

RESEARCH PAPER



Expression of circular RNAs during C2C12 myoblast differentiation and prediction of coding potential based on the number of open reading frames and N⁶-methyladenosine motifs

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ABSTRACT

The importance of circular RNAs (circRNAs) as regulators of muscle development and muscle-associated disorders is becoming increasingly apparent. To explore potential regulators of muscle differentiation, we determined the expression profiles of circRNAs of skeletal muscle C2C12 myoblasts and myotubes using microarray analysis. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses were performed to explore circRNA functions. We also established competing endogenous RNA (ceRNA) networks using bioinformatics methods and predicted the coding potential of differentially expressed circRNAs. We found that 581 circRNAs were differentially regulated between C2C12 myoblasts and myotubes. Bioinformatics analysis suggested that the primary functions of the linear transcripts of the circRNAs were linked with organization of the cytoskeleton, calcium signaling, cell cycle, and metabolic pathways. ceRNA networks showed that the myogenic-specific genes myogenin, myocyte enhancer factor 2a, myosin heavy chain (Myh)-1, Myh7, and Myh7b could combine with 91 miRNAs and the top 30 upregulated circRNAs, forming 239 edges. According to the number of open reading frames and N⁶-methyladenosine motifs, we identified 224 circRNAs with coding potential, and performed GO and KEGG analyses based on the linear counterparts of 75 circRNAs. We determined that the 75 circRNAs were related to regulation of the actin cytoskeleton and metabolic pathways. We established expression profiles of circRNAs during C2C12 myoblast differentiation and predicted the function of differentially expressed circRNAs, which might be involved in skeletal muscle development. Our study offers new insight into the functions of circRNAs in skeletal muscle growth and development.

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1. Introduction

Circular RNAs (circRNAs) are stable, highly conserved covalently closed RNA circles [1–3], and they seem to accomplish noncoding roles [4–6]. Although circRNAs were first discovered in the 1970s [5,7,8], they were initially considered to represent errors of the normal splicing process and were generally considered peculiarities of uncertain biological importance [9]. However, recent studies of circRNAs have resulted in the new understanding that circRNAs play an important role in cellular differentiation, development, and the onset and development of disease. In some cancers [10–12], circRNA expression differed compared with peritumoral tissue, suggesting circRNAs might be involved in cell proliferation, differentiation, and apoptosis.

Recent reports have shown that circRNAs play an important role in the differentiation and development of skeletal muscle [13–16], but the mechanism is not well understood [14,17].

It's reported that circRNAs play their role by acting as competing endogenous RNA (ceRNA) [13,15,16,18]. Coding and noncoding RNAs can regulate one another through their ability to compete for miRNA binding; these molecules have been termed ceRNA [18]. ceRNAs can sequester miRNAs, thereby protecting their target RNAs from repression. Circular RNA sponge for miR-7 (ciRS-7) acting as a miR-7 sponge probably engaged in the functioning of neurons and brain tumour development [6], and circular RNA MTO1 acting as the sponge of miR-9 could suppress hepatocellular carcinoma progression [19].

Recently, there is increasing evidence that circRNAs are important in the muscle regulatory network by ceRNA. circLMO7 was shown to promote primary bovine myoblasts proliferation and protect myoblasts from apoptosis by ceRNA [13]. Both circFUT10 sponging miR-133a [15] and circFGFR4 binding miR-107 [16] were shown to facilitate cell differentiation in bovine primary myoblasts.

While circRNAs were originally considered non-coding RNAs, recent studies have shown that circRNAs can be translated and have an important effect on cellular and muscle differentiation and development [20–22]. circFBXW7 could be translated into the protein FBXW7-185aa, which could induce cell cycle arrest and reduce proliferation in glioma cell [22]. Legnini et al. [14] firstly showed that circZNF609 regulated myoblast proliferation and could be translated, and the flagged protein derived from circZNF609 exhibited mainly nuclear localization, but its function and translation mechanism was unknown. Recently, it was reported that m⁶A motif was sufficient to drive translation initiation in circRNAs; and m⁶A motif-driven translation required eukaryotic translation initiation factor 4 gamma 2 (eIF4G2) and m⁶A reader YTH domain family protein 3 (YTHDF3) in human cells [21]. This was the first report of a circRNA with chemical m⁶A modification, which undoubtedly represented an important advance in circRNA research. Coding potential of the skeletal muscle circRNAs, and corresponding functions and mechanisms are currently in the development stage; it's necessary to make further study in the future.

Skeletal muscle is an important part of the motor system that maintains movement and support, and makes up approximately 40% of total body weight. The formation of skeletal muscle fibers is a multistep process that requires the fusion of myoblasts to produce multinucleated muscle fibers with contractile properties [23]. The muscle-specific proteins myogenic differentiation antigen (MyoD), myogenic factor (Myf)-5, myogenin (Myog), myogenic regulatory factor (MRF)-4, and the myocyte enhancer factor 2a-d (Mef2a-d) family, act at numerous points in the muscle lineage to collaboratively establish the skeletal muscle

phenotype by regulating proliferation, cell cycle, sarcomeric activation, and muscle-specific genes to promote differentiation and sarcomere assembly [23–25]. Myosin heavy chain (Myh) is a vital component of myosin, which is involved in the formation of the cytoskeleton, providing force for muscular contraction. Myh is divided into different subtypes, and Myh1 is the signature gene of fast-twitch muscle fibers [26], while Myh7 is important for slow-twitch muscle fibers [27]. Myh subtypes can change with external stimuli, and the contraction characteristics of muscle fibers can also change significantly when there is change in Myh subtypes.

While knowledge regarding the importance of circRNAs in the regulation of muscle development and muscle-associated disorders is increasing, little is known about the regulation of circRNAs during C2C12 myoblast differentiation. To explore potential regulators of muscle differentiation, we ascertained the expression profiles of C2C12 myoblast and myotube circRNAs using microarray analysis and predicted the function of differently expressed circRNAs. Our findings provide novel insight into the differentiation and development of skeletal muscle C2C12 myoblasts.

2. Results

2.1. Analysis of differentially expressed circRNAs between myoblasts and myotubes in mouse C2C12 cells

In total, 11,128 expressed circRNAs were detected using an Arraystar mouse circRNA microarray. Based on the circRNA expression profiles, differentially expressed circRNAs were distinguished between mouse C2C12 myoblasts and myotubes. Hierarchical clustering was performed to sort circRNAs according to their expression levels (Figure 1a). In the volcano plots (Figure 1b), differentially expressed circRNAs were grouped by fold change (FC) ≥ 2.0 , $P < 0.05$, and false discovery rate < 0.05 , which identified 581 markedly differentially expressed circRNAs in myotubes compared with myoblasts, with 346 upregulated and 235 downregulated circRNAs. The results suggest that the expression of circRNAs in myotubes differs from that in myoblasts.

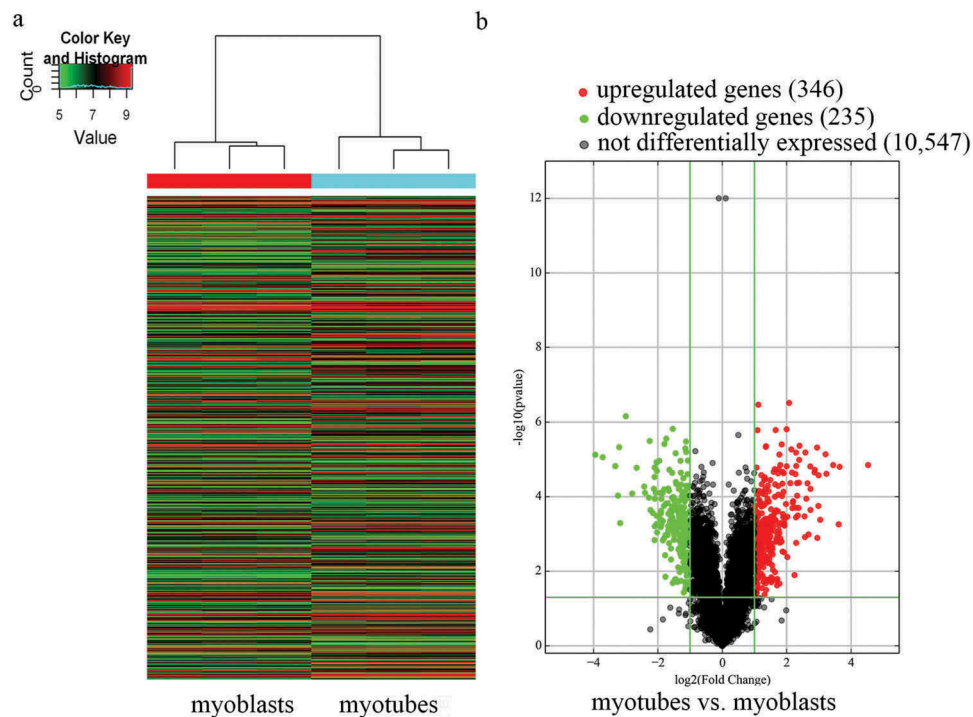


Figure 1. Characterization of circular RNA (circRNA) expression profiles of mouse C2C12 myoblasts and myotubes. **a)** Hierarchical clustering of the expression profiles of circRNAs. Red and green indicate high and low relative expression, respectively. **b)** Volcano plots were used to evaluate differences in circRNA expression between myotubes and myoblasts. The horizontal line corresponds to 2.0-fold (\log_2 scaled) up and down, respectively, and the vertical lines represent a P -value of 0.05 ($-\log_{10}$ scaled). The red and green points in the plot represent the differentially expressed circRNAs with statistical significance.

2.2. Quantitative real-time polymerase chain reaction (qRT-PCR) validation of differentially expressed circRNAs between myoblasts and myotubes in mouse C2C12 cells

We selected nine differentially expressed circRNAs, including five upregulated and four downregulated circRNAs, to validate the microarray analysis results in mouse C2C12 cells. The qRT-PCR results were consistent with the microarray assay except for circRNA_007438 (Figures 2a and b), verifying the predictive accuracy of the microarray analysis. These findings provided evidence that these circRNAs might be involved in the differentiation and proliferation of mouse C2C12 myoblasts.

2.3. Chromosomal distribution and types of differentially expressed circRNAs in mouse C2C12 cells

Based on our statistics, we found the differentially expressed circRNAs were widely distributed on all chromosomes of mouse C2C12 cells (Figure 3a), although they were commonly found on

chromosome 1 (chr1), chr2, chr4, chr5, chr7, and chr17. It suggests that each chromosome is related to varying frequency of differentially expressed circRNAs in C2C12 myoblast differentiation.

We summarized the types of circRNAs with different expression characteristics (Figure 3b) according to the relationship and genomic loci with their associated coding genes. The upregulated circRNAs included five distinct types: anti-sense (1), exonic (288), intergenic (3), intronic (17), and sense overlapping (37) (Figure 3b), while the downregulated circRNAs included four distinct types: antisense (7), exonic (177), intronic (11), and sense overlapping (40) (Figure 3b). Based on our analysis, most of the differentially expressed circRNAs originated from exons.

2.4. Gene ontology (GO) and kyoto encyclopedia of genes and genomes (KEGG) pathway analyses

To determine the involvement of circRNAs in gene regulation, we performed GO analysis of the linear counterparts that resulted in the differentially

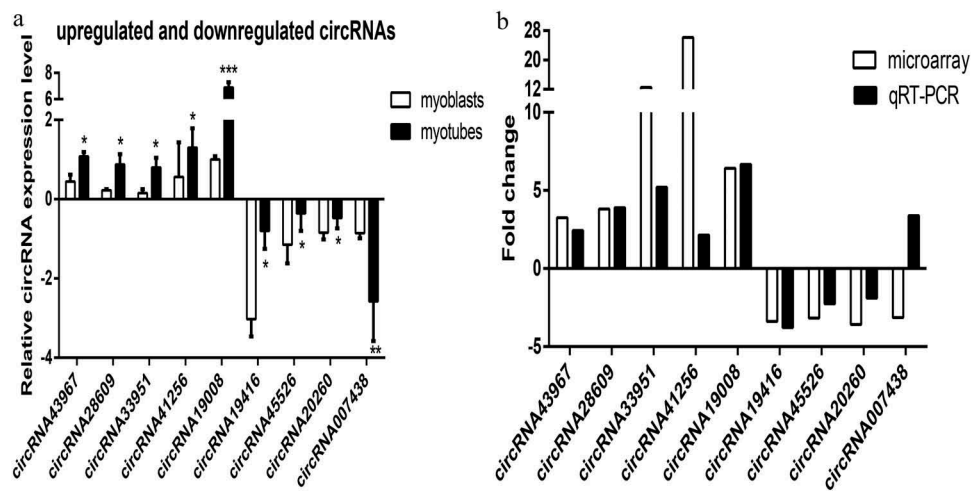


Figure 2. Quantitative real-time polymerase chain reaction (qRT-PCR) validation of nine differentially expressed circRNAs. **a)** Validation results of five upregulated and four downregulated circRNAs are shown. **b)** Comparison of qRT-PCR and microarray analysis. The heights of the columns represent fold changes (\log_2 transformed). Values are the mean \pm SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ myotubes vs. myoblasts.

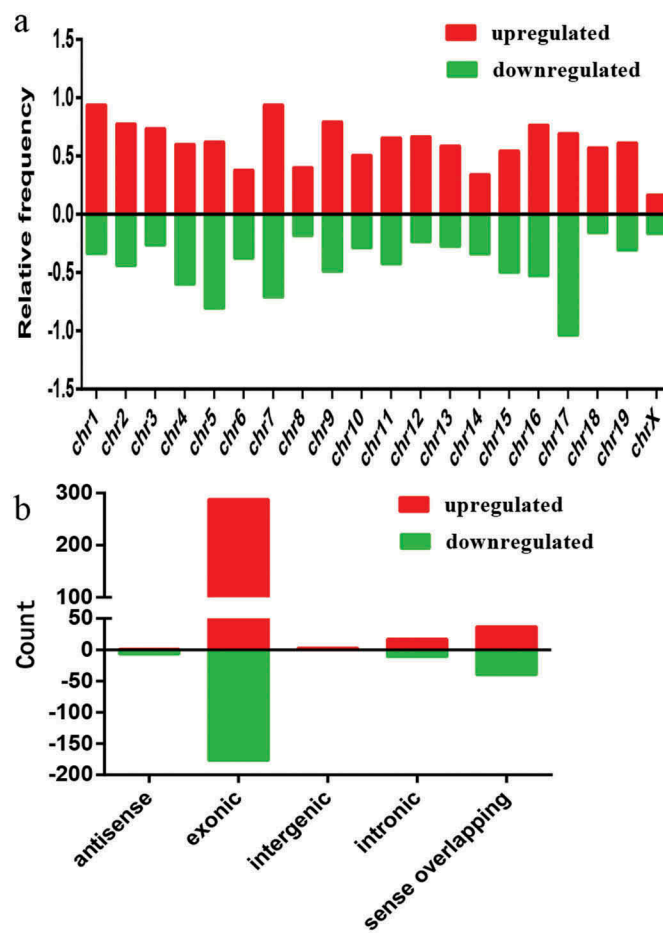


Figure 3. Chromosomal distribution and types of differentially expressed circRNAs. **a)** Relative frequency of differentially expressed circRNAs on mouse chromosomes. **b)** Types and counts of differentially expressed circRNAs detected by microarray (fold change ≥ 2.0 , $P < 0.05$). The circRNAs were classified into five or four types according to the relationship and genomic loci with their associated coding genes. Red and green indicate upregulated and downregulated circRNAs, myotubes vs. myoblasts, respectively.

expressed circRNAs. The results of the GO analysis were classified into the GO terms cellular component (CC), molecular function (MF), and biological process (BP). Our results suggested that the linear counterparts of the differentially expressed circRNAs in myotubes favored the endoplasmic reticulum, nucleic acid binding transcription factor activity, transmembrane transport activity, signal transduction, transcription factor activity, protein binding, and embryonic development compared with myoblasts. Whereas the GO terms of downregulated circRNAs were more commonly linked to chromosomes, DNA/RNA binding, ATPase activity, cell cycle, chromosome organization, cell division, organization of the cytoskeleton, cell proliferation, and translation (Figure 4a). These analyses suggest that the proposed pathways and molecular functions of these circRNAs may promote differentiation and proliferation of mouse myoblasts.

We then performed KEGG pathway enrichment analysis for linear transcripts that matched the differentially expressed circRNAs. Our data indicated that the calcium signaling pathway and the cell cycle were the top two related pathways of the upregulated linear transcripts; metabolic and mitogen-activated protein kinase (MAPK) signaling pathways were the related pathways of the downregulated linear transcripts (Figure 4b). In addition, several pathways with significant enrichment scores such as focal adhesion and adherens junction were involved in cell-cell and cell-extracellular matrix interactions, which are known to play crucial roles in modulating cell differentiation, migration, and proliferation [28].

2.5. Construction of the circRNA-microRNA (miRNA)-mRNA interaction network

RNA transcripts can compete for the same miRNA reaction elements to regulate one other by using microRNA response elements as letters of a new language [18,29]. According to the ceRNA hypothesis, circRNA could compete for the same miRNA response element and act as a molecular sponge for miRNA; miRNA could target on mRNA 3'UTR; hence circRNA could regulate the depression of target genes of the respective miRNA family. To investigate the functions of circRNAs during C2C12 myoblast differentiation, we constructed a ceRNA network in C2C12 myotubes based on our microarray data. The

differentially expressed circRNAs were predicted based on complementary miRNA matching sequences. We selected the myogenic-specific genes Myog, Mef2a, Myh1, Myh7, and Myh7b to construct a circRNA-miRNA-mRNA network of the differentially expressed circRNAs in mouse C2C12 myotubes. In total, 91 miRNAs could be combined with the selected myogenic-specific genes and the top 30 upregulated circRNAs, forming 239 edges (Figure 5 and Suppl. Table 1). Our data showed Myh1 could target three miRNAs and combine with three circRNAs; Myh7 could target three miRNAs and combine with three circRNAs; Myh7b could target seven miRNAs and combine with seven circRNAs; and Myog could target 42 miRNAs and combine with 22 circRNAs. In particular, Mef2a could target 75 miRNAs and combine with all of the top 30 upregulated circRNAs. Based on our results, circRNA_34451 can interact with Mef2a, Myh7b, and Myog, and circRNA_19008 can interact with Mef2a, Myh1, and Myog. circRNAs might play an important role in the process of C2C12 myoblast differentiation. These RNA interactions will provide novel insight into the differentiation and development of C2C12 myoblasts.

2.6. Prediction of coding potential and the presumed encoded polypeptide functions of differentially expressed circRNAs

It was recently reported that circRNAs can be translated into protein [14,20–22]. It is known that one m⁶A motif is required for circRNAs to encode polypeptides, and this m⁶A-driven translation requires eIF4G2 and YTHDF3 [21]. In this study, over the course of differentiation during C2C12 myoblast development, the relative mRNA expression level of Myog increased gradually (Figure 6a), verifying that C2C12 cells did undergo differentiation. The relative mRNA expression level of eIF4G2 (Figure 6b) and YTHDF3 (Figure 6c) also increased gradually during C2C12 myoblast differentiation.

We attempted to identify conceivable novel polypeptides encoded by putative circRNAs based on our microarray analysis. We screened the identified circRNAs for those with open reading frame (ORF) and at least one m⁶A

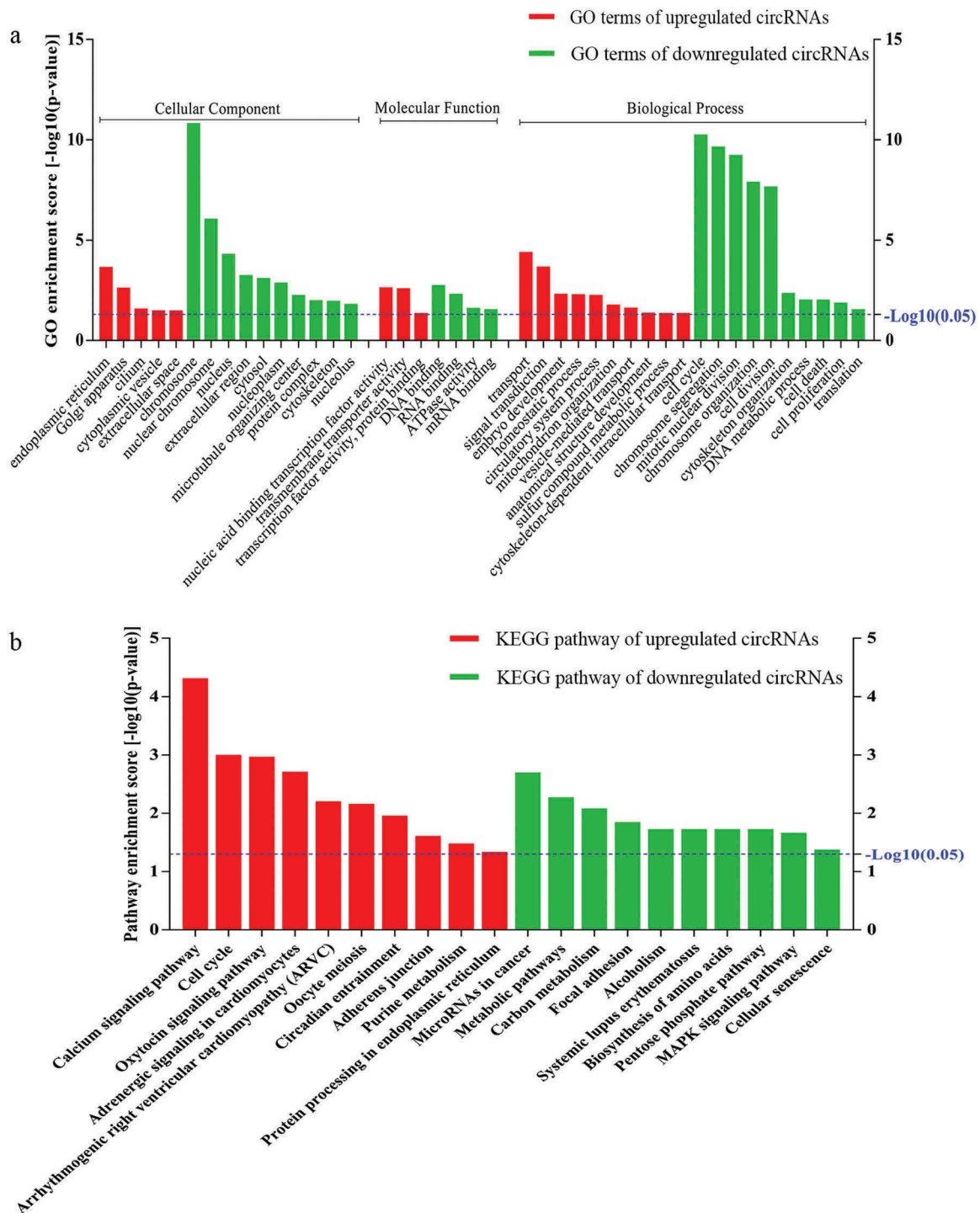


Figure 4. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses. **a)** GO analysis of genes related to differentially expressed circRNAs. GO annotations of the linear counterparts of upregulated and downregulated circRNAs in the cellular component (CC), molecular function (MF), and biological process (BP) terms. Red and green indicate GO terms of upregulated and downregulated circRNAs, myotubes vs. myoblasts, respectively. **b)** KEGG pathway enrichment analysis of the genes that produced upregulated and downregulated circRNAs. Red and green indicate KEGG pathways of upregulated and downregulated circRNAs, myotubes vs. myoblasts, respectively. Both upregulated and downregulated circRNAs were significantly altered with fold change ≥ 2.0 and $P < 0.05$.

motif in the sequence. From this screening, we identified 224 circRNAs with coding potential. A hierarchical clustering heat map was generated

to sort the top 20 upregulated and top 20 downregulated circRNAs among the 224 differentially expressed circRNAs with coding potential,

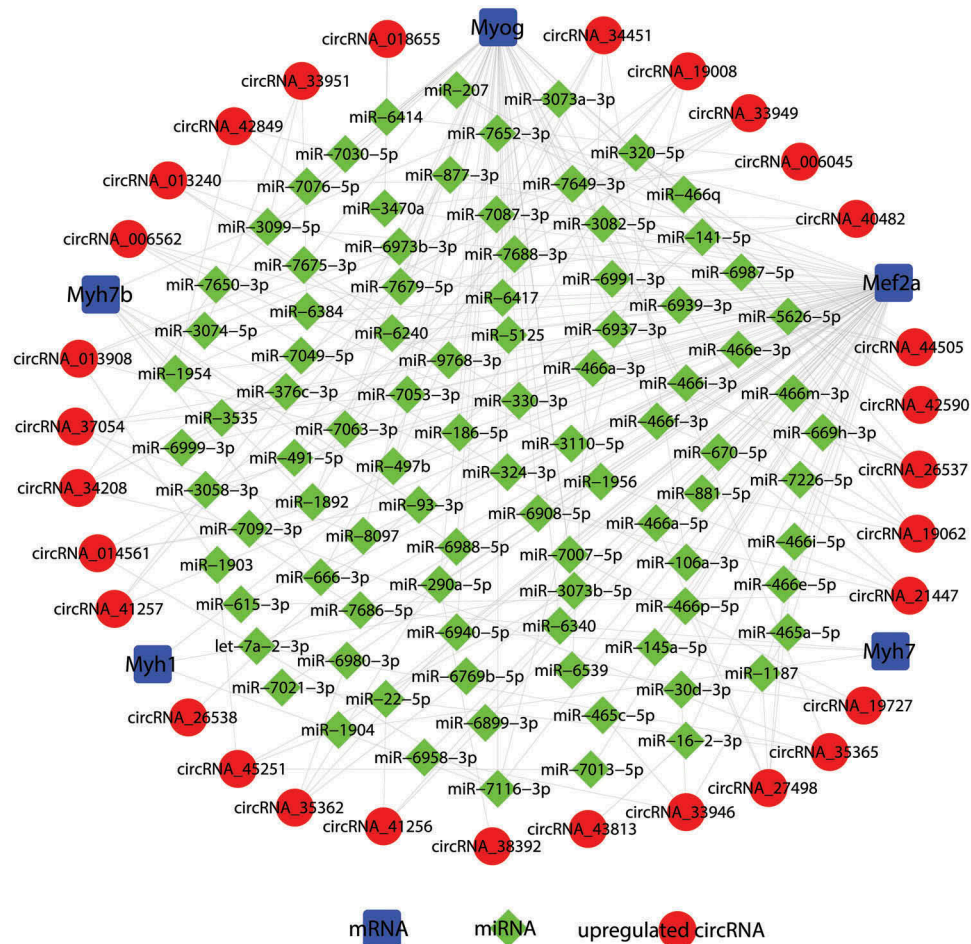


Figure 5. Construction of the circRNA-miRNA-mRNA competing endogenous RNA (ceRNA) regulatory network. In total, 91 miRNAs could be combined with the selected myogenic-specific genes and the top 30 upregulated circRNAs, forming 239 edges. Blue squares indicate myogenic-specific mRNAs, green diamonds indicate miRNAs, and red circles indicate upregulated circRNAs, myotubes vs. myoblasts.

Table 1. Primers for the detected mRNAs and circRNAs.

mRNA/circRNA	Forward primer (5' to 3')	Reverse primer (5' to 3')
18S	GTAACCGTTGAACCCATT	CCATCCAATCGGTAGTAGCG
Myog	GGCAATGCACTGGAGTTCG	AGCCCGCAGCAAATGATC
eIF4G2	AGTGCGATTGCAGAAAGGGG	GTGCTTCGTGCAGGAATCCA
YTHDF3	GATCAGCCTATGCCATATCTGAC	CCCCTGGTTGACTAAAAACACC
circRNA_43967	GAAGGCTCAAAGCAGTGGC	AGGGTCCGAGGTCTGGATG
circRNA_28609	GCTTTAGGAAGGATGATGGGA	TTGGGTAGGTTTTGAGATGGTG
circRNA_33951	AAGAATTTGGAAGACTCATGGTGG	AATCCCATTCTTTGCTTTGAC
circRNA_41256	TGAAACTCAAGGACATCGTAGGC	TGGTGGTCGTGTTCCAGTC
circRNA_19008	AATGGGCAACATAGTTAAACCC	GATGCAGCAGGAAGAGCAAA
circRNA_19416	AAGCAGCGGAGGATACAGCC	CCCTTCTCAGATTTCTCCAGT
circRNA_45526	GCATGAAATGGACATTCGCTAT	GGTTTGCTGGCTACCCAGT
circRNA_20260	GCTGTCAGTTAACGGGTGC	CAGGGTTCTGCTGGAGGTA
circRNA_007438	GTCAGACACCAGAACCCCTCG	TACCGTCATCATAGACTTTGACTCCT

according to their expression levels (Figure 6d). We then summarized the classification of the 224 circRNAs with different expressions. The circRNAs with coding potential included three distinct types: exonic (207), intronic (3), and

sense overlapping (14) (Figure 6e). Next, we counted the number of ORFs of the 224 circRNAs with coding potential. In total, 71 circRNAs had one ORF, 43 circRNAs had two ORFs, 32 circRNAs had three ORFs, and 78

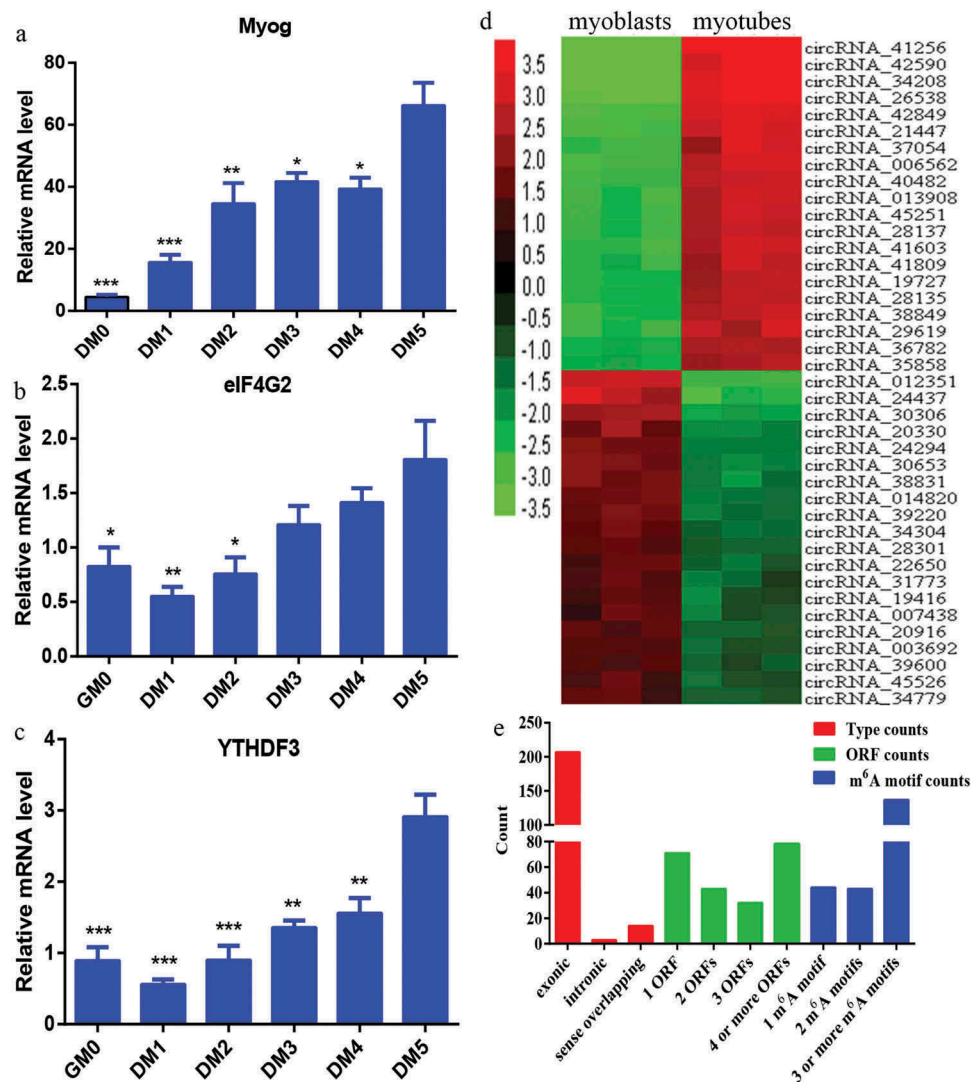


Figure 6. Prediction of the coding potential of differentially expressed circRNAs. **a)** Expression of the myogenic differentiation marker Myog was examined by qRT-PCR. **b, c)** eIF4G2 and YTHDF3 were examined by qRT-PCR. **d)** Hierarchical clustering heat map of the top 20 upregulated and top 20 downregulated circRNAs among the 224 differentially expressed circRNAs with coding potential. Red and green indicate high and low relative expression, respectively. **e)** The number of types, ORFs, and m⁶A motifs of the 224 circRNAs with coding potential. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ vs. after day 5 of differentiation.

circRNAs had four or more ORFs (Figure 6e). The number of m⁶A motifs in the 224 circRNAs with coding potential was also determined, which are required for circRNAs to encode polypeptides [21]. Briefly, 44 circRNAs had one m⁶A motif, 43 circRNAs had two m⁶A motifs, and 137 circRNAs had three or more m⁶A motifs (Figure 6e).

According to the screening of circRNAs with coding potential with known functional domains and more than 30 amino acids, we identified 75 circRNAs to perform GO and KEGG analyses based on the linear counterparts of the circRNAs. The predicted GO functions were ranked

according to the number of related genes and classified into the subcategories CC, MF, and BP (Figure 7a). Our results revealed a high proportion of genes of the 75 differentially expressed circRNAs were clustered in binding, catalytic activity, cellular process, and metabolic process categories (Figure 7a). Meanwhile, KEGG analysis of the identified 75 circRNAs revealed a number of pathways primarily related to regulation of the actin cytoskeleton and metabolic pathways (Figure 7b). These annotations could offer new insight into the investigation of specific processes, pathways, and functions of circRNAs in skeletal muscle growth and development.

3. Discussion

The study of gene regulation concentrated on protein-coding genes until the discovery of numerous non-coding RNAs, including circRNAs [29]. Recently, studies of circRNAs have resulted in the understanding that these non-coding RNAs play an important role in cellular differentiation, development, and the onset and development of disease. The development of skeletal muscle is a comprehensive process that requires the fusion of myoblasts to produce multinucleated muscle fibers with contractile properties [30]. However, analysis of the expression profiles of circRNAs in proliferative C2C12 myoblasts and differentiated C2C12 myotubes had not yet been reported. To explore the functions of circRNAs in C2C12 myoblast differentiation, we determined the expression profiles of circRNAs in proliferative C2C12 myoblasts and differentiated C2C12 myotubes using

microarray analysis, and predicted the function of differently expressed circRNAs by GO and KEGG analyses, ceRNA networks, and coding potential.

In the present study, we identified 581 circRNAs that were differentially regulated during C2C12 myoblast differentiation by microarray analysis. According to the relationship and genomic loci with their associated coding genes, the identified upregulated and downregulated circRNAs can be classified into five distinct types: antisense, exonic, intergenic, intronic, and sense overlapping. The most common type of differentially expressed circRNA was exonic, which is consistent with previous studies [31,32]. The second most common type was sense overlapping, which differs from results obtained during bovine myoblast differentiation [13].

GO analysis of the linear counterparts of differentially expressed circRNAs demonstrated that transmembrane transport activity, signal transduction,

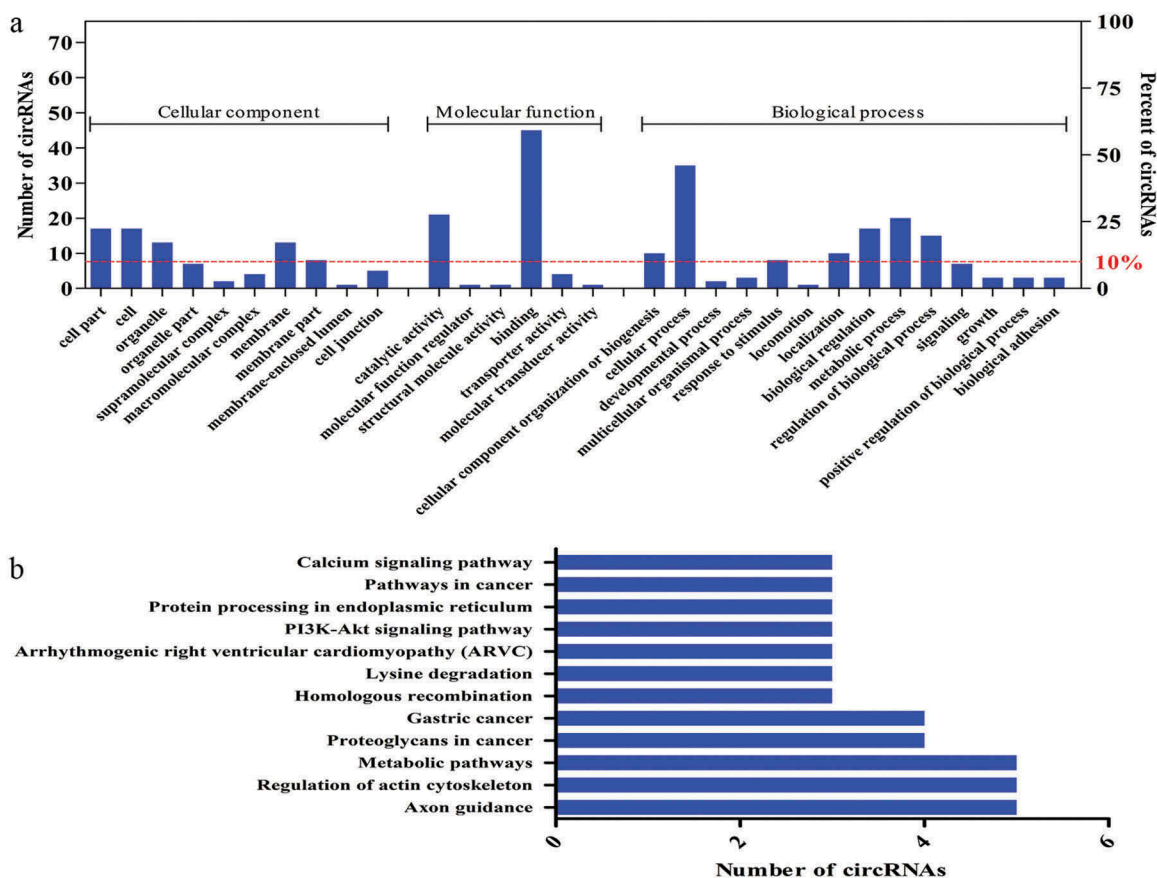


Figure 7. The presumed encoded polypeptide functions of differentially expressed circRNAs. **a)** GO analysis of the genes related to the 75 selected circRNAs with coding potential. GO annotations of the linear counterparts of the selected 75 circRNAs in the subcategories CC, MF, and BP. The left and right y-axes indicate the number of genes in a category and the percentage of a specific category of genes in the main category, respectively. **b)** KEGG pathway enrichment analysis of the genes that produced the selected 75 circRNAs. The y- and x-axes indicate the pathways and the number of circRNAs.

protein binding, and embryonic development related to coding genes contributed to C2C12 myoblast differentiation. KEGG pathway analysis uncovered 10 pathways that could have a significant effect on C2C12 myoblast differentiation, including calcium signaling, cell cycle, metabolic pathways, and MAPK signaling, indicating that circRNAs could play an important role by modulating the pathways involved in C2C12 myoblast differentiation. Calcium signaling has been reported to be significant for differentiation-dependent gene expression, and inhibition of the calcium-regulated phosphatase calcineurin could block myogenic gene expression and myogenic differentiation [33]. Lowering intracellular calcium levels could inhibit the differentiation of skeletal myoblasts into mature myotubes [34]. The MAPK signaling pathway could control fundamental cellular processes such as cell proliferation, cell migration, cell differentiation, embryogenesis, and cell death by transmitting largely unknown signals into the cell [35,36]. Liu et al. [37] reported that p38 α MAPK signaling was necessary for efficient expression of Myog in C2C12 myoblasts. p38 MAPK signaling impacted the activities of the Mef2 family and MyoD, and was involved in chromatin remodeling at specific muscle-regulatory regions [38].

Several recent studies have reported that some circRNAs targeting miRNAs via the ceRNA network were involved in myoblast differentiation. circLMO7 was shown to inhibit bovine myoblast differentiation, promote cell proliferation, and protect myoblasts from apoptosis by binding miR-378a-3p [13]. circFUT10 was shown to accelerate differentiation and decreased the proliferation of bovine myoblasts by inhibiting miR-133a activity [15]. circFGFR4 by binding miR-107 could promote cell differentiation via targeting Wnt3a in bovine primary myoblasts [16]. To date, the functions of most circRNAs are not well known. In this study, the myogenic-specific genes Myog, Mef2a, Myh1, Myh7, and Myh7b were selected to construct a circRNA-miRNA-mRNA network of differentially expressed circRNAs during mouse C2C12 myoblast differentiation. A total of 91 miRNAs could be combined with the selected myogenic-specific genes and the top 30 upregulated circRNAs, forming 239 edges. In particular, Mef2a could target 75 miRNAs and combine with all of the top 30 upregulated circRNAs. Our analysis also revealed that Myog and Mef2a could combine with

circRNA_21447 by targeting miR-670-5p, which has been shown to induce cell proliferation in hepatocellular carcinoma by targeting prospero-related homeobox 1 [39]. Furthermore, circRNA_19008 binding miR-186-5p may promote myoblast differentiation via targeting Myog and Mef2a in our analysis, and it was reported that the reduction in the levels of endogenous miR-186 resulted in increased differentiation of C2C12 and primary muscle cells of mouse through upregulation of Myog [40]. These data indicate that circRNAs could play key regulatory roles in skeletal muscle C2C12 myoblast differentiation. These precursory findings broaden our understanding of circRNA function during myoblast differentiation, although further research will be necessary to confirm our predictions.

Recently, it was reported that circRNAs can be translated into proteins [14,20–22]. m⁶A motifs were found to be enriched in circRNAs and one m⁶A motif was shown to be sufficient to drive translation initiation; this m⁶A-driven translation required the initiation factor eIF4G2 and the m⁶A reader YTHDF3 [21]. The depletion of eIF4G2 and YTHDF3 significantly reduced protein translation from circRNAs and overexpression of eIF4G2 and YTHDF3 increased protein translation from circRNAs [21]. In this study, the relative mRNA expression level of eIF4G2 and YTHDF3 increased gradually over the course of differentiation during C2C12 myoblast development. Therefore, to explore the coding potential of the differently expressed circRNAs in our microarray analysis, we identified 224 circRNAs with coding potential based on the number of ORFs and m⁶A motifs, and they could be classified into three types: exonic, intronic, and sense overlapping.

We further identified 75 circRNAs with coding potential, which have known functional domains and more than 30 amino acids [41], to perform GO and KEGG analyses. GO analysis of the 75 differentially expressed circRNAs demonstrated that binding, catalytic activity, cellular process, and metabolic process-related coding genes contributed to C2C12 myoblast differentiation. KEGG analysis of the identified 75 circRNAs revealed a number of pathways mainly related to regulation of the actin cytoskeleton and metabolic pathways. The functions of the actin cytoskeleton were most often aligned, allowing for consolidation and amplification of myofibroblast

differentiation signals [42]. Modulation of the actin cytoskeleton by disordering drugs could differentially modify mechanically induced signal transduction and mesenchymal stem cell differentiation [43]. These annotations could offer new insight into the investigation of specific pathways and coding potential of circRNAs in skeletal muscle development.

Studies of the functions of circRNAs are currently in the development stage. The present study predicted the functions and coding potential of differentially expressed circRNAs during skeletal muscle C2C12 myoblast differentiation; however, further work will be necessary to confirm our predictions in the future.

In summary, we established expression profiles of circRNAs during C2C12 myoblast differentiation and predicted the function of differentially expressed circRNAs by GO and KEGG analyses, ceRNA networks, and coding potential based on ORFs and m⁶A motifs. Our results suggest that circRNAs might be important in skeletal muscle development. Our study also offers new insight into the investigation of circRNA functions in skeletal muscle growth and development.

4. Methods

4.1. Cell culture and establishment of the C2C12 differentiation model

C2C12 cell line is a subclone of the mouse myoblast cell line established by D. Yaffe and O. Saxel in 1977 [44]. It serves as a manageable experimental model system for researching the molecular basis of skeletal muscle cell specification and development [45]. High glucose Dulbecco's modified Eagle's medium was used to culture the mouse myoblast cell line C2C12 (Stem Cell Bank, cat no. SCSP-505) in 5% CO₂ at 37°C. The markers of C2C12 differentiation model were myogenin and myosin heavy chain [46,47], and the differentiation model was also established in the early work of our laboratory [23,48,49].

4.2. RNA extraction and quantity control

Total RNA was extracted from mouse C2C12 myoblasts and myotubes using TRIzol reagent (Life Technologies, cat no.15596018) according to the

manufacturer's instructions. A NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) was used to assess the quantity and quality of purified RNA. RNA integrity was measured by conventional denaturing agarose gel electrophoresis.

4.3. Microarray analysis

To determine differentially expressed circRNAs, three pairs of mouse C2C12 myoblasts and differentiated myotubes were used for the microarray analysis. Sample labeling and mouse circRNA array hybridization were performed based on the standard protocols of the Arraystar Mouse circRNA Array V2 (Arraystar, Inc., Rockville, MD, USA). In brief, RNase R was used to digest total RNAs to remove linear RNAs and enrich circRNAs. Then, a random priming method was used to amplify the enriched circRNAs, which were transcribed into fluorescent cRNA using the Super RNA Labeling kit (Arraystar, Inc.). The labeled cRNAs were hybridized onto the Arraystar Mouse circRNA Array V2. The slides were washed and the arrays were scanned using the Agilent Scanner G2505C (Agilent Technologies, Santa Clara, CA, USA). Agilent Feature Extraction software (version 11.0.1.1) was used to analyze the acquired array images. The Gene Expression Omnibus accession number is GSE112014.

4.4. QRT-PCR

The myogenic-specific gene Myog was examined to verify the degree of muscle differentiation. eIF4G2 and YTHDF3 have been shown to be required for the translation of circRNAs. Five upregulated (mmu_circRNA_43967, mmu_circRNA_28609, mmu_circRNA_33951, mmu_circRNA_41256, and mmu_circRNA_19008) and four downregulated (mmu_circRNA_19416, mmu_circRNA_45526, mmu_circRNA_20260, and mmu_circRNA_007438) circRNAs were randomly selected for validation by qRT-PCR. Briefly, total RNA was extracted from myoblasts and myotubes and 2.5 µg of RNA was reverse-transcribed to cDNA using the PrimeScript RT Master Mix (TaKaRa, cat no. RR036A). According to the manufacturer's instructions, the abundance of circRNAs was measured with Takara SYBR Green Mix (TaKaRa, cat no. RR820A). The qRT-PCR

protocol was initiated at 95°C for 30 s, followed by 40 cycles of denaturation at 95°C for 5 s, annealing at 60°C for 30 s, and extension at 60°C for 1 min. 18S ribosomal RNA was used as a reference. The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative expression level of selected circRNAs. The examined mRNA and circRNA cross-junction site primers are shown in Table 1.

4.5. GO and KEGG pathway analyses

GO analysis based on the linear counterparts of the circRNAs was performed to investigate attributes of the differentially expressed circRNAs, including the GO terms CC, MF, and BP. The $-\log_{10}$ (P-value) indicated the enrichment score, representing the significance of GO term enrichment among genes producing differentially expressed circRNAs.

Pathway enrichment analysis helped identify significantly enriched metabolic or signal transduction pathways in differentially expressed genes compared with the whole genome background [50]. KEGG pathway analysis based on linear counterparts was used to harvest pathway clusters covering our knowledge of the molecular interaction and reaction networks in genes producing differentially expressed circRNAs [51].

4.6. CeRNA analysis

miRNA could target on mRNA 3'UTR; circRNA could act as a molecular sponge to regulate the de-repression of target genes of the respective miRNA family. In the present study, circRNA-miRNA-mRNA interactions were predicted with Arraystar's in-house miRNA target prediction software based on TargetScan (http://www.targetscan.org/vert_71/) and miRanda (<http://34.236.212.39/microrna/home.do>). We selected the myogenic-specific genes Myog, Mef2a, Myh1, Myh7, and Myh7b to construct a ceRNA network of the differentially expressed circRNAs in mouse C2C12 myoblasts and myotubes. An entire circRNA-miRNA-mRNA interaction network was defined using Cytoscape.

4.7. Prediction of coding potential of differentially expressed circRNAs based on the number of ORFs and m⁶A motifs

In a recent study, one m⁶A motif was determined to be sufficient to drive efficient initiation of circRNA protein translation in cells and m⁶A was required for circRNAs to encode polypeptides [21]. We attempted to identify possible novel polypeptides encoded by putative circRNAs based on our microarray analysis. The circRNAs were screened to identify sequences with at least one ORF and at least one m⁶A motif. This screening revealed 224 circRNAs with coding potential for the translation of polypeptides. To further predict the functions of the polypeptides presumably encoded by the circRNAs, we performed GO and KEGG analyses with the linear counterparts of the circRNAs with known functional domains and more than 30 amino acids [41].

4.8. Statistical analysis

GraphPad Prism 5.0 software (La Jolla, CA, USA) was used to perform statistical analysis. Student's *t*-test was applied for the comparison of two groups and $P < 0.05$ was considered to indicate statistical significance.

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Disclosure statement

The authors declare that they have no competing interests.

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