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lncRNA *IGF2 AS* Regulates Bovine Myogenesis through Different Pathways

Chengchuang Song,[1,2] Zhaoxin Yang,[1] Rui Jiang,[1] Jie Cheng,[1] Binglin Yue,[1] Jian Wang,[1] Xiaomei Sun,[1] Yongzhen Huang,[1] Xianyong Lan,[1] Chuzhao Lei,[1] and Hong Chen[1]

[1]Key Laboratory of Animal Genetics, Breeding and Reproduction of Shaanxi Province, College of Animal Science and Technology, Northwest A&F University, Yangling 712100, China; [2]Institute of Cellular and Molecular Biology, Jiangsu Normal University, Xuzhou 221116, China

The role of long non-coding RNA (lncRNA) in the regulation of bovine skeletal muscle development remains poorly understood. The present study investigated the function and regulatory mechanism of a novel lncRNA, insulin-like growth factor 2 antisense transcript (*IGF2 AS*), in bovine myoblast proliferation and differentiation. Gain or loss of *IGF2 AS* was performed using an expression plasmid or small interfering RNA (siRNA), respectively. Bovine myoblasts were used to investigate the biological function and mechanisms of *IGF2 AS in vitro*. Results were conjointly analyzed by celluar and molecular biology experiments as well as bioinformatics. Functionally, *IGF2 AS* could promote proliferation and differentiation of bovine myoblasts. The preliminary mechanism suggests, on the one hand, that *IGF2 AS* could complement the *IGF2* gene intron region and affect the stability and expression of *IGF2* mRNA. On the other hand, RNA pull-down and immunoprecipitation assays demonstrated that *IGF2 AS* could directly bind to the interleukin enhancer binding factor 3 (ILF3) protein and maybe partly though it to regulate myogenesis. In conclusion, the novel identified lncRNA *IGF2 AS* promoted proliferation and differentiation of bovine myoblasts through various pathways.

INTRODUCTION

Myogenesis is a highly orchestrated biological process that is initiated in the embryonic mesoderm,[1] and it continues to occur during skeletal muscle regeneration after birth. Through the processes of proliferation, differentiation, and fusion, the muscle progenitor cells or satellite cells differentiate into myoblasts and multinucleated myotubes, eventually forming mature muscle fibers.[2] Skeletal development in the trunk and limbs is mainly regulated by a series of transcription factors, including paired box protein 3/7 (*Pax*3/7), the myogenic regulatory factors (*MRFs*) family[3,4] and the myocyte enhancer factor 2 (*MEF*2) family.[2,5,6] Moreover, these transcription factors or regulators are directly or indirectly regulated by other protein-encoding and non-coding genes. Although the origin of skeletal muscle and the level of genetic hierarchy that determines its formation are clear, the regulatory mechanisms involved in this process are not yet well understood. Therefore, discovery of novel regulatory factors and the gaining of an improved understanding of the molecular mechanisms underlying skeletal muscle development remain urgent needs.

Long non-coding RNAs (lncRNAs) are a class of RNAs larger than 200 nt in length with no or little protein-encoding ability,[7] which are involved in a wide variety of cellular contexts and play integral roles in the regulation of gene activity through transcriptional and post-transcriptional processes.[8,9] lncRNAs have emerged as key regulators of major biological processes that impact proliferation and differentiation in every branch of life.[10,11] Interestingly, lncRNAs have also been reported to participate in the regulation of gene expression during skeletal muscle development.[12] lncRNAs typically have different regulatory mechanisms according to their cellular localization. In the nucleus, lncRNAs can be used as a molecular scaffold for the recruitment of transcription factors to mediate gene transcription. For instance, the lncRNA *SRA* can immunoprecipitate with MyoD to influence muscle differentiation.[13] The lncRNA *Dum* can recruit DNA methyltransferases to the promoter of its neighboring gene, *Dppa2* (developmental pluripotency-associated 2), resulting in *Dppa2* silencing and enhancement of myogenesis.[14] *Linc-RAM* is also able to promote myogenic differentiation by binding directly to MyoD.[15] In the cytoplasm, lncRNAs can act as molecular sponges and bind competitively to microRNAs (miRNAs), relieving the inhibitory effects of miRNAs on target genes. For instance, *lncMD* promotes bovine myoblasts differentiation via enhancement of the expression level of insulin-like growth factor 2 (IGF2) by sponging miR-125b[16]. During murine C2C12 myoblast differentiation, *lnc-mg* can also sequester the regulatory role of miR-125b at its target gene *IGF2*.[17] Antisense transcript lncRNA *sirt1 AS* promotes myoblast proliferation by stabilizing *Sirt1* mRNA via competitive binding to miR-34a.[18] For a detailed description, one is referred to a previous review regarding lncRNAs in the regulation of skeletal myogenesis and muscle diseases.[19] These studies provide a novel avenue for uncovering the regulatory mechanism of muscle development, in addition to the genes related to the network regulation. Although a relatively large number of functional lncRNAs involved in myogenesis have been well characterized in humans and mice, there remain a large number of lncRNAs yet to be discovered and investigated in cattle.

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Correspondence: Hong Chen, Key Laboratory of Animal Genetics, Breeding and Reproduction of Shaanxi Province, College of Animal Science and Technology, Northwest A&F University, Yangling 712100, China.
E-mail: chenhong1212@263.net

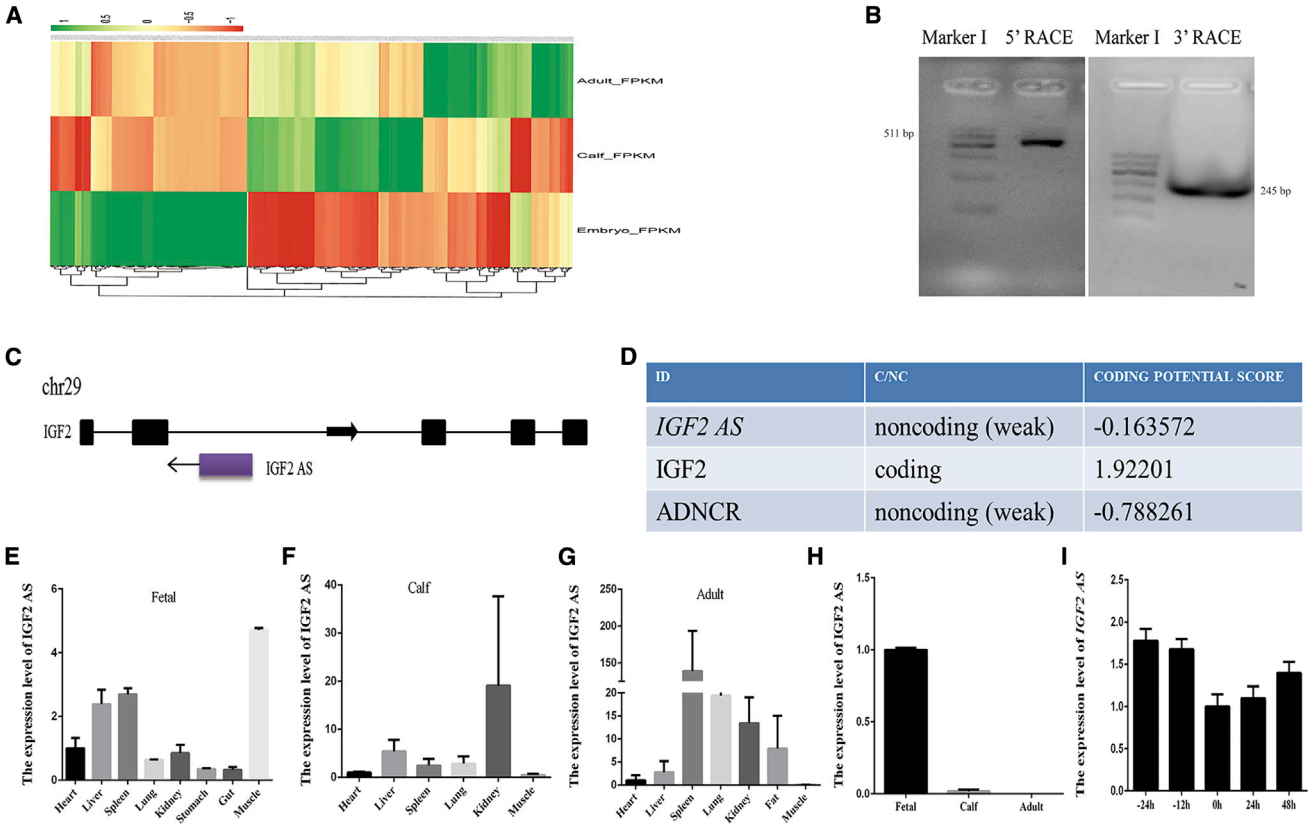

Figure 1. Screening and Identification of *IGF2 AS* and Analysis of Expression Characteristics

(A) Transcript heatmap analysis of the differential expression of skeletal muscle during the embryonic, postnatal, and adult stages. (B) 5′and 3′ RACE of *IGF2 AS* in bovine myoblasts. (C) Localization of *IGF2 AS* in the bovine genome. The thick black arrow indicates the direction of IGF2 gene transcription. (D) The coding potential of *IGF2 AS* was predicted by the coding potential calculator. (E) Expression of *IGF2 AS* in various tissues during the embryonic stage. (F) Expression of *IGF2 AS* in various tissues during the postnatal stage. (G) Expression of *IGF2 AS* in various tissues during the adult stage. (H) Comparison of the expression characteristics of *IGF2 AS* in skeletal muscle during the embryonic, postnatal, and adult stages. (I) Expression features of *IGF2 AS* in the growth stage (−24 h, −12 h) and differentiation stage (24 h, 48 h) of bovine myoblasts.

In the present study, a novel lncRNA transcript was identified, which was positioned in the intron region of *IGF2* in cattle, with the opposite transcriptional direction to that of *IGF2* mRNA; thus, it was named IGF2 antisense transcript (*IGF2 AS*). Considering the importance of *IGF2* in skeletal muscle development,[20] the production of *IGF2 AS* has greatly attracted our research interest. Of note, *IGF2 AS* is mainly expressed in the skeletal muscle of calf fetuses. The detailed data are derived from our previous skeletal muscle RNA sequencing results of lncRNAs during different developmental stages (fetal, calf, and adult) in cattle.[16] As mentioned previously, the embryonic stage is a critical period for the formation of skeletal muscle, which determines the number of muscle fibers after birth. Based on this, we hypothesized that *IGF2 AS* may regulate skeletal muscle development, and thus proceeded to investigate the impact of altered expression of *IGF2 AS* on bovine myoblast proliferation and differentiation. Mechanistically, on the one hand, it could affect *IGF2* expression by binding to the precursor sequence of *IGF2* mRNA; on the other hand, *IGF2 AS* can also bind to protein interleukin enhancer binding factor 3 (ILF3), possibly to regulate the proliferation and differentiation of myoblasts. In this study, we provide a possible avenue for the function and mechanisms of *IGF2 AS* in bovine skeletal muscle development.

RESULTS

Identification of *IGF2 AS* Genomic Features and Detection of Its Expression Profiles

To investigate the function of lncRNAs in skeletal muscle development, three representative stages (embryo, calf, and adult) of cattle skeletal muscle were used for RNA sequencing analysis.[16] These previously published data were used to further analyze differentially expressed lncRNAs, and it was found that a transcript is expressed mainly during the embryonic stage (Figure 1A). Aligning the known sequence with the reference genome revealed that it is located on the antisense strand of the gene *IGF2*. To clearly understand the genetic information in this transcript, 5′ rapid amplification of cDNA ends (RACE) and 3′ RACE were used to amplify the full-length transcript (Figure 1B). The transcript was identified by assembly splicing to a full length of 698 bases, consisting of only one exon (Figure S1A). Here, we named the transcript of the antisense strand of the *IGF2* gene, *IGF2* antisense lncRNA, referred to as *IGF2 AS* (Figure 1C).

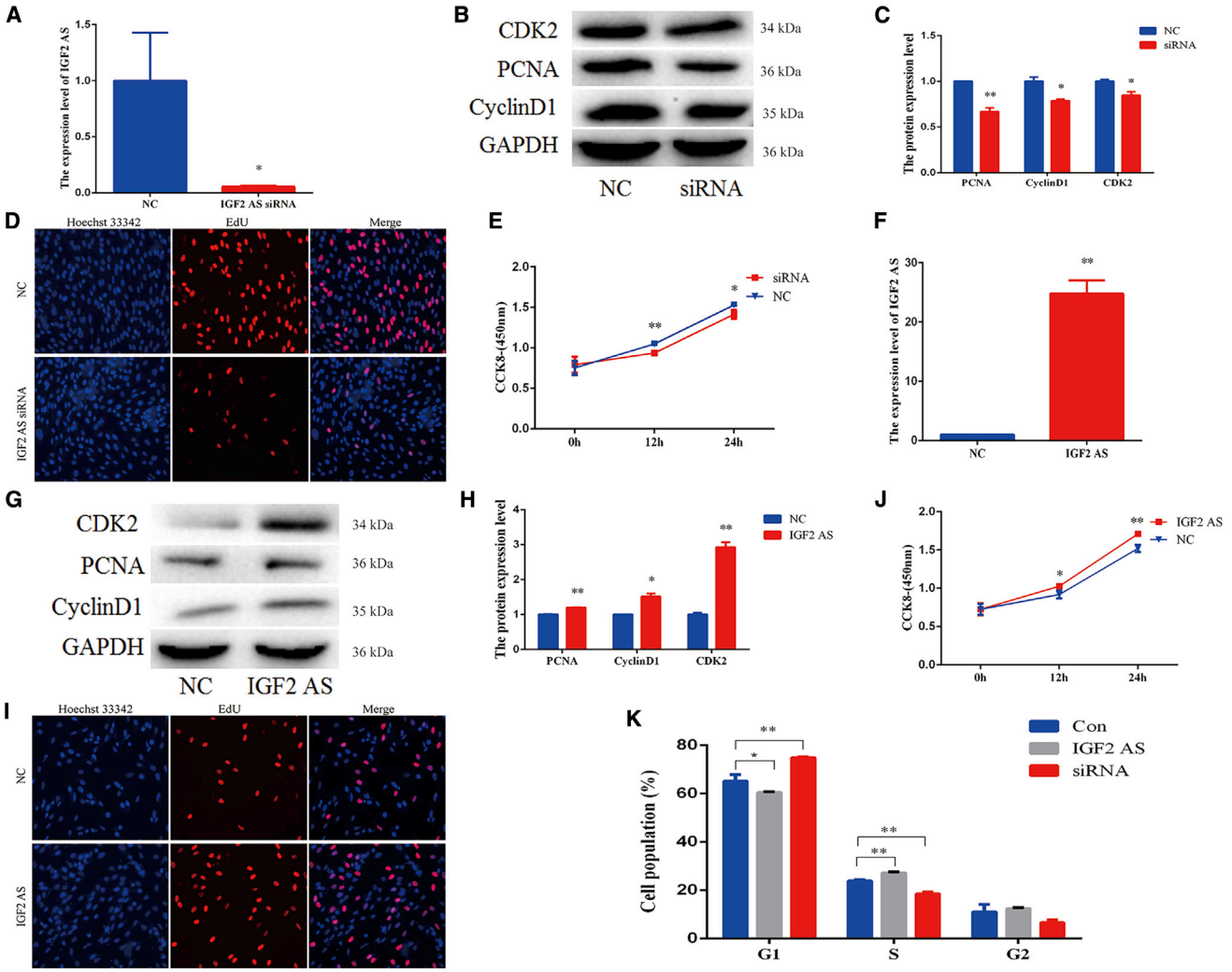

Figure 2. Functional Analysis of *IGF2 AS* in the Proliferative Phase of Bovine Myoblasts

(A) *IGF2 AS* siRNA was transfected into myoblast cells and the expression of *IGF2 AS* was detected 24 h later. NC stands for a scrambled siRNA. (B) Western blotting analysis of the expression of key genes involved in cell proliferation (*CDK2*, *PCNA*, and *CyclinD1*) following the loss of *IGF2 AS*; *GAPDH* acts as a control gene. (C) Western blotting images were quantitated using the ImageJ software. (D) The number of EdU-positive cells detected during the proliferative phase using an EdU kit following the loss of *IGF2 AS*. EdU staining (red) of S-phase cells and Hoechst staining (blue) of cell nuclei are shown. Original magnification, ×200. (E) OD values measured at 450 nm using CCK-8 reagent following the loss of *IGF2 AS* at 0, 12, and 24 h. (F) Detection of *IGF2 AS* expression levels following transfection of myoblasts with *IGF2 AS* expression plasmid after 24 h. NC stands for an empty vector. (G) Western blotting analysis of the expression of key genes involved in cell proliferation following gain of *IGF2 AS*. (H) Western blotting images were quantitatied using the ImageJ software. (I) The quantity of EdU-positive cells detected during the proliferative phase using an EdU kit following the gain of *IGF2 AS*. Original magnification, ×200. (J) OD values measured at 450 nm using CCK-8 reagent following the gain of *IGF2 AS* at 0, 12, and 24 h. (K) Detection of the cell cycle using flow cytometry following the gain or loss of *IGF2 AS*. Statistical analysis of cell numbers at various stages of the cell cycle, including the G_1 and S phases. Data are presented as the mean ± SEM; n = 3; *p < 0.05, **p < 0.01.

To further evaluate the coding potential of this transcript, the bioinformatics software CPC (coding potential calculator) was used for prediction. Analysis showed that this transcript score was lower, and the coding potential was weaker, than that of the *IGF2* gene (Figure 1D), and the coding ability was similar to that of another bovine non-coding RNA, *ADNCR*.[21] To further understand the expression level of *IGF2 AS* in different cattle tissues during different developmental stages, quantitative real-time PCR was used to detect its expression characteristics. The results indicated that *IGF2 AS* is mainly expressed in the skeletal muscle of calf fetuses (Figures 1E–1H). More importantly, in isolated bovine myoblasts, *IGF2 AS* was highly expressed during the proliferative phase and showed an increasing expression trend following the induction of differentiation (Figure 1I). Based on the genomic localization of *IGF2 AS* and its expression characteristics, we speculated that this lncRNA might play a crucial role in bovine skeletal muscle development.

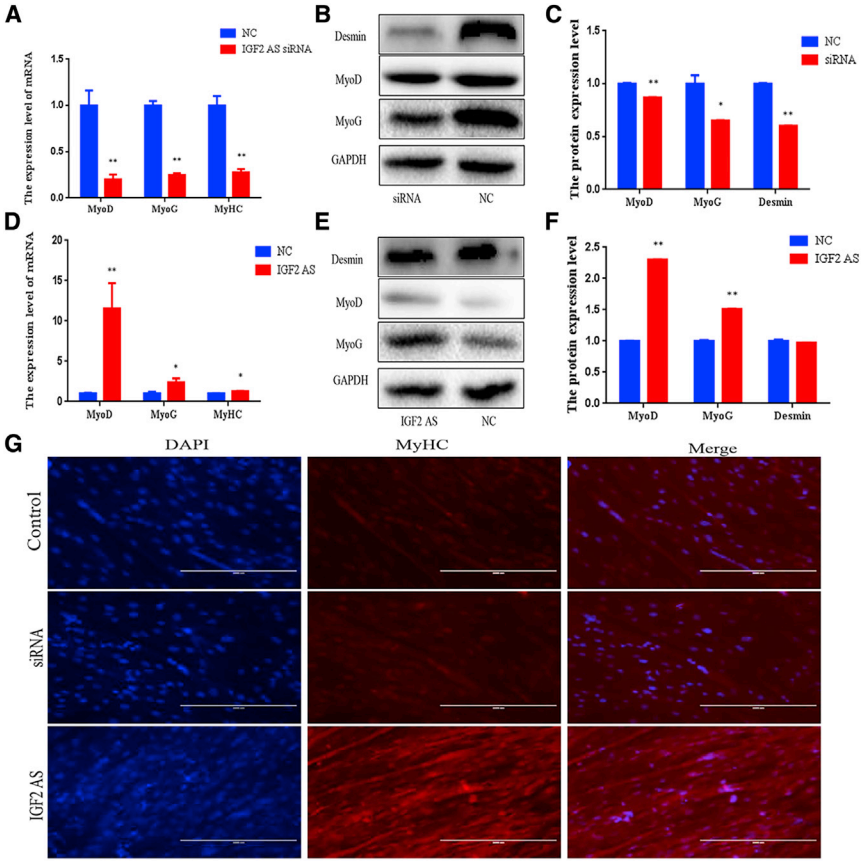

Figure 3. Functional Analysis of *IGF2 AS* during the Differentiation Stage of Bovine Myoblasts

(A) Detection of the mRNA expression levels of the myogenic genes *MyoD*, *MyoG*, and *MyHC* following the loss of *IGF2 AS*. (B) Detection of the protein expression levels of Desmin, MyoD, and MyoG by western blotting analysis following the loss of *IGF2 AS*. (C) Western blotting images were quantitated using ImageJ software. (D) Detection of the mRNA expression levels of the myogenic genes *MyoD*, *MyoG*, and *MyHC* following the gain of *IGF2 AS*. (E) Detection of the protein expression levels of Desmin, MyoD, and MyoG by western blotting analysis following the gain of *IGF2 AS*. (F) Western blotting images were quantitated using ImageJ software. (G) MyHC (red)-positive myotubes detected by immunofluorescence analysis following the loss or gain of *IGF2 AS*. Original magnification, ×200. Data are presented as the mean ± SEM; n = 3. *p < 0.05, **p < 0.01.

IGF2 AS Promotes Bovine Myoblast Proliferation

To explore the function of *IGF2 AS* during myoblast proliferation, its role was verified by loss or gain functionality. First, *IGF2 AS* small interfering RNA (siRNA) could significantly reduce the expression level of *IGF2 AS* (Figure 2A). Some key proteins involved in cell proliferation, such as CyclinD1, CDK2, and PCNA, were detected by western blot (Figure 2B). In comparison with the control group, knockdown of *IGF2 AS* significantly reduced the expression levels of these proteins (Figure 2C). The proliferation status of the myoblasts was detected using 5-ethynyl-2′-deoxyuridine (EdU) reagent; the quantity of EdU-positive cells following transfection of *IGF2 AS* siRNA was significantly decreased as compared with that in the control group (Figure 2D). The proliferation activity of cells was detected using Cell Counting Kit-8 (CCK-8) reagent, and the optical density (OD) value of the cells following loss of *IGF2 AS* was reduced as compared with that in the control group (Figure 2E). Second, transfection of myoblasts with *IGF2 AS* expression plasmid significantly enhanced the expression level of *IGF2 AS* (Figure 2F). Consistently, the protein expression levels of cell proliferation-related genes were elevated following overexpression of *IGF2 AS* (Figures 2G and 2H). Additionally, the number of EdU-positive cells and the OD value increased significantly (Figures 2I and 2J). To further elucidate the function of *IGF2 AS* in cell proliferation, flow cytometry was used to detect the precise changes in the cell cycle following overexpression or interference of *IGF2 AS*. The results showed that the number of myoblasts in the G_1 phase decreased and those in the S phase increased following *IGF2 AS* overexpression (Figures 2K and 2L; Figure S2). On the contrary, interference with *IGF2 AS* siRNA led to an increase in the number of cells in the G_1 phase and a decrease in the number of cells in the S phase (Figures 2K and 2L; Figure S2). These results demonstrated that *IGF2 AS* was acceptable to promote bovine myoblast proliferation.

IGF2 AS Promotes Bovine Myoblast Differentiation

The potential biological roles of *IGF2 AS* during bovine myoblast differentiation were investigated. Knockdown of *IGF2 AS* significantly reduced the mRNA expression levels of the myogenic genes *MyoD*, *MyoG*, and myosin heavy chain (*MyHC*) (Figure 3A), and the protein expression levels of MyoD, MyoG, and Desmin were also significantly downregulated (Figures 3B and 3C). In addition, the numbers of MyHC-positive myotubes in the interference group were reduced compared with the blank control group (Figure 3G). Conversely, overexpression of *IGF2 AS* significantly elevated the mRNA expression levels of *MyoD*, *myogenin*, and *MyHC* (Figure 3D), and the protein expression levels of MyoD and MyoG were significantly upregulated, but that of Desmin did not change (Figures 3E and 3F). Consistently, the number of MyHC-positive myotubes in the overexpression group was significantly increased compared with the blank control group (Figure 3G). To further clarify the role of *IGF2 AS* in regulating the differentiation of bovine myoblasts, transcriptome sequencing analysis was performed following the loss of *IGF2 AS* expression. In comparison with the control group, there were 681 differentially expressed genes, of which 234 were upregulated and 447 were downregulated

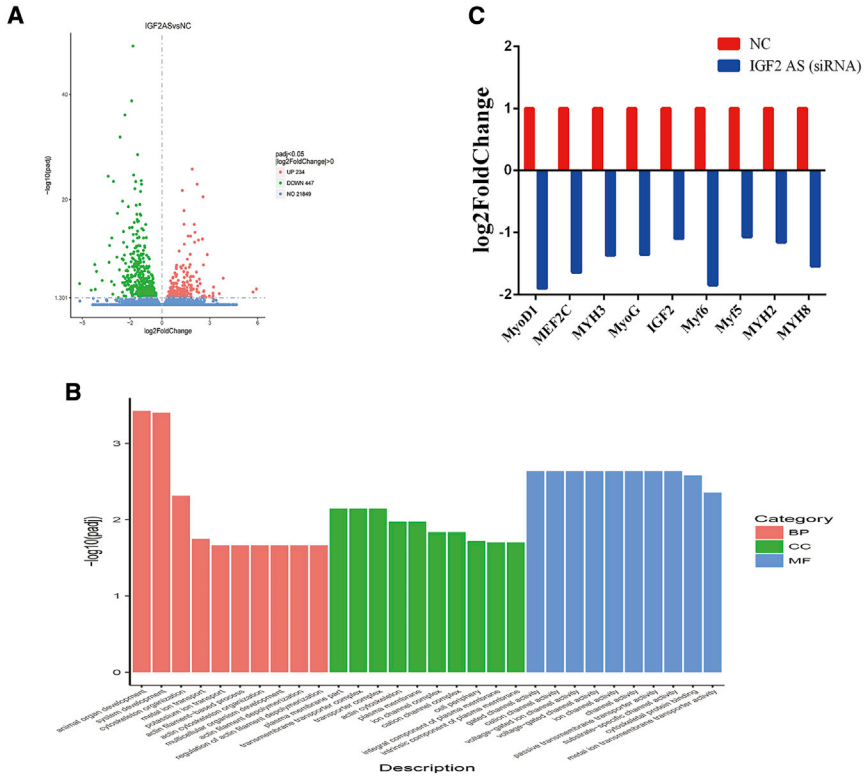

Figure 4. Transcriptome Analysis following Loss of *IGF2 AS* during Bovine Myoblast Differentiation

(A) The volcanic map shows the differential gene distribution. The x axis indicates the fold change in expression (log_2 fold change) of the genes in the *IGF2 AS* (siRNA) and negative control groups; the y axis indicates the level of significance of gene expression differences between the *IGF2 AS* (siRNA) and control groups. (B) GO enrichment analysis results of downregulated genes following the loss of *IGF2 AS*. The x axis indicates the GO term and the y axis indicates the level of significance of GO term enrichment. BP, biological process; CC, cellular component; MF, molecular function. (C) Expression of genes involved in myoblast differentiation following the loss of *IGF2 AS*.

(Figure 4A; Table S1). Gene Ontology (GO)-enriched analysis revealed that the downregulated genes are mainly involved in biological processes such as animal organ development (Figure 4B; Table S2). Among the significantly downregulated genes, some are closely related to skeletal muscle cell differentiation such as *MyoD*, *MyoG*, *MYH2*, *MYH3*, *MYH8*, *MEF2C*, *IGF2*, *Myf5*, and *Myf6* (Figure 4C). Collectively, our multi-level research results indicated that *IGF2 AS* promoted bovine myoblast differentiation.

IGF2 AS Affects IGF2 Expression by Binding to the Precursor Sequence of IGF2 mRNA

As described in the previous results, *IGF2 AS* is derived from the antisense strand of *IGF2* gene, and its sequence is complementary to that of the IGF2 mRNA precursor. These circumstances greatly aroused our research interest. To further investigate the relationship between *IGF2 AS* and *IGF2*, the expression of *IGF2 AS* was investigated during the proliferation (Figure 5A) and differentiation (Figure 5B) stages of bovine myoblasts by PCR. It was found that its expression could be detected in both the nucleus and cytoplasm during these two stages. In addition, fluorescence *in situ* hybridization (FISH) experiments also directly demonstrated that *IGF2 AS* was expressed in both the cytoplasm and nucleus (Figure 5C), although the signal in the cytoplasm was slightly stronger than that in the nucleus. Since *IGF2 AS* was expressed in the nucleus, we sought to determine whether the *IGF2 AS* sequence can bind to the precursor sequence of *IGF2* mRNA. For verification of this, DNase I and RNase A were jointly used to digest the newly extracted total RNA, and PCR amplification was then directly performed using this RNA as a template. It was found that *IGF2 AS* could still be detected (Figure 5D). More interestingly, both positions (regions 1 and 2) of the same intron region of the *IGF2* gene could be detected (Figure 5E; Figure S3A), and the sequencing results were correct when compared with the *IGF2* gene reference sequence (Figure 5F). However, the sequence derived from the genomic DNA gene, *BTF3*, was not detected (Figure 5E), which further supports our hypothesis. It is known that there are numerous transcript types of *IGF2*, and some intron regions of *IGF2* transcripts contain *IGF2 AS* and some transcripts do not intersect with it (Figure S3B). Furthermore, quantitative real-time PCR was used to detect the expression of partial transcripts of *IGF2* in fetal skeletal muscle (Figure S3C), as well as the expression of these transcripts during the proliferative (Figure S3D) and differentiation (Figure S3E) stages of myoblasts. Intriguingly, interference with *IGF2 AS* siRNA led to a decrease in the expression levels of these different *IGF2* transcripts during the proliferative phase (Figure 5G), whereas the expression of these transcripts was significantly decreased during the differentiation phase (Figure 5H) of bovine myoblasts. During the proliferation and differentiation stage of myoblasts, interference with *IGF2 AS* siRNA resulted in decreased IGF2 protein levels (Figures S3F and S3G). During the proliferative phase, bovine myoblasts were treated with actinomycin D (1.5–2.0 μg/mL) to evaluate the expression of *IGF2 AS* and *IGF2* at different time points. It was found that the expression level of *IGF2 AS* showed a slow declining trend as compared with that of *IGF2* (Figure 5I). Furthermore, overexpression of *IGF2 AS* and following treatment with actinomycin D (Figure 5J), it was found that the expression levels of the different *IGF2* transcripts were higher than those in the control group (Figure 5K). Taken together, these data supported the idea that *IGF2 AS* might regulate the proliferation and differentiation of bovine myoblasts by binding to the precursor sequence of *IGF2* mRNA and affecting its expression.

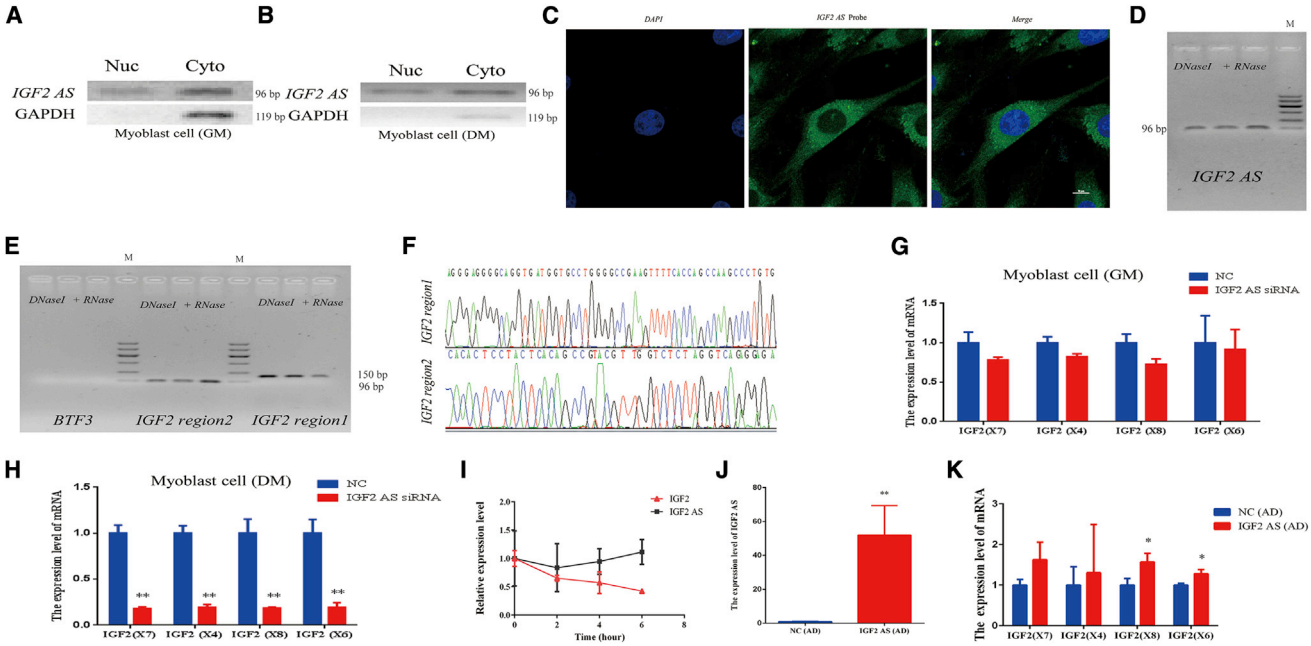

Figure 5. Subcellular Localization of *IGF2 AS* and Its Relationship with the *IGF2* Gene

(A) Distribution of *IGF2 AS* in the cytoplasm and nucleus during the myoblast growth stage. (B) Distribution of *IGF2 AS* in the cytoplasm and nucleus during the myoblast differentiation stage. (C) Subcellular localization of *IGF2 AS* by fluorescence *in situ* hybridization. Original magnification, ×400. (D) Detection of *IGF2 AS* by PCR following treatment of RNA with Dnase I and Rnase A. (E) Detection of *IGF2* gene introns in different positions by PCR following treatment of RNA with Dnase I and Rnase A; *BTF3* acts as a control gene. Introns 1 and 2 indicate the different positions in the same intron. (F) DNA sequencing analysis of amplification products at different positions in the *IGF2* gene intron. (G) Detection of the mRNA expression levels of different *IGF2* transcripts during the proliferative phase following the loss of *IGF2 AS*. X7, X4, X8, and X6 represent different transcript variants of the *IGF2* gene (NCBI). (H) Detection of the mRNA expression levels of different *IGF2* transcripts during the differentiation stage following the loss of *IGF2 AS*. (I) Expression of *IGF2* and *IGF2 AS* detected at different time points (0, 2, 4, and 6 h) following treatment with actinomycin D. (J) Detection of *IGF2 AS* after treatment with actinomycin D following the gain of *IGF2 AS*. (K) Different *IGF2* transcripts were detected after treatment with actinomycin D following the gain of *IGF2 AS*. Data are presented as the mean ± SEM; n = 3; *$p < 0.05$, **$p < 0.01$.

IGF2 AS Binds the ILF3 Protein to Regulate Bovine Skeletal Muscle Development

The mechanism of lncRNA reported in the current literature is complex and diverse, and it remains unclear whether *IGF2 AS* can exert its functions by other means. To seek a deeper understanding of the mechanism of *IGF2 AS*, RNA pull-down assays were used to screen its binding proteins. The results of silver staining showed that the molecular mass of the differential protein band was between 75 and 100 kDa (Figure 6A), which was demonstrated to be ILF3 by mass spectrometry (Figure S4). Conversely, the RNA-binding protein immunoprecipitation (RIP) results indicated that ILF3 protein antibodies can also capture *IGF2 AS* (Figure 6B). This fully demonstrated that *IGF2 AS* can bind to the ILF3 protein. It is noteworthy that during the proliferative phase of bovine myoblasts, interference or overexpression of *IGF2 AS* decreased or enhanced the expression of *ILF3*, respectively (Figures 6C and 6D). Similarly, during bovine myoblasts differentiation, interference or overexpression of *IGF2 AS* also decreased or enhanced *ILF3* expression, respectively (Figures 6E and 6F). These data indicated that the expression of *IGF2 AS* was consistent with that of *ILF3*; therefore, we speculated that *IGF2 AS* might exert its biological function through *ILF3*. Subsequently, the functional role of *ILF3* in the development of bovine skeletal muscle was investigated. Using siRNA to knock down the expression of *ILF3* during the proliferative phase (Figure 6H), it was found that the protein expression levels of PCNA and CDK2 decreased (Figure 6I). Knockdown of *ILF3* disrupted the cell cycle, resulting in an increase in the number of cells in the G_1 phase and a decrease in those in the S phase (Figure 6J; Figure S5A). In addition, the number of EdU-positive cells was also significantly decreased (Figure S5B). During the differentiation phase, successful knockdown of *ILF3* (Figure 6K) led to a reduction in the mRNA expression levels of *MyoG* and *MyHC* (Figure 6L). Moreover, the protein expression levels of the myogenic regulatory factors, MyoD and MyoG, were also reduced (Figure 6M). Taken together, these data demonstrated that *IGF2 AS* regulated bovine skeletal muscle development by binding to the *ILF3* protein.

DISCUSSION

In the present study, we identified a novel lncRNA, *IGF2 AS*, involved in skeletal muscle development based on data from transcriptome sequencing of bovine skeletal muscle.[16] Functionally, *IGF2 AS* promoted bovine myoblast proliferation and differentiation. Mechanically, in one aspect, the sequence of *IGF2 AS* was complementary to the *IGF2* mRNA precursor sequence, thereby affecting the expression of *IGF2*. Alternatively, *IGF2 AS* regulated the proliferation and differentiation

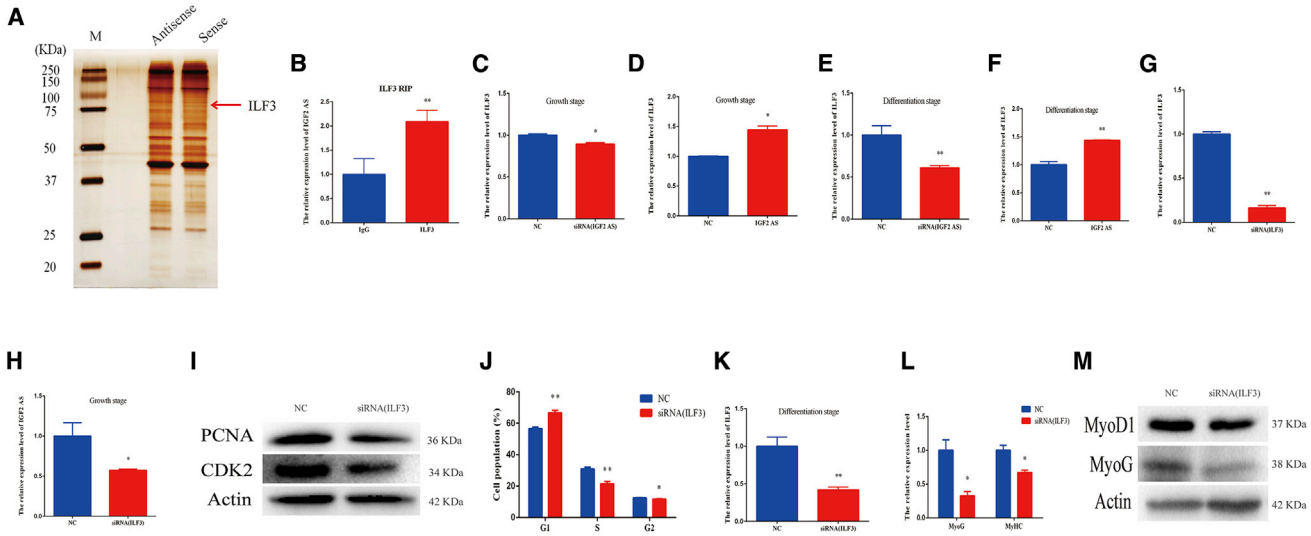

Figure 6. *IGF2 AS* Binds to the ILF3 Protein

(A) Detection of captured proteins by RNA pull-down using silver staining. The red arrow marks the protein band that differed between the sense and the control (antisense) groups; antisense RNA acts as a negative control. (B) RIP analysis indicates that *IGF2 AS* can be captured by the ILF3 protein. (C) The growth phase interferes with *IGF2 AS* siRNA detection of *ILF3* mRNA expression. (D) Detection of the mRNA expression of *ILF3* during the growth stage following the gain of *IGF2 AS*. (E) The differentiation stage interferes with *IGF2 AS* siRNA detection of *ILF3* mRNA expression. (F) Detection of the mRNA expression of *ILF3* during the differentiation stage following the gain of *IGF2 AS*. (G) Detection of ILF3 expression following the loss of *ILF3*. (H) Detection of *IGF2 AS* expression following the loss of *ILF3*. (I) Detection of the protein expression levels of PCNA and CDK2 by western blotting analysis following the loss of *ILF3*; β-actin acts as a control gene. (J) Statistical analysis of cell numbers at various stages of the cell cycle following the loss of *ILF3*. (K) During the differentiation stage, the expression of *ILF3* was detected following interference with *ILF3* siRNA. (L) Detection of the mRNA expression levels of the myogenic genes *MyoG* and *MyHC* following the loss of *ILF3*. (M) Detection of the protein expression levels of MyoG and MyoD by western blotting following the loss of *ILF3*; β-actin acts as a control gene. Data are presented as the mean ± SEM; n = 3. *p < 0.05, **p < 0.01.

of bovine myoblasts in concert with the ILF3 protein. Based on these results, we built a model to detail the functional mechanism of *IGF2 AS* in the regulation of bovine skeletal muscle development (Figure 7).

IGF2 AS was originally discovered to be aberrantly upregulated in Wilms' tumors.[22] Intriguingly, a natural antisense transcript exists at the IGF2 gene locus in mice,[23] humans,[21] and pigs.[24] Functional verification has revealed that transcripts in different species have different biological roles. For instance, *IGF2 AS* in humans is mainly involved in tumorigenesis. At the initial stage of mouse C2C12 myotube formation, the expression of *IGF2 AS* coincides with the expression of *IGF2* variant 3, suggesting that *IGF2 AS* may play a role in the formation of myotubes.[23] In the present study, we first identified a novel natural antisense transcript of the *IGF2* gene in cattle, also named *IGF2 AS*. In humans and pigs, *IGF2AS* has open reading frames (ORFs), but not in mice.[24] In the cattle *IGF2 AS*, we found two ORFs by ORF finder analysis, suggesting polypeptides of 109 and 76 aa residues (Figure S1B). However, *IGF2 AS* differs in the location of transcription among species, and the sequence is also poorly conserved, which may be the main reason for the functional differences. To date, natural antisense transcripts such as *IGF2 AS* have been identified and reported; *Sirt1 AS* can form an RNA duplex with *Sirt1* mRNA by competing with miR-34a to promote Sirt1 translation, thereby impeding muscle formation.[18] *ZFPM2-AS1* can bind to macrophage migration inhibitory factor (MIF) to impact the *p53* pathway and promote gastric carcinogenesis.[25] *MACC1-AS1* is involved in gastric cancer cell metabolic plasticity by promoting stability of *MACC1* mRNA through the direct binding mode.[26] The subcellular distribution based on *IGF2 AS* provides a prerequisite for its function mechanisms (Figures 5A–5C). In the nucleus, we had demonstrated that *IGF2 AS* can bind to the transcriptional precursor of *IGF2* gene and participate in the expression of IGF2. Whether the binding of *IGF2 AS* to the protein ILF3 occurs in the nucleus or cytoplasm remains to be further studied. In the cytoplasm, we have demonstrated that *IGF2 AS* can act a molecular sponge for miRNA, but studies are ongoing and incomplete.

IGF2 is essential for skeletal muscle growth and regeneration and myoblast differentiation.[27,28] Our data indicate that the sequence of *IGF2 AS* can be complementary to the precursor sequence of *IGF2* mRNA to form an RNA duplex. Treatment of bovine myoblasts with actinomycin D revealed that *IGF2 AS* was beneficial to the stability of *IGF2*, suggesting that *IGF2 AS* regulated skeletal muscle development partially through affecting IGF2 expression. However, to determine the specific mechanism of regulation requires further research and investigation. *ILF3* is a ubiquitously expressed protein in animals that contains two double-stranded RNA-binding domains (dsRBDs) and exists in both the nuclear and cytoplasmic fractions.[29] ILF3 is known to be associated with many biological functions involved in mRNA stabilization[30] and non-coding RNA biogenesis.[31]

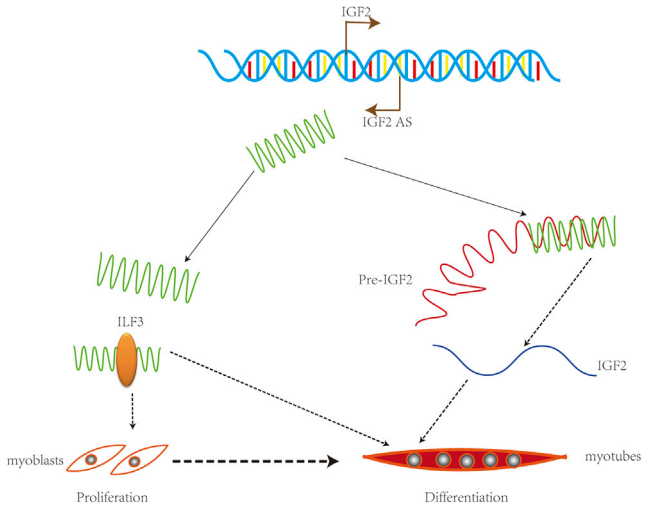

Figure 7. Proposed Model of *IGF2 AS* Regulation during Bovine Skeletal Muscle Development

IGF2 AS can be involved in the regulation of myoblast proliferation and differentiation by binding to the protein ILF3 (in left). *IGF2 AS* can affect the expression of *IGF2* by binding to the transcriptional precursor of the *IGF2* gene and participate in the regulation of myoblast differentiation (in right).

In the present paper, secondary structure prediction analysis by the RNAfold web server indicated that the *IGF2 AS* sequence could fold into many stem-loop motifs (Figure S1C), suggesting the possibility that *IGF2 AS* could be physiologically combined with the ILF3 protein. It is noteworthy that mice with a disrupted *ILF3* gene have skeletal muscle tissue disorders and die soon after birth due to a reduction in *MyoD* and *myogenin* mRNA levels, and partly through disruption of post-transcriptional mRNA stabilization.[29] In the present study, we also show that interference of *ILF3* led to the downregulation of myogenic regulatory factors, which is consistent with previous studies. Our data support the notion that *IGF2 AS* interacts with ILF3 and synergistically regulates the expression of key myogenic genes to mediate myogenesis. Indeed, we thought that *IGF2 AS* may act in concert with *ILF3* to perform the desired function, but the precise downstream target genes of *ILF3* involved in bovine skeletal muscle development remian elusive and require subsequent research (data not shown).

In conclusion, we identified that *IGF2 AS* is a differentially expressed lncRNA in the skeletal muscle of cattle at different developmental stages. *IGF2 AS* regulated the development of bovine myoblasts in a variety of ways, from affecting the stability of maternal genes *IGF2* to binding the specific protein ILF3. These data indicated that *IGF2 AS* regulated bovine skeletal muscle development through a variety of combined mechanisms.

MATERIALS AND METHODS

Isolation and Culture of Bovine Myoblasts

Three-month-old fetal calf (Shaanxi Kingbull Livestock, Baoji, China) hindlimb muscle or longissimus muscle was used to separate myoblasts. The brief steps related to cell separation are presented in the following description. The muscle tissue was digested with the concentration 2 mg/mL of collagenase I at 37°C in a water bath for about 1 h. Then, the digested muscle tissue was centrifuged at a low speed, the supernatant was discarded, and the remaining tissue was filtered through a 200-mesh filter. The filtrate was washed three times with PBS, and finally the cells were resuspended with growth medium containing 20% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Then, the cells were seeded at a certain density on the cell culture plate at 37°C in 5% CO_2. When the cells grew to contact adhesion, the growth medium was replaced with a differentiation medium containing 2% horse serum to induce cell differentiation. The cells isolation steps were performed as previously described.[32] Animal care and study protocols were approved by the Animal Care Commission of the College of Veterinary Medicine, Northwest A&F University.

5′ and 3′ RACE

A RACE experiment was performed to learn the complete nucleotide sequences of lncRNA *IGF2 AS* by using the SMARTer RACE cDNA amplification kit (Clontech, Palo Alto, CA, USA). Total RNA isolated from bovine skeletal myoblasts was used as a template. The gene-specific primers of 5′ RACE and 3′ RACE were 5′-TGGGTCC CCGGGCTTCCAGCCTTGC-3′ and 5′-ACTGATGCGGGAGG GATGCGCCTGC-3′, respectively. The detailed operating procedure refers to manual instructions.

Plasmid Construction and Cell Transfection

The full lengths of lncRNA *IGF2 AS* sequences were cloned and inserted into pcDNA3.1(+) to construct their expression plasmids. The plasmid sequence was confirmed by sequencing analysis. All primer sequences are listed in Table S3. Turbofect transfection reagent (Thermo Fisher Scientific, USA) was used for plasmid transfection according to the manufacturer's instructions.

Transcriptome Sequencing Analysis

After interfering with *IGF2 AS* siRNA (RiboBio, Guangzhou, China) into cells, the extracted RNA was used for transcriptome sequencing analysis (Novogene). Differentially expressed genes and GO enrichment analyses were performed as in our previous description.[33] Raw datasets have been uploaded to the NCBI Sequence Read Archive (SRA) database. All files can be found under NCBI SRA: PRJNA528202.

CCK-8 Assay

The proliferation state of bovine myoblasts was investigated with CCK-8 (Tiandz, Beijing, China). After transfection, the myoblasts in 96-well plates continued to be cultured for about 20 h, and 10 μL of CCK-8 reagent was added to each well. After 1–4 h, the absorbance value was detected at 450 nm. According to the absorbance value of the control group and the treatment group, the state of cell proliferation was evaluated. The detailed procedure follows the manufacturer's instructions.

EdU Assay

A Cell-Light EdU Apollo 567 *in vitro* imaging kit (RiboBio) was used to detect the number of bovine myoblasts at the S phase. The EdU reagent was diluted and added to the transfected bovine myoblasts. After 2–4 h, the cells were fixed with 4% paraformaldehyde solution for 30 min, and the solution was discarded. Then, the excess paraformaldehyde solution in the wells was neutralized with 2 mg/mL glycine solution and washed with PBS three times. 0.5% Triton X-100 was added and incubated for 10 min, then washed with PBS. Apollo 567 staining solution was added, incubated for 30 min in dark conditions, and washed with PBS three times. The nuclei were stained with Hoechst. The numbers of S-phase-positive cells in the control group and the treatment group were observed under a fluorescence microscope.

Cell Cycle Assay

Cell cycle was analyzed by using a cell cycle testing kit (Multisciences, Hangzhou, China). The bovine myoblasts were treated with 1 mL of DNA staining solution and 10 μL of permeabilization solution in dark conditions for 30 min, then detected using a FACSCalibur flow ytometer (Becton Dickinson, USA). For the detailed procedure, refer to the manufacturer's instructions.

Nuclear and Cytoplasmic RNA Fractionation

Nuclear and cytoplasmic RNA fractionation was performed in bovine myoblasts using a nuclear/cytoplasmic RNA extraction kit (Chunhe, Yangling, China). The detailed separation method is described in the manufacturer's instructions. Nuclear and cytoplasmic RNA was extracted and transcribed into cDNA for further analysis. The cellular localization of *IGF2 AS* was analyzed by PCR. Primer sequences for PCR are listed in Table S3.

FISH

FISH was performed per the manufacturer's instructions for *in situ* hybridization reagent (Servicebio, Wuhan, China). In brief, bovine myoblasts were cultured on glass slides and were treated with 4% paraformaldehyde solution for 20 min. The fixed cells were incubated with the pre-hybrid solution for 1 h at 37°C. Then, the fixed cells were incubated with the hybridization solution containing the *IGF2 AS* probe. After overnight incubation, the cells were washed multiple times with different concentrations of SSC solution. Finally, DAPI solution was added to the cell surface and incubated for 8 min in the dark. Then, these cells were observed by using a fluorescence microscope (Nikon, Japan). The *IGF2 AS* probe was 5′-FAM-GAG CAGGCGCATCCCTCCCGCATCAGT-FAM-3′.

RNA Extraction and Quantitative Real-Time PCR

Total RNAs of cells and tissues were extracted by using TRIzol reagent (TaKaRa, Dalian, China). Total RNAs were reverse transcribed using a PrimeScript RT reagent kit with genomic DNA (gDNA) Eraser to remove gDNA (TaKaRa). Quantitative real-time PCR was performed in triplicate using a SYBR Green kit (Genestar, Beijing, China) on a Bio-Rad CF96 system (Bio-Rad, USA). *GAPDH* was selected as the internal gene. The $2^{-\Delta\Delta Ct}$ method was performed to analyze the relative expression level of quantitative real-time PCR data. Primer sequences for quantitative real-time PCR are listed in Table S3.

RNA Stability Assay

Bovine myoblasts were treated with 1.5–2.0 μg/mL actinomycin D (Leagene Biotechnology, Beijing, China), which were harvested at 0, 2, 4, and 6 h after treatment. Quantitative real-time PCR was used to measure expression level change of *IGF2 AS* and *IGF2* mRNA. *GAPDH* was used as an internal control. For detailed steps, refer to the published experimental process.[34] Primer sequences for quantitative real-time PCR are listed in Table S3.

Western Blot

Proteins were extracted from cells using radioimmunoprecipitation assay (RIPA) buffer with 1% PMSF (Solarbio, Beijing, China). The protein concentration was measured using a bicinchoninic acid (BCA) kit (Beyotime, Shanghai, China). The proteins were separated in 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidine fluoride (PVDF) membranes. The membranes were exposed with enhanced chemiluminescence (ECL) Plus (Solarbio) and quantified with the ChemiDoc XRS+ system (Bio-Rad, USA). The detail experiment procedure was performed as previously described.[35] The primary antibody and secondary antibody are listed in Table S4.

Immunofluorescence

Bovine myoblasts in 12-well plates were washed three times with PBS, fixed with 4% formaldehyde solution for 20 min, and permeabilised with 0.5% Triton X-100 in sterile water for 10 min. The cells were blocked with 5% BSA in PBS for 30 min and then incubated at 4°C with myosin antibody (Abcam, USA) overnight. After washing cells with PBS, the cells were incubated with Cy3-conjugated secondary antibody (Beyotime) diluted 1:200 in 1% BSA in the dark at room temperature for 2 h. DAPI staining was done simultaneously to show the position of the nuclei. All images were taken on an inverted fluorescence microscope (AMG EVOS).

RNA Pull-Down Assay

The RNA pull-down assay procedure was performed as previously described.[36,37] The complete sequence fragment of the *IGF2 AS* gene was synthesized and cloned into pBluescript II SK(+) to prepare the plasmid template. This template was used to prepare biotinylated RNA probes. The sample was collected by magnetic beads, separated by SDS-PAGE gel, and then subjected to high-resolution silver staining, and different bands were cut for mass spectrometry analysis (Foregene, Chengdu, China).

RIP Assay

A RIP assay was used to identify specific RNA molecules (of many types) associated with ILF3 protein. Briefly, experiment procedures were as follows: (1) lyse cells in RIP lysis buffer, (2) immunoprecipitate with antibody to RBP of ILF3 (Abcam) with protein A/G magnetic beads, (3) immobilize magnetic beads bound to complexes

with magnet and wash off unbound materials, and (4) extract RNAs for quantitative real-time PCR analysis. Immunoglobulin G (IgG) protein was used as a control reference. The detailed procedure was performed per the manufacturer's instructions of the EZ-Magna RIP kit (Millipore, USA).

Statistical Analysis

Data are presented as the mean ± SEM. p values were calculated by a two-tailed Student's t test. $p < 0.05$ was considered statistically significant (*$p < 0.05$, **$p < 0.01$).

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.omtn.2020.07.002.

AUTHOR CONTRIBUTIONS

Conceptualization, C.S. and H.C.; Writing – Original Draft, C.S.; Writing – Review & Editing, C.S., Z.Y., R.J., J.C., B.Y., J.W., X.S., Y.H., X.L., and C.L.; Funding Acquisition, H.C.; Validation and Investigation, C.S., Z.Y., and R.J.; Supervision & Project Administration, H.C.

CONFLICTS OF INTEREST

The authors declare no competing interests.

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