



# Overexpression of goat *STEAP4* promotes the differentiation of subcutaneous adipocytes

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**Abstract. Objective:** The focus of this study was the six-transmembrane epithelial antigen of the prostate 4 (*STEAP4*) gene, on the basis of the cloned goat *STEAP4* gene sequence. Its molecular and expression characteristics were analyzed, and its influence on the differentiation of goat subcutaneous adipocytes was explored through overexpression. **Method:** Reverse-transcription PCR (RT-PCR) was used to clone the goat *STEAP4* sequence, and online tools were used to analyze the molecular characteristic. Real-time quantitative PCR (qPCR) was used to detect the expression level of *STEAP4* in goat tissues and subcutaneous adipocyte differentiation. Liposome transfection, BODIPY, Oil Red O staining, and qPCR were used to explore the effect of overexpression of *STEAP4* on adipocyte differentiation. **Results:** The cloned goat *STEAP4* gene sequence was 1388 bp, and the complete coding sequence (CDS) region was 1197 bp, which encoded a total of 398 amino acids. Compared with the predicted sequence (XM\_005679300.3), there were three base mutations in the CDS region of goat *STEAP4*, A188G, T281C, and A507G. Among them, A507G changed the amino acid at position 170 from Ile to Val. Analysis of the physical and chemical properties of the protein showed that *STEAP4* was a stable hydrophilic basic protein. *STEAP4* gene expression level was highest in goat liver tissue ( $P < 0.01$ ), followed by lung and back subcutaneous adipose tissue. *STEAP4* showed different expression levels in goat subcutaneous adipocytes at different times during the induction of differentiation. The expression in the late stage of differentiation was higher than that before differentiation and lowest at 12 h ( $P < 0.01$ ). Overexpression of *STEAP4* promoted the accumulation of intracellular lipid droplets; *C/EBPβ* (CCAAT enhancer binding protein) was extremely significantly up-regulated ( $P < 0.01$ ), and *aP2* (fatty acid binding protein) was significantly up-regulated ( $P < 0.05$ ). **Conclusion:** Overexpression of *STEAP4* could promote the differentiation of goat subcutaneous preadipocytes. This study lays the foundation for an in-depth study of the role of *STEAP4* in goat lipid deposition.

## 1 Instruction

Goats can provide humans with important living resources such as cashmere, milk, and chevon and are an important breed of livestock. The Jianzhou Da'er goat is the second independently selected breed of goat for meat in China, which has the advantages of resistance to rough feeding and strong adaptability. The taste of chevon is susceptible to flavor substances, which are mainly deposited in adipose tissue, and fat

traits are important economic traits of livestock (Kong et al., 2021). Therefore, analyzing the mechanism of goat fat tissue deposition is helpful for breeders to regulate lipid accumulation at the molecular level, thereby improving meat quality. Lipid deposition mainly depends on the clonal proliferation and expansion of adipocytes, and a variety of genes and transcription factors participate in these processes (Lefterova and Lazar, 2009).

*STEAP* (*STEAP1*) was first discovered in 1999, and because of its high expression in early prostate cancer, it was named the prostate six-transmembrane epithelial antigen (Huber et al., 1999). It has a highly conserved six-span membrane structural domain. Later, the genes of *STEAP2*, *STEAP3*, and *STEAP4* (six-transmembrane epithelial antigen of the prostate 4), which also have a conserved six-pass transmembrane structure, were classified into the *STEAP* family. Ohgami et al., (2005, 2006) did a systematic study on the sequence, subcellular localization, tissue expression, and metal reductase activity of the human *STEAP* family. All four members of this family have six transmembrane domains and carboxy-terminal sequences, which mediate the cell reduction of  $\text{Fe}^{3+}$  and promote the absorption of  $\text{Fe}^{3+}$  and  $\text{Cu}^{2+}$  (Oosterheert et al., 2018). *STEAP* family genes are widely present in eukaryotes and participate in their life activities, such as cell proliferation, differentiation, apoptosis, and other physiological processes (Qin et al., 2010; Kim et al., 2016; Valenti et al., 2010). *STEAP4* (six-transmembrane epithelial antigen of the prostate) is the newest member of the prostate six-transmembrane epithelial antigen family (Moldes et al., 2001). It has the strongest metal reductase activity in the *STEAP* family and plays an important role in cancer cell proliferation and metastasis (Tanaka et al., 2012; Korkmaz et al., 2005). For example, *STEAP4*, as a target of *miR-1265*, was related to focal adhesion kinase (FAK) phosphorylation and epithelial–mesenchymal transition (EMT). It was involved in the metastasis of bladder cancer (Yan et al., 2019). The *STEAP4* gene was located on the long arm of human chromosome 7 (Moldes et al., 2001), which was known to be a susceptible area for type 2 diabetes and insulin resistance, and obesity was closely related to the occurrence of these diseases. Studies have found that *STEAP4* was highly expressed in the subcutaneous and visceral adipose tissue of obese patients (Ozmen et al., 2016). Functional studies have shown that the *STEAP4* gene is highly expressed in human preadipocytes, gradually down-regulated with adipocyte differentiation and maturation. It can promote adipocyte differentiation, maturation, and lipid accumulation, which may be related to the occurrence of obesity (Chen et al., 2010). In addition, studies have found that mutations in fat content were related to obesity-related genes *FTO*, melanocortin receptor-4 (*MCR4*), histamine, leptin receptor (*LEPR*), and *STEAP4* (Millon-Frémillon et al., 2008). But the effect of *STEAP4* on goat adipocyte differentiation and lipid deposition is unclear.

Therefore, this study took the Jianzhou Da'er goat as the research object, cloned the *STEAP4* gene in goat sequence by reverse-transcription PCR (RT-PCR), and analyzed its physical and chemical properties. The expression of *STEAP4* was detected by qPCR in multiple goat tissues and adipocytes at different stages of differentiation. Further, the constructed pcDNA-3.1-*STEAP4* vector was transfected into subcutaneous adipocytes for overexpression. On a morphological and molecular level, the effect on the expression of subcutaneous adipocytes was obvious. This study will provide a

basis for elucidating the biological function of the *STEAP4* gene in goat and provide a reference for goat molecular breeding.

## 2 Materials and methods

### 2.1 Sample collection

The tissue samples cryopreserved in liquid nitrogen were taken from a 1-year-old Jianzhou Da'er male goat (Sichuan Jianyang Dageda Animal Husbandry Co., Ltd, Sichuan, China) (heart, liver, spleen, lung, kidney, longissimus dorsi, back subcutaneous adipose tissue, and abdominal subcutaneous adipose tissue,  $n = 3$ ) (He et al., 2021), and TRIzol reagent (TaKaRa, Tokyo, Japan) was used to extract the total RNA from the tissue. The concentration and quality of the RNA sample were detected by the Implen NanoPhotometer® N60, 1  $\mu\text{g}$  of qualified RNA was taken, and the reverse-transcription kit (Thermo Fisher Scientific, Waltham, USA) was used to reverse-transcribe it into cDNA and store it at  $-20^\circ$  for later use.

### 2.2 Cell culture

The primary subcutaneous adipocytes from longissimus dorsi were isolated and cryopreserved as in previous work (He et al., 2021). Briefly, the subcutaneous adipose tissue was isolated from 7 d old Jianzhou da'er goats ( $n = 3$ ) and digested with collagenase type I (Sigma, St. Louis, MO, USA) at  $37^\circ\text{C}$  for 60 min. The goat primary subcutaneous adipocytes were resuscitated, cultured in a  $37^\circ$  incubator with 5%  $\text{CO}_2$ , and were passed when the confluence reached 90%. When the  $\text{F}_3$  generation cell confluence reached 80%, a complete medium containing  $50\mu\text{mol L}^{-1}$  oleic acid (Sigma, St. Louis, MO, USA) was added to induce differentiation, and they were induced and collected separately at 0, 24, 48, 72, 96, and 120 h. RNA was extracted and reverse-transcribed to obtain cell temporal cDNA.

### 2.3 Cloning and analysis of the goat *STEAP4* gene

According to the predicted sequence of the goat *STEAP4* gene on NCBI (XM\_00567900.3), primers were designed using Primer Premier 5.0 software (Table 1) and were synthesized by Sangon Biotech (Shanghai, China). The PCR reaction system included 12.5  $\mu\text{L}$  Primer STAR Max DNA Polymerase (TaKaRa, Tokyo, Japan), 1.0  $\mu\text{L}$  ( $10\mu\text{mol L}^{-1}$ ) sense and antisense primers, 1.0  $\mu\text{L}$  goat liver tissue cDNA, and 9.5  $\mu\text{L}$  ddH<sub>2</sub>O. The PCR amplification program was as follows:  $98^\circ\text{C}$  for 3 min,  $94^\circ\text{C}$  for 30 s,  $58^\circ\text{C}$  for 15 s, and  $72^\circ\text{C}$  for 30 s and 38 cycles. The PCR products were purified and recovered by electrophoresis identification, and the recovered product was ligated with PMD-19T (TaKaRa, Tokyo, Japan) and transformed into DH5 $\alpha$  (Tiangen, Beijing, China). The positive colonies were screened by an AMP-

resistant solid medium, and the gene sequence was obtained by Sangon Biotech. The molecular characteristics of the obtained gene sequence are analyzed, and the analysis items and tools are shown in Table 2.

## 2.4 Construction of goat pcDNA-3.1-*STEAP4* vector and cell transfection

*Kpn* I and *Not* I restriction sites were selected to construct the goat *STEAP4* expression vector by double-restriction enzyme digestion. Subcloning primers were designed according to the cloned *STEAP4* sequence, S: 5'CGGGGTACCATGGAGAAAATTTCTACAGATGC3' and A: 5'TATTCTTAGCGGCCGCTCAGGATCCTTTCATG3'. The pcDNA-3.1 plasmid and the subcloned product were each digested with double enzymes, and the fragments were purified. The fragments were ligated with T4 ligase (TaKaRa, Tokyo, Japan) overnight at 16 °C. After the enzyme digestion was verified, they were transformed into DH5 $\alpha$  to screen positive colonies for sequencing. The bacteria solution with correct sequencing was amplified, and then the plasmid was extracted. F<sub>3</sub> generation cells were seeded into a 12-well plate with 4  $\times$  10<sup>4</sup> cells per well. The master mix was configured according to the transfection reagent instructions and incubated for 15 min at room temperature. The cells were cultured with oleic acid induction solution after 14 h of transfection. The total RNA was collected after 48 h to obtain cDNA by reverse transcription, and the negative control group (NC) was transfected with empty pcDNA-3.1. Three biological replicates in each group.

## 2.5 BODIPY and Oil Red O staining

Cells used for staining were cultured in 24-well plates. The transfection system corresponds to 12-well plates halved. The culture medium was discarded after 48 h of induction, washed three times with phosphate-buffered saline (PBS), and fixed with 10 % neutral formaldehyde at room temperature for 30 min. It was then washed with PBS three times, 200  $\mu$ L BODIPY working solution (BODIPY stock solution: PBS = 1 : 1000) (Thermo Fisher Scientific, Waltham, USA) was added to each well under dark conditions, and the lipid droplets were observed after 30 min. For Oil Red O staining, Oil Red O working solution (Oil Red O storage solution: ddH<sub>2</sub>O = 3 : 2) (Solarbio, Beijing, China) was added, and the red lipid droplets were observed after dyeing for 30 min. ImageJ was used to quantify the staining results.

## 2.6 Quantitative polymerase chain reaction (qPCR) detection

According to the cloned goat *STEAP4* sequence, specific qPCR primers were designed (Table 1) to detect its expression. qPCR was used to detect the expression of *STEAP4* and to detect the expression of adipogenic differentiation marker

genes after overexpression of *STEAP4*. The primer information is shown in Table 1. With *UXT* as the internal reference gene, the qPCR system included 2  $\times$  10  $\mu$ L SYBR<sup>®</sup> Premix Ex Taq<sup>™</sup> (TaKaRa, Tokyo, Japan), 1  $\mu$ L each of the sense and antisense primers, 1  $\mu$ L cDNA, and ddH<sub>2</sub>O filled to 20  $\mu$ L. The qPCR procedure was as follows: 95° predenaturation for 3 min, 95° denaturation for 10 s, 60° annealing for 10 s, and 72° extension for 15 s for 40 cycles.

## 2.7 Data analysis

The results of qPCR data were processed by the 2<sup>- $\Delta\Delta$ Ct</sup> method, expressed as mean  $\pm$  standard deviation. SPSS 18.0 one-way analysis of variance (ANOVA) was used to test the significance. The *T* test was used in multiple comparisons. *P* < 0.05 indicates a significant difference, and *P* < 0.01 indicates a extremely significant difference.

# 3 Results

## 3.1 Cloning and sequence analysis of goat *STEAP4*

PCR amplification obtained fragments consistent with the expected target product size (Fig. 1a). The length of the goat *STEAP4* gene was 1388 bp, including the complete coding sequence (CDS) region of 1197 bp, which was consistent with the predicted sequence (XM\_005679300.3). In comparison, there were three base mutations in the CDS region, namely A188G, T281C, and A507G (Fig. 1b). A188G and T281C did not affect protein translation, while A507G changed the amino acid at position 170 from Ile to Val. The *STEAP4* gene in goats encodes a total of 398 amino acids, including 48 leucine (L), at the highest proportion (12.1 %), followed by 32 proline (V) (8.0 %) (Fig. 1c).

## 3.2 Analysis of goat *STEAP4* protein characteristics

ExPASy was used to analyze the physical and chemical properties of the goat *STEAP4* protein. The results showed that the molecular weight of goat *STEAP4* protein was 45.327 kD. The total average theoretical isoelectric point, instability index, and hydrophilicity were 9.08, 36.55, and 0.130, respectively, indicating that the goat *STEAP4* protein is a stable, hydrophilic basic protein. Since there were more positively charged amino acids than negatively charged amino acids, the goat *STEAP4* protein was positively charged. There were 33 potential phosphorylation sites for goat *STEAP4* protein, including 17 serine (Ser) phosphorylation sites, 11 threonine (Thr) phosphorylation sites, and 4 tyrosine (Tyr) phosphorylation sites (Fig. 2a). The NetNGlyc tool was used to predict that *STEAP4* had two N-glycosylation sites, located at positions 158 and 323, without O glycosylation sites and no signal peptide.

SOPMA was used to predict the secondary structure of *STEAP4*, and this showed that it contains 42.71 %  $\alpha$  helix,

**Table 1.** Primer information for PCR and qPCR.

Gene name	Sequence	Purpose	Product length (bp)	$T_m$ (°)	Accession
<i>STEAP4</i>	S: GCACCTCTCCCGCAGTTAT A: GCTTAGTCACCCTCTCATGG	Clone	1388	58	XM_005679300.3
<i>STEAP4</i>	S: CACTGCTCGCCTTGTTTA A: TTGCCTGGGTAGCGTTCT	qPCR	231	60	
<i>Pref-1</i>	CCTGAAAATGGATTCTGCGACG GACACAGGAGCACTCGTACTG	qPCR	255	60	KP686197.1
<i>PPAR<math>\gamma</math></i>	S: AAGCGTCAGGGTCCACTATG A: GAACCTGATGGCGTTATGAGAC	qPCR	197	60	NM_001285658.1
<i>C/EBP<math>\alpha</math></i>	S: CCGTGGACAAGAAGCAAGC A: AGGCGGTCATTGTCCTGGT	qPCR	142	58	XM_018062278.1
<i>C/EBP<math>\beta</math></i>	CAAGAAGACGGTGGACAAGC AACAAGTTCGCGAGGGTG	qPCR	204	66	XM_018058020.1
<i>LPL</i>	S: TCCTGGAGTGACGGAATCTGT A: GACAGCCAGTCCACCACGAT	qPCR	174	60	NM_001285607.1
<i>FASN</i>	S:TGTGCAACTGTGCCCTAG A:GTCCTCTGAGCAGCGTGT	qPCR	111	57	NM_001285629.1
<i>aP2</i>	S:TGAAGTCACTCCAGATGACAGG A:TGACACATTCCAGCACCAGC	qPCR	143	58	NM_001285623.1
<i>UXT</i>	S: GCAAGTGGATTTGGGCTGTAAC A: ATGGAGTCCTTGGTGAGGTTGT	qPCR	180	60	XM_005700842.2

S – sense primer. A – antisense primer.

**Table 2.** Sequence analysis content and corresponding analysis tools.

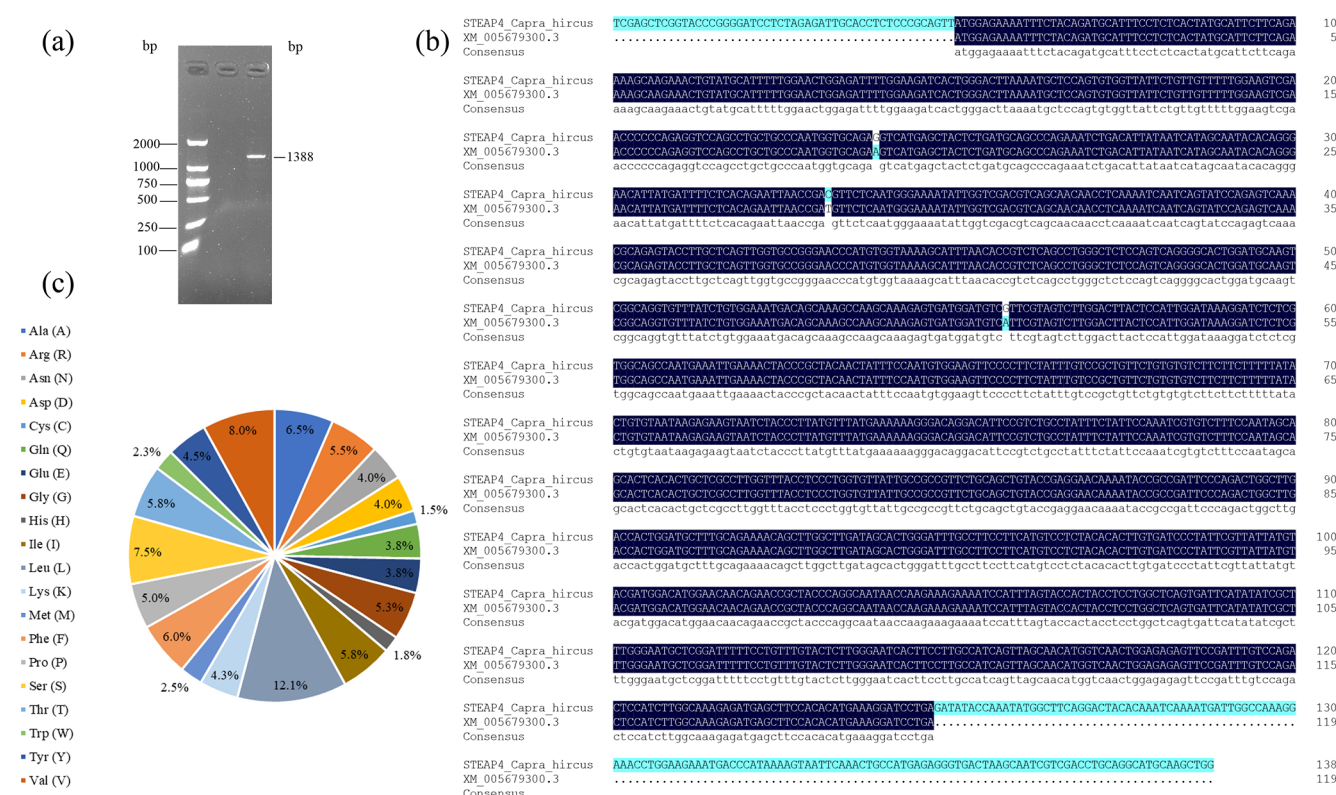
Analysis content	Software tools used
Looking for open reading frames (ORFs)	ORFfinder
Align with the predicted sequence of goat and other species	BLAST
Construction of phylogenetic tree	MAGA 7.0
Protein physical and chemical property analysis	ProtParam
Prediction of signal peptide site	Signalp-5.0 server
Prediction of transmembrane domain	TMHMM 2.0
Analysis of SMART domain	SMART
Subcellular location analysis	PSORT II server and Targetp 1.1 server
Prediction of phosphorylation and glycosylation site	NetPhos, NetOGlyc, NetNGlyc
Protein topology prediction	Protter
Protein secondary-structure prediction	SOPMA
Protein tertiary-structure prediction	SWISS-MODEL
Interacting protein prediction	STRING

34.17 % random coil, 4.02 %  $\beta$  turn, and 19.10 % stretched chain (Fig. 2b). SWISS-MODEL was used to predict the tertiary structure shown in Fig. 2c, and this was consistent with the results of the secondary-structure prediction.

Using WoLF PSORT to predict subcellular localization showed that *STEAP4* was mainly distributed in the endoplasmic reticulum membrane, with a small amount in the cytoplasm. Then, Protter was used to predict the transmem-

brane structure of *STEAP4*, showing that *STEAP4* crosses the membrane four times through the  $\alpha$  helix. And there were two N-glycosylation sites (Fig. 2d), which was consistent with NetNGlyc's prediction results. SMART domain analysis and TMHMM transmembrane structure prediction both showed that goat *STEAP4* contains four-transmembrane structures (Fig. 2e, f).





**Figure 1.** Sequence analysis of the goat *STEAP4* gene. **(a)** Goat *STEAP4* gene amplification results. **(b)** *STEAP4* gene sequence alignment with predicted sequence. **(c)** Amino acid composition of *STEAP4* protein.

Predicting interacting proteins through the STRING database showed that goat *STEAP4* had strong interactions with proteins such as MECR, TNIN2, and CDKL2 (Fig. 2g).

### 3.3 Conservative analysis of goat *STEAP4*

BLAST was used to compare the amino acid similarity of goat *STEAP4* with other species. The results showed that the sequence identity of goat *STEAP4* and sheep reached 99.48 %, followed by deer and cattle (Fig. 3a). Based on the amino acid sequence of *STEAP4*, MEGA7.0 was used to construct the phylogenetic tree. The tree showed that goat and sheep were closely related, and cattle and deer were relatively closely related (Fig. 3b).

### 3.4 Analysis of the goat *STEAP4* expression pattern

#### 3.4.1 Tissue distribution of goat *STEAP4*

The expression level of *STEAP4* in different tissues of goats was detected by qPCR. The expression profile showed that *STEAP4* expression was significantly higher in goat liver tissue than other tissues ( $P < 0.01$ ), followed by lungs, also higher in back subcutaneous fat tissues and abdominal subcutaneous fat tissues (Fig. 4).

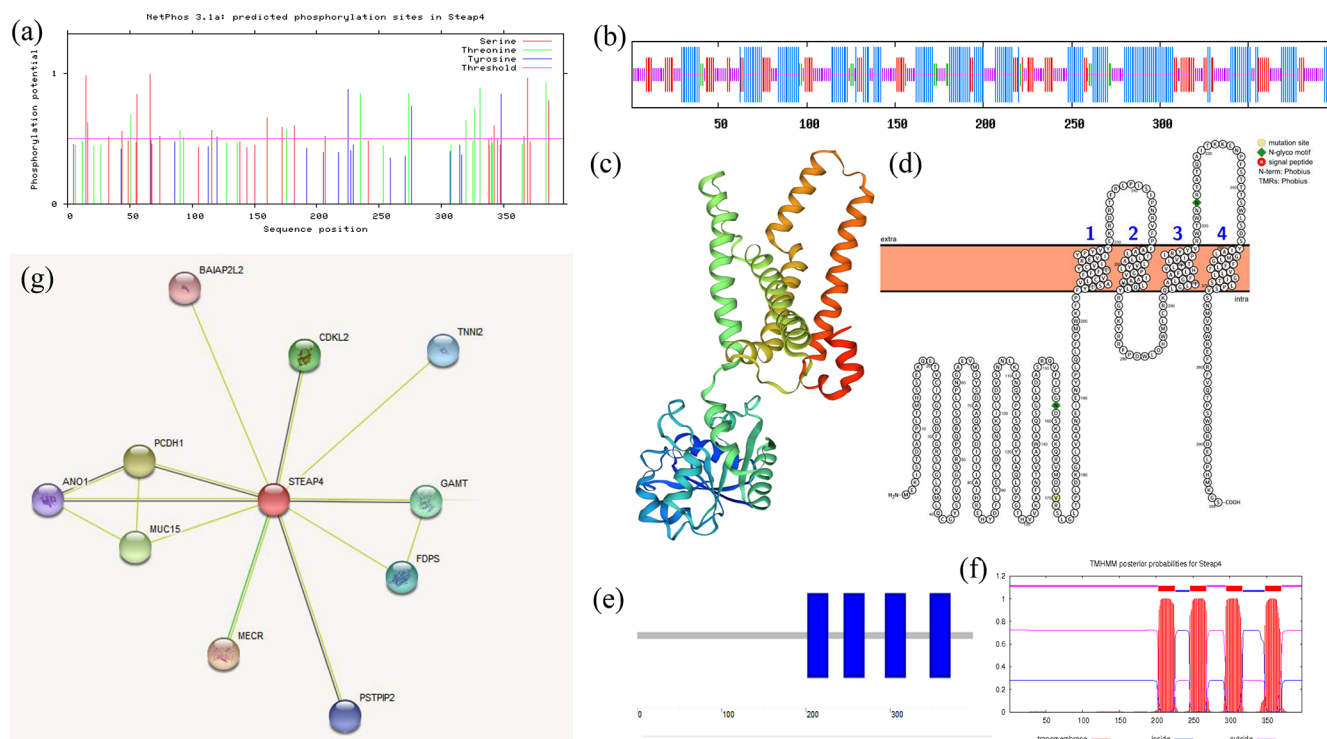
Different capital letters in the figure indicate that the difference is extremely significant ( $P < 0.01$ ), and different lowercase letters indicate significant difference ( $P < 0.05$ ), the same as below.

#### 3.4.2 Cell temporal expression profile of goat *STEAP4*

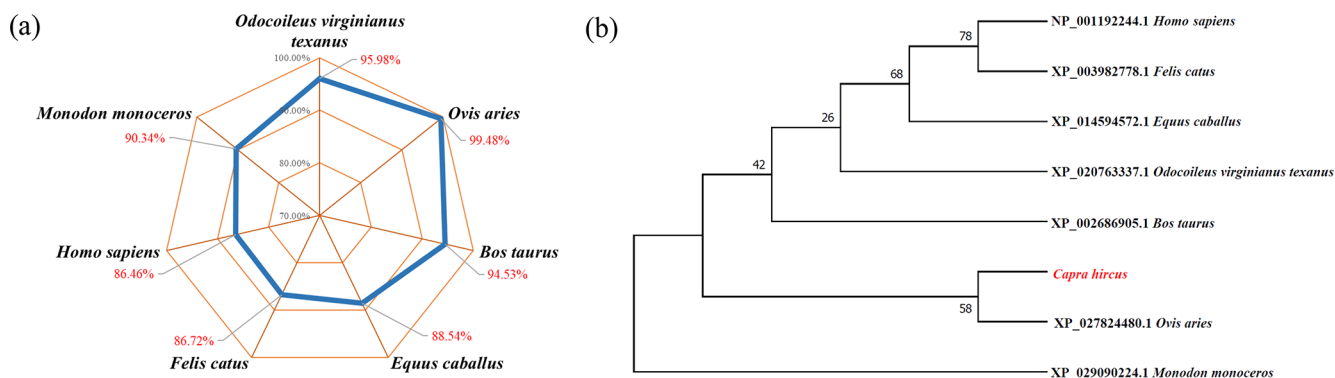
The expression level of *STEAP4* was expressed at different levels at different times of subcutaneous adipocyte differentiation in goats. The expression of *STEAP4* showed a decreasing and then an increasing trend, reaching the lowest level at 24 h after differentiation (Fig. 5).

### 3.5 Overexpression of goat *STEAP4* promotes the differentiation of subcutaneous preadipocytes

The successfully constructed pcDNA-3.1-*STEAP4* plasmid (Fig. 6a) was transferred into goat subcutaneous preadipocytes. *STEAP4* expression was detected to be up-regulated by about 8900 times (Fig. 6b), indicating the successful establishment of *STEAP4* cell overexpression model. Using BODIPY and Oil Red O staining methods to observe the formation of intracellular lipid droplets, it was found that overexpression of *STEAP4* promoted lipid droplet accumulation in subcutaneous adipocytes (Fig. 6c, d, e). Detection of the expression of differentiation marker genes showed that



**Figure 2.** Analysis of goat *STEAP4* protein characteristics. (a) *STEAP4* protein phosphorylation site prediction. (b) Secondary-structure prediction. (c) Tertiary-structure prediction. (d, e, f) *STEAP4* protein transmembrane structure prediction. (g) Interaction protein prediction of goat *STEAP4*.

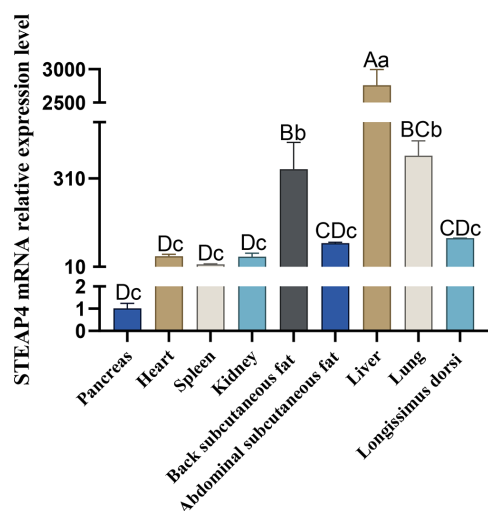


**Figure 3.** Analysis of amino acid sequence similarity (a) and phylogenetic tree (b) of goat *STEAP4*.

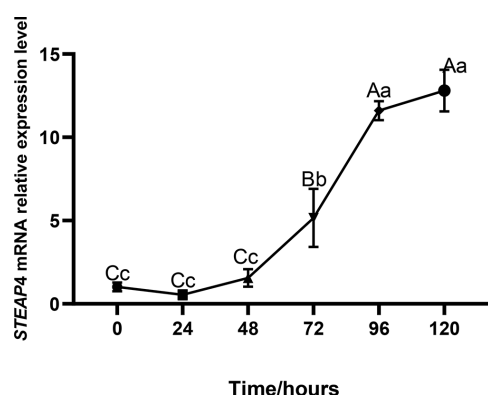
*C/EBP $\beta$*  (CCAAT enhancer binding protein) was extremely significantly up-regulated ( $P < 0.01$ ), *aP2* was significantly up-regulated ( $P < 0.05$ ), *PPAR $\gamma$*  (peroxisome proliferator-activated receptor) and *Pref-1* (the preadipocyte factor) were significantly down-regulated ( $P < 0.05$ ), and *LPL* (lipoprotein lipase) was extremely significantly down-regulated ( $P < 0.01$ ).

## 4 Discussion

*STEAPs* can play an important role in growth and development and the occurrence of various diseases by regulating their target genes (Qin et al., 2010). In this study, we cloned the goat *STEAP4* sequence of 1388 bp, including the complete CDS of 1197 bp. The goat *STEAP4* gene collectively encoded 398 amino acids, while human *STEAP4* contains 459 amino acids, sheep *STEAP4* contains 384 amino acids, cattle contains 470 amino acids, and pig *STEAP4* also contains 470 amino acids (Wang et al., 2013), indicating that the length and composition of the amino acid sequence of



**Figure 4.** Distribution of *STEAP4* in different tissues of goats.



**Figure 5.** Cell temporal expression profile of *STEAP4* in goat subcutaneous adipocytes.

*STEAP4* differ among different species. This is compared with the predicted sequence in NCBI (XM\_005679300.3). There were three base mutations in the CDS region of the goat *STEAP4* gene, which are A188G, T281C, and A507G. A507G changed the amino acid at position 170 from Ile to Val. Whether it is unique to the Jianzhou Da'er goat and whether the mutation causes changes in protein function still needs further research.

This study used online tools to predict that goat *STEAP4* was an stable hydrophilic basic protein with a molecular mass of 45.327 kD and a theoretical isoelectric point (pI) of 9.08. The subcellular location showed that *STEAP4* was mainly located in the plasma membrane, which was consistent with a number of studies (Zhang et al., 2008; Wellen et al., 2007; Xue et al., 2017) and conforms to the membrane location characteristics of the *STEAP* family. Different tools were used to predict that goat *STEAP4* contains four- transmembrane domains, which was different from the six-transmembrane structure conserved in the *STEAP* family.

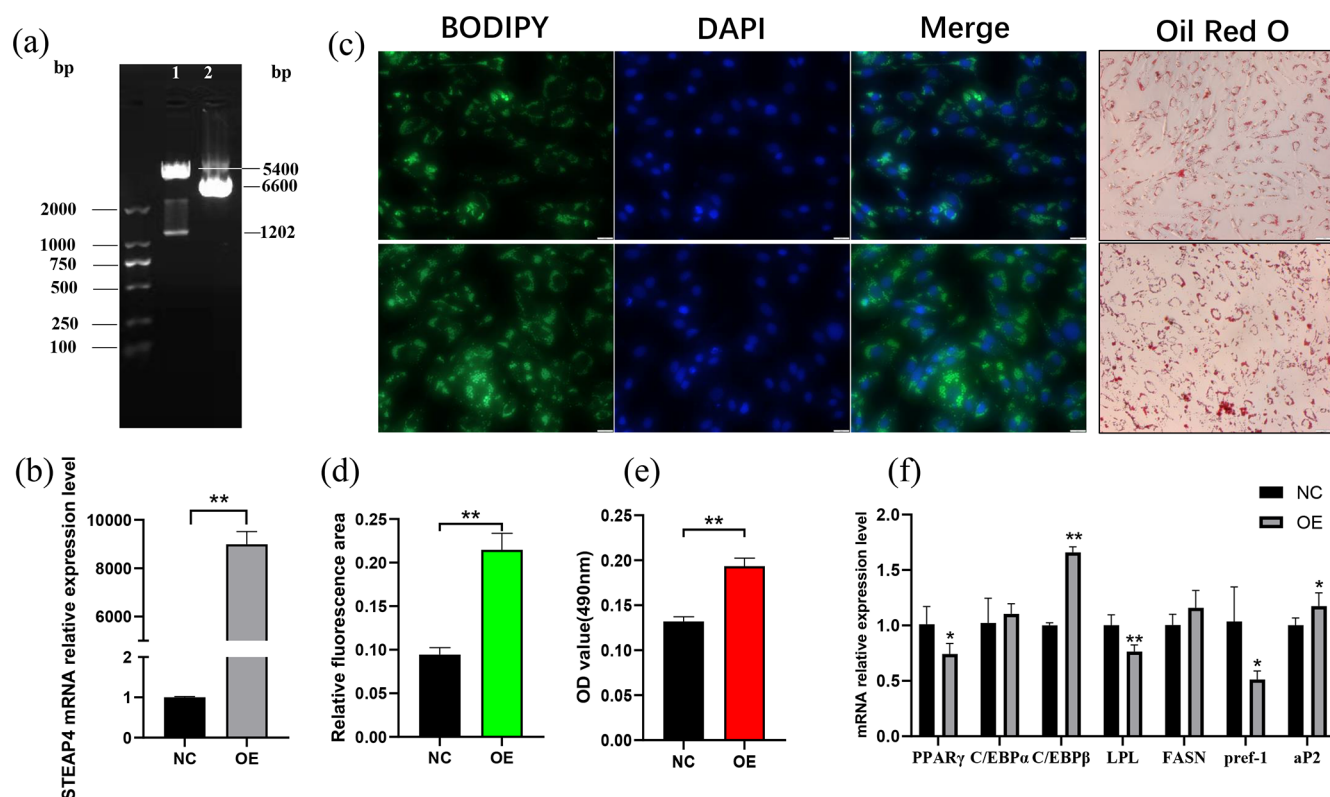
The predecessor obtained a variant of *STEAP4*, *STEAP4v*, when cloning the pig *STEAP4* gene, which contained only four-transmembrane structure regions (Wang et al., 2013). The results of this study were similar to it. Therefore, we speculated that there may be cleavage of goat *STEAP4* at the post-translational level, which leads to truncation of goat *STEAP4*. But what kind of functional changes may cause this cleavage still needs further study.

The goat *STEAP4* gene was expressed in pancreas, heart, spleen, kidney, back subcutaneous fat tissue, abdominal subcutaneous fat tissue, liver, lung, and longissimus dorsi. But the relative expression level was the highest in the liver, followed by the lung. There were also higher expressions in the subcutaneous fat tissues of different parts. Moldes et al. (2001) reported that *STEAP4* was expressed in large amounts in rat white fat, brown fat, heart, liver, and muscle. Human *STEAP4* was highly expressed in bone marrow, placenta, fetal liver, and adipose tissue (Korkmaz et al., 2005). Pig *STEAP4* was highly expressed in adipose tissue, kidney, cerebellum, heart, and lung, while *STEAP4v* was highly expressed in lung and adipose tissue (Wang et al., 2013). Combined, the results of this experiment show that *STEAP4* was expressed at a high level in adipose tissues of different species, indicating that the tissue distribution of *STEAP4* had a certain consistency, and there were also species differences.

The high expression of *STEAP4* in adipose tissue suggested that it may regulate lipid accumulation in animals. Therefore, this study continues to detect the expression of *STEAP4* in goat subcutaneous adipocytes. The results showed that *STEAP4* was widely expressed during adipocyte differentiation and showed a decreasing and then an increasing trend, which decreased in the early stage of differentiation, and then it showed a trend of continuous increase. In 3T3-L1 preadipocytes, *STEAP4* showed an upward trend with differentiation (Moldes et al., 2001), and the results of this experiment were similar. The expression trend in human adipocytes was opposite to that of 3T3-L1 and goat subcutaneous adipocytes. Human *STEAP4* was highly expressed in preadipocytes, and its expression decreased with the induction of differentiation, being its lowest when adipocytes were mature (Chen et al., 2010). This may be due to differences in species and cell types. The above results suggested that *STEAP4* may have different roles in the differentiation process of adipocytes of different species, and the role of *STEAP4* in the differentiation process of goat subcutaneous adipocytes still needs further study.

Therefore, this study constructed a goat *STEAP4* expression vector. After overexpression, it was observed that the accumulation of intracellular lipid droplets increased, and the mRNA expression of adipogenic differentiation marker genes, CCAAT enhancer binding protein (*C/EBPβ*), and fatty acid binding protein (*aP2*) was extremely and significantly up-regulated. *C/EBPα* and fatty acid synthase (*FASN*) mRNA levels also showed an upward trend. The expression of the preadipocyte factor (*Pref-1*), the peroxisome





**Figure 6.** The effect of overexpression of goat *STEAP4* on the differentiation of subcutaneous preadipocytes. (a) pcDNA-3.1-*STEAP4* restriction digestion diagram. (b) Efficiency of *STEAP4* overexpression. (c) Image of BODIPY and Oil Red O staining. (d) BODIPY fluorescence quantification. (e) Oil Red O staining lipid droplet quantification. (f) Adipocytes differentiation-related gene expression. “\*\*\*” means  $P < 0.01$ , and “\*” means  $P < 0.05$ .

proliferator-activated receptor (*PPARγ*), and lipoprotein lipase (*LPL*) was down-regulated. Adipocyte differentiation was a complex process involving the coordinated action of multiple transcription factors. A number of studies have shown that *C/EBPs* and *PPARs* played a central role in adipocyte differentiation (Morrison and Farmer, 1999; Rosen and Spiegelman, 2000; Shao and Lazar, 1997). There was also a cascade of expression between them. *C/EBPβ* was necessary for the early stage of adipogenesis, and its expression could activate *PPARγ* and *C/EBPα* (Schwarz et al., 1997; Park et al., 2004; Ayala-Summano et al., 2008). In this experiment, *C/EBPβ* was extremely significantly up-regulated after *STEAP4* overexpression, and *C/EBPα* also showed an up-regulation trend, suggesting that the cells were in the process of transitioning from the initial stage of differentiation. *Pref-1* was a marker of preadipocytes, which could inhibit adipocyte differentiation to maintain the phenotype of preadipocytes (Smas and Sul, 1993). *LPL* regulated systemic lipid distribution and can hydrolyze triglycerides (Merkel and Eckel, 2002). *FASN* participated in fatty acid synthesis (Song et al., 2018), and *aP2* was a fatty acid binding protein and the main target gene of *PPARγ* (Boord et al., 2002; Furuhashi and Hotamisligil, 2008). In addition,

*aP2* can also negatively regulate the level of *PPARγ* (Garin-Shkolnik, 2014). So, the down-regulation of *PPARγ* in this experiment may be negatively regulated by the up-regulation of *aP2*. Combined with the experimental results of this study, it was speculated that the fatty acid synthesis increases, and the hydrolysis pathway was blocked after the overexpression of *STEAP4*, which leads to the accumulation of lipid droplets. According to reports by Korkmaz et al. (2005), *C/EBPα* can directly bind to and promote the promoter activity of human and mouse *STEAP4*, while *PPARγ* does not directly act on the promoter of *STEAP4*. But whether goat *STEAP4* was directly regulated by *C/EBPs* or *PPARs* to promote the differentiation of goat subcutaneous adipocytes still needs further study.

## 5 Conclusion

In this study, the goat *STEAP4* gene sequence containing the complete CDS region was cloned. The protein physicochemical analysis showed that it was a basic hydrophilic protein. Goat *STEAP4* has the highest amino acid sequence identity with sheep and has the closest genetic relationship with sheep. After overexpression of *STEAP4* in goat, it was com-



prehensively demonstrated through morphology and molecular biology that overexpression positively regulated the differentiation of preadipocytes.

**Data availability.** The original data are available upon request to the corresponding author.

**Author contributions.** YaqL designed and supervised the study. XL, HZ, JZ, and YW curated and analyzed the data. YanL and YX managed the figures. XL wrote the paper. YaqL and RL reviewed and revised the paper. All authors have read and agreed to the published version of the paper.

**Competing interests.** The contact author has declared that none of the authors has any competing interests.

**Ethical statement.** All experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee, Southwest Minzu University (Chengdu, Sichuan, China).

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