circFLT1 and IncCCPG1 Sponges miR-93 to Regulate the Proliferation and Differentiation of Adipocytes by Promoting IncSLC30A9 Expression

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Although many circular RNAs (circRNAs) and long non-coding RNAs (lncRNAs) have been discovered in adipocytes, their precise functions and molecular mechanisms remain poorly understood. Based on existing circRNA and IncRNA sequencing data of bovine adipocytes, we screened for the differential expression of circFLT1 and IncCCPG1 in preadipocytes and adipocytes and further analyzed their function and regulation during adipogenesis. The overexpression of circFLT1 and IncCCPG1 together facilitated adipocyte differentiation and suppressed proliferation. Computationally, the RNA hybrid showed that circFLT1 and IncCCPG1 had multiple potential binding sites with miR-93. Additionally, luciferase reporting experiments verified that circFLT1 and IncCCPG1 may interact with miR-93. We also demonstrated that overexpressed miR-93 effectively suppresses the expression of IncSLC30A9. Signaling pathway enrichment analysis, luciferase activity assay, and expression analysis revealed that IncSLC30A9 inhibits proliferation by inhibiting the expression of AKT protein and promotes differentiation by recruiting the FOS protein to the promoter of peroxisome proliferator-activated receptor gamma (PPARG). In sum, our results elucidate the regulatory mechanisms of circFLT1 and IncCCPG1 as miR-93 sponges in bovine adipocytes.

INTRODUCTION
Qinchuan cattle are among the best local cattle breeds in China. The breed has the advantages of tall stature, strong bones, well-developed muscles, and favorable meat production performance. Generally, the main evaluation indexes of beef quality include marbling, juiciness, tenderness, color, and texture of fat as well as flavor, water content, and pH. Most of these indexes are related to the growth and development of adipose tissue. Therefore, research on beef fat, especially on the molecular mechanisms of bovine adipocyte proliferation, apoptosis, and differentiation, is crucial to bovine breeding in China.

Adipose tissue is an important tissue, as it can be used for energy storage and supply and can participate in physiological and pathological regulation. Adipose tissue is mainly composed of adipose stem cells (precursor cells to generate new fat cells) and adipose cells (fat storage cells). Their main function is to maintain the energy balance in the body by storing triglycerides and facilitating fat mobilization.1,2 Adipogenesis is generally divided into two stages. The first stage is the differentiation of embryonic stem cells into mesenchymal stem cells with multi-differentiation potential. The second stage is terminal differentiation, whereby the preadipocytes have the characteristics of mature adipocytes and acquire lipid droplets and the ability to respond to hormones, such as insulin.3 These phases are associated with the processes of adipocyte apoptosis, differentiation, and proliferation, which are regulated by a series of transcription factors and adipokines and lead to adipocyte development.4,5

Much progress has been made in the study of genetic polymorphism and the function of adipogenesis genes. In the field of non-coding RNA, microRNAs (miRNAs) are known to play important regulatory roles in adipogenesis. For example, miR-204 facilitates adipocyte differentiation by targeting Sirt1,6,7 and miR-93 can regulate adipocyte differentiation by inhibiting expression of Sirtuin-7 and T-box3.7

IncRNA regulation of adipogenesis has also attracted increasing attention at the transcriptional, epigenetic, and post-transcriptional levels. The IncRNA ADNCR promotes Sirt1 gene expression through adsorption of miR-204 and thereby inhibits adipocyte differentiation.7 The IncRNA IMFNCR promotes the expression of the promoter of adipogenic differentiation-related gene (PPARG) through adsorption of miR-128-3p and miR-27b-3p, thereby promoting adipocyte differentiation.7

The role of circRNA in the regulation of fat development and its mechanisms remains unclear. In light of previous studies of miRNAs and IncRNAs and the lack of research on circRNAs, this study aimed to analyze the molecular mechanisms of circRNA and IncRNA adsorption of miRNA to regulate adipogenesis and define the regulatory network of bovine fat development. Early screening high-throughput sequencing was used to evaluate Qinchuan cattle fat cells at different periods of differentiation and their associated IncRNAs and circRNAs. Key IncRNAs and circRNAs are known to be involved in the generation and regulation of fat cells in Qinchuan cattle.

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Received 22 May 2020; accepted 11 September 2020; https://doi.org/10.1016/j.omtn.2020.09.011.
However, further study is required to determine the mechanisms of proliferation and differentiation. At the non-coding RNA level, we aimed to characterize the regulation of fat development and the origin of excellent meat quality traits in Qinchuan cattle. The use of non-coding RNA as a molecular marker in Chinese beef cattle breeds may provide a new theoretical basis for the selection of superior genetic traits.

RESULTS

Identification of miR-93 in Bovine Adipocytes

Laboratory data for Qinchuan cattle, including adipocyte differentiation on days 0, 6, and 9; the results of cell transcriptome sequencing; and random screening of differentially expressed miRNAs revealed an association between adipose differentiation and the differential expression of six miRNAs. The real-time qPCR results were basically consistent with the results of sequencing (Figure S1). We selected adipocytes with the highest levels of expression of miR-93 and the most significant differences in the expression of miR-93 for further analysis to explore the role of miR-93 in fat development (Figure 1A).

Primary bovine fat cells were isolated for induced differentiation. Successful differentiation was verified by detecting the expression level of differentiation marker genes and Oil Red O staining (Figures 1B–1E). We then detected changes in the expression of miR-93 during differentiation (days 0, 2, 4, 6, 8, and 10). The trend in expression was consistent with that observed in sequencing, with the highest expression level at day 6 of differentiation (Figure 1F). Similarity analysis indicated that the miR-93 seed sequence was conserved among cows, mice, and humans (Figure 1G). Thus, we speculate that miR-93 might be a potential regulator during adipogenesis.

Effects of miR-93 on Adipocyte Proliferation, Apoptosis, and Differentiation

To determine whether miR-93 regulates adipocyte proliferation and apoptosis, we conducted the 5-ethynyl-20-deoxyuridine (EdU) assay, flow cytometry, cell counting kit-8 (CCK-8) assay, real-time-PCR, and western blotting. Detection of overexpression efficiency showed that miR-93 expression was markedly increased (Figure 2A). We used the EdU incorporation assay to detect cell proliferation and observed that miR-93 promoted the mitotic activity of adipocytes (Figure 2B). Flow cytometry demonstrated that the number of S phase cells was increased upon miR-93 overexpression (Figure 2C). Subsequently, CCK-8 detection produced similar findings (Figure 2D). In addition, we observed that the proliferation-related markers, including cyclinD, cyclinE, and proliferating cell nuclear antigen (PCNA) expression levels were significantly increased following the overexpression of miR-93 (Figures 2E and 2F). Further assay detection showed that miR-93 significantly increased both mRNA and protein expression levels of Bcl-2 (Figures 2G and 2H). Conversely, knockdown of miR-93 inhibited cell proliferation and promoted cell apoptosis (Figures S2A–S2G). These results indicate that miR-93 stimulates proliferation and suppresses apoptosis. To further explore the involvement of miR-93 in adipocyte differentiation, changes in its expression at days 0, 2, 4, 6, 8, and 10 of differentiation were detected. The results showed that miR-93 expression was first increased and then reduced, and its highest expression occurred on day 6 of differentiation (Figure 1B). Detection of overexpression efficiency showed that miR-93 expression was markedly increased (Figure 2I). After transfection with pcDNA3.1-miR-93 or pcDNA3.1, the mRNA and protein expression levels of the adipose differentiation markers PPARG, CEBPA, and FABP4 were also decreased on day 6 of differentiation (Figures 2J and 2K). Fewer lipid droplets were detected in the pcDNA3.1-miR-93-transfected adipocytes via Oil Red O staining on day 6 of differentiation (Figure 2L). Interference with miR-93 promoted cell differentiation (Figures S2H–S2K). Collectively, these results indicate that miR-93 suppresses adipocyte differentiation. Given that miR-93 is highly conserved among cows, mice, and humans, we also assessed its function in 3T3-L1 cells. The overexpression of miR-93 promoted 3T3-L1 cell proliferation and inhibited 3T3-L1 cell differentiation (Figure 3F). These findings relating to 3T3-L1 cells are consistent with our bovine adipocyte findings.

LncSLC30A9 Is a Target IncRNA of miR-93

To explore the molecular mechanism by which miR-93 regulates adipogenesis, we used the bioinformatics software TargetScan and considered three candidate genes associated with adipogenesis. The luciferase reporter assay showed that when the psi-Sirt7-3’UTR or psi-SLC30A9-3’UTR wild type was co-transfected with pcDNA-miR-93, the luciferase activity was significantly reduced. No significant change was noted in luciferase activity in the co-transfected mutant vector group (mutation seed region – 3~6 site). However, luciferase activity was significantly decreased when psi-PDGFRα-3’UTR wild type or psi-PDGFRα-3’UTR mutant type was co-transfected with pcDNA-miR-93 (Figure 3A). This observation may stem from the fact that the mutations were unsuccessful, suggesting that PDGFRα may be a target gene of miR-93.

As several studies have now clarified the effects of Sirt7 on fat function, we focused on the effects of SLC30A9 on fat function.7 We found that the SLC30A9 3’ end could produce two lncRNAs, which were differentially expressed both before and after differentiation.8 The rapid amplification of cDNA ends (RACE) analysis of adipocytes showed that LncSLC30A9 had two transcripts, named lncSLC30A9a and lncSLC30A9b, which were 1296 nt and 848 nt in length, respectively (Figures 3B and 3C).

The bioinformatics software, Coding Potential Calculator (CPC), was used to evaluate the coding potential. IncSLC30A9 had a very low coding potential, which was similar to that of another bovine non-coding RNA, MEG9 (Figure 3D). We then overexpressed miR-93 and detected a significant reduction in IncSLC30A9 mRNA expression in bovine adipocytes (Figure 3E).

However, we detected no significant differences in miR-93 expression among IncSLC30A9-overexpressed cells (Figure 3F). To confirm the binding between IncSLC30A9 and miR-93, we performed dual-luciferase assays, which showed that luciferase activity in the IncSLC30A9 wild type was reduced (Figure 3G). The results of luciferase activity at different doses further demonstrated the relationship between
Figure 1. The Expression of miR-93 in Bovine Adipocytes

(A) Expression of six miRNAs in adipocytes of Qinhuian cattle. QC-0, QC-6, and QC-9 represents induction of adipocyte differentiation in Qinhuian cattle for 0 days, 6 days, 9 days, respectively. (B–E) The mRNA expression of marker genes PPARG (B), CEBPA (C), FABP4 (D) at different stages of differentiation and Oil Red O staining (E). (F) Expression of miR-93 in Qinhuian cattle adipocytes at different stages of differentiation. (G) The similarity analysis of miR-93 in cow, human, and mouse. Values are mean ± SEM for three biological replicates.
IncSLC30A9 and miR-93 (Figure 3H). Furthermore, the semiquantitative PCR results demonstrated that IncSLC30A9 was expressed in the nucleus and cytoplasm (Figure 3I). The above data revealed that IncSLC30A9 is a potential target of miR-93.

**Effects of IncSLC30A9 on Adipocyte Proliferation, Apoptosis, and Differentiation**

We examined the expression of IncSLC30A9 and its maternal gene (SLC30A9) in different tissues of the fetus, calf, and adult cattle during different differentiation periods. IncSLC30A9 was mainly expressed in the spleen of calves, while SLC30A9 was mainly expressed in the liver of the fetus and lung of adults (Figures S4A and S4B). However, their expression patterns were consistent during adipogenic differentiation and showed an overall increasing trend (Figures S4C and S4D). In addition, we found that overexpression of IncSLC30A9 did not affect the expression of the maternal gene (SLC30A9; Figure S4E). However, when SLC30A9 was knocked down, the expression of IncSLC30A9 decreased significantly (Figures S4F and S4G).

We overexpressed IncSLC30A9a and IncSLC30A9b and performed the EdU incorporation assay to detect cell proliferation. Detection of overexpression efficiency showed that IncSLC30A9a and IncSLC30A9b expression was markedly increased (Figure 4A). The data showed that IncSLC30A9a reduced mitotic activity in adipocytes (Figure 4B). The results of flow cyt!metry were consistent with these findings (Figure 4C). Furthermore, CCK-8 detection showed that IncSLC30A9a markedly inhibited cell viability (Figure 4D). In addition, the proliferation-related markers, including cyclinD, cyclinE, and PCNA mRNA and protein levels were significantly reduced after cells were transfected with pcDNA- IncSLC30A9a (Figures 4E and 4F).

Further detection assays showed that IncSLC30A9b significantly reduced the expression of Bcl-2 (Figures 4G and 4H). We also found that overexpressed IncSLC30A9a and IncSLC30A9b increased the expression levels of three adipocyte markers, PPARγ, CEBPA, and FABP4, at day 6 (Figures 4I–4K). Consistent results were obtained with the Oil Red O staining assay (Figure 4L). These findings indicate that IncSLC30A9 may have an important function in adipocyte development.

**Overexpression of circFLT1 and IncCCPG1 Serves as a Competing Endogenous miRNA-93 Sponge to Attenuate Its Inhibition of IncSLC30A9**

Based on the high-throughput sequencing data of circRNAs and lncRNAs in Qinchuan bovine adipocytes, we randomly selected 12 differentially expressed circRNAs that may interact with miR-93. The results of real-time qPCR analysis showed that the expression of three circRNAs was much higher in preadipocytes compared with mature adipocytes (Figure S5A; Figure 5A). Furthermore, only circFLT1 (bta_circ_002673) cDNA was obtained via a divergent primer (Figure S5B; S.Z., unpublished data). The size and sequence of amplified PCR products were determined by Sanger sequencing (Figure 5B). We isolated total RNA of adipocytes treated with actinomycin D to analyze the stability and localization of circFLT1. The data illustrated that the half-life of circFLT1 surpassed 12 h. However, the half-life of linear FLT1 was less than 4 h (Figure 5C). We also screened and identified a significantly upregulated IncRNA, IncCCPG1, according to its host gene cell cycle progression 15 (Figure 5D). Through bioinformatics analysis, we found that IncCCPG1 has a large number of miR-93 binding sites. DNA AF4-CCPG1 IncRNA also exists in humans. The RACE assay showed that IncCCPG1 is a 2,397 nt transcript, and the CPC predicted that IncCCPG1 has extremely low coding potential (Figures S5C–S5E).

The semiquantitative PCR results demonstrated that circFLT1 and IncCCPG1 were mainly expressed in the cytoplasm (Figures 5E and 5F). Based on their subcellular location (in the cytoplasm), we speculated they employed the mechanism of miRNA adsorption to regulate gene expression. Therefore, we used the RNAhybrid tool to evaluate the capacity of circFLT1 and IncCCPG1 to bind to miRNAs, and the results revealed the presence miR-93 binding sites (Figures S5F and S5G). CircFLT1 overexpression significantly reduced the expression of miR-93 in preadipocytes, and IncCCPG1 knockdown significantly increased the expression of miR-93 in preadipocytes/adipocytes (Figures S5H–S5K). Moreover, the luciferase assay showed that miR-93 inhibited RLuc expression of psiCHECK2-circFLT1-WT and psiCHECK2-IncCCPG1-WT constructs (Figures 5G and 5H). The sensor assays further confirmed the above results (Figures S5L–S5K). To determine whether the potential mechanisms of circFLT1 and IncCCPG1 during adipogenesis were related to miR-93-mediated regulation of IncSLC30A9, we used psiCHECK2-IncSLC30A9 as a sensor. As expected, the results were consistent with previous findings (Figures 5L and 5M). circFLT1 overexpression significantly reduced the expression of IncSLC30A9, and IncCCPG1 knockdown significantly increased the expression of IncSLC30A9 (Figures S5L and S5M). Thus, circFLT1 and IncCCPG1 acted as molecular sponges to relieve the inhibitory effects of miR-93 on the expression of IncSLC30A9.

**Effects of circFLT1 and IncCCPG1 on Cell Proliferation, Apoptosis, and Differentiation**

To explore the functions of circFLT1 and IncCCPG1 in adipogenesis, we overexpressed circFLT1 and knocked down IncCCPG1 to detect cell proliferation (Figures 6A and 6B). The EdU incorporation assay showed that circFLT1 reduced the mitotic activity of adipocytes, whereas the
overexpression of miR-93 slightly reversed these effects (Figure 6C). In contrast, si-lncCCPG1 promoted cell proliferation (Figure 6D). The results of flow cytometry, CCK-8, real-time qPCR, and western blotting were all consistent (Figures 6E–6L). Considering that miR-93 can inhibit apoptosis, we further investigated whether circFLT1 and lncCCPG1 can affect the apoptosis of adipocytes by regulating miR-93. Notably, circFLT1 also reduced the expression of Bcl-2, which was increased after co-transfection with miR-93 (Figures 6M and 6N). However, lncCCPG1 had no significant effects on apoptosis (Figures 6O and 6P). Furthermore, circFLT1 overexpression increased the expression of PPARG, CEBPA, and FABP4, and miR-93 overexpression slightly reversed these effects (Figures 7A–7C). However, si-lncCCPG1 showed the opposite effect (Figures 7D–7F). Consistent results were obtained via the Oil Red O staining assay (Figure 7G and 7H). Furthermore, lncCCPG1 overexpression inhibited cell proliferation and promoted differentiation (Figure S6).

To explore the regulatory mechanism of lncCCPG1 at the transcriptional level, we predicted its activity with the aid of the UCSC Genome Browser. We found that H3K4me1 and H3K27ac were used in the upstream region of lncCCPG1 (Figure S7A). Therefore, five reporter genes containing different lncCCPG1 promoter fragments were constructed for luciferase detection; however, no promoter active region was detected (Figure S7B).

We then connected the upstream region (−1416~−721) to the enhancer region to detect its activity and found this region to show significant enhancer activity (Figure S7C). The combined predicted results from

Figure 3. IncSLC30A9 is a Target of miR-93
(A) HEK293T cells were cotransfected with the wild-type or mutant Sirt7/SLC30A9/PDGFRα dual-luciferase vector and PCDNA-miR-93, and the relative luciferase activity was analyzed 24 h after transfection. (B) Genomic structure of IncSLC30A9. (C) The 5′RACE and 3′RACE analyses demonstrated the full length of IncSLC30A9. (D) The RNA sequences of IncSLC30A9α/IncSLC30A9β were processed by the Coding Potential Calculator (CPC) program. (E and F) The mRNA expression of IncSLC30A9α and IncSLC30A9β was measured via RT-qPCR. (G) The PCDNA-miR-93 was cotransfected with pCK-lncSLC30A9 into HEK293T cells. Then, the relative luciferase activity was analyzed 24 h after the transfection. (H) The miR-93 sensor was cotransfected with the PCDNA-miR-93 and/or pCDNA-PPARG into HEK293T cells. Double luciferase activity analysis. (I) Distribution of IncSLC30A9 after separation of nucleus and cytoplasm. Values are presented as mean ± SEM for three biological replicates. *p < 0.05, **p < 0.001. N.S., not significant.
the UCSC and JASPAR databases showed that the FOXA1 and FOS protein binding sites might exist in this region. Following the overexpression of FOS and knockdown of FOXA1, we observed that FOS significantly enhanced the fluorescence activity of this region, while FOXA1 showed no significant change in the fluorescence activity in this same region (Figure S7/D). Further detection of changes in the expression of IncCCPG1 revealed that FOS significantly promoted the expression of IncCCPG1, while si-FOXA1 did not significantly alter the expression of IncCCPG1 (Figure S7/E). The transcription factor, FOS, can bind to the enhancer region of IncCCPG1 to promote the expression of IncCCPG1.

CircFLT1/IncCCPG1 Sponges the miR-93-IncSLC30A9-AKT/ FOS Axis to Regulate Adipocyte Proliferation and Differentiation

To further explore the mechanisms of circFLT1/IncCCPG1, the PI3K-AKT signaling pathway was screened using the Kyoto Encyclopedia of Genes and Genomes (Figure 8A). Moreover, IncSLC30A9a and IncSLC30A9b overexpression significantly inhibited AKT phosphorylation. However, no significant change was detected at day 6 of differentiation (Figure 8B). Based on these results, we hypothesized that IncSLC30A9 inhibits proliferation by inhibiting the activation of the AKT signaling pathway.

Studies have shown that some lncRNAs play a functional role by interacting with specific proteins. The nuclear localization of IncSLC30A9 suggests that lncRNA might play a regulatory role by interacting with proteins. Therefore, the potential IncSLC30A9 binding protein was predicted using the RNA-Protein Interaction Prediction (RPISeq) tool. The results showed that IncSLC30A9 can bind the FOS protein (Figure 8C). The FOS overexpression vector was also transfected into adipocytes, and the IncSLC30A9 expression level was significantly upregulated (Figure 8D). However, the overexpressed IncSLC30A9a and IncSLC30A9b vectors were transfected into fat cells, and the FOS gene showed no significant change in mRNA levels but showed significantly increased protein levels (Figures 8E and 8F).

The FOS protein was also expressed at different periods of fat differentiation in Qinshuan cattle, showing a tendency to increase and then decline; its highest expression was observed on day 6 of differentiation (Figure 8G). Previous studies have shown that FOS can bind to the PPARG promoter. In differentiated adipocytes, the binding activity of FOS to the PPARG promoter was significantly increased, revealing the potential role of FOS in the transcriptional regulation of PPARG in adipocyte differentiation.9

We further examined the effects of FOS on the proliferation and differentiation of adipocytes. The real-time qPCR and western blotting analyses showed that FOS had no significant effects on cell proliferation but was able to significantly promote cell differentiation. More lipid droplets were detected in the pcDNA-FOS-transfected adipocytes via Oil Red O staining on day 6 of differentiation (Figure S8). Overall, these results indicated that circFLT1 and IncCCPG1 regulate the proliferation and differentiation of bovine adipocyte by the circFLT1/IncCCPG1-miR-93-IncSLC30A9-AKT/FOS axis (Figure 9).

DISCUSSION

Previous studies have underlined the significance of ncRNAs in adipogenesis, especially in regulating the proliferation and differentiation of adipocytes.16,17 The expression of ncRNAs is specific to cells and tissues and may differ between various stages of differentiation. This observation suggests that ncRNAs may be fine-tuning factors of cell fate. Research on ncRNAs has been mainly focused on tumorigenesis and the pluripotency of stem cells. However, much remains to be learned about the functions of ncRNAs in adipocyte formation and particularly the regulation of adipocyte differentiation.

In this study, we found three adipocyte differentiation-related ncRNAs (circFLT1, IncCCPG1, and IncSLC30A9) that facilitate adipogenesis. By functioning as competing endogenous RNAs (ceRNAs), circFLT1 and IncCCPG1 overexpression released the inhibitory effects of miR-93 on IncSLC30A9 through the adsorption of miR-93 and thereby increased the expression of IncSLC30A9. This increase in IncSLC30A9 expression further activates the IncSLC30A9-AKT/FOS axis to regulate adipocyte proliferation and differentiation.

Previous work suggests that miRNAs play an important role in regulating biological processes.14,15 However, the functions of many miRNAs in bovine fat production are not entirely understood. In this study, a miRNA was identified from high-throughput miRNA sequencing data from various differentiation intervals in Qinshuan cattle. Compared with other miRNAs, miR-93 expression was significantly upregulated at day 6 of differentiation. Therefore, we speculated that miR-93 may regulate adipogenesis.

Previous studies have shown that miR-93 can regulate many cancers, but, to our knowledge, there are no reports on its effects on bovine fat development.16,17 Cioffi et al. reported that miR-93 controls obesity by inhibiting Sirt7 and Tbx3.7 The current study showed that miR-93 regulates both the proliferation and differentiation of bovine adipocytes. Consider that miR-93 is highly conserved among cows, mice, and humans. Therefore, we also checked its function in 3T3-L1 cells (a well-characterized model of mouse adipogenesis). We noted that the findings were entirely consistent with those of bovine adipocyte studies. These results may
provide a new theoretical basis for the functions of miR-93 in adipogenesis. To our knowledge, this is the first study to identify miR-93 as a potential miRNA that can negatively regulate bovine fat production.

TargetScan analysis showed that miR-93 can directly target IncSLC30A9. The accuracy of this result was further verified by the dual-luciferase reporting system, and the molecular mechanism of miR-93 regulation of fat generation was preliminarily explored. To explore the function of IncSLC30A9 in bovine adipocytes, we overexpressed IncSLC30A9 in vitro and transfected it into bovine adipocytes. The overexpressed IncSLC30A9 may promote adipocyte differentiation and inhibit proliferation by increasing the expression of PPARG,
CEBPA, and FABP4, which is consistent with the function of overexpressed miR-93. Although the regulatory effects of miR-93 on fat production in Qinchuan cattle have been preliminarily revealed, additional studies on its mechanism of action are needed.

Previous studies have shown that circRNAs and lncRNAs can participate in post-transcriptional regulation through the competitive binding of miRNAs. Some studies have shown that circRNAs can bind to many miRNAs to participate in normal growth and development. However, only a few bovine circRNAs, such as circSNX29, have been shown to play a key role in growth. Studies on the role of circRNAs in fat development have been rarely conducted. Although lncRNAs reportedly regulate adipocyte differentiation, the details of many regulatory mechanisms remain unclear.

In this study, sequencing data on circRNAs and lncRNAs revealed that circFLT1 was downregulated, and lncCCPG1 upregulated, during adipogenic differentiation. Nucleosomal separation revealed that both circFLT1 and lncCCPG1 were located mainly in the cytoplasm. Therefore, we analyzed the potential miRNA of circFLT1 and lncCCPG1 using bioinformatics tools and confirmed that they both had direct adsorption targets in miR-93 bovine fat cells using the dual-luciferase reporting system.

In addition, we found that circFLT1 and lncCCPG1 play a regulatory role in the proliferation of fat cells by regulating the removal of miR-93 from fat cells. More importantly, we found that the overexpression of circFLT1 and lncCCPG1 significantly promotes adipocyte differentiation, while the overexpression of miR-93 slightly reverses these effects. In addition, interference with lncCCPG1 significantly promotes the expression of miR-93 and further inhibits adipocyte differentiation. Therefore, the overexpression of circFLT1 and lncCCPG1 leads to reduced activity of miR-93, disabling the inhibitory effect of miR-93 on the downstream target gene lncSLC30A9.

Therefore, circFLT1 and lncCCPG1 may indirectly increase the mRNA and protein levels of PPARG, CEBPA, and FABP4 and thereby promote adipocyte differentiation. In sum, these results further confirm that circFLT1 and lncCCPG1 may play a role in regulating the proliferation and differentiation of adipocytes through the competitive binding of miR-93. However, the present study only preliminarily discussed whether circFLT1 and lncCCPG1 can competitively adsorb miR-93 to regulate the proliferation and differentiation of adipocytes. Further in-depth studies on systematic mechanisms are necessary.

Previous studies have reported that some signaling pathways also participate in the regulation of cell proliferation and differentiation and that circRNAs and lncRNAs in the nucleus may play a role by interacting with proteins. The present study showed that circFLT1/lncCCPG1 could significantly increase the expression of its downstream target gene, lncSLC30A9, through the competitive adsorption of miR-93, leading to the question as to whether lncSLC30A9 can also activate certain signaling pathways or recruit certain proteins to function within fat cells.

Generally, adipogenic differentiation is rigidly regulated by the activity of transcription factors, which can either activate or inhibit each other. The C/EBP family members (C/EBPA, C/EBPB, and C/EBPD) and PPARG are major transcription factors that are involved in adipogenesis. The present study showed that overexpression of circFLT1 or lncCCPG1 in bovine fat cells could disarm the inhibitory effects of miR-93 on lncSLC30A9.

Furthermore, lncSLC30A9 can be regulated by the recruitment of the FOS protein. The FOS protein can reportedly bind to the PPARG promoter. In differentiated adipocytes, the binding activity of c-fos to the PPARG promoter is significantly increased, indicating the potential role of c-fos in the transcriptional regulation of PPARG in adipocyte differentiation. The potential lncSLC30A9 binding proteins were predicted using the RPISeq algorithm, revealing that lncSLC30A9 could bind the FOS protein.

In addition, the FOS overexpression vector was transfected into adipocytes, and the lncSLC30A9 expression level was significantly upregulated, whereas the overexpression of lncSLC30A9a and lncSLC30A9b was significantly upregulated. The FOS gene showed no significant change at the mRNA level but was significantly increased at the protein level. More importantly, overexpression of circFLT1 or lncCCPG1 may inhibit proliferation by inhibiting the AKT signaling pathway and, in combination with miR-93, may promote differentiation by recruiting the FOS protein to the PPARG promoter.

In sum, we identified the adipogenesis-associated circFLT1 and lncCCPG1 and found that they can promote adipocyte differentiation and inhibit proliferation in vitro. Specifically, overexpression of circFLT1 or lncCCPG1 can act as a ceRNA, adsorb miR-93 to remove its inhibitory effects on lncSLC30A9, and increase the expression of lncSLC30A9. Furthermore, lncSLC30A9 inhibits proliferation by inhibiting the activation of the AKT signaling pathway and promotes differentiation by recruiting the FOS protein to the PPARG promoter. However, additional studies are needed to determine whether circFLT1 and lncCCPG1 are associated with other regulatory processes and whether their regulatory approaches to adipocyte differentiation are conserved in humans and mice.
MATERIALS AND METHODS

Sample Preparation

All animal experiments in this study were approved by the Faculty Animal Policy and Welfare Committee of Northwest A&F University (No. NWAFAC1008). The care and use of experimental animals were in full compliance with local animal welfare laws, guidelines, and policies.

Tissue samples were collected from the slaughterhouse in Xi’an. Subcutaneous adipose tissue was resected and immediately placed in...
Dulbecco’s modified Eagle’s medium at 37°C and taken back to the laboratory for primary adipose cell separation.

**Cell Culture**

Adipocytes were isolated from bovine subcutaneous adipose tissue. The specific separation was performed following the methods of a previous study.²⁴ The cells were seeded in a medium containing 20% fetal bovine serum and 1% antibiotics in 5% CO₂ at 37°C. HEK293T (human embryonic kidney 293) cells and 3T3-L1 cells (a preadipocyte cell line derived from the Swiss 3T3 mouse fibroblast cell line) were cultured in 10% fetal bovine serum and 1% penicillin-streptomycin, which were provided by our laboratory.

**RNA Isolation and Real-Time qPCR**

Total RNA from tissues and cell lysates was isolated using the TRIzol reagent and was stored at −80°C (Takara, Dalian, China).²⁵ Real-time qPCR was conducted using the SYBR green detection dye and CFX96 Real-Time PCR Detection System. The PCR protocol was similar to that described by Li et al.²⁶ Primer information is presented in Table S1. The 2^−ΔΔCt method was used to analyze the relative expression levels of real-time qPCR data.

**RACE**

RACE amplification was conducted to determine the complete fragments of lncCCPG1 and lncSLC30A9 using the SMARTer RACE cDNA Amplification Kit (Clontech, Palo Alto, CA, USA). Total
RNA was obtained from bovine adipocytes per the manufacturer’s instructions.

**Plasmid Construction**

The full lengths of circFLT1, lncCCPG1, and lncSLC30A9, including miR-93, were cloned into pLCDH-circRNA or pcDNA3.1(+) vectors, respectively. The circFLT1 and lncCCPG1 3’ UTR fragments were amplified by PCR and included the miR-93-binding site. The fast enzymes, Not I and Xho I, were used to connect two fragments to the psiCHECK-2 (pCK) vector, which was then ligated by T4 DNA ligase. We consistently generated the plasmids of psiCHECK2 (pCK)-circFLT1-WT, psiCHECK2 (pCK)-lncCCPG1-WT, psiCHECK2 (pCK)-lncSLC30A9-WT, psiCHECK2 (pCK)-Sirt7-WT/Mut, and psiCHECK2(pCK)-PDGFRA-WT/Mut. The wild-type fragment was directly amplified by PCR amplification, including the miR-93-binding site. The seed sequence was randomly mutated to prevent the binding of miR-93, and the mutant sequence was designed with the reverse primer. Primer information is provided in Table S1.

**Luciferase Activity Assay**

The HEK293T cells were inoculated in 96-well plates. When cell fusion was approximately 50%, cells were co-transfected with miR-93 and pCK-circFLT1-Wild or pCK-lncCCPG1-Wild. The cells were then cultured in twice the volume of medium for 24 h. The miR-93 sensor was constructed by inserting two miR-93 complementary sequences into the pCK vector. Similarly, adipocytes were inoculated in 96-well plates and co-transfected with a wild-type construct of lncSLC30A9/lncSLC30A9/Sirt7/PDGFRA with or without miR-93. Luciferase activity was determined per the manufacturer’s instructions for the luciferase reporter assay kit (Promega).

**Cell Proliferation Assay**

In the CCK-8 assay, the preadipocytes were seeded into a 96-well plate and further cultured until the cell density was approximately 50%. Plasmids (100 ng) were then transfected into the cells and cultured for 24 h with double complete medium. When the cell density reached approximately 75%, 10 μL of CCK-8 reagent was added to each well, and cells were cultured for 2–4 h. After gentle shaking, the optical density was immediately detected at a wavelength of 450 nm for further statistical analyses. In the EdU assay, preadipocytes were inoculated in a 48-well plate. When the cell density was 50%, plasmids (200 ng) were transfected into the cells, which were then cultured for 24 h. The EdU medium was added to each well, and cells were fixed after 3 h of culture. Cells were detected with the aid of a microscope. For the cell cycle assay, preadipocytes were cultured in a 60-cm² culture dish, to which 4 mL of medium was added. The cells were treated and transfected with plasmids (3 μg). After 24 h, adipocytes were fixed with 70% ethanol (ethanol:PBS = 7:3) overnight at −20°C. The cell suspension was evaluated with a cell cycle testing kit.

**Adipogenic Differentiation and Oil Red O Staining**

When cell confluence reached 100%, the medium was replaced with the induced differentiation medium. After 2 days, cells were incubated in the same differentiation medium, which was changed every 2 days. After adipocyte differentiation was achieved, the medium was discarded, and cells were washed with PBS. Cells were fixed with 4% paraformaldehyde and left at room temperature for 30 min. The 4% paraformaldehyde solution was then discarded, and cells were washed three times and stained for 1 h in Oil Red O solution. The Oil Red O dye was then discarded, and the cells were immediately washed four times with PBS. Images were captured under a light microscope.
Western Blotting Analysis
After the medium in the cell culture dish was discarded, the cells (about 10^6) were washed with PBS, digested with trypsin, and collected into a 1.5-mL centrifuge tube. After centrifugation, the supernatant was removed. The radioimmunoprecipitation assay cell lysate containing the phenylmethanesulfonyl fluoride protease inhibitor was added at a ratio of 4:1 (Solarbio, Beijing, China). The protein was heated with sodium dodecyl sulfate (SDS)-loading buffer to 98°C for 10 min. The samples were added to SDS-polyacrylamide gel for electrophoretic separation and then transferred to 0.2-μm polyvinylidene difluoride (PVDF) membranes. After electrophoresis, the PVDF membranes were removed, washed with Tris-buffered saline + Tween 20 (TBST) solution for 10 min, and sealed with sealant for 2 h. The membranes were again washed three times with TBST solution, each time for 10 min. The membranes were incubated overnight with primary antibodies at 4°C and then washed three times with TBST. The PVDF membrane was then incubated with the secondary antibody at room temperature for 2 h. Details of the antibodies are presented in Table S2.

Statistical Analysis
GraphPad Prism 8.0 (GraphPad, USA) and SPSS 24.0 (IBM Corp., NY, USA) were used for data analyses. All data were analyzed using one-way ANOVA or t tests. When p < 0.05, the difference was considered statistically significant. Data were presented as mean ± SEM.

SUPPLEMENTAL INFORMATION
Supplemental Information can be found online at https://doi.org/10.1016/j.omtn.2020.09.011.

AUTHOR CONTRIBUTIONS
X.L. and Z.K designed research. Z.K., S.Z., E.J., X.W., and Z.W. performed experiments and analyzed data. Z.K. wrote the paper. E.J. and X.W. contributed new analytic tools. X.L. and H.C. helped modify the language of this manuscript.

CONFLICTS OF INTEREST
The authors declare no competing interests.

ACKNOWLEDGMENTS
This work was supported by the National Natural Science Foundation of China (No. 31872331; No. 31672400). We greatly appreciated the Life Science Research Core Services (LSRCS) of Northwest A&F University (Northern Campus) for providing us with the platform.

REFERENCES
1. Ali, A.T., Hochfeld, W.E., Myburgh, R., and Pepper, M.S. (2013). Adipocyte and adipogenesis. Eur. J. Cell Biol. 92, 229–236.
2. Berry, D.C., Stenesen, D., Zeve, D., and Graff, J.M. (2013). The developmental origins of adipose tissue. Development 140, 3939–3949.
3. Cristancho, A.G., and Lazar, M.A. (2011). Forming functional fat: a growing understanding of adipocyte differentiation. Nat. Rev. Mol. Cell Biol. 12, 722–734.
4. White, U.A., and Stephens, J.M. (2010). Transcriptional factors that promote formation of white adipose tissue. Mol. Cell. Endocrinol. 318, 10–14.
24. Li, M.X., Sun, X.M., Zhou, Y., Wei, X.F., Sun, Y.J., Lan, X.Y., Lei, C., and Chen, H. (2015). Nicotinamide and resveratrol regulate bovine adipogenesis through a SIRT1-dependent mechanism. J. Funct. Foods 18, 492–500.

25. Li, W., Liu, D., Tang, S., Li, D., Han, R., Tian, Y., Li, H., Li, G., Li, W., Liu, X., et al. (2019). A multiallelic indel in the promoter region of the Cyclin-dependent kinase inhibitor 3 gene is significantly associated with body weight and carcass traits in chickens. Poult. Sci. 98, 556–565.

26. Li, W.Y., Jing, Z.Z., Cheng, Y.Y., Wang, X.N., Li, D.H., Han, R.L., Li, W., Li, G., Tian, Y., Liu, X., et al. (2020). Analysis of four complete linkage sequence variants within a novel IncRNA located in a growth QTL on chromosome 1 related to growth traits in chickens. J. Anim. Sci 98, skaa122.

27. Song, C., Yang, J., Jiang, R., Yang, Z., Li, H., Huang, Y., Lan, X., Lei, C., Ma, Y., Qi, X., and Chen, H. (2019). miR-148a-3p regulates proliferation and apoptosis of bovine muscle cells by targeting KLF6. J. Cell. Physiol. 234, 15742–15750.