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Assessment of myoblast circular RNA dynamics and its correlation with miRNA during myogenic differentiation

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ABSTRACT

Myoblast differentiation is a highly complex process that is regulated by proteins as well as by non-coding RNAs. Circular RNAs have been identified as an emerging new class of non-coding RNA in the modulation of skeletal muscle development, whereas their expression profiles and functional regulation in myoblast differentiation remain unknown. In the present study, we performed deep RNA-sequencing of C2C12 myoblasts during cell differentiation and uncovered 37,751 unique circular RNAs derived from 6943 hosting genes. The ensuing qRT-PCR and RNA fluorescence *in situ* hybridization verification were carried out to confirm the RNA-sequencing results. An unbiased analysis demonstrated dynamic circular RNA expression changes in the process of myoblast differentiation, and the circular RNA abundances were independent from their cognate linear RNAs. Gene ontology analysis showed that many down-regulated circular RNAs were exclusive to cell division and the cell cycle, whereas up-regulated circular RNAs were related to the cell development process. Furthermore, interaction networks of circular RNA-microRNA were constructed. Several microRNAs well-known for myoblast regulation, such as miR-133, miR-24 and miR-23a, were in this network. In summary, this study showed that circular RNA expression dynamics changed during myoblast differentiation. Circular RNAs play a role in regulating the myoblast cell cycle and development by acting as microRNA binding sites to facilitate their regulation of gene expression during myoblast differentiation. These findings open a new avenue for future investigation of this emerging RNA class in skeletal muscle growth and development.

1. Introduction

Circular RNA (circRNA) is a type of widespread and diverse endogenous noncoding RNA. CircRNA was first reported decades ago. It is usually considered to be of low abundance and likely represents exon shuffling or errors in splicing (Al-Balool et al., 2011; Quinlan and Hall, 2010; Sanger et al., 1976). In recent years, with the advances in RNA sequencing (RNA-seq) technology, it has become clear that circRNAs express across a broad range of species, and some of these circRNAs are even more abundant than their linear counterparts (Jeck et al., 2013; Memczak et al., 2013). CircRNA is recognized as a novel class of RNA with regulatory functions in normal physiology (e.g., brain, heart, and liver) as well as in many disease conditions (e.g., Alzheimer's disease and cancer) (Hansen et al., 2013; Khan et al., 2016; Lukiw, 2013; Salzman et al., 2013). CircRNA is generated from pre-mRNA by 'back-splicing', where a 5′ splice donor joins an upstream 3′ splice acceptor, resulting in a covalently closed, single-stranded RNA molecule that lacks poly (A) tail (Starke et al., 2015; Zhang et al., 2014). Regarding its potential function, recent studies have proven that circular RNA can act as a microRNA sponge. For example, circ-HIPK3 directly binds to miR-124 and thus acts as a miR-124 antagonist (Zheng et al., 2016). Furthermore, a decoy or an antagonist is another emerging circRNA function. Circ-Foxo3 binds to cyclin-dependent kinase 2 and p21, forming an RNA-protein complex that disrupts the interactions of CDK2 with cyclins A and E, thus blocking cell cycle progression (Du et al., 2016).

The skeletal muscle constitutes ~40% of the animal body mass and plays an important role in locomotion and metabolism. Muscle wasting due to cancer (cachexia), genetic factors (muscular dystrophy), and aging (sarcopenia) severely compromises the quality of life of humans

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(Yang, 2014; Zhang et al., 2015). Muscle growth is a highly coordinated process that includes fine regulation of muscle stem cell activation, proliferation, differentiation, and fusion, during which both coding RNA and non-coding RNA can regulate myogenesis (Ballarino et al., 2016). It has been reported that circRNA expression was regulated in the process of skeletal muscle development and growth, such as in sheep, chicken, and pig (Li et al., 2017a; Liang et al., 2017b; Ouyang et al., 2017). Furthermore, it was found that circ-ZNF609 could control myoblast proliferation and circ-QKI regulated myoblast differentiation (Legnini et al., 2017). Despite these findings, the expression profile of circRNAs in skeletal muscle stem cells and the biological function remain largely unknown.

In the current study, we sought to globally identify novel circRNAs during myogenesis. We identified thousands of circRNAs that were actively regulated in myoblasts. Furthermore, we determined the independence of circRNAs' expression with their host genes and performed GO analysis of the circRNA host genes. We also predicted the interactions between circRNAs and microRNAs, supporting the argument that circRNAs may act as microRNA sponges to regulate gene expression.

2. Materials and methods

2.1. Cell culture

The C2C12 mouse myoblasts were maintained in growth medium consisting of DMEM supplemented with 10% fetal bovine serum. Once the cells became confluent, the medium was switched to DMEM supplemented with 2% horse serum to promote myoblasts differentiation into myocytes and subsequent fusion to myotubes.

2.2. Isolation of total RNA

Total RNA was isolated from C2C12 cells at the following time points: proliferating (70–80% confluence, D0), 3 days (D3), and 6 days (D6) after induction of differentiation. Total RNA was isolated using TRIzol (Invitrogen, Cat#15,596,018) according to the manufacturer's instructions. The quality of RNA was determined by an Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, US). Circular RNA library construction and Illumina sequencing were done as follows. To enrich circRNAs, the total RNA was treated with the RNA Clean XP Kit (Beckman Coulter Inc, Cat# A63987) and the RNase-Free DNase Set (QIAGEN, Cat#79,254) following the manufacturer's instructions. Next, 1000 ng was used as the input for the VAHTSTM mRNA-seq v2 Library Prep Kit for Illumina (Vazyme, Inc), and the sequencing libraries were created according to the manufacturer's protocol. The libraries were sequenced using an Illumina Hiseq 4000 instrument with a Paired-End module at Shanghai Biotechnology Co., Ltd. (Shanghai, China). All samples for each condition were sequenced in biological triplicates.

2.3. Identification of circRNA

For all raw sequencing data of each sample, the adapter reads and low-quality reads were removed using Seqtk. The filtered data were aligned to the reference genome GRCm38.p4 (mm10) using BWA-MEM (Li, 2013). Identification of circRNAs was performed using CIRI (Gao et al., 2015). The expression levels of circRNAs were measured by "back-splicing junction reads per billion mapping" (SRPBM).

2.4. Target miRNA prediction, pathway and network analysis

All exonic circRNAs were used to predict miRNAs potential binding sites using miRanda, as previously described (Enright et al., 2003). The interaction network of microRNA and circular RNA was illustrated with Cytoscape 3.5.1. All parental genes of differentially expressed circRNAs were subjected to Gene Ontology (GO) enrichment.

2.5. cDNA synthesis and quantitative real-time PCR (qRT-PCR)

Total RNA was converted to cDNA using random primers and the M-MLV Reverse Transcriptase PrimeScript RT Master Mix (TaKaRa, Cat#RR036A). Real-time PCR was carried out with a Roche Lightcycler 96 PCR System using FastStart Essential DNA Green Master (Roche, Cat#06,924,204,001). The 18S rRNA was used as internal control. The fold-changes of indicated genes were calculated using the $2^{-\Delta\Delta Ct}$ method. All experimental data are presented as the means ± SEM. The comparisons were made by the unpaired two-tailed Student's *t*-test. A value of $P < 0.05$ was considered statistically significant.

2.6. FISH and confocal microscopic analysis

Probes were designed to specifically detect individual circRNAs by targeting junction regions. The probes were commercially synthesized and labeled at the 3′ ends with digoxigenin (Takara, Dalian, China). CircRNA expression was detected by FISH, using the Ribo™ Fluorescent *in situ* hybridization kit (RiboBio, Guangzhou, China) following the manufacturer's instructions. Briefly, the cell slide was blocked with prehybridization buffer at 37 °C for 60 min after washing and fixation. The slide was incubated with 0.5 μmol/L circRNA FISH Probe Mix at 37 °C overnight. The signals of biotin-labeled probes were detected using anti-digoxigenin fluorescein fab (Roche, Cat#11,207,741,910). The nuclei were counterstained with DAPI and observed by confocal microscopy with appropriate fluorescence filter sets (Leica, Germany).

2.7. Accession numbers

Raw fastq files from the RNA-seq data are deposited at NCBI, and the accession number is GSE108505.

3. Results

3.1. Mapping circRNAs from total RNA-seq data of growth and differentiation myoblasts

To investigate the global profile of circRNAs in the process of skeletal muscle stem cell differentiation, we performed deep RNA-seq on ribosomal-depleted total RNA extracted from murine C2C12 myoblast on day 0 (D0), day 3 (D3) and day 6 (D6) after differentiation (Fig. 1A). For all conditions, sequencing was done in biological triplicates. We obtained an average of 92.07 million reads per sample (Supplementary Table 1). Back-spliced junctions were detected using CIRI for de novo circRNA identification (Gao et al., 2015). In total, we identified 37,751 circRNA candidates with a minimum of two reads spanning the back-splice junction. Among them, 36,724 circRNAs were novel (97.28%) and not included in the circBase (Supplementary Table 2).

3.2. Distribution of the myoblast circRNAs

We noticed that 19,764 circRNAs were detected on D0, 19,428 on D3 and 17,723 on D6. Among them, 6366 circRNAs were detected at all three-time points (Fig. 1B). According to the host gene locations, the 37,751 circRNAs were widely scattered on all chromosomes except chromosome Y, since C2C12 cells were derived from the female mouse. Chromosome 2 produced the most circRNAs (3283), and Chromosome X generated the fewest circRNAs (819), while most other chromosomes generated 1000 to 2000 circRNAs (Fig. 1C). Of note, the number of circRNAs from each chromosome seemed to be related to the chromosome length ($R = 0.51$, $P < 0.05$, Supplementary Fig. 1). Furthermore, we found a stronger correlation between the circRNA number and the mRNA number for each chromosome ($R = 0.77$, $P < 0.05$, Fig. 1D).