Circular RNA TTN Acts As a miR-432 Sponge to Facilitate Proliferation and Differentiation of Myoblasts via the IGF2/PI3K/AKT Signaling Pathway

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Circular RNAs (circRNAs) are ubiquitous endogenous RNA found in various organisms that can regulate gene expression in eukaryotes. However, little is known about potential roles for circRNAs in muscle development. We analyzed circRNA sequencing data of bovine skeletal muscle tissue and found differential expression of circTitin (circTTN) in fetal and adult bovine muscle tissue. We then further studied the role of circTTN in bovine muscle development. Overexpression and inhibition of circTTN together elicited its promoting roles in proliferation and differentiation of bovine primary myoblasts. Mechanistically, circTTN showed interaction with miR-432 by luciferase screening and RNA immunoprecipitation (RIP) assays. Additionally, miR-432 is a regulator of insulin-like growth factor 2 (IGF2), as indicated by luciferase activity, quantitative real-time PCR, and western blotting assays. Increased miR-432 expression inhibited the expression of IGF2, but this effect was remitted by circTTN. Conclusively, our results showed that circTTN promoted proliferation and differentiation of bovine primary myoblasts via competitively combining with miR-432 to activate the IGF2/phosphatidylinositol 3-kinase (PI3K)/AKT signaling pathway.

INTRODUCTION
Circular RNAs (circRNAs) are widespread and diverse endogenous RNAs with covalently closed continuous loop structures, generated by back-splicing of a single pre-mRNA, making these RNAs more stable than linear RNA.1,2 The first circRNA was identified in human cells in the early 1990s.3 Although circRNAs were discovered decades ago, they were originally considered byproducts of spliceosome-mediated splicing errors and thought to lack any significant function.4 However, recent high-throughput sequencing and novel computational approaches have identified a large number of circRNAs within the transcriptome, suggesting potential roles for these RNAs in development.5–9 These circRNAs have extremely abundant microRNA (miRNA) binding sites and thus act as a competitive endogenous RNA (ceRNA) to regulate miRNA expression.10,11 For example, two circRNAs, ciRS-7/CRI4as and Sry, were reported to contain multiple miRNA binding sites.6,12 Besides, it has been proposed that some circRNAs may “sponge” other factors, such as RNA binding proteins (RBPs).13 Recently, a novel subclass of circRNAs has been described as exon-intron circRNAs (EicRNAs). These EicRNAs interact with RNA polymerase II and U1 small nuclear ribonucleoproteins (snRNP) and can act in cis to induce host-gene transcription in the nucleus.14

Skeletal muscle is one of the most dynamic and plastic tissues in the human body, playing a crucial role in movement, metabolism, and homeostasis, accounting for about 40% of adult body weight.15,16 Skeletal muscle fibers are formed by the fusion of multiple mononuclear myoblasts17 in a process that is regulated by multiple factors during myogenesis. Myogenesis is regulated by myogenic regulatory factors (MRFs)18,19 and various noncoding RNAs, such as miRNAs and long noncoding RNAs (lncRNAs).20–23 Recent work has examined potential roles for circRNAs during myogenesis in a variety of organisms. For example, the mouse ortholog circZfp609 acts as a decoy for miR-194-5p to promote expression of BCLAF1 and thereby suppress myoblast differentiation;24 circFGFR4 inhibits bovine primary myoblast differentiation and apoptosis by sponging miR-107;25 and chicken circSVIL promotes myoblast proliferation and differentiation by sponging miR-203 and increasing expression of targets c-JUN and MEF2C.26 Studies also indicated that circFUT10 and circLMO7 could regulate myogenesis.27,28 Interestingly, circZNF609 is an endogenous circRNA that may be associated with polyribosomes for translation, thus promoting myoblast proliferation.29 Overall, additional study on the regulation by circRNAs of bovine skeletal muscle development is of great significance for the beef production industry.

To explore the role of circRNAs in bovine skeletal muscle development, we obtained and analyzed the circRNA sequencing data of bovine muscle tissue from NCBI: GSE87908 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=atglausktpsjlel&acc=GSE87908).

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We noticed that circTitin (circTTN) was differentially expressed in fetal and adult bovine muscle tissues and selected it as a candidate circRNA. The full length of circTTN was 675 nt and was named after its host gene Titin (TTN), which is located on chromosome 2 (Text S1). Our results showed that circTTN is able to contribute dramatically to bovine primary myoblast proliferation and differentiation. Further examination revealed that circTTN acts as a sponge of miR-432 and activates the insulin-like growth factor 2 (IGF2)/phosphatidylinositol 3-kinase (PI3K)/AKT signaling pathway. Our research may provide new insights into complex RNA regulation with implications for the beef cattle industry in China.

RESULTS

Characterization of Bovine circTTN

To confirm the circular nature of circTTN, we designed two pairs of primers, oriented in the divergent direction and in the convergent direction (Figure 1A), and then used amplified cDNA (RNase R treated) or genomic DNA (gDNA) as templates for amplification. The results showed that the divergent primers amplified the expected band from the cDNA but not from the gDNA. With the use of the convergent primers, products were amplified from either the cDNA or the gDNA samples (Figure 1B). We verified the putative circTTN junction by Sanger sequencing (Figure 1C). RNase R assay was performed to determine circTTN subcellular localization. Blue indicates nuclei stained with DAPI; green indicates the RNA probe that recognizes circTTN. Scale bar, 50 μm. (H) The expression of circTTN in the cytoplasm and nucleus was detected by semiquantitative PCR. (I) The expression of circTTN in myoblasts differentiated for 0, 1, 3, and 5 days is shown. Data are presented as means ± SEM for three individuals.

Figure 1. Characterization of Bovine circTTN

(A) Schematic view illustrating the design of primers for circTTN. A divergent primer was also used in quantitative real-time PCR. (B) A convergent primer and divergent primer were used to confirm the circular nature of circTTN. (C) The circular junction of circTTN was identified by using a divergent primer on Sanger sequencing. (D) RNase R detected the presence of circTTN. (E) The expression of circTTN and TTN mRNA in myoblasts treated with RNase R was determined by quantitative real-time PCR. (F) Quantitative real-time PCR for the abundance of circTTN and TTN mRNA in bovine primary myoblasts treated with Actinomycin D at the indicated time points. (G) RNA-FISH assay was performed to determine circTTN subcellular localization. Blue indicates nuclei stained with DAPI; green indicates the RNA probe that recognizes circTTN. Scale bar, 50 μm. (H) The expression of circTTN in the cytoplasm and nucleus was detected by semiquantitative PCR. (I) The expression of circTTN in myoblasts differentiated for 0, 1, 3, and 5 days is shown. Data are presented as means ± SEM for three individuals.

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exhibited a half-life of <4 h (Figure 1F). To investigate the cellular localization of circTTN, we performed RNA fluorescent in situ hybridization (RNA-FISH) assay with an RNA probe that specifically recognizes the back-splicing junction region of circTTN to determine its subcellular localization (Figure 1G). We also detected the expression of circTTN in the nucleus and cytoplasm by semiquantitative PCR and nucleoplasmic separation (Figure 1H). These two results both suggested that circTTN is mainly localized in the cytoplasm, suggesting that circTTN may regulate gene expression at the post-transcriptional level. We found that circTTN is generally expressed in various fetal (Figure S5A) and adult cattle tissues (Figure S5B) but showed upregulated expression in fetal, calf, and adult bovine muscle tissue (Figure S5C). The expression of circTTN was higher in the differentiation period compared with the level during the proliferation period and was also upregulated during myoblast differentiation (Figure 1I). Taken together, our results suggested that circTTN may be a positive regulating and stable circRNA for muscle development.

Effect of circTTN on Bovine Primary Myoblast Proliferation
To determine the role of circTTN in the proliferation of bovine primary myoblasts, we transfected PCD2.1-circTTN into myoblasts to overexpress circTTN significantly (Figure 2A). To assess its function, we used cell counting kit-8 (CCK-8), 5-ethyl-2′-deoxyuridine (EdU), flow cytometry, quantitative real-time PCR, and western
First, we detected the effect of circTTN on expression of the cell proliferation-related genes Cyclin D1 and proliferating cell nuclear antigen (PCNA) and found that circTTN significantly increased the expression of these genes at both the mRNA and protein levels (Figures 2B and 2C). The cell-cycle analysis revealed that overexpression of circTTN increased the proportion of myoblasts in the S phase and decreased the number of cells in the G0/G1 phase (Figures 2D and 2E and S1). The results of the EdU staining also showed a higher number of EdU-positive cells relative to that in the control group. Additionally, both the CCK-8 assay (Figure 2H) and the EdU-staining positive cell population (Figures 2F and 2G) were lower in the si-circTTN-treated cells compared to those in the control. These results demonstrated that circTTN promotes proliferation of bovine primary myoblasts.

Next, small interfering RNAs (siRNAs) were designed to target the back-splicing junction of circTTN. After bovine primary myoblasts were transfected with siRNA1, siRNA2, and siRNA3, the expression of circTTN was detected by quantitative real-time PCR, and we selected the most effective siRNA2 (Figure 3A). We found that the knockdown of circTTN significantly decreased the expression of the cell proliferation-related genes PCNA, cyclin-dependent kinase 2 (CDK2), and Cyclin D1 at both the mRNA and protein levels (Figures 3B and 3C). The cell-cycle analysis revealed that circTTN siRNAs (si-circTTN) reduced the proportion of myoblasts in the S phase and increased the number of cells in G0/G1 and G2/M phases (Figures 3D and 3E and S2). Additionally, both the CCK-8 assay (Figure 3H) and the EdU-staining positive cell population (Figures 3F and 3G) were lower in the si-circTTN-treated cells compared to those in the control. These results demonstrated that circTTN promotes proliferation of bovine primary myoblasts.
Effect of circTTN on Bovine Primary Myoblast Differentiation

To investigate the involvement of circTTN in bovine myoblast differentiation, the expression levels of established myogenic differentiation markers, myogenic factor 5 (MyF5), myogenic differentiation 1 (MyoD1), myogenin (MyoG), and myosin heavy chain (MyHC), were detected in primary cattle myoblasts treated with PCD2.1-circTTN for 4 days during differentiation. As shown in Figure 4A, the overexpression of circTTN efficiently enhanced the expression of MyF5, MyoD1, MyoG, and MyHC at the mRNA level (Figure 4A).

Moreover, the protein levels of MyoD1, MyoG, and MyHC were also significantly increased (Figures 4B and 4C). In contrast, we used a specific siRNA (si-circTTN) targeting circTTN to examine the influence of circTTN on bovine myoblasts differentiation. The knockdown of circTTN significantly suppressed the expression of myogenic markers MyF5, MyoD1, MyoG, and MyHC at the mRNA level (Figure 4E), as well as that of MyoD1, MyoG, and MyHC proteins (Figures 4F and 4G). As shown in the immunofluorescence assay results presented in Figures 4D and 4H, circTTN overexpression facilitated
not only the expression of MyoD1 and MyHC but also myotube formation (Figure 4D); however, the knockdown of circTTN reduced expression of MyoD1 and MyHC and myotube formation (Figure 4H). Collectively, these results indicated that circTTN acts to promote bovine myoblast differentiation.

**circTTN Acts as a Sponge for miR-432**

Our results showed that circTTN can promote the proliferation and differentiation of bovine myoblasts. Recent work has suggested that different classes of noncoding RNAs may interact with each other, and this interaction regulates their expression. CircRNAs are non-coding RNAs that can act as competing endogenous sponges to regulate the expression of miRNAs. Thus, we next analyzed the complementarity of the circTTN sequence with miRNA using the RNAhybrid bioinformatics program. We found that circTTN has perfect target site complementarity with miR-432, and the two binding sites are shown in Figure 5A. The luciferase assay revealed that miR-432 significantly inhibited Renilla luciferase (RLuc) activity in HEK293T cells (Figure 5B). We also generated a miR-432 sensor by inserting two copies of the miR-432 complementary sequence into the psiCHECK-2 vector (Figure 5C). The result showed that miR-432 dramatically reduced RLuc activity of the miR-432 sensor in HEK293T cells. However, overexpression of the circTTN partially restored the reduced...
Rluc activity induced by binding miR-432 in a dose-dependent manner (Figure 5D). We next performed an RNA immunoprecipitation (RIP) experiment, and quantitative real-time PCR showed successful enrichment of miR-432 (Figure 5E) and circTTN (Figure 5F) in the Argonaute 2 (Ago2) pull-down samples compared to the negative control, suggesting that circTTN binds to miR-432 via the Ago2 protein. Next, we transfected PCD2.1-circTTN into bovine primary myoblasts and significantly decreased the abundance of miR-432 (Figure 5G). There was higher expression of miR-432 during the proliferation period compared with the level in the differentiation period. Additionally, expression of miR-432 was downregulated during myoblast differentiation (Figure 5H). Interestingly, the expression levels of miR-432 and circTTN showed the opposite trend. Altogether, these findings indicated that circTTN interacts with miR-432.

Effects of miR-432 on Proliferation and Differentiation of Bovine Primary Myoblasts

Next, we elucidated the functional role of miR-432 in proliferation and differentiation in bovine primary myoblasts. For cultured bovine primary myoblasts, we transfected miR-432 mimic into cells and confirmed significant overexpression of miR-432 (Figure 6A). The forced expression of miR-432 efficiently attenuated proliferation marker genes PCNA and Cyclin D1 at the mRNA and proteins levels, and these effects were abolished by overexpression of circTTN (Figures 6B and 6C). Cell-cycle analysis revealed that the miR-432 mimic reduced the number of myoblasts in the S and G2/M phases and increased the proportion of cells in the G0/G1 phase, suggesting that miR-432 may inhibit bovine myoblast proliferation (Figures 6D and 6E and S3). Detection of CCK-8 and EdU showed that

Figure 6. Effect of miR-432 on Proliferation of Bovine Primary Myoblasts

(A) The expression level of miR-432 in bovine primary myoblasts transfected with miR-432 mimic is shown. (B and C) The expression of PCNA and Cyclin D1 was detected by quantitative real-time PCR (B) and western blotting (C). β-Actin acts as an internal control gene. (D and E) Bovine primary myoblasts were transfected with miR-432 mimic and/or PCD2.1-circTTN, and cell phases were analyzed by flow cytometry (D) and counted (E). (F and G) The number of EdU-positive bovine primary myoblasts was detected by EdU after transfection of miR-432 mimic and/or PCD2.1-circTTN (F) and counted using ImageJ (G). EdU staining (red) for positive cells. DAPI staining (blue) for the cell nuclei. Scale bars indicate 200 μm. (H) Cell proliferation index was detected by cell counting kit-8 (CCK-8) assay. Data are presented as means ± SEM for three individuals. *p < 0.05; **p < 0.01.

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overexpression of miR-432 significantly downregulated cell proliferation (Figures 6F–6H). However, we found that cotransfection of miR-432 mimic and PCD2.1-circTTN into cattle primary myoblasts resulted in negligible effects on cell proliferation, suggesting that circTTN relieved the effect of miR-432 on cell proliferation (Figures 6A–6H).

To assess further the effect of miR-432 on myoblast differentiation, we transfected miR-432 mimic or PCD2.1-circTTN into cattle primary myoblasts for 4 days of differentiation. After overexpression of miR-432, the results showed decreased mRNA expression of myogenic differentiation marker genes MyF5, MyoD1, MyoG, and MyHC (Figure 7A), with significantly decreased protein levels of MyoD1, MyoG, and MyHC (Figures 7B and 7C). However, cotransfection with PCD2.1-circTTN and miR-432 showed somewhat restored expression of MyF5, MyoD1, MyoG, and MyHC (Figures 7A–7C). Immunofluorescence assay revealed that miR-432 inhibited MyoD1, MyHC expression, and myotube formation, but this effect was reversed to some extent by circTTN overexpression (Figure 7D).

In summary, our above results confirmed that miR-432 could negatively regulate proliferation and differentiation of bovine primary myoblasts, but these effects can be eliminated by overexpression of circTTN. Additionally, these results also demonstrated that circTTN promotes myogenesis by binding miR-432.

circTTN Serves as a ceRNA for miR-432 to Attenuate Its Inhibitory IGFR

To elucidate the potential molecular regulatory mechanism of miR-432 to inhibit proliferation and differentiation of bovine primary myoblasts, we used the bioinformatics software program, TargetScan 7.2. IGF1 and IGF2 were considered as two potential target genes of miR-432 (Figure 8A). Next, we constructed IGF1-3′ UTR and IGF2-3′ UTR (wild-type [WT] and mutant-type [MUT]) luciferase reporter vectors containing potential binding sites of miR-432. The two plasmids were separately cotransfected with miR-432 mimic into HEK293T cells. The luciferase activity of IGF2-WT was markedly reduced by miR-432 but had no effect on IGF2-MUT (Figure 8B). Moreover, the overexpression of circTTN recovered the reduced luciferase activity induced by miR-432 (Figure 8D). Similarly, we found that miR-432 markedly suppressed the expression of IGF2 at the protein level in proliferation and differentiation stages, but these effects were abrogated by forced expression of circTTN (Figures 9C and 9F). Additionally, the luciferase activities of IGF1-WT and IGF1-MUT were unaffected by miR-432 (Figure 8C). Together, these findings revealed that circTTN acts as a bait to alleviate miR-432-mediated inhibition of IGF2.

Effects of IGF2 on Proliferation and Differentiation of Bovine Primary Myoblasts

Next, we used a specific siRNA of IGF2 (si-IGF2) to examine the roles of IGF2 in myoblast proliferation and differentiation. After
transfection with si-IGF2 for myoblasts, the IGF2 mRNA and protein expression levels were significantly reduced (Figures 5A and SSC and S6B). We also detected the expression of PCNA, CDK2, and CyclinD1 by quantitative real-time PCR and western blot assays and found that the knockdown of IGF2 efficiently attenuated the expression of these genes Figures S5B and SSC. Cell-cycle analysis revealed that si-IGF2 reduced the proportion of myoblasts in the S and G2/M phase and increased the number of cells in the G0/G1 phase (Figures S4 and S5D and S5E). Moreover, the results of the EdU-staining positive cell population (Figures S5F and S5G) and CCK-8 (Figures S4 and S5D and S5E). Moreover, the results of the EdU-staining positive cell population (Figures S5F and S5G) and CCK-8 assay (Figure S5H) were lower than those of the control.

The suppression of IGF2 also significantly reduced the expression of myogenic differentiation markers Myf5, MyoD1, MyoG, and MyHC at the mRNA level (Figure S6A). Additionally, the expression of MyoD1, MyoG, and MyHC was significantly decreased at the protein level (Figures S6B and S6C). Consistently, similar results were obtained by immunofluorescence assay (Figure S6D). Taken together, these data suggested that IGF2 promotes cattle myoblast proliferation and differentiation.

circTTN Regulates the Activity of the IGF2/PI3K/AKT Signaling Pathway

Previous studies had shown the essentiality of IGF2 for muscle growth and development. In addition, IGF2 has closely related to the PI3K/AKT signaling pathway. Therefore, we hypothesized that circTTN may promote proliferation and differentiation of bovine myoblasts through the IGF2/PI3K/AKT signaling pathway. To test our hypothesis, western blot analysis was performed to analyze the changes of relevant gene (IGF2, IRS1, PI3K, PDK1, AKT) levels in the IGF2/PI3K/AKT pathway during the proliferation and differentiation phases. For the proliferation stage, the forced expression of circTTN remarkably increased the expression of IGF2, IRS1, PI3K, PDK1, and AKT, whereas the knockdown of circTTN decreased the level of IGF2, IRS1, PI3K, PDK1, and AKT (Figures 9A and 9B). To determine further whether circTTN regulates the IGF2/PI3K/AKT pathway by affecting miR-432, we transfected miR-432 mimic or PCD2.1-circTTN into bovine primary myoblasts. It was obvious that the overexpression of miR-432 reduced the expression of IGF2, IRS1, PI3K, PDK1, and AKT at mRNA and protein levels, but circTTN overexpression alleviated these effects (Figure 9C). Analogously, we took out the semblable consequence in the differentiation stage (Figures 9D–9F). Overall, these results indicated that circTTN regulates proliferation and differentiation of bovine primary myoblasts by the circTTN-miR-432-IGF2/PI3K/AKT axis (Figure 10).

DISCUSSION

Large numbers of circRNAs exist in the human and animal transcriptome, and more and more evidence suggests that circRNA has important roles in regulating cellular functions. circTTN might be used as an exogenous molecule to treat muscle-related diseases. Here, we used bovine primary myoblasts as a model to study the role of circTTN in muscle growth and development, given the fact that cattle, as large animals, could be used as a model for human disease research. ATP2A1 is the causative gene for pseudomyotonia in Chianina cattle, and this new bovine genetic disease is a suitable large-animal model for human Brody disease. Genome-wide analysis of alternative splicing (AS) in cows found that AS plays a major role in the effects of diseases in both humans and cow and is suitable as a model to investigate more serious diseases, such as spinal muscular atrophy and colon cancer.

The bovine parasite Onchocerca ochengi is closely related to the pathogenic factor (O. volvulus) of human river blindness, sharing the same vector with it. In addition, livestock provides many advantages for the discovery of causative variants influencing complex traits. For species, such as cattle, individual polymorphisms may explain a larger...
proportion of the variation in these populations compared to the proportion in humans because of intense artificial selection. Overall, cattle can serve as a good model organism for many more human diseases.

To the best of our knowledge, TTN is the largest known protein. It is encoded by 364 exons and plays key structural, developmental, mechanical, and regulatory roles in the heart and skeletal muscle. Mutations in the TTN gene have been linked to multiple skeletal and cardiac myopathies. The circTTN is derived from five exons (exon 110–exon 114) of the TTN gene, and sequencing data reveal that it is differentially expressed between fetal and adult bovine muscle tissues through sequencing data. However, the role of circTTN in bovine myogenesis has not been previously described. In this study, we found that circTTN could facilitate proliferation and differentiation of bovine primary myoblasts. Mechanistically, we confirmed

Figure 9. circTTN Regulates the Activity of the IGF2/P3K/AKT Signaling Pathway

(A and D) Bovine primary myoblasts transfected with the PCD2.1-circTTN, IGF2/P3K/AKT pathway relevant genes (IGF2, IRS1, P3K, PDK1, and AKT) were detected by quantitative real-time PCR and western blot analysis in proliferation (A) and differentiation (D) stage. (B and E) Bovine primary myoblasts transfected with si-circTTN, IGF2, IRS1, P3K, PDK1, and AKT were detected by quantitative real-time PCR and western blot analysis in the proliferation (B) and differentiation (E) stage. (C and F) Bovine primary myoblasts were transfected with miR-432 mimic or PCD2.1-circTTN + miR-432 mimic; IGF2, IRS1, P3K, PDK1, and AKT were detected by quantitative real-time PCR and western blot analysis in proliferation (C) and differentiation (F) stage. β-Actin acts as an internal control gene. Values are means ± SEM for three individuals. *p < 0.05; **p < 0.01.
that circTTN acts as a decoy for miR-432 to relieve the miRNA inhibition effect on IGF2 and to activate further the IGF2/PI3K/AKT signaling pathway.

Previous work has shown that circRNA exhibits multiple mechanisms of action, including acting as a ceRNA,12 acting on RNA transcription,39 participating in protein translation,40 and interacting with proteins.6 The ceRNA hypothesis is a previously unknown pattern for gene expression regulation that was formally proposed in 2011.41 ceRNAs are transcripts that can regulate each other at the post-transcription level by competing for shared miRNAs.42 Various types of ceRNA have been found, including mRNA, pseudogene transcripts, lncRNA, and circRNA.11,43–45 However, circRNAs, as particular endogenous RNAs, have become a new focus among the ceRNA family after lncRNAs. circRNA can act as sponges to block and inhibit miRNAs from binding to their target sites. Here, in order to determine potential downstream effectors of circTTN-mediated regulation of myogenesis, we screened miR-432. RNAhybrid prediction analysis showed that circTTN includes a perfect target site complementarity with miR-432. miR-432 targeting E2F3 and P53PIK inhibits proliferation and differentiation of myoblasts.46 In disease, miR-432 inhibits proliferation of lung adenocarcinoma cells by targeting E2F3 and is highly expressed in skeletal muscle and has been previously identified as a quantitative trait locus (QTL) of muscle mass, as change in a single base of pig IGF2 intron 3 is a major QTL affecting muscle growth, heart size, and fat deposition.55 During differentiation of, IGF2 is regulated by mammalian target of rapamycin (mTOR) and by amino acid sufficiency through IGF2 promoter 3 and a downstream enhancer.56 In yak, IGF2 promotes fibroblast proliferation by inhibiting the expression of IGF1R and PI3KCG.57 The binding of IGFs (IGF1 and IGF2) to their receptors induces autophosphorylation of its tyrosine residues, which in turn, activate a variety of intracellular signaling pathways, including the PI3K/AKT and mitogen-activated protein kinase (MAPK) pathways that trigger various biological processes, such as cell proliferation, differentiation, and apoptosis.58 IGF2 promotes metabolism and proliferation of rainbow trout myocytes by activating PI3K/AKT and MAPK signaling pathways.59 In terms of cancer, IGF2 mRNA-binding protein 2 (Imp2) regulates the activity of IGF2, which further activates PI3K/AKT signaling to promote glioblastoma (GBM) malignancy.32 Forced circTTN expression increased the protein expression of IGF2 and genes (IRS1, PI3K, PDK1, and AKT) involved in the PI3K/AKT pathway, but knockdown of circTTN has the opposite effect. Furthermore, overexpression of miR-432 significantly inhibited the expression of IGF2, IRS1, PI3K, PDK1,
and AKT, but these effects were abolished by increased circTTN. These results demonstrated that IGF2, IRS1, PI3K, PDK1, and AKT are downstream molecules of miR-432 in bovine myoblasts.

In summary, this study demonstrated the role and regulatory mechanism of circTTN in bovine primary myoblasts. Our research identified for the first time that circTTN regulates proliferation and differentiation of bovine myoblasts by competing with miR-432 as a ceRNA, which activates the IGF2/PI3K/AKT signaling pathway. This helps elucidate the molecular events associated with muscle development and muscle disease and unravels the complex molecular mechanism of myogenesis.

MATERIALS AND METHODS

Sample Preparation

Fetal bovines were obtained from a local slaughterhouse in Xi’an, China. Six tissues of Qinchuan cattle at embryonic and adult stage (heart, liver, spleen, lung, kidney, and muscle) were obtained and stored at −80°C in a freezer until use. A total of nine muscle tissue samples of Qinchuan cattle were collected from three developmental states (fetal, 90 days; calf, 6 months; and adult, 24 months). Animal samples used in this study were approved by the Animal Care and Use Committee of Northwest A&F University.

RNA Preparation and Quantitative Real-Time PCR

Total RNA samples of cells or tissues were extracted using TRIzol Reagent (Takara, Dalian, China) as the manufacturer’s instructions. RNA was reverse transcribed into cDNA using the PrimeScript RT Reagent Kit with gDNA Eraser (Takara, Dalian, China). The nuclear and cytoplasmic fractions were separately extracted using a nucleo-plasmatic separation kit (PARIS kit; Life Technologies, Carlsbad, CA, USA). For RNase R treatment, 1 μg of total RNA was incubated for 15 min at 37°C with 2 units μg−1 of RNase R and subsequently purified using an RNeasy MinElute Cleaning Kit (QIAGEN, Hilden, Germany). The quantitative real-time PCR analyses were performed with the SYBR Green Kit (Genestar, Beijing, China), and quantitative real-time PCR was performed on three biological replicates at each time point. GAPDH and U6 (for miRNA) genes were used as internal controls for normalizazation of the data. All spanning the distal ends of circRNAs with sequence specificity was detected using Primer-BLAST (NCBI). The relative expression level was calculated by the comparative cycle threshold (2−ΔΔCt) method. The primers in our study are listed in Table S1.

Vector Construction

The whole length of circTTN was cloned into the overexpression vector of PCD2.1 using the two times TSINGKE Master Mix (blue) (TSINGKE, Beijing, China) and was designated PCD2.1-circTTN. The fragment of the IGF2 3′ UTR-WT, including the binding site of miR-432, was amplified and inserted into the psiCHECK-2 vector (IGF2-WT) (Promega, Madison, WI, USA) at the 3′ end of the Renilla gene using restriction enzymes XhoI and NotI (Takara, Dalian, China) and T4 DNA ligase. The mutant type psiCHECK-2-IGF2-3′ UTR-MUT (IGF2-MUT) was generated by mutating complementary to the seed region of the miR-432 using overlapping PCR. Finally, IGF1-WT and IGF1-MUT were inserted into the psiCHECK-2 vector in the same way. A miR-432 sensor (with a perfect miR-432 binding site) was generated by insertion of two sequences that are completely complementary to the mature sequence of miR-432 after the psiCHECK-2 vector RLuc. Therefore, it can respond to miR-432 reflecting by the fluorescence activity alteration. Similarly, the vector of PCK-circTTN was obtained using the same method. Primer sequences are shown in Table S2, and all constructs were verified by sequencing (Sangon Biotech, Shanghai, China).

Cell Culture and Treatment

Bovine primary myoblasts were isolated and cultured from bovine longissimus muscle as previously described.60 We plated bovine primary myoblasts at the stage of 80% confluence at a density of 5 × 10⁵ cells per well in six-well plates in 2 mL 20% fetal bovine serum (FBS) culture medium per well or 1 × 10⁵ cells per well in 96-well plates (NEST, Wuxi, China) in 100 μL culture medium per well and incubated them, as described previously. After growth to ~80% confluence, the cells were transfected with PCD2.1-circTTN (2 μg/mL), IGF2/PI3K/AKT

RNA-FISH assay

A RNA-FISH assay was performed in bovine primary myoblasts following the instructions of the probe manufacturer (RiboBio, Guangzhou, China). The probe sequence for circTTN is 5′-AGCTTTTTTGGTCACCTTGTGGTACCTGAAAC-3′. The cells were plated into cover-glass in the 6-well plates and were cultured to 70%~80% confluence and then fixed. After 0.1% Triton X-100 transmembrane treatment, the cells were incubated with 20 mg/mL of circTTN probe overnight at 37°C. Nuclei were counterstained with DAPI. Images were acquired using an FV1200 laser confocal microscope (Olympus).

Flow Cytometry for the Cell Cycle

Cell-cycle assay kits were used to analyze cell cycles in different treatment groups (Multisciences, Hangzhou, China). We cultured bovine primary myoblasts in 6-well plates (1 × 10⁶ cells/well) with 2 mL culture medium. We collected cells and washed them once with PBS buffer after treatment for 24 h. Next, 1 mL of DNA strain solution and 10 μL of permeabilization solution were added to the resuspended cells. Flow cytometry was next performed using the cell suspensions after incubation for 30 min in the dark at room temperature (FACSCanto II; BD Biosciences, USA). Each treatment group had three independent replicates.
**CCK-8 Assay**
The cells were plated into 96-well culture plates in 100 µL of culture medium per well, and each treatment group had seven independent replicates. After 24 h of incubation at 37°C, 10 µL of CCK-8 reagent (Tianzö, Beijing, China) was added to each well and incubated at 37°C for 4 h. The absorbance of each sample was detected at 450 nm using an enzyme standard instrument (Molecular Devices, Sunnyvale, CA, USA).

**EdU Assay**
The proliferation circumstances of bovine primary myoblasts were probed by Cell-Light EdU Apollo 567 In Vitro Imaging Kit (RiboBio, Guangzhou, China), according to the manufacturer’s instructions, and each treatment group had three independent replicates.

**Luciferase Activity Assay**
HEK293T cells were used to validate interactions and were cultured in DMEM with 10% FBS. When the cell confluence reached about 80%, the miR-432 mimics and PCK-circTTN or miR-432 sensor were cotransfected into HEK293T cells using Lipofectamine 2000. Similarly, the miR-432 mimics and IGF2-WT or IGF2-MUT were cotransfected into cells. In addition, the miR-432 mimics, PCD2.1-circTTN, and IGF2-WT were cotransfected into cells. After incubation for 24 h, the cells were washed with PBS and collected using 100 µL passive lysis buffer (PLB). Dual-Luciferase Reporter (DLR) Assay System Kit (Promega, Madison, WI, USA) was performed to analyze firefly luciferase activity and Rluc activity, according to the manufacturer’s instructions. The firefly luciferase activity was used as a control to normalize the signal value.

**RIP**
The Magna RIP RNA-Binding Protein Immunoprecipitation Kit was used to perform the RIP assay (Millipore, Bedford, MA, USA), following the manufacturer’s protocol. Bovine primary myoblasts at the stage of 80% confluence were collected and lysed using RIP lysis buffer. Then, Ago2 immunoprecipitation was performed using an anti-Ago2 antibody (Abcam, Cambridge, UK), and an immunoglobulin G (IgG) antibody was used as a negative control. Finally, the immunoprecipitated RNA was isolated, and the abundance of circTTN and miR-432 in bound fractions was evaluated by quantitative real-time PCR analysis.

**Western Blotting Analysis**
Total proteins of bovine primary myoblasts were extracted from the different treatment groups using the protein lysis buffer radioligand precipitation assay (RIPA) containing 1% PMSF (Solarbio, Beijing, China). The protein concentration was determined by a bicinechonic acid kit (Beyotime, Shanghai, China). The collected proteins were boiled with 25% five times protein loading buffer (2-mercaptoethanol [2-ME]) (HAT, Xi’An, China) at 98°C for 10 min, and 20–40 µg total protein was loaded and separated by SDS-PAGE and then transferred to a polyvinylidene fluoride (PVDF) membrane, blocked in 5% skim milk for about 2 h at room temperature, and incubated with primary antibodies diluted in Primary Antibody Dilution Buffer (HAT, Xi’An, China) overnight at 4°C. anti-β-actin (1:1,000; KM9001T; SangeneBiotech, Tianjin, China); anti-PCNA (1:1,000; D220014; SangeneBiotech, Shanghai, China); anti-CyclinD1 (WL01435a), anti-CDK2 (WL02028), anti-IGF2 (WL02665), anti-IR5 (WL03123), anti-P3K (WL02240), anti-PDK1 (WL00707), and anti-AKT (WL0003b) (1:500; Wanleibio, Shenyang, China); anti-MyoD1 (ab16148) and anti-MyoG (ab124800) (1:1,000; Abcam, Cambridge, UK); anti-heavy chain cardiac myosin (1:1,000; MyHC, GTX20015; GeneTex, USA). After that, we washed the PVDF membranes with Tris-buffered saline Tween 20 (TBST) buffer and then incubated with horseradish peroxidase (HRP)-conjugated secondary antibody for 1.5 h at room temperature. The HRP-conjugated secondary antibodies were as follows: goat anti-mouse IgG HRP (M21001S; Abmart, Shanghai, China); goat anti-rabbit IgG (H+L)-HRP conjugate (BA1054; Boster, Wuhan, China). Finally, antibody-reacting bands were detected using ECL Super Sensitive Kit (DiNing, Beijing, China).

**Immunofluorescence Staining**
Bovine primary myoblasts were differentiated for 4 days and then were washed three times with PBS buffer (pH 7.4), followed by fixation with 4% paraformaldehyde for 30 min. Then, cells were permeabilized for 15 min in PBS containing 0.5% Triton X-100. Cells were stained overnight at 4°C using MyoD1 antibody (1:500; ab16148; Abcam, Cambridge, UK) and MyHC antibody (1:250; GTX20015; GeneTex, USA), diluted in 1% BSA. We then incubated them at room temperature for 2 h with the corresponding secondary antibody goat anti-mouse IgG (heavy chain and light chain [H&L])-Alexa Fluor 594 (1:500; RS3608; Immunoway, USA), diluted in 1% BSA in PBS. Nuclei were visualized by DAPI stain. Finally, we washed them three times with PBS and observed under a fluorescence microscope (DM5000B; Leica, Germany).

**Statistical Analyses**
The experimental results were expressed as the mean ± SEM. All data in this study were analyzed by t tests using GraphPad Prism 7.0 software. p < 0.05 was considered statistically significant among means, and p < 0.01 was considered highly significant.

**SUPPLEMENTAL INFORMATION**
Supplemental Information can be found online at https://doi.org/10.1016/j.omtn.2019.10.019.

**AUTHOR CONTRIBUTIONS**
X.W. and H.C. designed and coordinated the project. X.W. performed the experiments and drafted the manuscript. X.C. modified the manuscript and provided valuable advice. D.D., X.S., J.C., R.J., Z.Y., and S.P. helped perform the experiments and analyzed the data. X.L. and C.L. provided suggestions to the experiments. Y.H. and I.E.E. helped collect tissue sample.

**CONFLICTS OF INTEREST**
The authors declare no competing interests.
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