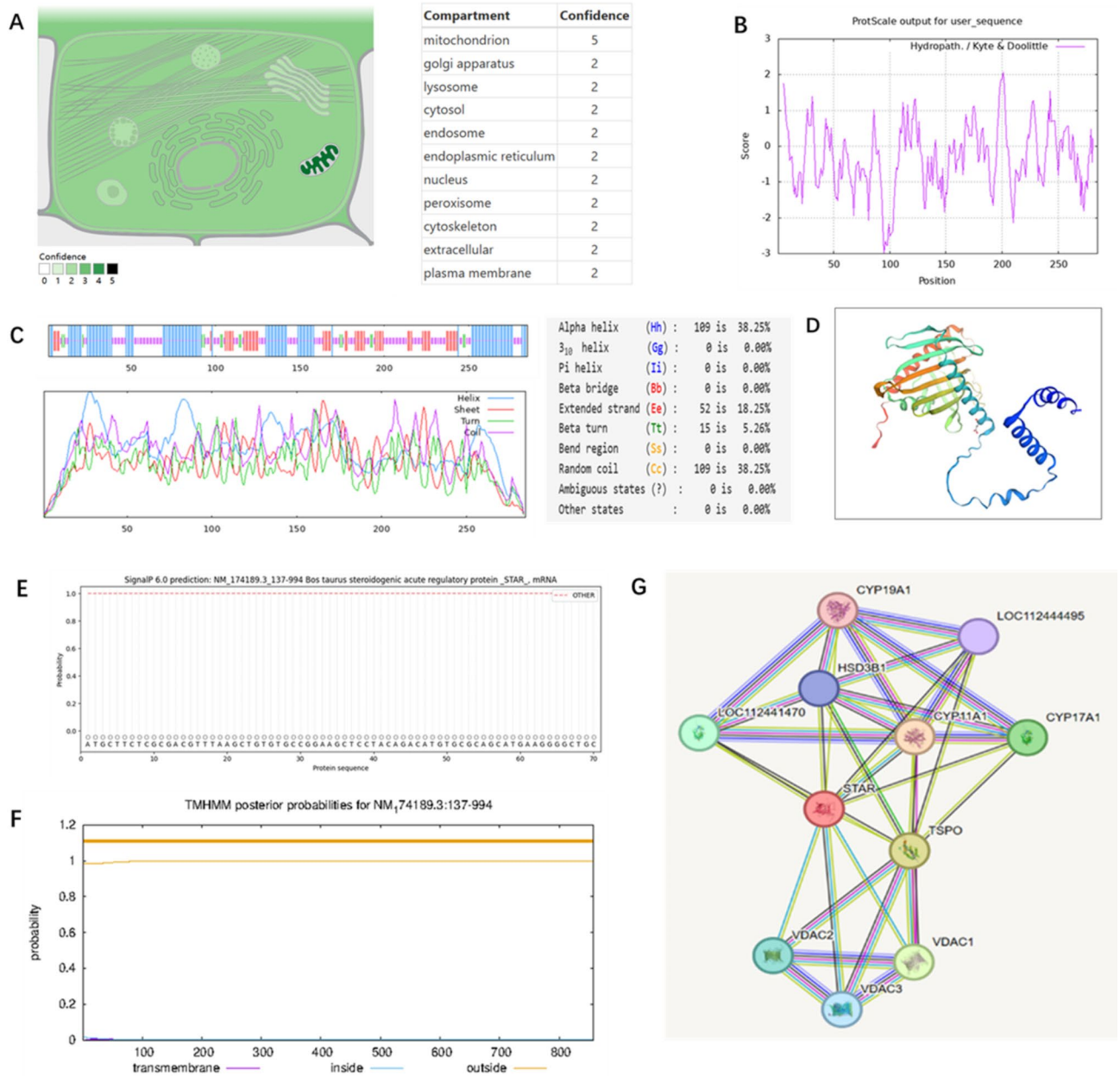
#### Effect of STAR overexpression on progesterone synthesis level in bovine luteal cells

The pLV-CMV-STAR plasmid was transiently transfected into bovine luteal cells for 24 h. RT-qPCR analysis showed that STAR overexpression significantly upregulated the expression of *STAR* and progesterone synthesis-related genes *3βHSD* and *CYP11A1* compared to the control group ( $P < 0.01$ ) (Fig. 7-A). Enzyme-linked immunosorbent assay (ELISA) revealed that the progesterone content in the supernatant of the overexpressed STAR group (the intra-assay coefficient was 2.0% and the inter-assay coefficient was 5.0%) was significantly higher than that of the control group (the intra-assay coefficient was 2.3% and the inter-assay coefficient was 3.1%). ( $P < 0.01$ ) (Fig. 7-B). Western blot assay was performed to analyze the expression levels of key enzymes involved in progesterone synthesis (Fig. 7-C). Image-J grayscale statistical analysis showed that STAR overexpression significantly upregulated the protein levels of STAR and *3βHSD* ( $P < 0.01$ ) (Fig. 7-D).

#### Discussion

The steroidogenic acute regulatory protein (STAR), a mitochondrial phosphorylated protein, is rapidly synthesized in response to hormones such as adrenocorticotrophic hormone (ACTH) and luteinizing hormone (LH). It serves as the primary regulatory protein during the acute synthesis phase of steroid hormones<sup>13,14</sup>. The

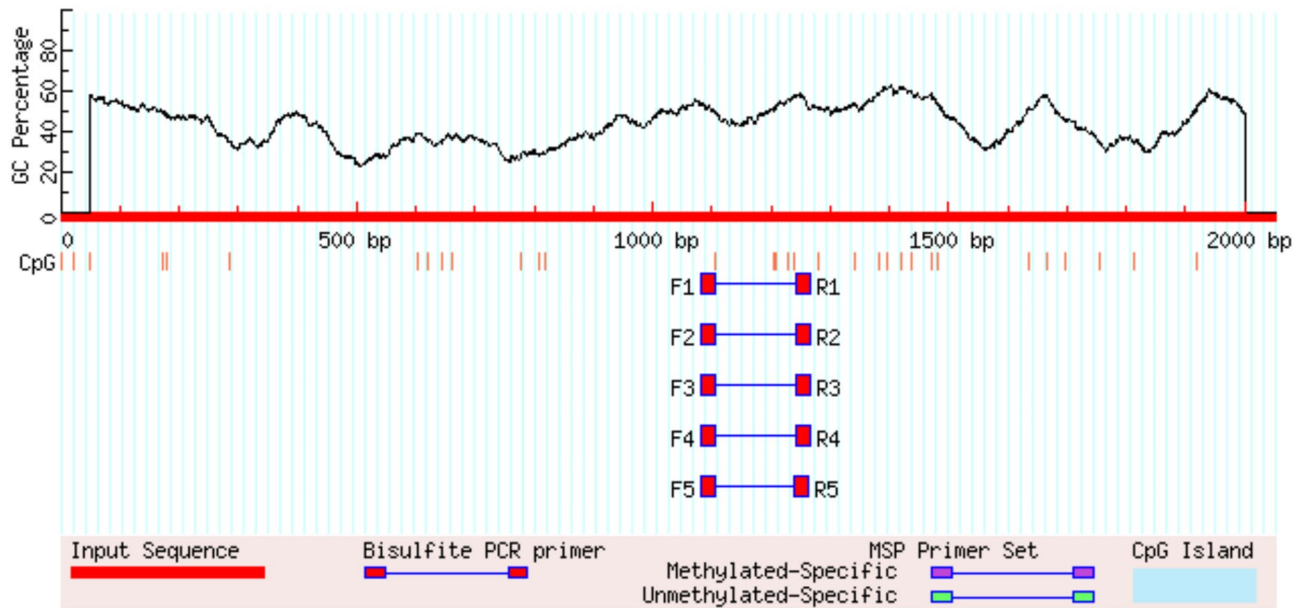




**Fig. 2.** Prediction analysis of cattle STAR protein structure. **A:** Subcellular localization of STAR protein; **B:** Hydropobicity analysis of STAR protein; **C:** Prediction of Secondary Structure of STAR Protein; **D:** STAR protein tertiary structure prediction; **E:** STAR protein signal peptide prediction; **F:** STAR protein transmembrane region prediction; **G:** STAR protein interaction analysis.

Start/bp	End/bp	Promoter sequence	Score
151	201	TCTATGGGGTCACAAAAGAGTCGGACATGAGCGAGCAACTAAACAAGTGG	0.66
314	364	TTGTGATAAATAAATAAAAAGGATAACCAACAAGGACCCACTGCACAGG	0.74
419	469	TGGATACATGTATATGTATGACTGAGTCCCTTTGCTGTACTACCTGAAATT	0.72
1916	1966	TTGATTCGTGTAGAAGTGGGGTGGGGTGGCCTCTGCCAGCTCCAGCAGA	0.90

**Table 5.** TSS prediction of 5' flanking sequence of cattle STAR gene.



**Fig. 3.** Methylation analysis of bovine STAR promoter. The 5'UTR of the bovine STAR gene was downloaded from NCBI, and the CPG region of the gene was predicted using Meth Primer.

Segments:

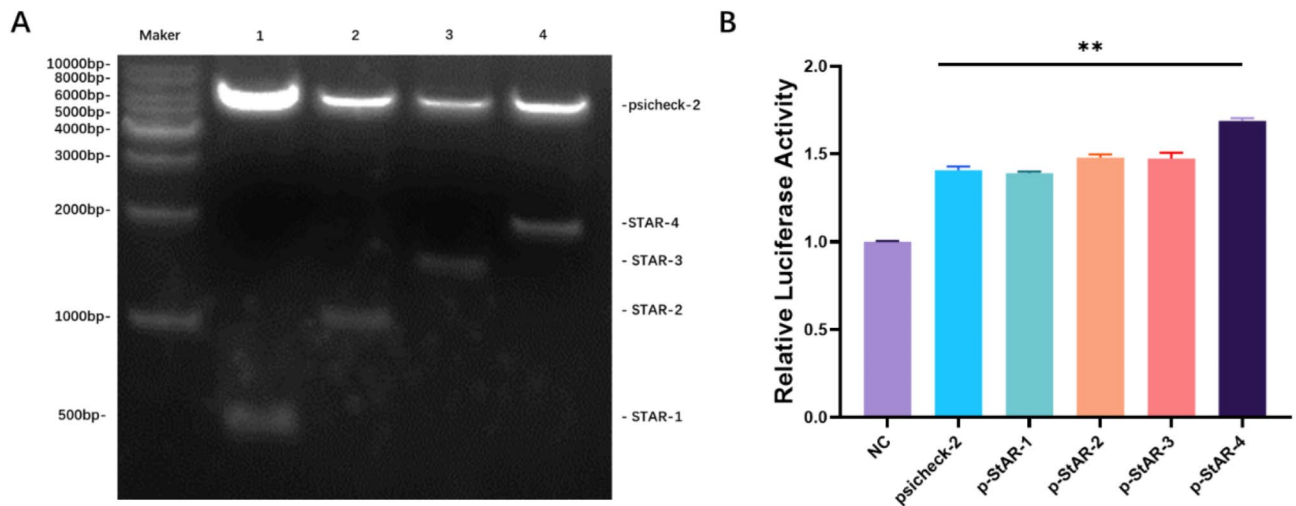
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seq( 1320.. 1379)    aaccagagagctgtgagtttccgctttatctgggggcttctctgaagacctgaccctggga
Segments:
9.9.590    1349 1360                                ==NF-kappaB=
seq( 1620.. 1679)    ggtctgggggtaagacgctgtgctcccaatgaagagggcagggttcgatccctggatg
Segments:
2.3.1.0    1651 1664                                =====Sp1=====
seq( 1740.. 1799)    ctctaaggactcatttcgctgtttttgacagtgacagcaaaaaaacaacatgata
Segments:
9.9.539    1772 1783                                =====NF-1=====
2.3.2.2    1778 1789                                =====LyF-1=====
seq( 1920.. 1979)    tcgtgtagaactgggggtggggtggcctctgccagctcccagcagatgcctgagcactct
Segments:
2.3.1.0    1931 1942                                =====Sp1=====
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**Fig. 4.** Prediction of transcription factor binding sites in the bovine STAR promoter region. The 5'UTR of bovine STAR gene was downloaded from NCBI, and the transcription factor binding sites of the gene were predicted using AliBaba2.1.

core function of STAR lies in its ability to efficiently transfer cholesterol from the cytoplasm to the mitochondria, a step that directly determines the rate and efficiency of steroid hormone synthesis<sup>3,15</sup>. Given its specific role in cholesterol transport, the expression level of STAR becomes a key rate-limiting factor for regulating steroid hormone synthesis. The high expression of STAR in tissues such as the adrenal glands, corpus luteum, ovaries, and testes further underscores its critical role in the reproductive process, while its dysregulation is often accompanied by disorders of reproductive endocrine function, affecting embryo development and sperm production<sup>16,17</sup>. In mouse models, knockout of the STAR gene directly leads to a significant decrease in adrenal steroid synthesis, further confirming STAR's central regulatory position in steroid hormone synthesis<sup>18</sup>.

To comprehensively analyze the biological characteristics of the bovine STAR gene, this study conducted homology comparisons, physicochemical property analyses, and protein structure predictions based on the bovine



**Fig. 5.** Double enzyme digestion of bovine STAR promoter deletion plasmid. **AA:** Double enzyme digestion of bovine STAR promoter deletion plasmid; The four lanes from 1 to 4 in the figure are psicheck-2-STAR-1 (-472/-1), psicheck-2-STAR-2 (-1003/-1), psicheck-2-STAR-3 (-1499/-1), and psicheck-2-STAR-4 (-1990/-1) was digested by BglII and NheI **B:** Changes in luciferase activity after transfection of bovine STAR gene promoter truncation into cells. \*  $p < 0.05$ , \*\*  $p < 0.01$ . Statistical analyses were performed using one-way ANOVA (analysis of variance) with Duncan's test. The data shown are expressed as the mean  $\pm$  SEM of three biological replicates.

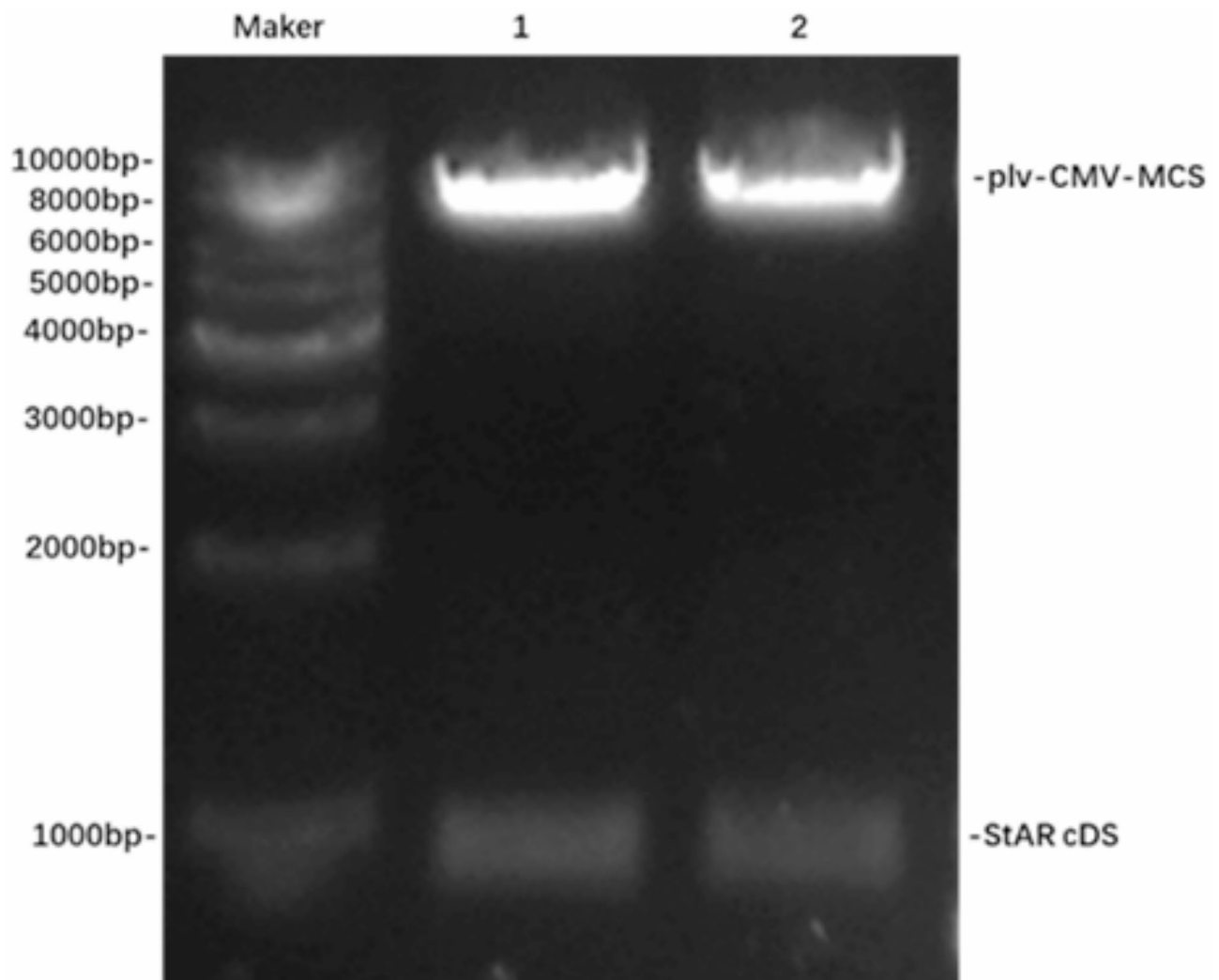
STAR amino acid sequence. The results showed that the STAR in cattle shares the closest genetic relationship with that of sheep and the most distant relationship with rats and mice, in accordance with evolutionary patterns. Moreover, as a lipid-soluble non-secretory protein, there exists a complex interaction network among STAR, key enzymes for progesterone synthesis, and cholesterol transport proteins, further indicating that STAR plays an important role in cholesterol transport and the catalysis of progesterone synthesis<sup>19</sup>.

By constructing the plv-CMV-STAR overexpression vector and successfully transfecting it into bovine corpus luteum cells, this study not only verified the effectiveness of the vector but also observed a significant increase in the expression levels of key enzymes such as CYP11A1 and 3 $\beta$ HSD for steroid synthesis, directly proving the crucial role of STAR in the synthesis of progesterone in corpus luteum cells<sup>20</sup>. Cholesterol, the precursor for progesterone synthesis, is primarily sourced via endocytosis or selective uptake. Cholesterol within cytosolic lipid droplets migrates along the cytoskeleton to reach the mitochondria. The rate-limiting step in steroidogenesis is the transport of cholesterol from the outer mitochondrial membrane to the inner mitochondrial membrane, a process executed by the steroidogenic acute regulatory protein, STAR<sup>21,22</sup>. STAR acts as a transporter protein that facilitates the transfer of cholesterol into the mitochondria<sup>4</sup>. Progesterone synthesis involves the conversion of cholesterol to pregnenolone by CYP11A1 located on the inner mitochondrial membrane, and subsequently, the conversion of pregnenolone to P<sub>4</sub> by 3 $\beta$ HSD in the smooth endoplasmic reticulum. In this experiment, we observed that overexpression of STAR in luteal cells led to a corresponding increase in the expression levels of both 3 $\beta$ HSD and CYP11A1. This may be due to the increased availability of cholesterol, the raw material for progesterone synthesis, which in turn stimulates the activity of the key enzymes 3 $\beta$ HSD and CYP11A1 involved in progesterone synthesis, enabling more cholesterol to be utilized and converted into progesterone.

To investigate the transcriptional activity of the STAR gene promoter and its regulatory mechanism, this experiment adopted the method of constructing promoter deletion fragments<sup>23,24</sup> to clone and analyze the 5' flanking region sequence of the bovine STAR gene. The results showed that there are multiple transcription initiation sites and CpG regions (cytosine - C, phosphate - P, guanine - G) in this region. Although there is no typical CpG island, the high GC content suggests that it may be involved in the binding of transcriptional regulatory factors and the initiation of gene expression<sup>25</sup>. Using the dual luciferase reporter system, this study further confirmed the active region of the STAR gene promoter, providing strong support for in-depth research on the subsequent transcriptional regulatory mechanism.

Transcription factor prediction revealed the presence of binding sites for transcription factors NF- $\kappa$ B, Sp1, NF-1, and LyF-1 in the STAR promoter region. These transcription factors play key roles in gene expression regulation. For example, NF- $\kappa$ B not only participates in the regulation of multiple genes but also affects cholesterol metabolism and steroid synthesis through complex signaling pathways<sup>26-30</sup>; Sp1, with its high affinity binding characteristic to GC-rich motifs, widely regulates the expression of mammalian genes<sup>31,32</sup>. The next step of the research will focus on constructing eukaryotic expression vectors of these transcription factors and further revealing their regulatory effects on STAR gene transcription through co-transfection experiments with the STAR promoter.

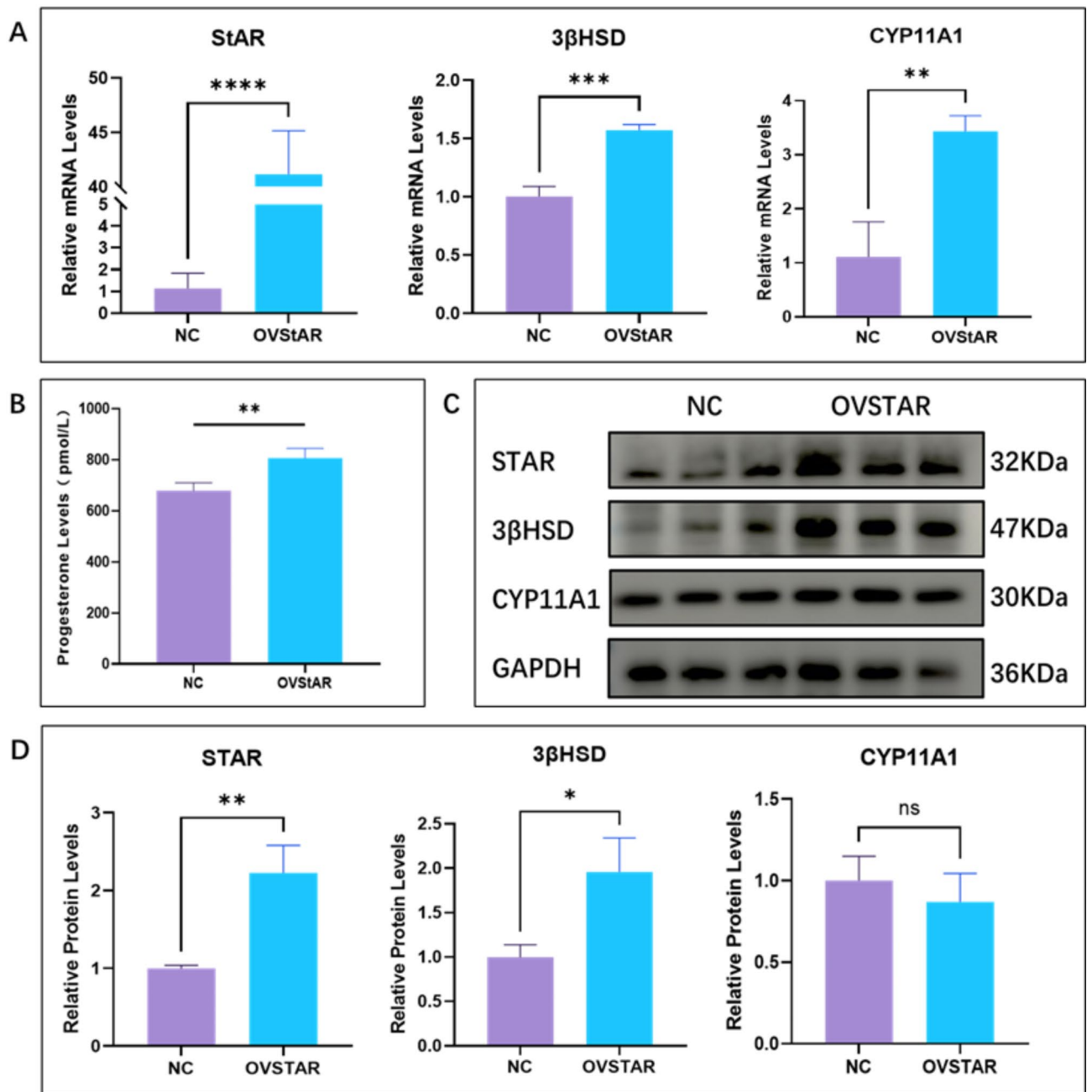




**Fig. 6.** Double enzyme digestion of bovine STAR overexpression plasmid. Lane 1 and lane 2 in the figure are the products of plv-CMV-STAR digested by EcoR I and NoT I.

## Conclusions

In this study, we successfully cloned the bovine STAR gene and constructed an efficient overexpression vector, which realized the overexpression of the STAR gene in bovine luteal cells and significantly enhanced the expression level of the key enzyme for progesterone synthesis. Core promoter analysis of the promoter region of the STAR gene was performed using the dual luciferase reporter gene system, and the core promoter region was identified as being located in the  $-1990/-1$  region, which is consistent with the online prediction results. In addition, several key transcription factor binding sites, including NF- $\kappa$ B, Sp1, NF-1 and LyF-1, were predicted and identified, laying a solid foundation for the subsequent in-depth study of the transcriptional regulatory mechanism of STAR gene. The results of this study demonstrate that overexpression of STAR gene significantly promotes progesterone synthesis in luteal cells, which is of great significance for progesterone secretion by the bovine corpus luteum and the maintenance of pregnancy. The study on the promoter activity and transcriptional regulatory mechanism of the bovine STAR gene not only contributes to a deeper understanding of the biological function of this gene but also provides new insights and approaches for improving the reproductive performance of bovine through genetic regulation.



**Fig. 7.** Effect of overexpression of STAR on progesterone synthesis in bovine somatic cells. **A:** Changes in expression levels of progesterone related genes after overexpression of STAR; **B:** Changes in luciferase activity after transfection of bovine STAR gene promoter truncation into cells; **C–D:** Changes in expression levels of progesterone related proteins after overexpression of STAR. \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ . Statistical analyses were performed using one-way ANOVA (analysis of variance) with Duncan's test. The data shown are expressed as the mean  $\pm$  SEM of three biological replicates.

### Data availability

The datasets generated and/or analyzed during the current study are not publicly available due research design but are available from the corresponding author on reasonable request. Data is provided within the manuscript or supplementary information files. The STAR gene and protein sequences are from NCBI and Genebank. The STAR gene accession number is NM\_174189.3, and the STAR protein accession number is NP\_776614.2.

Received: 1 November 2024; Accepted: 27 February 2025

Published online: 05 March 2025

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## Acknowledgements

This work was supported by grants from the National Natural Science Foundation of China (Grant number: 32172811, 32372969), PR-China.

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## Declarations

### Competing interests

The authors declare no competing interests.

### Ethical statement

The bovine luteal cells used in this study were derived from ovarian tissues of cows slaughtered at commercial abattoirs (non-experimental euthanasia).

### Additional information

**Supplementary Information** The online version contains supplementary material available at <https://doi.org/10.1038/s41598-025-92446-2>.

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