SGK1 Affects the Phosphorylation of FOXO1/FOXO3 Promoting Bovine Fat Deposition via the PI3K/Akt Signaling Pathway

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Abstract

Background: Improving the intramuscular fat content of beef not only could improve our living standard, but decrease the the risk of the disease from the aspect of human nutrition and diet. Serum/glucocorticoid-inducible kinase 1 (SGK1) is a critical kinase involved in regulating multiple metabolic processes.

Results: In the present study we reveal the molecular mechanism by which *SGK*1 affects the phosphorylation of FOXO1 and FOXO3 thus regulating bovine fat deposition via the PI3K/Akt signaling pathway. *SGK*1 was characterized primarily in the bovine kidney, where it peaked at day 6 of preadipocyte differentiation. Overexpression of *SGK*1 in bovine preadipocytes promoted mRNA expression of adipose-specific genes, while cell cycle-related genes were repressed. Adipocyte differentiation-related proteins were not significantly changed, but proliferation-related proteins were markedly inhibited. Moreover, mRNA expression of adipose-specific genes were down-regulated while cell cycle-related genes were up-regulated in response to loss-of-function *SGK*1. The expression of adipose differentiation-related proteins were significantly inhibited but proliferation-related proteins were not significantly changed. Overexpression of *SGK*1 in bovine adipocytes significantly changed the gene expression enriched in the PI3K/Akt signaling pathways through RNA-seq. Subsequently, we found that overexpression of *SGK*1 increased phosphorylation of FOXO1/FOXO3 and reduced protein expression, whereas *SGK*1 deletion had opposite effects. Overall, we show that overexpression of *SGK*1 promotes adipogenesis mainly via inhibiting preadipocyte proliferation, whereas the loss-of-function of *SGK*1 represses adipogenesis mainly through inhibition of adipocyte differentiation. Mechanistically, *SGK*1 changes the phosphorylation of two key genes located downstream in the PI3K/Akt signaling pathway, FOXO1/FOXO3, thus promoting fat deposition in cattle.

Conclusion: These data reveal that *SGK*1 is a positively regulatory during intramuscular adipogenesis of cattle, which expands the candidates regulating bovine adipogenesis and provides the security for human healthy from the aspect of high-quality beef.

Introduction

Intramuscular fat (IMF) content is favored by consumers as since it determines sensory characteristics and palatability [1], and also contains fatty acids and proteins that cannot be synthesized [2]. Adipose tissue helps in energy storage [3], and in maintaining homeostasis [4]. Adipogenesis grows by two mechanisms, hyperplasia and hypertrophy, and is regulated by other tissues via paracrine and endocrine mechanisms. Exploring the mechanism by which adipose tissue develops, which depends on transcription factors, hormones, enzymes and non-coding RNAs [5-8], may improve genetic improvement and beef quality. Meanwhile, revealing more regulatory mechanism of IMF will provide theoretical basis for improving beef quality and healthy diet of human.

SGK1 is one of the most conserved proteins in mammals and a member of the serine/threonine kinase AGC family [9]. SGK1 was first cloned in rat breast cancer cells stimulated by serum and glucocorticoid. Functionally, It is similar to the phosphorylated mammalian target of rapamycin (mTOR)[10]. SGK1 inhibits autophagy in red muscle by acting upstream of ULK1, which initiates autophagosome formation [11, 12]. SGK1 also controls renal tubular transport by inhibiting the degradation of ENaC [13]. The inhibitory role of SGK1 in angiogenesis is regulated by BMP9 and leads to endothelial cell proliferation [14]. SGK1 also plays a key role in regulating adipocyte differentiation and in maintaining glycolipid homeostasis. Both its mRNA and protein levels are high in white adipose tissue, and this has been linked to obesity and type 2 diabetes [15]. Expression of *SGK*1 in subcutaneous and omental adipose tissue is regulated by obesity-associated inflammation, and SGK1 expression is stimulated in response to inflammatory signals [16]. SGK1 is a mediator of glucocorticoids and high-fat feeding, and induced insulin resistance in adipocytes by phosphorylation of FOXO1 in db/db mice [17].

Multiple pathways participate in the regulation of adipogenesis [18], among which the PI3K/Akt signaling pathway plays a pivotal role in maintaining lipid metabolism and regulating insulin [19]. The family of forkhead box O (FOXO) transcription factors has emerged as a central player that is located downstream of the PI3K/AKT signaling and is negatively regulated by phosphorylated AKT [20, 21]. Genetic mutations in FoxO genes, or abnormal expression of FoxO proteins, are associated with metabolic disease, cancer or altered lifespan in humans and animals [22]. FOXO1 is a downstream target of Akt, regulating the cell cycle of adipocytes and lipogenesis through PPARγ [23]. Moreover, FOXO1 and FOXO3 may be targets for in anti-obesity treatments. Indeed, a FOXO1 antagonist increased adipocyte autophagy and inhibited obesity [24], whereas FOXO3 knockdown significantly inhibited autophagy and lipid accumulation, reducing LPS-induced inflammation [25].

After previous RNA-seq data that showed that *SGK*1 is differentially expressed in bovine adipose tissue, herein, we have explored the role of *SGK*1 in bovine adipogenesisin. We demonstrate that *SGK*1 affects the PI3K/Akt signaling pathway and further changes the phosphorylation of downstream genes FOXO1/FOXO3. This contributes to expand the number of candidates in a network regulating bovine adipogenesis and elucidate the role of *SGK*1 in promoting beef IMF.

Results

Establishment of a model of bovine preadipocytes differentiated into mature adipocytes

Primary preadipocytes from bovine subcutaneous adipose tissue were isolated using collagenase Type I digestion. Primary preadipocytes were induced to differentiate into mature adipocytes using insulin when the confluence of seeded preadipocytes reached ~90%-100%. There were many lipid droplets present in adipocytes from day 2 to 6 (Figure 1A). Oil Red O staining results showed that mature adipocytes contained more lipid droplets than primary preadipocytes (Figure 1B). The absorbance of lipid droplets (measured at 480 nm) of mature adipocytes was also significantly higher than in preadipocytes (Figure 1C). Consistent with the above morphological observation, the relative expression level of adipocyte-

specific genes, e.g., *peroxisome proliferative activated receptor gamma* (*PPARγ*), *CCAAT enhancer binding protein alpha* (*C/EBPα*), *Lipoprotein Lipase* (*LPL*) and *fatty acid-binding protein* 4 (*FABP*4), increased with adipocyte differentiation (Figure 1D-G). These results indicate a successful induction of primary preadipocytes into mature adipocytes.

Expression pattern detection of bovine SGK1

Compared to heart, *SGK*1 was more expressed in kidney ($P < 0.01$), followed by liver, lung, spleen and fat tissues, whereas expression was lowest in muscle ($P < 0.05$) (Fig. 2A). The relative expression level of *SGK*1 peaked at day 6 of differentiation during adipocyte differentiation (Fig. 2B). Thus, we successfully obtained the expression pattern of bovine *SGK*1 gene in different tissues and during adipocyte differentiation as well as we speculated *SGK*1 might a hub gene during lipid metabolism.

Overexpression of SGK1 promotes bovine adipogenesis

The 1296 bp-long CDS region of full length bovine *SGK*1 gene was amplified without mutations. The recombinant plasmid was digested with Pac I enzyme, producing two expected fragments (Supplementary Fig. 1A). The digestion products were transfected into taraka cells. Taraka cells grew well 24 h after transfection (Supplementary Fig. 1B-1) and cells expressed green fluorescent protein (GFP) that appeared forming a scattered pattern (Supplementary Fig. 1B-2). GFP expression increased after day 5 (Supplementary Fig. 1B-3) and appeared the shape of comet-like tail (Supplementary Fig. 1B-4). GFP covered the whole plate and detached from plate after day 8. Subsequently, collected cells with culture medium and repeatedly infected 4-5 times to obtain high-titer virus solution. The virus titers of OE-*SGK*1 and OE-NC were 2.51*1010 and 3.16*1010 pfu/mL, respectively. Therefore, the overexpression virus was successfully packaged.

Bovine preadipocytes were infected with OE-*SGK*1 and OE-NC. Adipocytes grew well, reaching ~90% confluence and GFP covered the visual field after 48 h (Fig. 3A). In OE-*SGK*1, expression of mRNA was 664 times higher than in OE-NC (Fig. 3B) and SGK1 protein expression was much higher and formed more lipid droplets than in OE-*SGK*1 (Fig. 3C-D). RNA integrity was measured by electrophoresis on agarose gel (Supplementary Fig. 1C). The results of qPCR revealed that mRNA expression of adipocyte-specific genes, e.g., PPARγ and C/EBPα, increased in OE-*SGK*1 compared with OE-NC (Fig. 3E) whereas cell cycle-related genes decreased (Fig. 3F). Western blots showed no differences in PPARγ and C/EBPα, whereas CDK2 and CCND2 were down-regulated when *SGK*1 was overexpressed (Fig. 3G and 3H). Thus, *SGK*1 promoted bovine adipogenesis mainly via inhibition of preadipocyte proliferation.

Loss-of-function SGK1 represses bovine adipogenesis

Three 61 bp shRNAs were inserted in pENTR/U6 (Supplementary Fig. 2A) and the coding region of *SGK*1 was joined with psicheck II (Supplementary Fig. 2B). A dual-luciferase reporter assay identified shRNA1 as the best interfering sequence (Supplementary Fig. 2C). Thus, shRNA1 was used to package lentivirus

and the recombinant plasmid c-shRNA1 was constructed (Supplementary Fig. 2D). The titers of sh-*SGK*1 and sh-NC were 108 and 3 x 108 TU/mL, respectively.

Bovine preadipocytes infected with sh-*SGK*1 and sh-NC grew well and confluence reached ~90%, with GFP covering the whole visual field after 72 h (Fig. 4A). In sh-*SGK*1, reduction of mRNA (Fig. 4B), SGK1 protein expression (Fig. 4C) and lipid droplets (Fig. 4D) were observed, compared with sh-NC. RNA integrity was measured by electrophoresis on agarose gel (Supplementary Fig. 2E). In sh-*SGK*1, results qPCR revealed reduction in mRNA expression of adipocyte-specific genes (Fig. 4E) and increase in cell cycle related genes (Fig. 4F) compared to sh-NC. This is opposite to the results observed under *SGK*1 overexpressed. Expression of PPARγ and C/EBPα were down-regulated and no difference was observed in CDK2 and CCND2 (Fig. 4G and 4H). Hence, loss-of-function *SGK*1 repressed bovine adipogenesis mainly through repressing adipocytes differentiation.

SGK **1 affected the gene expression in PI3K/Akt signaling pathway**

RNA-seq was used to further investigate the effect of SGK1 in the lipid metabolism of bovine adipocytes. A total of 29, 404 genes were detected (Fig. 5A). The sequencing data is shown in Supplementary table 2. A total of 1704 differentially expressed genes (DEGs) were detected, 1126 of which were up-regulated and 578 were down-regulated (Fig. 5B). k-means cluster analysis was performed on DEGs (Fig. 5C). Ten clusters were obtained and key genes were screened at the vertex or inflection points of each cluster (Supplementary Table 3). By GO annotation, DEGs were assigned to metabolic, development and biological processes, among others (Fig. 5D). Subsequently, the terms related to metabolism and development, positive regulation of MAPK cascade, triglyceride biosynthetic process and cellular response to cAMP were selected to draw a GO chord graph, whereas genes of interest in the selected terms were sorted according to their logFC value (Fig. 5E). KEGG enrichment analysis revealed that DEGs were significantly enriched in PI3K/Akt, MAPK and lipic acid metabolism signaling pathways, among others (Fig. 5F). Genes involved in signaling pathways related to lipid metabolism, and those related to PI3K/Akt, MAPK and related-lipid metabolism signaling pathways were screened to perform visual analysis (Fig. 5G).

Subsequently, qRT-PCR was used to verify the reliability of RNA-seq sequencing results (primers shown in Supplementary Table 4). Six up-regulated genes and four down-regulated genes were randomly selected for qRT-PCR. Expression change of up-regulated (*CDKL*4, *LIF*, *SGK*2, *CDK*1, *KLF*5 and *IL*6) and down-regulated (*ELOVL*6, *FAP*, *PLIN*4 and *FABP7*) genes was consistent with RNA-seq results (Fig. 5H).

SGK **1 indirectly affects the phosphorylation of FOXO1 and FOXO3 via the PI3K/Akt signaling pathway**

It has been reported that SGK1 affects the phosphorylation of FOXO1 and FOXO3 [26, 27]. Indeed, FOXO1 and FOXO3 are downstream genes of the PI3K/Akt signaling pathway [20]. STRING predicts an interaction between SGK1 and FOXO family proteins (Fig. 6A-B). Indeed, mRNA levels of FOXO1 and FOXO3 were inhibited after *SGK*1 was overexpressed, and opposite results were obtained for loss-of-function *SGK*1 (Fig. 6C). Protein expression and phosphorylation of FOXO1 and FOXO3 were detected by

Western blot after *SGK*1 was overexpressed or inhibited. Compared with OE-NC, OE-*SGK*1 led to reduced expression of FOXO1 and FOXO3, with increased phosphorylation (Fig. 6D-E). Compared with sh-NC, expression of FOXO1 and FOXO3 were up-regulated in response to *SGK*1 loss-of-function, wheras phosphorylation of FOXO3 was down-regulated (Fig. 6F-G). The regulatory network by which *SGK*1 regulates bovine adipogenesis through phosphorylation of FOXO1 and FOXO3 was mapped through the PI3K/Akt signaling pathway (Fig. 6H).

Discussion

SGK **1 is a critical factor for bovine adipogenesis.**

Understanding how regulators control beef IMF content can suggest candidates that promote adipocyte differentiation and lipid deposition. A model of bovine preadipocytes differentiated into mature adipocytes was established as described [28]. The role of *SGK*1 during bovine adipogenesis was examined by overexpression adenovirus and short hairpin RNA lentivirus packaging. Viral vectors are easily manipulated and mass reproduced in cells. Also, they are widely used in various cell types as they provide strong infection and efficiency, compared with DNA-liposome mixture transfections [29, 30]. Our study reveals that *SGK*1 is a positive regulatory factor during bovine adipocyte differentiation, regulating the expression of specific-adipocyte genes and proliferation genes. *SGK*1 changes the gene expression in PI3K/Akt and other signaling pathways related to lipid metabolism. Moreover, *SGK*1 indirectly affected the phosphorylation level of FOXO1/FOXO3 via the PI3K/Akt signaling pathway. Thus, our research expands the number of candidates involved in a network of regulating bovine adipogenesis.

A previous research reported that SGK1 is mainly expressed in kidney where it regulates multiple renal ion channels [31]. Our study also shows that expression of *SGK*1 in cattle is higher in kidney and adipose tissue than in heart. The latter is consistent with results in mice [32]. Moreover, *SGK*1 was highly expressed at day 6 during adipogenic differentiation. Preadipocytes were induced to differentiate into mature adipocytes at day 7, leading to deposition of lipid droplets [33]. It has been reported that SGK1 is mainly expressed at the last stage of adipocyte differentiation [32]. Therefore, together with previous available evidence, we propose that *SGK*1 regulates fat deposition and promotes lipid accumulation during the late stages of bovine adipocyte differentiation.

SGK **1 affects the expression of genes related to adipocyte differentiation and proliferation in cattle.**

We successfully packaged the overexpression adenovirus and short hairpin RNA lentivirus of *SGK*1 to verify the function of *SGK*1 in the regulation of bovine adipogenesis. Expression of adipocyte differentiation-related genes, e.g., *PPARγ* and *C/EBPα*, increased when *SGK*1 was overexpressed. As a key regulator of adipogenesis, PPAR*γ* controls the transcription of numerous genes related to adipocyte differentiation and lipid accumulation [34, 35], and a high level of the C/EBPα gene maintains adipocytes in a fully differentiated state [36, 37]. Loss-of-function *SGK*1 produced opposite results. Consistently, OE-*SGK*1 produced more lipid droplets than the control. It has been reported that transgene SGK1 increased the lipid droplets formation in 3T3-L1 cells compared with wild type cells [38], whereas expression of

proliferation-related genes decreased in OE-*SGK*1, e.g., *PCNA*, *CCND*2 and *MCM*6, which are marker genes regulating cell proliferation [39, 40].

Overexpression of *SGK*1 did not change the expression of differentiation-related proteins PPARγ and C/EBPα, but decreased that of proliferation-related proteins CCDN2 and CDK2. This suggests that overexpression of *SGK*1 positively regulates bovine adipogenesis mainly via inhibition of adipocyte proliferation. Inhibition of *SGK*1 expression by short hairpin RNA lentivirus led to reduced expression of adipogenic differentiation genes and increased expression of cell proliferation genes. The inhibition of insulin signalling by dexamethasone and oleic acid was reversed by lv-sh*SGK*1 [41], whereas we observed that loss-of-function *SGK*1 led to down-regulation of proteins related to differentiation (PPARγ and C/EBPα) and no changes in proteins related to proliferation (CCDN2 and CDK2). This suggests that sh-*SGK*1 repressed bovine adipogenesis mainly by inhibiting adipocytes differentiation. Therefore our study proves that *SGK*1 promotes bovine adipogenesis through adipogenic differentiation and repressing preadipocyte proliferation. We also show that overexpression of *SGK*1 promotes bovine adipogenesis mainly through inhibiting adipocyte proliferation, whereas loss-of-function *SGK*1 repressed adipogenesis mainly via inhibiting adipocyte differentiation.

SGK **1 regulates gene expression related to lipid metabolism in bovine adipocytes**

1704 DEGs were found when *SGK*1 was overexpressed. GO annotation analysis found DEGs related to metabolic process, development process and biological process activation. KEGG enrichment analysis revealed DEGs significant enriched in the PI3K/Akt signaling pathway, MAPK signaling pathway and lipic acid metabolism signaling pathway.

The MAPK/PI3K/Akt signaling pathway is important in regulating lipid metabolism, energy homeostasis and cell proliferation [42–44]. Overexpression of *SGK*1 up-regulated FGF23, a key factor in the MAPK and Akt signaling pathway. FGF23 is a key endocrine factor involved in the regulation of systemic homeostasis and lipid metabolism [45]. The FOXO signaling pathway is essential in cellular energy production, oxidative stress resistance, and cell viability and proliferation [46]. *SGK*1 down-regulated PCK1, located in the FOXO and PI3K/Akt signaling pathway. Tissue-specific knock out of PCK1 in mice produced a phenotype of obesity, lipodystrophy, fatty liver, and death [47]. Thus, SGK1 might promote bovine adipogenesis through inhibiting PCK1 on the PI3K/Akt signaling pathway. Our results show that DEG colony-stimulating factor 3 (CSF-3) was enriched in the PI3K/Akt signaling pathway. The function of CSF-3 is closely related to adipose tissue and whole-body insulin sensitivity and glucose tolerance in human [48]. Furthermore, *SGK*1 also affects expression of genes enriched in the lipid metabolism signaling pathway. In conclusion, *SGK*1 mainly regulates the expression of PCK1 in the PI3K/Akt signaling pathway and up-regulates FGF23, FGFR3, promoting mitosis, cell growth and adipogenesis in cattle.

SGK **1 phosphorylation of FOXO1 and FOXO3 via PI3K/Akt signaling pathway.**

SGK1 affect many multiple physiologic processes via phosphorylation of downstream proteins [49–51]. SGK1 promotes adipocyte differentiation and controls cell proliferation by regulating the phosphorylation level of FOXO1 and FOXO3, respectively [52, 53]. FOXO1 directly inhibited the expression of PPARγ and interfered with promoter DNA occupancy of the receptor via direct protein-protein interaction [23, 54]. Our results show that *SGK*1 affects the expression of the PI3K/Akt signaling pathway. FOXO family proteins are located downstream of this signaling pathway and are regulated by phosphorylated AKT [21]. After overexpression of *SGK*1, mRNA and protein expression level of *FOXO*1 and *FOXO*3 were down-regulated, whereas inhibition of *SGK*1 showed opposite results in our research. Overexpression of FOXO1 inhibited bovine adipogenesis and induced apoptosis [55], and FOXO1 interacted with PPARγ and repressed its transcription during adipogenesis[56]. We found that overexpression of *SGK*1 elevates the phosphorylation of FOXO1 and FOXO3, and loss-of- function *SGK*1 had opposite effects. Notably, the phosphorylation level of FOXO1 did not change in sh-*SGK*1 and sh-NC. FOXO1 is located in the nucleus where it may control the cell cycle [57]. Phosphorylation of FOXO1 by *SGK*1 may lead to the loss of its transcriptional activity by nuclear exclusion [58]. However, shRNA is synthesized in an nucleus of cells and is transported to the cytoplasm [59]. This may explain the poor interference efficiency of sh-*SGK*1 and may have resulted in unchanged phosphorylation level of FOXO1.

The SGK1/Akt signaling pathway affects adipogenesis in mouse white adipose tissue via phosphorylation of FOXO1 [32, 60] and the interaction between SGK1 and FOXO3 has been described in several disease contexts [61, 62]. Our study shows that *SGK*1 can also affect FOXO3 in bovine adipose tissue. Although we show that *SGK*1 regulates bovine adipogenesis by phosphorylation FOXO1 and FOXO3 via PI3K/Akt signaling pathway, the interaction is not direct.

Conclusion

Overall, *SGK*1 is a positive regulatory factor during bovine adipogenesis. In mechanism *SGK*1 indirectly affects the phosphorylation levels of FOXO1/FOXO3 via the PI3K/Akt signaling pathway. Our study provides clues to the regulatory role of *SGK*1 during bovine adipogenesis and in improving beef quality.

Materials And Methods

Ethics statement

Three adult Nanyang cattles were slaughtered from Biyin cattle farm Nanyang city, Henan province. Subcutaneous adipose tissue was obtained from calves for isolation of preadipocytes. The Animal Ethics Committees of Ningxia University approved the experimental design and the animal sample collection for the present study (permit number NXUC20211168). This study is reported in accordance with the ARRIVE guidelines.

Isolation and induced differentiation of bovine preadipocytes

Bovine preadipocytes were isolated using collagenase digestion, as described [63]. Briefly, adipose tissue without blood vessels and connective tissues was minced into cubes with size ~1 mm^3 and digested in 1 mg/mL collagenase type I (Sigma, C0130) in a water bath for 90 min at 37 ℃. Subsequently, preadipocytes were seeded and cultured on a 10 cm^2 plate with a growth medium containing Dulbecco's modified eagle medium (DMEM), 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S) at 37 ℃ with 5% CO_2 for 24 h. Cells were then washed with PBS three times and fresh medium was replaced every two days.

When density reached 90%, cells were transferred to 6-well cell culture plates using trypsin. Subsequently, adipocytes were differentiated by feeding induction medium (IM) containing DMEM, 10% FBS, 10 μg/mL insulin, 1 μM rosiglitazone, 1 μM dexamethasone (DEXA, Sigma) and 0.5 mM 1-methyl-3-isobutylxanthine (IBMX, Sigma). Three days later, the medium was replaced with maintenance medium which was changed every two days until maturation.

Sequence analysis of bovine SGK1

Primers were designed to amplify the CDS region of *SGK*1 (Genbank No. NM_001102033.1) (Supplementary Table 1) and interacting proteins were predicted using STRING.

Recombinant adenovirus packaging

Endonucleases Kpn I (Takara, Dalian, China) and Hind III (Takara) were added to forward and reverse primers, respectively. Subsequently, the CDS region was inserted into the shuttle vector pAd-tract-CMV and was transformed into competent cells BJ5183. The recombinant vector was transfected into taraka (HEK293a). Subsequently, cells and culture medium were collected named (OE-*SGK*1) with the and negative control named (OE-NC) eight days after transfection. pAd-track-CMV and taraka were preserved in our laboratory.

Dual luciferase report analysis

Three short hairpin RNAs (shRNA1, shRNA2 and shRNA3) were designed using online software (http://rnaidesigner.thermofisher.com.rnaiexpress/) (Table 1) and were synthesized. They were connected to the pENTR/U6 plasmid and were named pENTR/U6-shRNA1, pENTR/U6-shRNA2 and pENTR/U6-shRNA3. The recombinant plasmid was constructed with psicheck II and the CDS region of *SGK*1 (psicheck II-*SGK*1). Cell transfection was performed using Lipofectamine 3000 (Thermo, Waltham, MA, USA) according to the manufacturer's instructions. pENTR/U6-shRNA or pENTR/U6-NC were dissolved using Opti-MEM (Sigma, St. Louis, Missouri, USA) and incubated with psicheckII-*SGK*1 to form a DNA-liposome mixture. Subsequently, the DNA-liposome mixture was added to the culture medium and was incubated 48 hrs at 37°C and 5% CO_2. The luciferase activity in 24 wells containing HEK293T cells were measured.

Table 1 short hairpin RNA sequences information of *SGK*1

Names of sequence
ShRNA1-F 5'-GATCC GCCAATAACTCCTATGCATGC***TCAAGAG***GCATGCATAGGAGTTATTGGC TTTTTT C -3'
ShRNA1-R 5'-TCGAG AAAAAA GCCAATAACTCCTATGCATGC***CTCTTGA***GCATGCATAGGAGTTATTGGC G -3'
ShRNA2-F 5'-GATCC GGAATGTTCTCCTGAAGAACG*TCAAGAG*CGTTCTTCAGGAGAACATTCC TTTTTT C -3'
ShRNA2-R 5'-TCGAG AAAAAA GGAATGTTCTCCTGAAGAACG***CTCTTGA***CGTTCTTCAGGAGAACATTCC G -3'
ShRNA3-F 5'-GATCC GCCGAAACACAGCTGAGATGT***TCAAGAG***ACATCTCAGCTGTGTTTCGGC TTTTTT C -3'
ShRNA3-R 5'-TCGAG AAAAAA GCCGAAACACAGCTGAGATGT***CTCTTGA***ACATCTCAGCTGTGTTTCGGC G -3'

Note: GATCC and TCGAG were the sequence of endonuclease BamHl and Xhol, italic sequence indicated loop sequence.

Short hairpin RNA lentivirus packaging

The selected shRNA1 for single target lentivirus packaging and the negative control were named sh-*SGK*1 and sh-NC, respectively. Recombinant vector c-shRNA1 and helper vectors psPAX2 and pMD2G were co-transfected with Lipofectamine 3000 into HEK293T cells according to the manufacturer's instructions. Fluorescence was detected with an inverted microscope after 24 h. The supernatant were collected at 48 h and 72 h to concentrate and purify the virus.

Cell infection

Preadipocytes were seeded onto 6-well plates overnight to reach 70% confluence. The OE-*SGK*1 and sh-*SGK*1 virus solutions were added to adipocyte culture plates (n = 3) with an optimal MOI (multiplicity of infection) value determined in a preliminary experiment, using OE-NC and sh-NC, respectively, as controls. After 48 h incubation at 37°C and 5% CO2, fluorescence was detected with an inverted microscope after 24 h and the medium was replaced by high glucose medium containing 10% FBS.

Total RNA extraction and RT-qPCR

Total RNA was extracted from adipocytes or tissues using Trizol reagent. cDNA was synthesized by using a Primescript^{TM} RT reagent kit (TaKaRa) and SYBR premix Ex Taq II kit (TaKaRa) was used to perform the RT-qPCR reaction on a Bio-Red CFX 96 Touch instrument (Bio-Rad, Hercules, CA, USA). The $2^{-\Delta\Delta Ct}$ method was used to analyze the data. Primers of adipogenic and cell cycle genes were designed by Primer Premier 5 according to the primer designing criteria (Supplementary Table 1). Relative expression levels were normalized with the internal control GAPDH [64].

Total protein extraction and immunoblotting

Total protein was extracted using whole cell Lysis Assay (KeyGEN BioTECH, Nanjing, China) and quantified using a BCA protein assay kit (KeyGEN BioTECH, Nanjing, China). Equal amounts of protein (10 μg per lane) were run on 5% and 12% or 8% SDS-PAGE and were transferred onto a polyvinylidene fluoride (PVDF) membrane (Millipore Corporation, Bedford, MA, USA). Membranes were blocked with 3% bovine serum albumin (Sigma, WXBD4881V) for 2 h at room temperature and incubated with the primary antibody overnight at 4°C. Primary antibodies were: anti-CDK2 (abbexa 1:500, cat. no. abx009457), anti-PPARγ (abbexa, 1:500, cat. no. abx104516), anti-C/EBPα (abbexa, 1:500, cat. no. abx009679), anti-Cyclin D2 (Abmart, 1:500, cat. no. TA5410), anti-FOXO1 (Abmart, WB: 1:500, cat. no. PA1431; pS256: 1:500, cat. no. PA5293), anti-FOXO3 (Abmart, WB: 1:500, cat. no. PA5311; pS322+S325/pS318+S321: 1:500, cat. no. PA5855) and anti-GAPDH (ZSGB-BIO, 1:500, cat. no. ZB-2301). Then membranes were incubated with anti-rabbit IgG (ZSGB-BIO, 1:5000, ZB-2301) secondary antibodies for 2 h at room temperature. Finally, blots were visualized by ECL reagent and captured using the Tanon-5200 imaging system (Shanghai, China).

RNA-seq analysis

Adipocytes infected by OE-*SGK*1 or OE-NC and induced differentiation for 6 days. Subsequently, cells were used to perform deep sequencing (n = 4) using Illumina xten completed by BioMarker Co (Qingdao, China).

RNA-seq data analysis

RNA-seq data was analyzed in R (R x64 4.1.2) using the R package (such as pheatmap and GOplot) and visual analysis was performed in Cytoscape (cytoscape_3.9.0).

Statistical analysis

For each group at least three independent experiments were performed and data was expressed as mean ± standard deviation (SD). GraphPad Prism v8.0.2 (GraphPad Software, Inc., La Jolla, CA, USA) was used to analyze the experimental data. Comparisons among multiple groups were performed with two-way analysis of variance (ANOVA). $P<0.05$ was considered to indicate a statistically significant difference, and $P<0.01$ was considered to indicate that the difference was extremely significant.

Declarations

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Authors' contributions

Conceived and designed the research:YM and ZXL; Analyzed the data and conducted the experiment: ZXL; Wrote the paper: ZXL; Modified manuscript: YM, DWW, YFM, LT, SZW, CLP. All authors read and approved the final manuscript.

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Availability of data and materials

All data are available within the article or Supplementary Information. The RNA-seq data reported in this paper are available in NCBI BioProject ID: PRJNA786433 or access link: https://submit.ncbi.nlm.nih.gov/subs/bioproject/SUB10763814/overview. Source data are provided with this paper.

Ethics approval and consent to participate

The Animal Ethics Committees of Ningxia University and Biyin cattle farm all approved the experimental design and animal sample collection for the present study (permit number NXUC20211168). Animal experiments complied with the requirements of the directory of the Ethical Treatment of Experimental Animals of China. Animal experiments were conducted strictly followed the guidelines of the Regulations for the Administration of Affairs Concerning Experimental Animals (Ministry of Science and Technology, China, 2004). This study is reported in accordance with the recommendations put forward by the ARRIVE guidelines.

Consent for publication

Not applicable.

Competing interests

There is no conflict of interest exits in this manuscript, and the manuscript is approved by all authors for publication.

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Figures

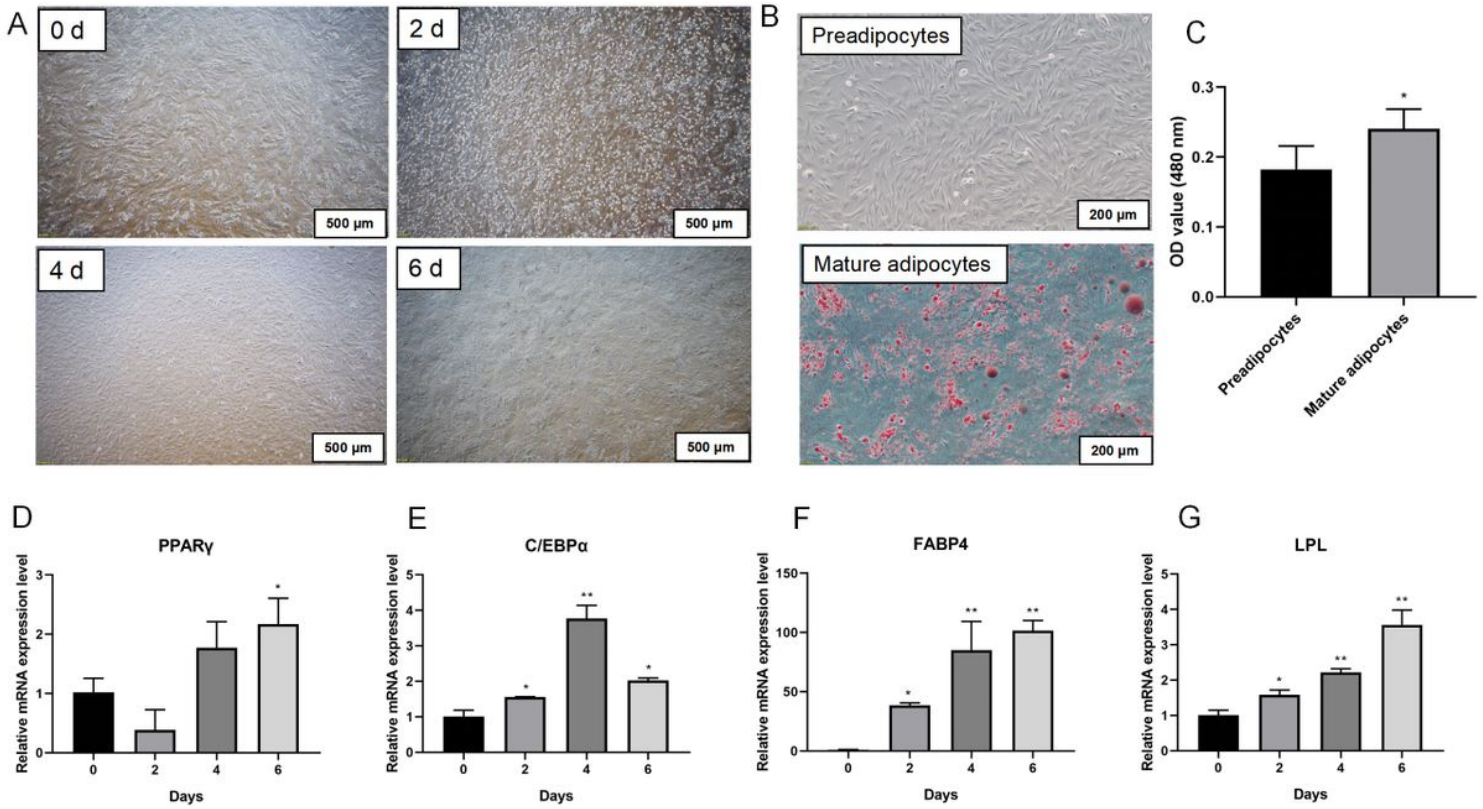

Figure 1

Bovine preadipocytes induced differentiation into mature adipocytes.

A, Lipid droplet accumulation during differentiation 0 d, 2 d, 4 d, 6 d (40×); B, Oil Red O staining of bovine preadipocyte (above, 100×) and differentiated adipocyte (below, 100×); C, OD value measured of lipid droplets in preadipocytes and Mature adipocytes; D-G: detected the relative mRNA expression level of PPARγ, C/EBPα, FABP4 and LPL, respectively. ** indicated the differentiation is extremely significant ($P<0.01$) and * indicated significant ($P<0.05$).

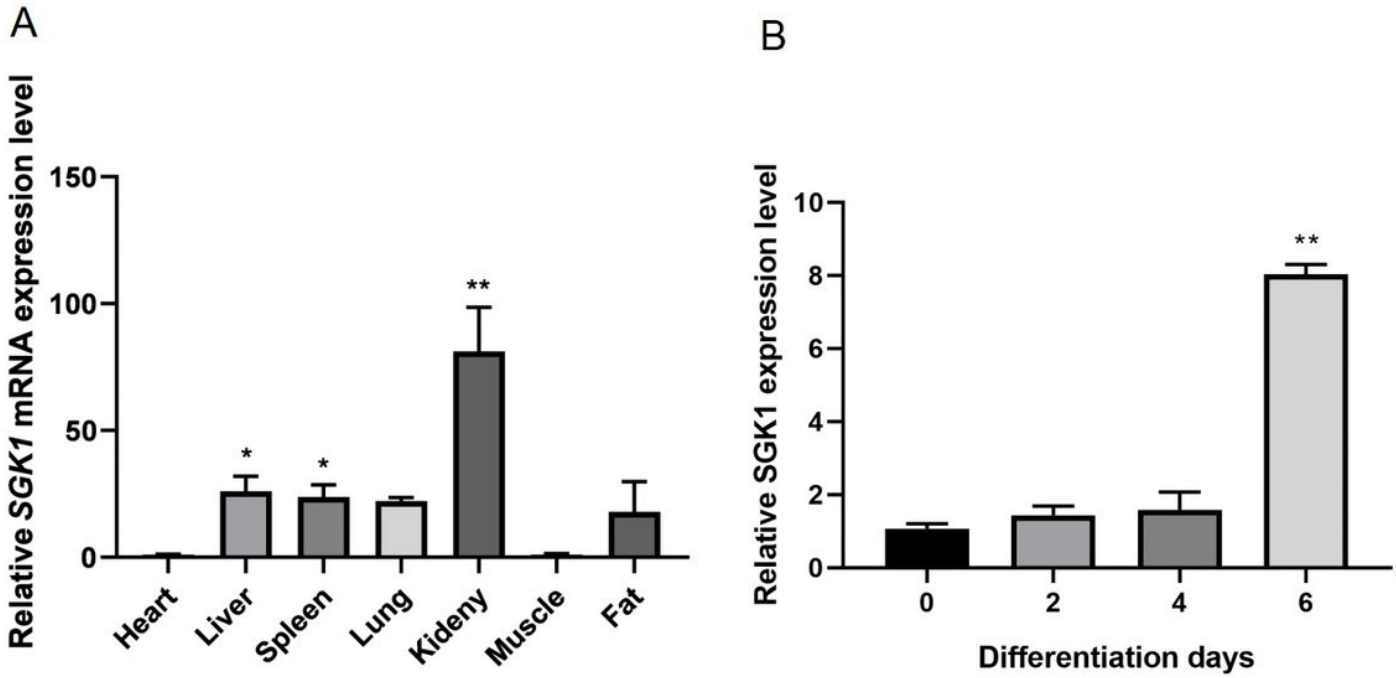

Figure 2

Expression pattern of bovine *SGK*1

A, Expression pattern in several tissues; B, changing expression pattern of *SGK*1 during differentiation.

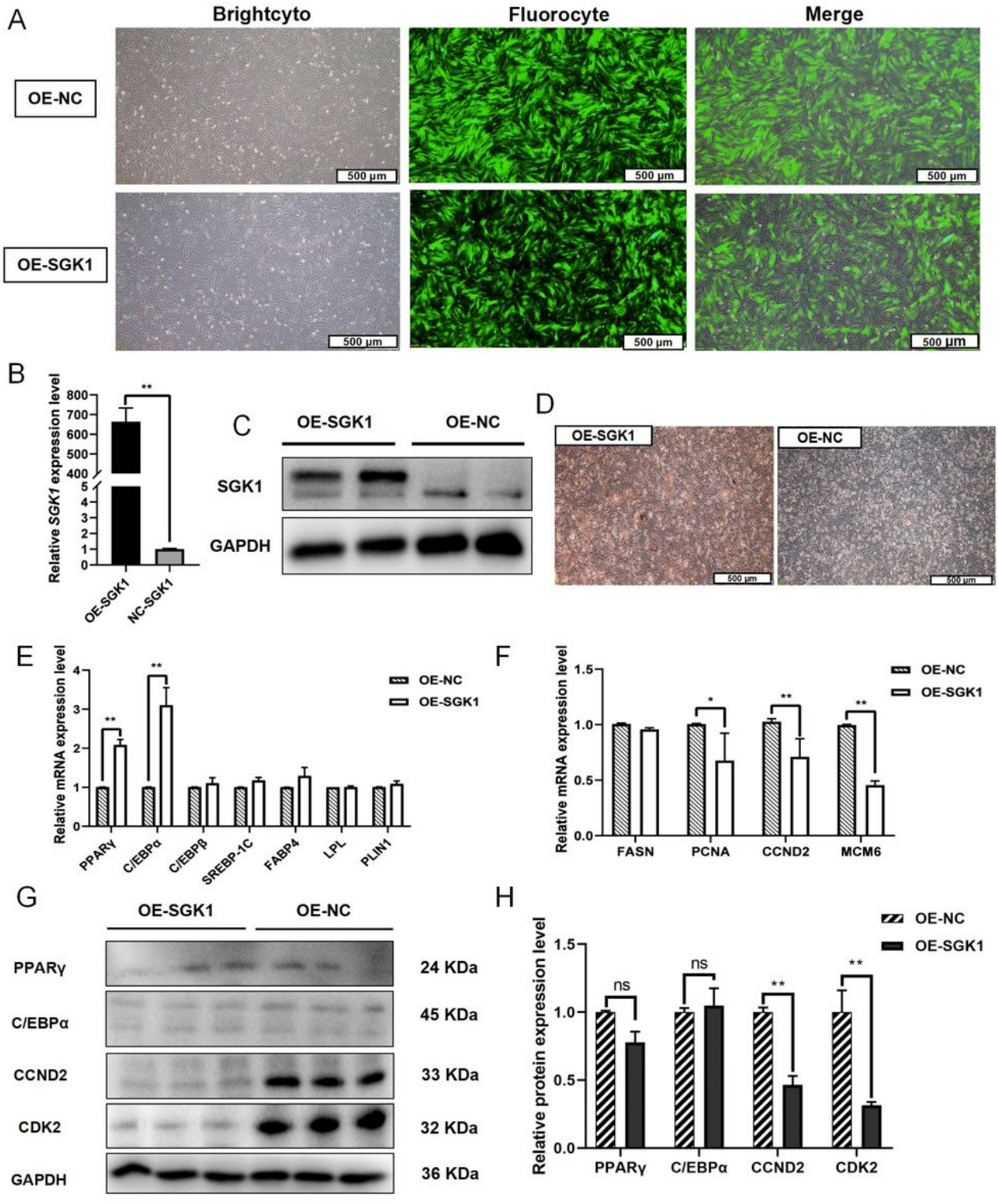

Figure 3

Overexpression of *SGK*1 promotes bovine adipogenesis

(A), cell growth status and expression in OE-*SGK*1 and OE-NC 48 h after infection; (B) In OE-*SGK*1 and OE-NC: mRNA levels of *SGK*1; (C) protein expression of SGK1 in OE-*SGK*1 and OE-NC; (D) oil red O staining; (E) mRNA expression of adipocyte differentiation-related genes; (F) mRNA expression of cell cycle

related genes; (G) expression of adipogenic-differentiation proteins (PPARγ and C/EBPα) and proliferation-related proteins CDK2 and CCND2; (H), protein quantitative.

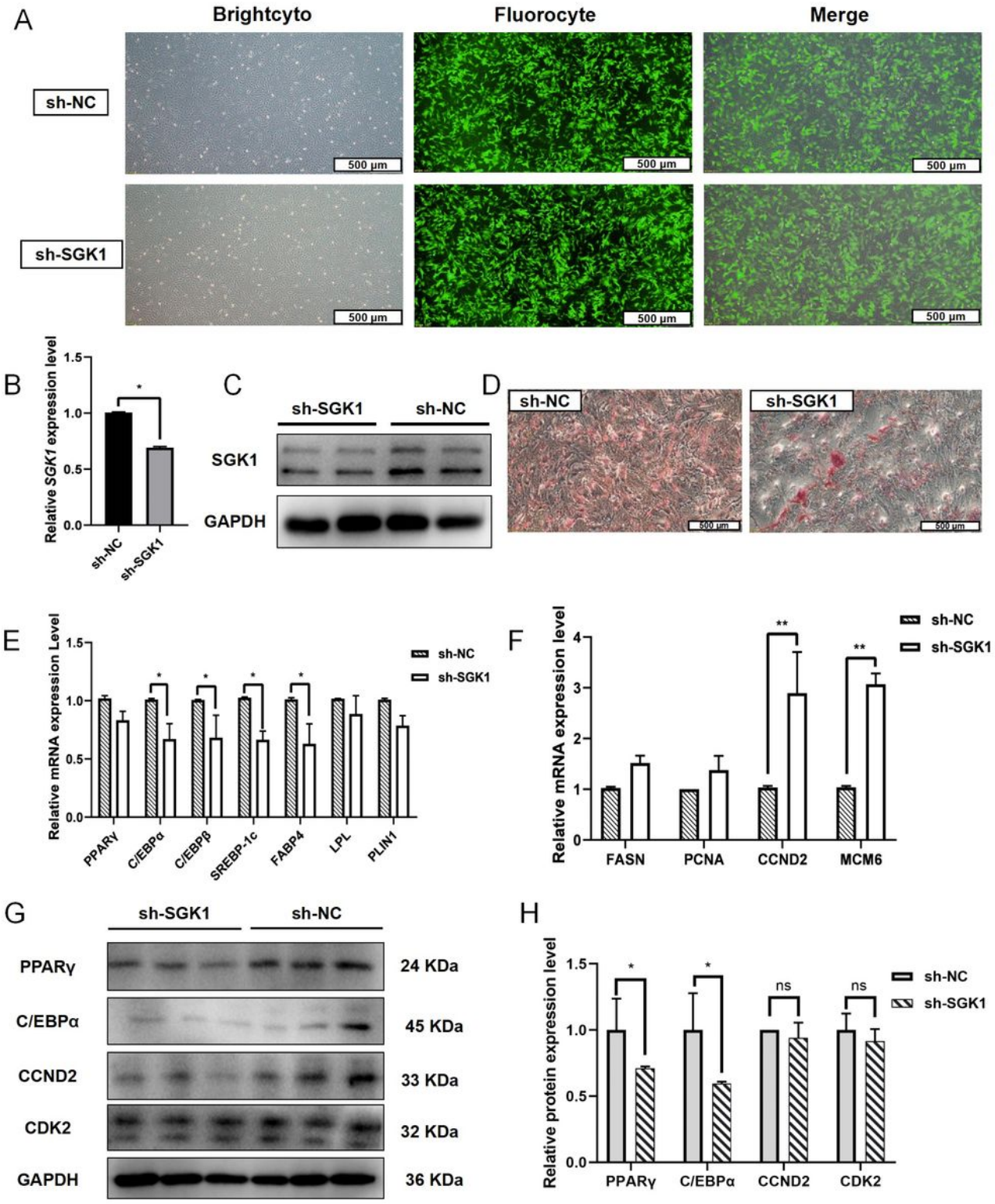

Figure 4

Same as Fig. 3, for sh-*SGK*1 and sh-NC, instead of OE-*SGK*1 and OE-NC.

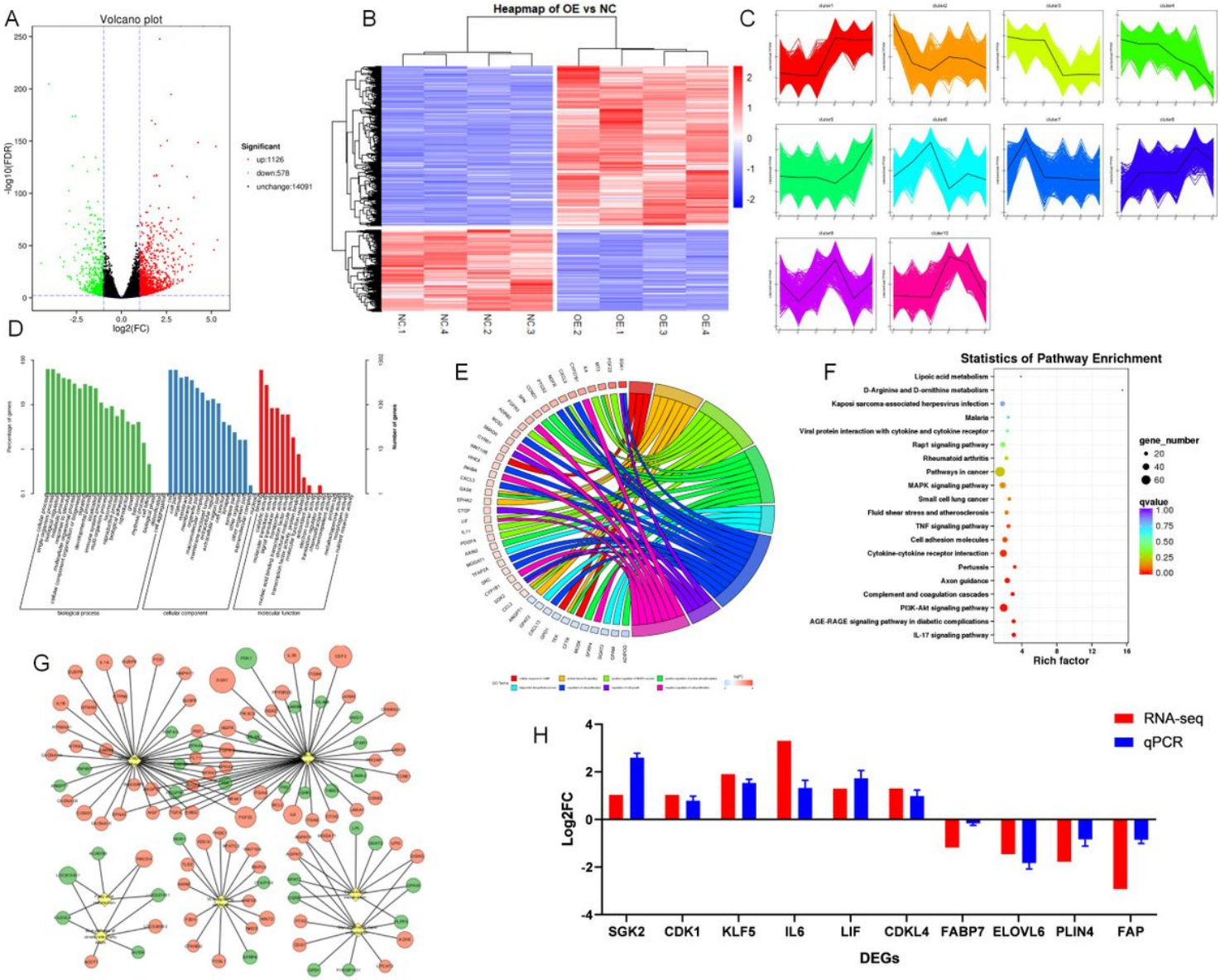

Figure 5

*SGK*1 regulates lipid metabolism in bovine adipocytes

A, volcano plots of RNA-seq; B, heatmap depicting expression of DEGs (red and blue indicate up-regulation and down-regulation, respectively); C, k-means cluster analysis; D, GO function note analysis; E, KEGG signaling pathway analysis; F, GO chord graph terms and DEGs of interest; G, visual analysis of signaling pathway and DEGs of interest, where red and green indicate up-regulated and down-regulated genes, respectively. Node size indicates fold changes and larger circles means larger fold change; H, comparison of expression of DEGs by qRT-PCR and RNA-seq.

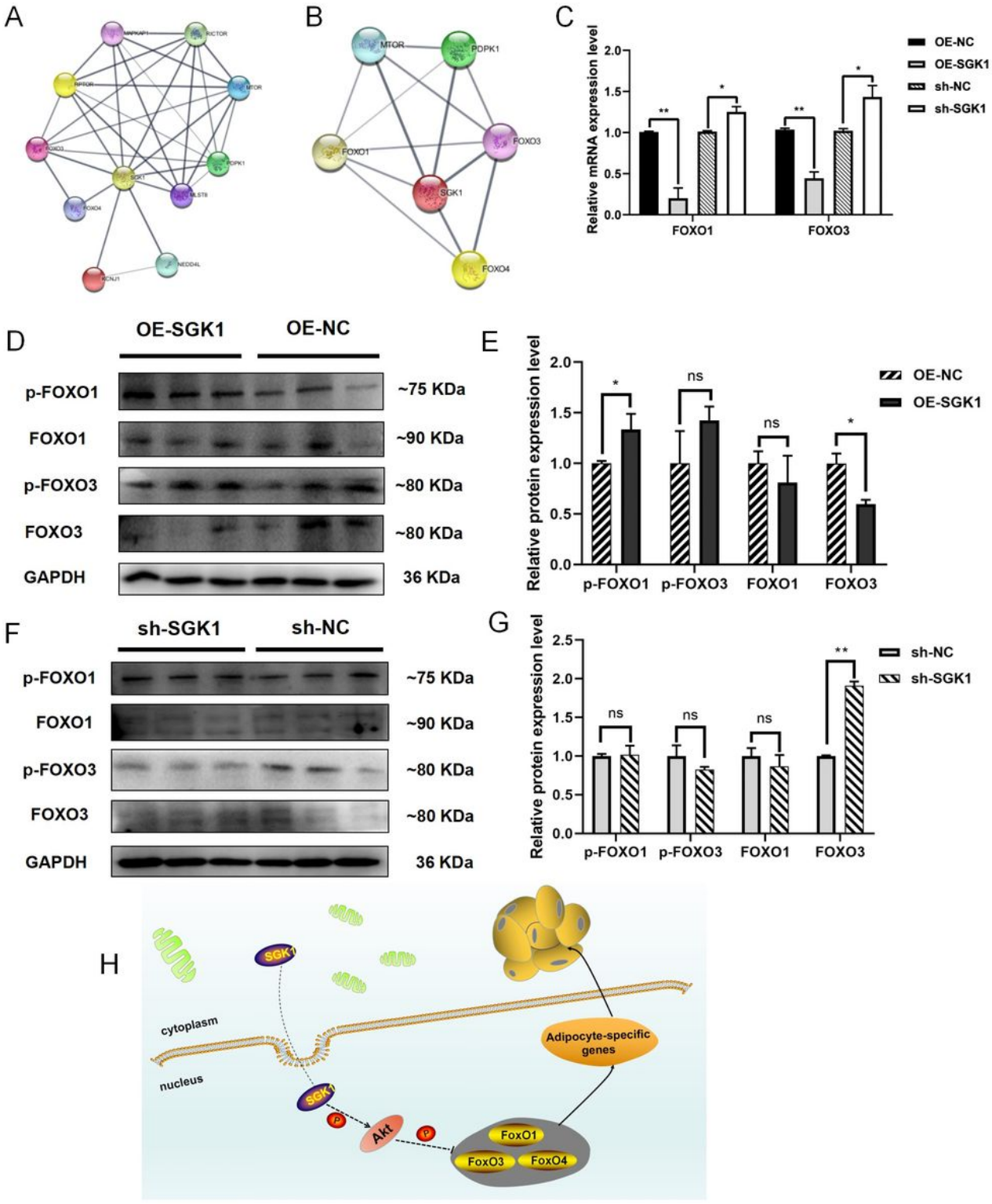

Figure 6

*SGK*1 affects FOXO1 and FOXO3 phosphorylation

A-B, interaction prediction between *SGK*1 and FOXO family; C, mRNA expression of FOXO1 and FOXO3 after overexpression and interference of SGK1; D-E, expression and phosphorylation of FOXO1 (pS256) and FOXO3 (pS322+pS325/pS318+pS321) in OE-*SGK*1 and OE-NC; F-G, expression and phosphorylation

of FOXO1 (pS256) and FOXO3 (pS322+pS325/pS318+pS321) in sh-*SGK*1 and sh-NC; H, interaction mechanism of *SGK*1 and FOXO1/FOXO3 in bovine adipocytes.

Supplementary Files

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