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CircRNA profiling of skeletal muscle satellite cells in goats reveals circTGFβ2 promotes myoblast differentiation

Siyuan Zhan[1,2†], Rui Jiang[1,2†], Zongqi An[1,2], Yang Zhang[1,2], Tao Zhong[1,2], Linjie Wang[1,2], Jiazhong Guo[1,2], Jiaxue Cao[2], Li Li[2] and Hongping Zhang[1,2*]

Abstract

Background Circular RNAs (circRNAs) function as essential regulatory elements with pivotal roles in various biological processes. However, their expression profiles and functional regulation during the differentiation of goat myoblasts have not been thoroughly explored. This study conducts an analysis of circRNA expression profiles during the proliferation phase (cultured in growth medium, GM) and differentiation phase (cultured in differentiation medium, DM1/DM5) of skeletal muscle satellite cells (MuSCs) in goats.

Results A total of 2,094 circRNAs were identified, among which 84 were differentially expressed as determined by pairwise comparisons across three distinct groups. Validation of the expression levels of six randomly selected circRNAs was performed using reverse transcription PCR (RT-PCR) and quantitative RT-PCR (qRT-PCR), with confirmation of their back-splicing junction sites. Enrichment analysis of the host genes associated with differentially expressed circRNAs (DEcircRNAs) indicated significant involvement in biological processes such as muscle contraction, muscle hypertrophy, and muscle tissue development. Additionally, these host genes were implicated in key signaling pathways, including Hippo, TGF-beta, and MAPK pathways. Subsequently, employing Cytoscape, we developed a circRNA-miRNA interaction network to elucidate the complex regulatory mechanisms underlying goat muscle development, encompassing 21 circRNAs and 47 miRNAs. Functional assays demonstrated that circTGFβ2 enhances myogenic differentiation in goats, potentially through a miRNA sponge mechanism.

Conclusion In conclusion, we identified the genome-wide expression profiles of circRNAs in goat MuSCs during both proliferation and differentiation phases, and established that circTGFβ2 plays a role in the regulation of myogenesis. This study offers a significant resource for the advanced exploration of the biological functions and mechanisms of circRNAs in the myogenesis of goats.

Keywords Goat, circRNA sequencing, Differential expression, circTGFβ2, Differentiation

†Siyuan Zhan and Rui Jiang contributed equally to this work.

*Correspondence:
Hongping Zhang
zhp@sicau.edu.cn

[1]Key Laboratory of Livestock and Poultry Multi-omics, Ministry of Agriculture and Rural Affairs, College of Animal and Technology, Sichuan Agricultural University, Chengdu 611130, P. R. China
[2]Farm Animal Genetic Resources Exploration and Innovation Key Laboratory of Sichuan Province, Sichuan Agricultural University, Chengdu 611130, P. R. China

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Introduction

Skeletal muscle constitutes the primary component of food animals, directly influencing both the quantity and quality of meat [1–3]. Consequently, the investigation of skeletal muscle growth and development holds significant importance for breeding practices within the field of animal husbandry. Nonetheless, the process of skeletal muscle growth and development is intricate, encompassing the activation of skeletal muscle satellite cells, the proliferation and differentiation of myoblasts, the fusion of myoblasts into myofibers, and the expression of a diverse array of genes [4, 5]. The introduction of high-throughput sequencing technologies has facilitated the identification of an expanding array of noncoding RNAs involved in the regulation of muscle growth and development, including microRNAs (miRNAs), long noncoding RNAs (lncRNAs), and circular RNAs (circRNAs) [6–9]. Importantly, these noncoding RNAs have the potential to exert both direct and indirect effects on the process of myogenesis [10, 11], although the specific regulatory mechanisms remain largely undefined. As a result, a considerable number of noncoding RNAs require further investigation.

CircRNAs represent a recently identified class of single-stranded non-coding RNAs characterized by a covalently closed loop structure generated through back-splicing [12, 13]. These circRNAs can originate from various genomic regions, including exons, introns, untranslated regions, non-coding RNA loci, as well as intergenic and antisense transcripts [14]. Compared to their linear RNA counterparts, circRNAs exhibit enhanced structural stability and resistance to ribonuclease R (RNase R) [14]. CircRNAs are ubiquitously present in cells and tissues and play a crucial role in regulating gene expression through diverse mechanisms. These mechanisms include functioning as miRNA sponges, modulating RNA-binding proteins, and serving as templates for protein translation [15–17]. Emerging evidence underscores the significant role of circRNAs in the regulation of muscle growth and development, primarily through their function as molecular sponges for miRNAs [18, 19]. For example, circSGCB has been shown to inhibit myoblast proliferation and promote differentiation by sequestering bta-miR-27a-3p [20]. Similarly, circNDST1 acts as a competitive endogenous RNA, modulating the proliferation and differentiation of bovine myoblasts via the miR-411a/Smad4 axis [21]. Additionally, circLRRFIP1 serves as a sponge for the miR-15 family, facilitating the proliferation and differentiation of chicken skeletal muscle satellite cells [22]. In addition to the well-documented sponge effect, alternative regulatory mechanisms of circRNAs in myogenesis have been proposed. For instance, the protein encoded by circKANSL1L has been shown to interact with Akt, thereby enhancing FoxO3 transcriptional activity to regulate skeletal myogenesis [23]. Furthermore, another study suggests that the binding of PUR proteins to circSamd4 facilitates muscle development by inhibiting MHC transcription [24]. Despite these advances, the dynamic expression of circRNAs throughout the formation of goat skeletal muscle are still unclear, and their functions in myogenesis deserve further exploration.

In this study, we performed circRNA sequencing on skeletal muscle satellite cells cultured under both proliferative and differentiative conditions to explore the roles of circRNAs in the development of goat skeletal muscle. We identified differentially expressed circRNAs and subsequently constructed a DEcircRNA-miRNA interaction network to elucidate the miRNA-sponge functions of circRNAs. Notably, we discovered a novel circRNA, circTGFβ2, which exhibited a skeletal muscle-specific expression pattern and facilitated the differentiation of goat skeletal muscle satellite cells. These findings will facilitate the compilation of a comprehensive catalog of circRNAs involved in goat muscle development and enhance our understanding of the regulatory mechanisms underlying the development of goat skeletal muscle.

Results

Overview of circRNA sequencing data

To identify potential circRNAs involved in the regulation of skeletal myogenesis in goats, we conducted RNA sequencing to profile circRNA expression in skeletal muscle satellite cells (MuSCs) across different stages (GM, DM1 and DM5). The flowchart for this study is shown in Fig. 1A. A total of 721,950,292 raw reads were generated from nine samples. Post quality filtering, which involved the removal of low-quality reads and adaptor sequences, yielded 720,121,930 clean reads that were subsequently aligned to the goat genome (Supplementary Table 1). Quality control metrics indicated that each sample achieved a Q20 score of ≥97.82% and a Q30 score of ≥93.74%, with an average GC content of 50.29% (Supplementary Table 1). Alignment to the goat reference genome ARS1 revealed that over 90% of the reads were uniquely mapped. A total of 2,094 circRNAs were identified, including 4, 9, and 15 circRNAs specifically expressed in GM, DM1, and DM5, respectively, along with 1,465 circRNAs common to all three phases (Fig. 1B) (Supplementary Table 2). To ensure the reproducibility of the sequencing data, three replicates of each sample were collected. Principal Component Analysis (PCA) revealed high sample similarity (Fig. 1C), indicating the reliability of the sequencing data. Subsequent statistical analysis demonstrated that the majority of circRNA types were exon circRNAs, with only a minor proportion comprising intron, antisense, and intergenic

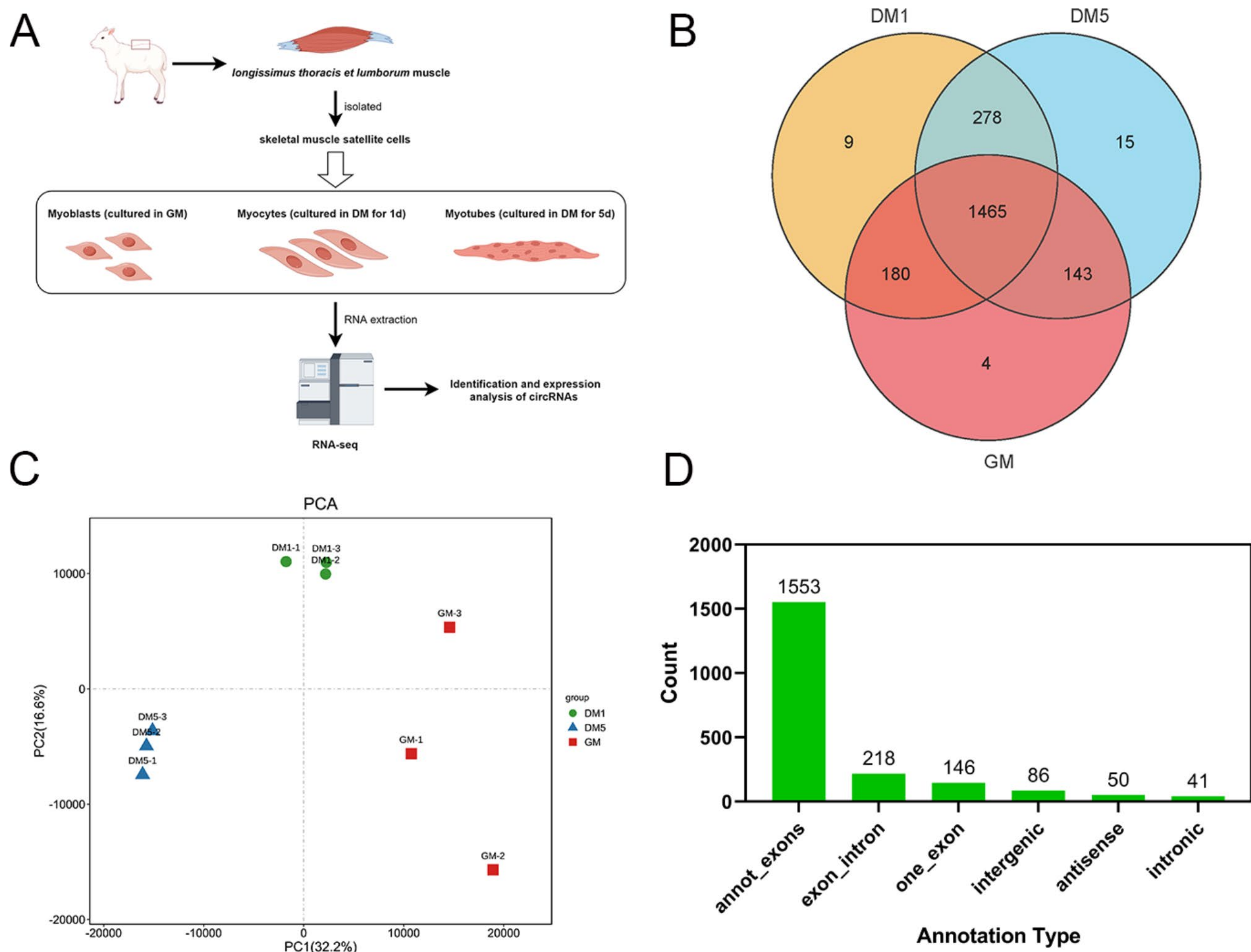

Fig. 1 Identification and characteristics of circRNAs in goat skeletal muscle satellite cells (MuSCs). (**A**) Schematic illustration of the experimental design. (**B**) Venn analysis of circRNAs detected at each time point. (**C**) Principal component analysis based on the expression of circRNAs. (**D**) Types of all circRNAs

region circRNAs (Fig. 1D). Taken together, these results confirmed that the sample and sequencing data generated in this study were of good quality and were reliable for subsequent bioinformatic analysis.

Identification of differentially expressed circRNAs

To elucidate the differences in circRNA expression patterns during myogenic differentiation in goats, the edgeR package was employed to identify DEcircRNAs across various sample pairs. A total of 84 DEcircRNAs were identified across the three stages, with 25, 55, and 22 DEcircRNAs detected in the GM-vs-DM1, GM-vs-DM5, and DM1-vs-DM5 comparison groups, respectively (Fig. 2A) (Supplementary Table 3). Further analysis using a Venn diagram revealed 14, 38, and 14 stage-specific DEcircRNAs in the GM-vs-DM1, GM-vs-DM5, and DM1-vs-DM5 comparisons, respectively (Fig. 2B). Notably, no DEcircRNAs were common across all three comparisons. Finally, hierarchical clustering analysis was used to assess the expression patterns of DEcircRNAs to explore the similarities and to compare the relationships between the different libraries, and it was found that samples within the same group were well clustered together (Fig. 2C).

Functional enrichment analysis of DEcircRNA host genes

To investigate the potential functions of the identified DEcircRNAs, GO and KEGG pathway enrichment analyses were conducted for the host genes of DEcircRNAs across the DM1-vs-DM5, GM-vs-DM1, and GM-vs-DM5 comparison groups. The results indicated significant enrichment of DEcircRNAs in 603, 375, and 543 GO terms, respectively, encompassing a wide array of biological processes (Supplementary Table 4). Specifically, in the DM1-vs-DM5 comparison group, the top 10 enriched biological process terms, as illustrated in Fig. 3A, revealed that the host gene functions were predominantly associated with muscle-related biological processes, including the regulation of striated muscle contraction,

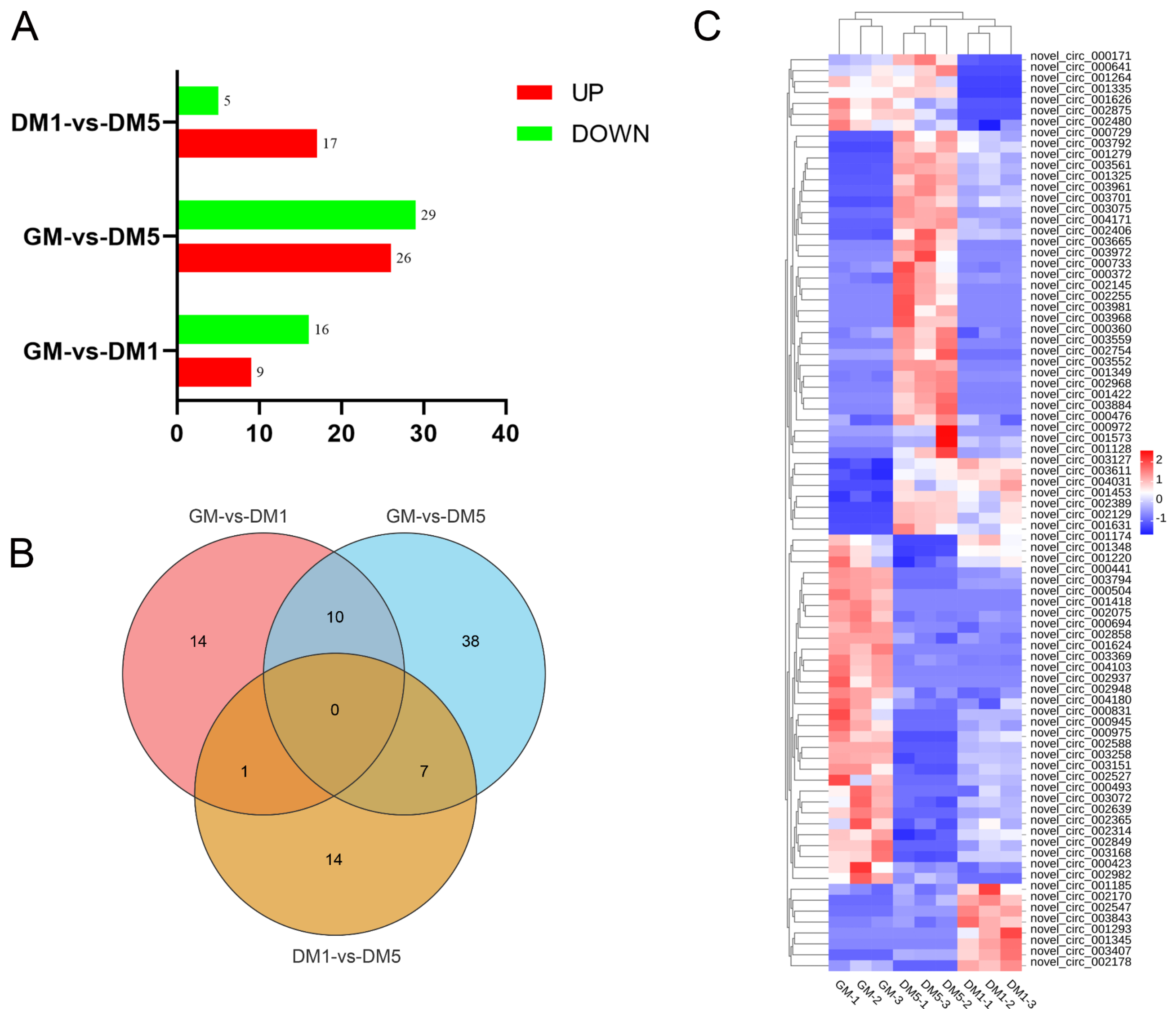

Fig. 2 Analysis of DEcircRNAs in goat MuSCs across different stages. (**A**) Numbers of up-regulated and down-regulated circRNAs in goat MuSCs across three stages. (**B**) Venn diagram showing the DEcircRNAs at the three comparisons. (**C**) Hierarchical clustering heat map of all DEcircRNAs. Data are expressed as RPM. Red represents relatively high expression, blue represents relatively low expression

regulation of skeletal muscle contraction, regulation of cell adhesion, muscle tissue development, and regulation of muscle system processes. In the GM-vs-DM1 group, the top 10 terms for biological processes included nucleic acid metabolic process, developmental process, and striated muscle contraction (Fig. 3B). Conversely, in the GM-vs-DM5 group, the identified host genes predominantly exhibited established roles in muscle biology, encompassing skeletal muscle contraction, musculoskeletal movement, striated muscle hypertrophy, muscle system processes, and cell differentiation (Fig. 3C).

To elucidate the functions of significantly enriched parental genes, a pathway analysis was performed using the KEGG pathway database (Supplementary Table 5). In the DM1-vs-DM5 comparison group, a substantial proportion of source genes for differentially expressed circRNAs (DEcircRNAs) exhibited significant enrichment in the Hippo signaling pathway and the MAPK signaling pathway (Fig. 3D), both of which are intimately linked to muscle development. Furthermore, pathways such as the adherens junction, TGF-beta signaling pathway, and FoxO signaling pathway, which rank among the top ten identified pathways, also play essential roles in muscle development (Fig. 3D). In a similar manner, within the GM-vs-DM1 group, several of the top ten pathways, such as the Hippo signaling pathway, Rap1 signaling pathway, TGF-beta signaling pathway, adherens junction, and tight junction, are associated with muscle development (Fig. 3E). Additionally, the parental genes of DEcircRNAs in the GM-vs-DM5 group were significantly

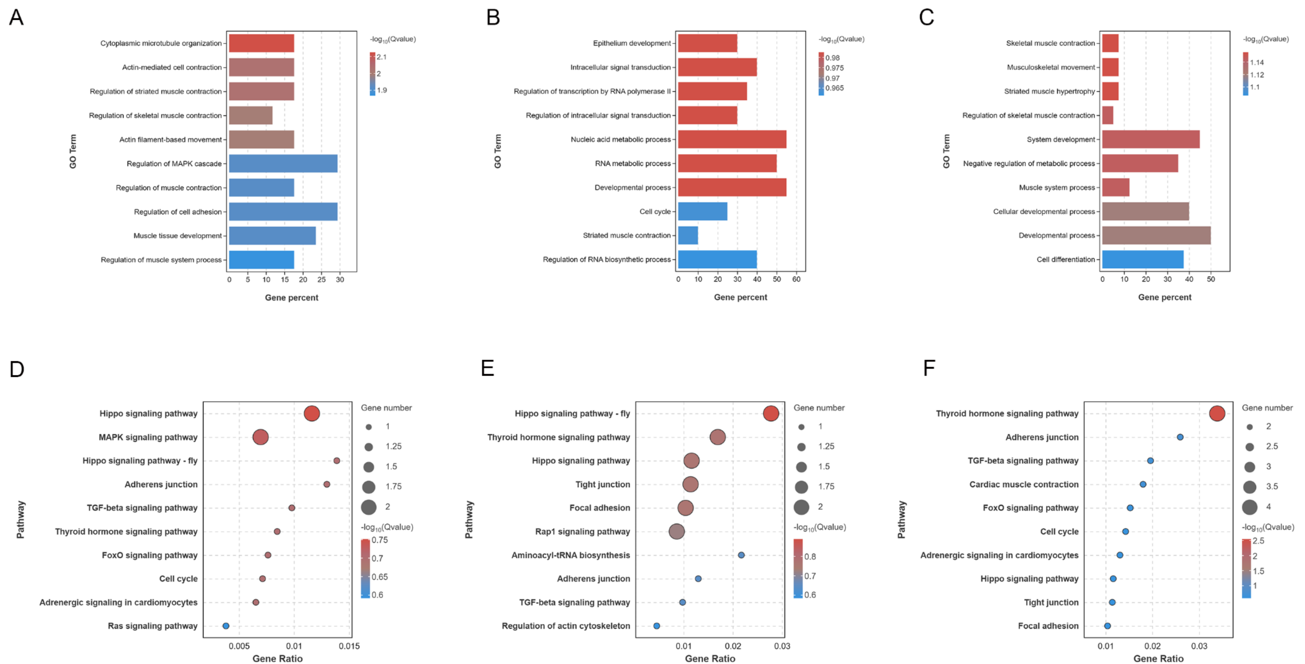

Fig. 3 Functional enrichment analysis for host genes of differentially expressed circRNAs. (**A**) The top 10 significance terms of biological process in DM1 vs. DM5. (**B**) The top 10 significance terms of biological process in GM vs. DM1. (**C**) The top 10 significance terms of biological process in GM vs. DM5. (**D**) The top 10 significance pathways in DM1 vs. DM5. (**E**) The top 10 significance pathways in GM vs. DM1. (**F**) The top 10 significance pathways in GM vs. DM5

enriched in the thyroid hormone, TGF-beta, FoxO, and Hippo signaling pathways (Fig. 3F).

Construction of the circRNA-miRNA network

More and more evidence suggests that circRNAs can act as miRNA sponges to competitively bind miRNA and regulate the expression of miRNA target genes, thereby participating in related biological processes [25–29]. To predict interactions between circRNAs and miRNAs, we utilized the miRanda and TargetScan algorithms. By integrating the interaction data with the differential expression profiles of circRNAs, we constructed interaction network data files, which were subsequently imported into Cytoscape software for visualization and analysis. Our analysis identified a total of 21 DEcircRNAs and 47 miRNAs, culminating in 71 interaction pairs between miRNAs and circRNAs (Fig. 4) (Supplementary Table 6). Notably, novel_circ_003559 was found to interact with 18 miRNAs, whereas novel_circ_002145 interacted with 14 miRNAs. Moreover, specific miRNAs, such as chi-miR-7-5p, chi-miR-487a-5p, and chi-miR-130b-5p, were found to interact with multiple circRNAs. In addition, our analysis identified several miRNAs implicated in skeletal muscle development within this interaction network, including chi-miR-206, chi-miR-381, chi-miR-380-5p, chi-miR-16b-5p, and chi-miR-15b-5p [30–32]. These observations indicate that these circRNAs may exert a regulatory influence on skeletal muscle development through their binding to miRNAs.

Validation of DEcircRNAs

To validate the reliability of RNA-Seq data, six highly expressed DEcircRNAs (novel_circ_000360, novel_circ_000805, novel_circ_002075, novel_circ_001349, novel_circ_003418, and novel_circ_004171) were selected for validation experiments, and divergent primers corresponding to circRNAs were designed (Supplementary Table 7). The PCR product was examined by sanger sequencing to confirm the back-splicing junction site (Fig. 5A). Subsequently, we performed reverse transcription polymerase chain reaction (RT-PCR) to evaluate the exonuclease resistance of circRNAs in comparison to linear RNA. Post RNase R digestion, circRNA remained detectable via RT-PCR, whereas the linear RNA, GAPDH, was rendered undetectable. These findings suggest that, relative to the control group, the six circRNAs demonstrated resistance to RNase R digestion as illustrated in Fig. 5B. Furthermore, divergent primers specific to each circRNA successfully amplified the anticipated fragments from complementary DNA (cDNA), but failed to produce PCR products when genomic DNA (gDNA) was used as a template (Fig. 5B), thereby confirming the presence of back-site junctions. Furthermore, we analyzed the expression patterns of these circRNAs across three phases of MuSCs using qRT-PCR. The results demonstrated that the expression profiles were consistent with the trends observed in the RNA-seq data (Fig. 5C). This concordance confirms the reliability of the

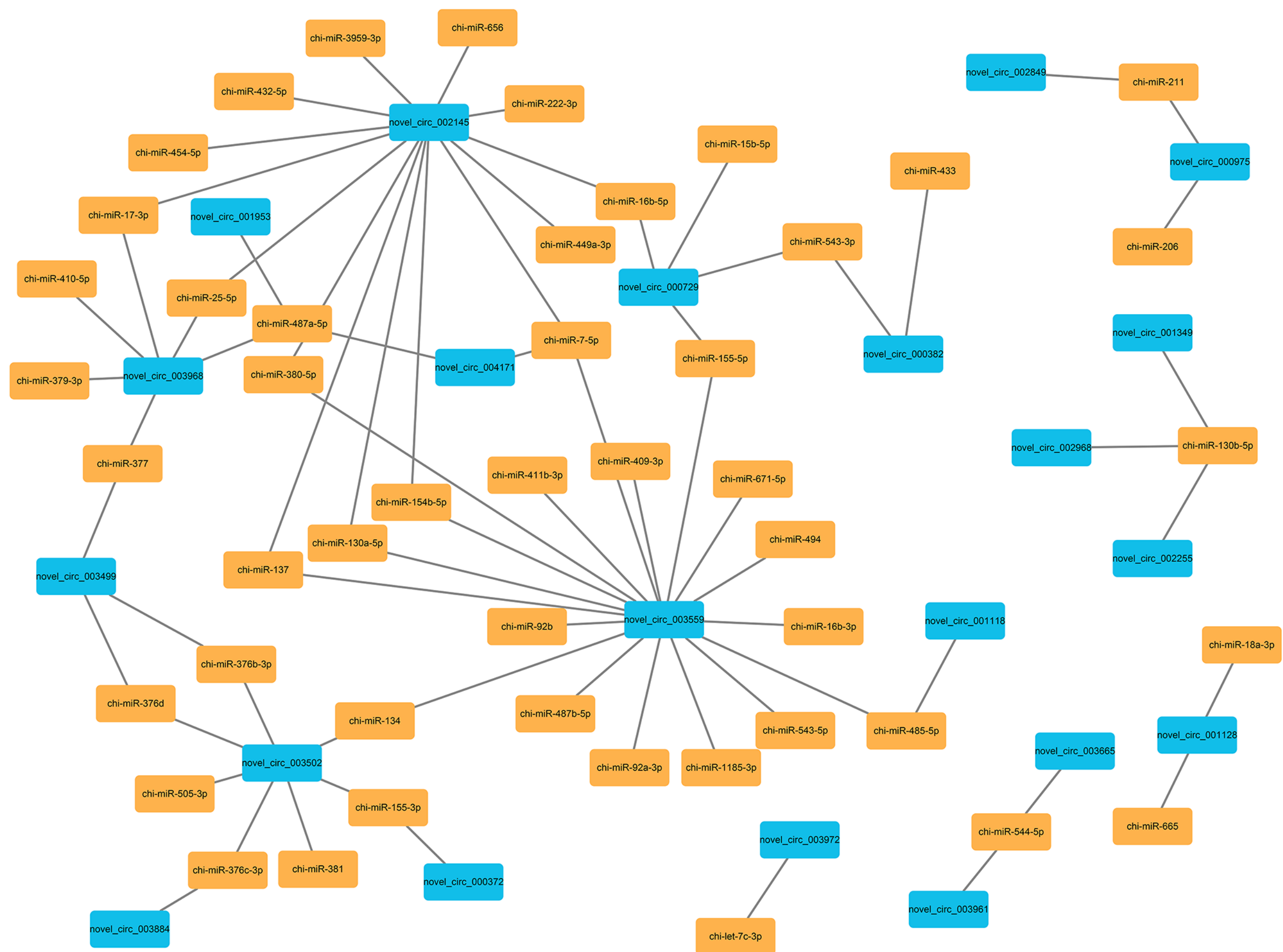

Fig. 4 The circRNA-miRNA interaction networks, including 21 DEcircRNAs and 47 miRNAs. Blue represent DEcircRNAs and yellow represent miRNAs

RNA-seq data and the expression of the circRNAs identified in this study.

The expression and characteristics of circTGFβ2

From the aforementioned network, we selected novel_circ_000975 (designated as circTGFβ2) for further investigation, hypothesizing its potential role in regulating myoblast differentiation through the predicted circTGFβ2-chi-miR-206/chi-miR-211 pathway. CircTGFβ2 is an circRNA derived from exons 4 to 7 of the TGFβ2 gene. The back-splice junction sequence of circTGFβ2 was validated via Sanger sequencing (Fig. 6A). Subsequently, we performed RT-PCR using cDNA synthesized with either random primers or Oligo(dT) primers. The absence of bands in the Oligo(dT) group indicated that circTGFβ2 lacks a 3’ poly-A tail (Fig. 6B). Divergent primers successfully amplified the predicted fragments utilizing cDNA, but failed to generate PCR products when gDNA was employed as a template (Fig. 6B), thereby corroborating the presence of back-site junctions. Additionally, we assessed the exonuclease resistance of circTGFβ2 using RT-PCR and qRT-PCR methodologies. Treatment with RNase R demonstrated that circTGFβ2 exhibited resistance to RNase R-mediated degradation, in contrast to linear TGFβ2 mRNA, which was predominantly degraded (Fig. 6B and C). The expression of circTGFβ2 was detected in various tissues, with the highest expression levels observed in skeletal muscle tissues, followed by the heart and liver (Fig. 6D). We performed a comprehensive analysis of circTGFβ2 expression during the differentiation of MuSCs, employing growth medium for 1 and 2 days, and differentiation medium for 1, 3, and 5 days. As depicted in Fig. 6E, the expression levels of circTGFβ2 escalated significantly during the differentiation phase, suggesting its pivotal role in muscle differentiation regulation. In addition, we investigated the subcellular localization of circTGFβ2 via RNA-FISH. The results indicated that circTGFβ2 predominantly resides in the cytoplasm (Fig. 6F), corroborating its hypothesized function as a miRNA sponge. A dual-luciferase reporter assay was conducted to confirm the interactions between circTGFβ2 and chi-miR-206/chi-miR-211. The findings indicated that chi-miR-206 and chi-miR-211 significantly decreased luciferase

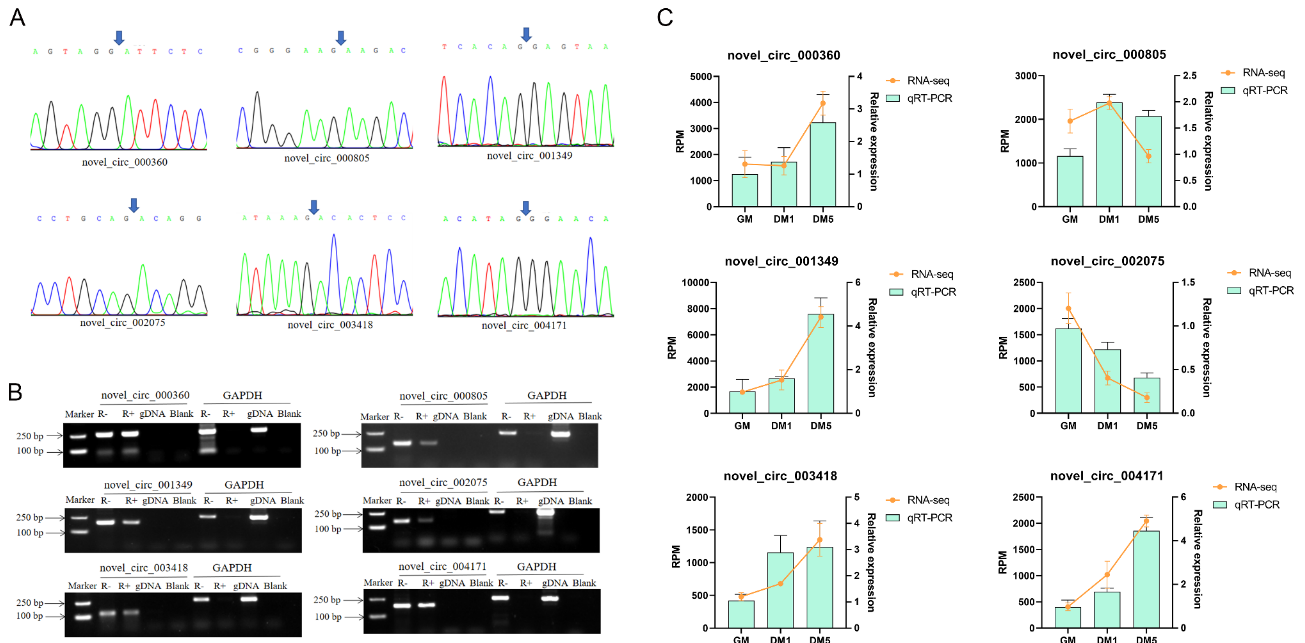

Fig. 5 Experimental validation of circRNAs. (**A**) Sanger sequencing shows head-to-tail junctions. Blue arrow shows back spliced junctions. (**B**) Agarose gel electrophoresis test for PCR product of six circRNAs using different templates. gDNA represent genomic DNA; R + indicates the samples were treated with exonuclease. (**C**) Expression profiles of the six randomly selected circRNAs based on RNA-Seq and qRT-PCR

activity in comparison to the mutant group (Fig. 6G and H), thereby substantiating the binding relationship between chi-miR-206/chi-miR-211 and circTGFβ2.

The function of circTGFβ2 in MuSC differentiation

To elucidate the function of circTGFβ2 in MuSC differentiation, we engineered an overexpression vector for circTGFβ2 (pLV-circTGFβ2) and designed three small interfering RNAs (siRNA-1, -2, -3) targeting the back-spliced junction of circTGFβ2. Quantitative reverse transcription PCR (qRT-PCR) analysis verified that the pLV-circTGFβ2 vector successfully facilitated the overexpression of circTGFβ2 (Fig. 6I), Conversely, all three small interfering RNAs (siRNAs) markedly reduced circTGFβ2 expression in MuSCs (Fig. 6J). Importantly, the silencing of circTGFβ2 did not lead to any significant changes in TGFβ2 mRNA expression levels (Fig. 6K). Furthermore, we examined the effects of circTGFβ2 overexpression and knockdown on the differentiation of myoblasts. The expression levels of well-established myogenic differentiation markers, namely MyoD, MyoG, and MyHC, were quantified in goat MuSCs subsequent to transfection with pLV-circTGFβ2 and siRNA constructs (si-1, si-2, si-3). The overexpression of circTGFβ2 resulted in a marked upregulation of the mRNA expression of MyoD, MyoG, and MyHC (Fig. 6L). In contrast, the knockdown of circTGFβ2 led to a significant downregulation of the mRNA expression of these markers (Fig. 6M). These findings suggest that circTGFβ2 exerts a promotive effect on the differentiation of myoblasts in goats.

Discussion

Myogenesis is a process including myoblast proliferation, differentiation and myotube formation and is controlled by a series of myogenic regulatory factors [33, 34]. In recent years, an increasing number of non-coding RNAs capable of regulating myogenesis have been identified. As a unique form of non-coding RNA family, circRNAs are distinguished by their high abundance, high degree of stability, and tissue/spatiotemporal-specific expression, all of which play crucial roles in a variety of biological processes [18, 35, 36]. Emerging evidence suggests that circRNAs play significant roles in the growth and development of skeletal muscle across multiple species, including pigs [37, 38], cattles [28, 39], and chickens [40, 41]. Despite recent advancements, the expression profiles and biological functions of circRNAs in goat skeletal muscle stem cells remain largely unexplored. To elucidate the roles of circRNAs in myoblast differentiation, we conducted a comprehensive RNA sequencing analysis to profile circRNA expression in both proliferative and differentiated goat skeletal muscle satellite cells. We further predicted the functions of differentially expressed circRNAs through GO and KEGG pathway analyses, complemented by interaction network assessments.

In this study, a total of 2,094 circRNAs were identified, with 84 DEcircRNAs observed across the three stages. GO enrichment analysis indicated that the source genes of the DEcircRNAs were significantly enriched in processes related to the regulation of striated muscle contraction, skeletal muscle contraction, cell adhesion,

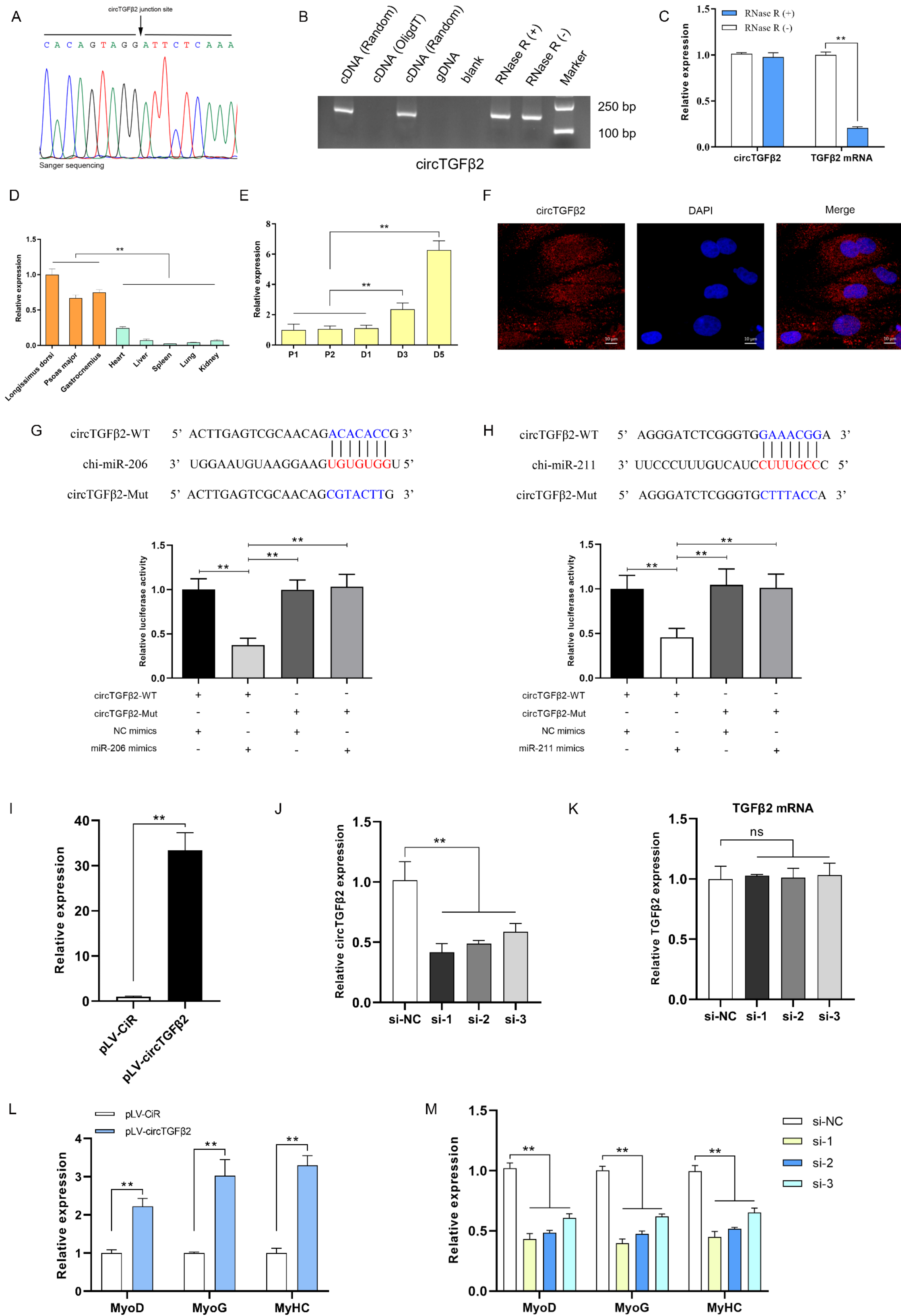

Fig. 6 (See legend on next page.)

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Fig. 6 The characterization and function of circTGFβ2 in MuSC differentiation. (**A**) The back-site junction of circTGFβ2 was identified using a divergent primer and sequenced by Sanger sequencing. (**B**) Agarose gel electrophoresis test for PCR product of circTGFβ2 using different templates. Random shows the cDNA template synthesized using random primers for RT-PCR, while Oligo(dT) denotes the cDNA synthesized using Oligo(dT) primers; gDNA represent genomic DNA; RNase R + indicates the samples were treated with exonuclease. (**C**) qRT-PCR analysis of the expression of circTGFβ2 and TGFβ2 in goat myoblasts treated with RNase R. (**D**) The abundance of circTGFβ2 in various tissues of goats on the third day after birth. (**E**) The expression level of circTGFβ2 during proliferation and differentiation of goat skeletal muscle satellite cells. (**F**) RNA FISH assay was performed to determine the subcellular localization of circTGFβ2 in goat skeletal muscle cells, blue indicates nuclei stained with DAPI; red indicates RNA probes recognizing circTGFβ2. Scale bar, 10 μm. (**G**) Luciferase reporter activity of circTGFβ2 in MuSCs co-transfected with chi-miR-206 mimics or mimics NC. (**H**) Luciferase reporter activity of circTGFβ2 in MuSCs co-transfected with chi-miR-211 mimics or mimics NC. (**I**) CircTGFβ2 was successfully overexpressed in MuSCs of goat. (**J**) CircTGFβ2 was successfully inhibited in MuSCs of goat. (**K**) The expression of TGFβ2 mRNA was detected by qRT-PCR after circTGFβ2 knockdown. (**L**) The expression of MyoD, MyoG, and MyHC was detected by qRT-PCR after transfection with pLV-circTGFβ2 and pLV-CiR vector. (**M**) The expression of MyoD, MyoG, and MyHC was detected by qRT-PCR after transfection with si-1, si-2, and si-3. *$P < 0.05$, **$P < 0.01$

muscle tissue development, and striated muscle hypertrophy, all of which are closely associated with muscle growth and development. In addition, KEGG pathway enrichment analysis indicated that the source genes of the DEcircRNAs were involved in Hippo, MAPK, TGF-beta and FoxO signaling pathways. Research has demonstrated that the Hippo signaling pathway plays a pivotal role in the regeneration and functional regulation of skeletal muscle [42–44]. Moreover, previous studies have established that the MAPK signaling pathway functions as the principal pathway during skeletal muscle differentiation [45, 46]. Additionally, the TGF-beta signaling pathway is acknowledged for its complexity and importance in the regulation of various cellular processes [47]. It is well-characterized as a potent inhibitor of muscle cell differentiation due to its suppressive effect on the transcriptional activity of myogenic regulatory factors (MRFs) [48]. Furthermore, the FoxO family of proteins, which are highly conserved transcription factors, play pivotal roles in regulating skeletal muscle plasticity. Specifically, FoxO1 and FoxO3 are essential for maintaining muscle energy homeostasis by modulating glycolytic and lipolytic fluxes as well as mitochondrial metabolism [49]. Collectively, these findings suggest that the corresponding circRNAs perform critical regulatory functions in the development of goat skeletal muscle. Additionally, the reliability of the circRNAs identified through RNA sequencing was corroborated by RT-PCR and qRT-PCR validation of six randomly selected DEcircRNAs.

Previous studies have established that circRNAs can act as miRNA sponges by competitively binding to miRNAs, thereby influencing the activity of their target genes [14, 50]. In order to elucidate the role of circRNAs in the differentiation of goat myoblasts, a circRNA-miRNA interaction network was constructed to predict potential interactions between circRNAs and miRNAs. Analysis of this network indicated that circRNAs are capable of interacting with multiple miRNAs. For instance, novel_circ_003559 was found to be associated with 18 miRNAs, whereas novel_circ_002145 was linked to 14 miRNAs. While none of the circRNAs have been annotated in myoblasts, our observations indicate that several miRNAs, such as chi-miR-206, chi-miR-381, chi-miR-16b-5p, and chi-miR-15b-5p, have previously been shown to play functional roles in myogenesis [30–32]. These findings suggest that circRNAs may regulate gene expression related to myogenesis through interactions with miRNAs.

Consequently, we selected circTGFβ2, a circRNA derived from the goat TGFβ2 gene, for validation experiments in MuSCs, due to its interaction with chi-miR-206, a well-established miRNA specific to skeletal muscle [51–53]. Before initiating the myoblast experiments, we employed RT-PCR, Sanger sequencing, and RNase R treatments to verify the presence of circTGFβ2. The results demonstrated that circTGFβ2 is abundantly expressed in skeletal muscle tissues and that its expression rapidly increases during the differentiation of MuSCs, suggesting a potential role in muscle differentiation. Furthermore, the subcellular localization of circRNAs is crucial in determining their molecular functions. Within the cytoplasm, circRNAs can function as competitive inhibitors of miRNAs by binding to them, thereby alleviating the miRNA-mediated repression of their target genes [14, 17]. In the present study, circTGFβ2 was predominantly localized in the cytoplasm, suggesting a possible regulatory function through miRNA competition. We subsequently investigated the role of circTGFβ2 in the differentiation of goat myoblasts. The findings demonstrated that circTGFβ2 promotes myogenic differentiation in these myoblasts. Recent studies have elucidated the critical role of circRNAs in myogenesis. Specifically, circMEF2As [54], circACTA1 [55], circFgfr2 [56], and circPTPN4 [57] have been confirmed to enhance myogenesis by positively regulating myoblast differentiation. In contrast, circMEF2D [58], circHUWE1 [59], circTAF8 [60], and circCPE [61] have been identified as inhibitors of differentiation. Collectively, these findings suggest that circTGFβ2 promotes myogenic differentiation in goats, potentially via a miRNA sponge mechanism.

Conclusions

In summary, this study characterized the expression profiles of circRNAs during the differentiation of myoblasts in goats. Enrichment analyses revealed that DEcircRNAs were significantly linked to a range of biological processes and pathways relevant to muscle development, such as muscle contraction, muscle tissue development, and the TGF-beta and MAPK signaling pathways. Moreover, the circRNA-miRNA interaction networks elucidated in this study provide a valuable resource of candidate circRNAs involved in myogenesis. Furthermore, we identified and validated a novel circRNA, circTGFβ2, which was observed to promote myogenic differentiation in goats. The findings of this study provide a foundation for future investigations into the mechanisms by which circRNA influence the proliferation and differentiation of goat skeletal muscle.

Materials and methods

Ethics statement

The Animal Care and Use Committee of the College of Animal Science and Technology, Sichuan Agricultural University, Sichuan, China, approved all of the animal care, slaughter, and experimental procedures in accordance with the Regulations for the Administration of Affairs Concerning Experimental Animals (Ministry of Science and Technology, China) [Approval No. SAU20231023].

Sample preparation

All experimental Chengdu Ma goats were managed and raised on a farm managed by the Chengdu Xilingxue Agricultural Development Co., Ltd. (Sichuan, China). Prior to sample collection, the goats were administered Jingsongling (batch number 870721, produced by Shandong Zibo Veterinary Medicine Factory, Shandong, China) via intramuscular injection in the hip region at a dosage of 2 mg/kg to achieve deep anesthesia. Once full anesthesia was confirmed, the goats were euthanized through arterial exsanguination, performed by trained slaughterhouse personnel (this method complies with euthanasia guide of the Chinese Society of Laboratory Animals, No. T/CALAS 31–2017). The primary skeletal muscle satellite cells (MuSCs) were isolated and cultured from *longissimus dorsi* muscle derived from a newborn goat (Chengdu Ma goat, female), as described previously [62, 63]. The MuSCs were seeded in 6-well plates (at a density of $\sim 2\times10^5$ cells/well) and cultured in Dulbecco's Modified Eagle Medium (DMEM) in a 5% CO_2 incubator at 37℃, supplemented with 10% FBS (Gibco, Invitrogen, Carlsbad, CA, USA) and 2% penicillin/streptomycin (Solarbio, Beijing, China). When the confluence reached about 80–90%, the growth medium (GM) was replaced with differentiation medium (DM) containing DMEM, 2% horse serum (Gibco) and 2% penicillin/streptomycin in order to induce MuSC differentiation. The medium was replaced with fresh medium every 48 h. Proliferating MuSCs were labelled GM samples, while MuSCs differentiated for 1 and 5 days were labelled as DM1 and DM5 samples (three biological replicates at each time point). In addition, three female Chengdu Ma goats were sacrificed humanely on the third day after birth. Three skeletal muscles (longissimus dorsi muscle, psoas major muscle, and gastrocnemius muscle) and five internal organs (heart, liver, spleen, lung, and kidney) were collected. All of the samples were kept at -80℃ before RNA extraction.

RNA extraction, library construction, and sequencing

Total RNAs was extracted using Trizol reagent kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. RNA quality was assessed on an Agilent 2100 Bioanalyzer (Agilent Technologies, PaloAlto, CA, USA) and checked using RNase free agarose gel electrophoresis. After extracted, total RNAs were treated with RNase R to degrade the linear RNAs, and purified using RNeasy MinElute Cleanup Kit (Qiagen, Venlo, The Netherlands). Next, strand-specific library was constructed using VAHTS Total RNA-seq (H/M/R) Library Prep Kit (Vazyme, Nanjing, China) for Illumina following the manufacture's instructions. Briefly, ribosome RNAs were removed to retain circRNAs. The enriched circRNAs were fragmented into short fragments by using fragmentation buffer and reverse transcribed into cDNA with random primers. Second-strand cDNA were synthesized by DNA polymerase I, RNase H, dNTP (dUTP instead of dTTP) and buffer. Next, the cDNA fragments were purified with VAHTSTM DNA Clean Beads (Vazyme, Nanjing, China), end repaired, poly(A) added, and ligated to Illumina sequencing adapters. Then UNG (Uracil-N-Glycosylase) was used to digest the second-strand cDNA. The digested products were purified with VAHTSTM DNA Clean Beads, PCR amplified, and sequenced using Illumina Novaseq60000 by Gene Denovo Biotechnology Co. (Guangzhou, China).

Quality control and circRNAs identification

Raw reads were filtered to acquire clean reads by removing adapter-containing reads, poly-N-containing reads (over 10%), and low-quality reads (Q-value $\leq$ 20) using the fastp (version 0.18.0) [64]. The clean reads were aligned to the goat reference genome (ARS1, GCF_001704415.1) using the TopHat2 (version 2.1.1) [65]. CircRNAs were then identified using find_circ [66] and CIRI [67]. The anchor alignments were then extended such that the complete read aligns and the breakpoints were flanked by GU/AG splice sites. A candidate circRNA was called if it was supported by at least two unique back spliced reads at least in one sample. The identified circRNAs were

subjected to statistical analysis of type, chromosome distribution and length distribution. The expression level of each annotated circRNA was normalized by calculating the Reads Per Million mapped reads (RPM). The edgeR package (version 3.12.1) was applied to identify differentially expressed circRNAs (DEcircRNAs) in each comparison, with $|\log_2(\text{Fold Change})| > 1$ and $P<0.05$.

GO Enrichment, KEGG Pathway, and CircRNA-miRNA network

Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses were performed using the DAVID tool [68, 69]. In the DAVID tool, the Benjamini method requests adjusted p-values using the linear step-up procedure of Benjamini and Hochberg, which was utilized to identify statistically significant GO terms and KEGG pathways [70]. The genome of the goat (*Capra hircus*) served as the background parameter for this analysis. The hypergeometric test was employed to evaluate statistical significance, with a threshold significance level set at $P<0.05$ for identifying significantly enriched GO terms and KEGG pathways. CircRNAs are proposed to regulate gene expression through competing endogenous RNAs acting as miRNA sponges [29]. The target miRNAs, which could be bound to the circRNAs, were predicted using the miRanda [71] and TargetScan [72] software with a set of default parameters. Cytoscape [73] was used for network visualization.

Validation of CircRNAs by qRT-PCR, sequencing, and RT-PCR

The circRNA sequencing data was validated by randomly selecting six differentially expressed circRNAs to assess the reliability of the obtained results. Given the unique head-to-tail junction characteristic of circRNAs, their authenticity was confirmed using RT-PCR. The RT-PCR amplicons were analyzed via 2% agarose gel electrophoresis and subsequently sequenced using Sanger sequencing. To evaluate the sensitivity of circRNAs to RNase R, RT-PCR was conducted on RNA samples treated with and without RNase R. Additionally, qRT-PCR was employed to verify the expression levels of circRNAs, using GAPDH as the reference gene. Total RNA was extracted from tissues or cells using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. cDNA was synthesized using a RT reagent kit (Takara, Dalian, China) and qRT-PCR for circRNA and mRNA was performed on the CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) using SYBR Premix Ex Taq™ II (Takara, Dalian, China). Each experiment was conducted independently three times with three biological replicates. The $2^{-\Delta\Delta Ct}$ method [74] was used to calculate relative expression levels. The primer sequences used in this study are listed in Supplementary Table 7.

Vector construction and siRNA synthesis

To construct the novel_circ_000975 (designated as circTGFβ2) overexpression vector, exons 4, 5, 6, and 7 of the caprine TGFβ2 gene were amplified utilizing cDNA derived from MuSCs and subsequently inserted into a pLV-CiR vector (Geneseed, Guangzhou, China) at the *EcoRI* and *BamHI* restriction sites. Additionally, three small interfering RNAs (siRNAs) targeting circTGFβ2 were designed and synthesized by RiboBio (Guangzhou, China). The sequence of siRNAs are listed in Supplementary Table 7.

Cell culture and transfection

The goat MuSCs were seeded in 6-well ($\sim 2\times10^5$ cells per well) plates containing growth medium (GM) (DMEM containing 10% FBS, Hyclone) and cultured at 37 °C in a 5% CO_2 atmosphere. When MuSCs achieved 80% confluence, GM was replaced with differentiation medium (DM) containing 2% horse serum (Hyclone) to stimulate myoblast differentiation. The medium was replaced every 48 h. For transfection, MuSCs were seeded in 6-well plates and allowed to reach 70–80% confluence. Transfection was performed using Lipofectamine 3000 (Invitrogen, USA) according to the manufacturer's protocol, with a minimum of three replicates. After 24 h, the transfected cells were collected for qRT-PCR assay. The final concentration of the pLV-circTGFβ2 vector and siRNAs used in transfections was 4 μg/mL and 50 nM, respectively.

RNA FISH

For the synthesis of RNA-FISH probes and the subsequent RNA-FISH assay on goat MuSCs using the FISH assay kit (Gefan Biotechnology Co., Shanghai, China), cells were seeded into 6-well plates and cultured until they reached 60 ~ 70% confluence. The cells were then fixed with 4% paraformaldehyde. Following fixation, the cells were permeabilized with 0.3% Triton X-100 and incubated with a 20 mg/mL probe at 65 °C for 48 h. The nuclei were counterstained with DAPI, and fluorescence images were captured using a fluorescence microscope (Olympus, Tokyo, Japan).

Luciferase activity assay

To further elucidate the binding interaction between circTGFβ2 and chi-miR-206/chi-miR-211, both wild-type and mutant circTGFβ2 fragments were amplified and subcloned into the psiCHECK-2 vector. The circTGFβ2 mutant (circTGFβ2-Mut) was generated by altering the binding site using the TaKaRa MutanBEST Kit. A list of primers utilized for plasmid construction is provided in

Supplementary Table 7. In the luciferase reporter assays, MuSCs were cultured in 24-well plates and subsequently co-transfected with either circTGFβ2-WT or circTGFβ2-Mut constructs, along with chi-miR-206/chi-miR-211 mimics or a negative control mimic (NC mimics). Following a 48-hour post-transfection period, the activities of firefly and renilla luciferases were quantified using a dual luciferase reporter assay kit (Promega, Madison, WI, USA), in accordance with the manufacturer's protocol.

Statistical analysis

All data were expressed as mean ± standard error, with at least three biological replicates. The student's t-test was used to analyze statistical significance for two-group comparisons, and one-way ANOVA with Tukey's post-hoc test for multiple-group comparisons using SAS software version 9.2 (SAS, Cary, NC, USA). The significance level was set at $^*P<0.05$ and $^{**}P<0.01$.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12864-024-11008-4.

Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

Supplementary Material 4

Supplementary Material 5

Supplementary Material 6

Supplementary Material 7

Supplementary Material 8

Supplementary Material 9

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Author contributions

SZ: Writing - original draft, Conceptualization, Funding acquisition. RJ: Investigation, Validation. ZA and YZ: Formal analysis, Validation. TZ and LW: Methodology, Software. JG and JC: Methodology, Visualization. LL: Data curation, Resources. HZ: Writing - review & editing, Supervision. All authors have read and approved the final manuscript.

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Data availability

Sequence data that support the findings of this study have been deposited in the NCBI with the primary accession code PRJNA779184. The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

The Animal Care and Use Committee of the College of Animal Science and Technology, Sichuan Agricultural University, Sichuan, China, approved all of the animal care, slaughter, and experimental procedures in accordance with the Regulations for the Administration of Affairs Concerning Experimental Animals (Ministry of Science and Technology, China) [Approval No. SAU20231023]. Informed consent was obtained from the owner of Chengdu Xilingxue Agricultural Development Co., Ltd, before sampling Chengdu Ma goats for this study.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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