Differentially expressed coding and noncoding RNAs in CoCl₂-induced cytotoxicity of C2C12 cells

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Aim: We aimed to explore potential regulators of coding and noncoding RNAs (ncRNAs) in Co(II) ion-induced myo cytotoxicity. **Materials & methods:** We confirmed the toxic effects of Co(II) on mouse skeletal C2C12 myotubes by CoCl₂, and performed the expression profiles of circular RNAs (circRNAs), long noncoding RNAs (lncRNAs) and mRNAs using microarray analysis. We constructed co-expression, competing endogenous RNA and *cis/trans* regulation networks for ncRNAs, and filtered 71 candidate circRNAs with coding potential. **Results:** We identify 605 differentially expressed circRNAs, 4409 long noncoding RNAs and 3965 mRNAs. We also provided several ncRNAs regulation networks and presumed functions of circRNAs with coding potential. **Conclusion:** Our findings may reveal novel regulatory mechanisms underlying the noxious effects of CoCl₂ in skeletal muscle.

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It has been well documented in the literature that both local and systemic adverse reactions are induced following cobalt administration [1]. Cobalt primarily accumulates in the skin, heart, lung, liver, pancreas, kidney and locomotor system [2–5]. Additionally, there is sufficient evidence supporting the contribution of cobalt in the development of some cancers [2,6]. *In vitro* test systems have been used to demonstrate that Co(II) ions are acutely toxic and can induce apoptosis, necrosis, inflammation or genotoxicity [3,7,8]. On November 3, 2016, the US Department of Health and Human Services released the 14th Report on Carcinogens, and cobalt and compounds that release cobalt ions in the body were described as reasonably anticipated to be human carcinogens.

Cobalt-chrome alloys are used as a material in prosthetics. Of note, severe poisoning of skeletal muscle has been reported in orthopedic joint replacements in humans, especially in metal-on-metal bearings in hip joint arthroplasty [5]. Materials containing cobalt-chrome alloys tend to produce soluble metal ions and metal debris on polyethylene and ceramic implants in nanometric size, resulting in bone and muscle destruction via the lymphatic and systemic vascular systems [1]. Several case reports have described patients who presented with heart failure or muscle atrophy following a metal-on-metal hip replacement [4,5]. Francesca Rovetta *et al.* indicated that cobalt might impact skeletal muscle homeostasis by increasing the expression of stress proteins and other typical ‘pro-atrophic’ molecular hallmark [9]. Our previous study also showed that CoCl₂ promoted the autophagy of C2C12 myotubes with increased expression of the E3 ligase parkin and the muscle-specific ubiquitin ligase Atrogin1 [10].

Recent developments in RNA sequencing have led to renewed interest in noncoding RNAs (ncRNAs), which include circular RNAs (circRNAs), long noncoding RNA (lncRNAs) and miRNAs. Few studies have investigated the role of lncRNAs in skeletal muscle development and muscle-related diseases. For instance, linc-MD1 was severely reduced in Duchenne muscular dystrophy patients, together with the unrestricted accumulation of miR-

135 and its target Mef2C [11]. Malat1, linc-YY1 and Dum have been shown to be increased in dystrophic muscles in a Duchenne muscular dystrophy animal model [12–14].

circRNAs were discovered in skeletal muscle tissues as well as human, monkey, bovine and mouse cell lines [15–18]. A recent study has systematically identified circRNAs in the skeletal muscle of the rhesus monkey as a function of advancing age [15]. circLMO7, circFUT10 and circFGFR4 were identified in bovine myoblasts, where they affected proliferation and differentiation by binding miRNAs [16,17,19]. circZNF609, which functions in myogenesis, was the first reported translated circRNA [18]. However, few studies have addressed the role of ncRNAs in CoCl₂-induced toxicity in skeletal muscle.

In the present study, we hypothesized that the dysregulation of ncRNAs is involved in the cytotoxic effects of CoCl₂ in myotubes. Here, we investigated differentially expressed circRNAs and lncRNAs in C2C12 myotubes following CoCl₂ treatment, which may further improve our current understanding of the underlying molecular mechanisms.

Materials & methods

Chemicals

CoCl₂ (Sigma-Aldrich, MO, USA) was dissolved in Dulbecco's Modified Eagle Medium (DMEM) as a 2000 × concentrate and stored at -20°C. The final concentrations were diluted at 10, 50, 100 or 200 μM in differentiation medium as previously reported [10].

Cell culture

The mouse myoblast C2C12 cell line was kindly provided by the Stem Cell Bank (Chinese Academy of Sciences, Shanghai, China) and cultured in high glucose DMEM (Gibco-BRL, NY, USA) containing 10% fetal bovine serum (HyClone, UT, USA) at 37°C and 5% CO₂. When the cells reached 80–90% confluency, DMEM supplemented with 2% horse serum (HyClone) was used to induce myoblast differentiation. After 3 days of differentiation induction, increasing doses of CoCl₂ were added to cells for 48 h prior to cell collection as previously described [20].

Cell viability assays

To determine cell viability using the Cell Counting Kit (CCK)-8 assay (Beyotime Institute of Biotechnology, Jiangsu, China), 0.2 × 10⁴ cells/well were seeded in 96-well plates. Quantification was performed 1 h following the addition of CCK-8 solution. Data are presented as a percentage of the control [21].

Apoptosis assays

CoCl₂-induced cell apoptosis was measured using an Annexin V-fluorescein isothiocyanate apoptosis detection kit (Sony Biotechnology Inc., CA, USA). Briefly, cells were incubated with CoCl₂ for 48 h, digested and washed. Subsequently, the cells were incubated with 5 μl of Annexin V and 5 μl of 7-amino-actinomycin D in the dark for 15 min.

Western blotting

Proteins (20 μg) were separated by electrophoresis and transferred onto polyvinylidene fluoride membranes. The membranes were incubated with primary antibodies targeting myogenin (Myog; 1:500; Millipore, MA, USA), phosphoinositide 3-kinase (PI3K; 1:1000; Cell Signaling Technology, MA, USA), phosphorylated AKT serine/threonine kinase (p-AKT; 1:10,000; Abcam, Cambridge, UK), AKT (1:1000; Cell Signaling Technology) and tubulin (1:5000; ABclonal, MA, USA). Band intensity was determined using a chemiluminescent imaging system (Tanon Sciences and Technology Co., Ltd., Shanghai, China).

Cytoskeleton staining

Cells were fixed with a 4% paraformaldehyde solution and stained with phalloidin (Beyotime Institute of Biotechnology), and 4',6-diamidino-2-phenylindole (KeyGEN Biotech, Jiangsu, China) was used to stain nuclei. Fluorescence images were acquired using a Leica DMIL LED microscope (Leica Microsystems Inc., Wetzlar, Germany).

Microarray analysis

Differentially expressed circRNAs, lncRNAs and mRNAs of three pairs of control (control group) and 200 μM CoCl₂-treated (CoCl₂ group) myotubes were subjected to microarray analysis according to the manufacturer's

instructions (Agilent Technology, CA, USA). Briefly, total RNAs were purified, amplified, transcribed into fluorescent cRNA, and hybridized onto the Arraystar Mouse circRNA Array (V2.0) and Arraystar Mouse lncRNA Expression Microarray (V3.0). Finally, the arrays were scanned using the Agilent Scanner G2505C and analyzed using Agilent Feature Extraction software (version 11.0.1.1). circRNAs, lncRNAs, and mRNAs with fold changes ≥ 2 and p-values ≤ 0.05 were selected as significantly differentially expressed. The Gene Expression Omnibus accession number is GSE115090.

Gene ontology & Kyoto Encyclopedia of Genes & Genomes pathway analyses

Gene ontology (GO) analysis was performed to annotate the identified genes and gene products into molecular function (MF), biological process (BP) and cellular component (CC) categories. The $\log_{10}$ (p-value) denotes the enrichment score, which indicates the significance of the GO term enrichment among the differentially expressed genes. Kyoto Encyclopedia of Genes and Genomes (KEGG) mapping pathways were used to determine pathway clusters of the molecular interaction networks of the differentially regulated genes. The $\log_{10}$ (p-value) denotes the enrichment score representing the significance of the pathway correlations.

Quantitative real-time polymerase chain reaction

A number of circRNAs and lncRNAs were randomly selected for validation by quantitative real-time polymerase chain reaction. We also detected 12 specific mRNAs involved in muscle differentiation (*Myog*, *Mef2a*, creatine kinase and ACTN2), muscle atrophy (*Murf1* and *Atrogin1*), development (*Igf1* and *Egfr*), glucose metabolism (hexokinase 2), autophagy (BNIP3) and translation initiation (*eIF4G2* and *YTHDF3*). Briefly, total RNA was extracted and reverse-transcribed to cDNA with PrimeScript RT Master Mix (Takara Biotechnology, Otsu, Japan). circRNA, lncRNA, and mRNA abundance was measured with SYBR[®] Green Mix (Takara Biotechnology). Quantitative real-time polymerase chain reaction was performed as follows: denaturation 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. The expression of 18 S ribosomal RNA was used as an internal control. The primers used for the examined circRNAs, lncRNAs and mRNAs are shown in Supplementary Table 1.

Correlation & co-expression analysis

Pearson correlation coefficients were used as co-expression measures to construct gene co-expression networks based on mRNA and lncRNA expression levels. The absolute value of the parameter Pearson correlation coefficient ≥ 0.995 ($p < 0.01$) and the false discovery rate (FDR) < 0.01 were selected to construct the network.

Prediction of *cis*- & *trans*-regulation

lncRNAs can mediate *cis* and *trans*-regulation of gene expression. *Cis*-regulation, which regulates the transcription of nearby genes located on the same chromosome, is vital for gene expression, whereas *trans*-regulation functions by participating in various pathways regulated by transcription factors (TFs). *Cis* and *trans* predictions were performed as previously described [22].

Competing endogenous RNA analysis

According to the competing endogenous RNA (ceRNA) hypothesis, lncRNAs and circRNAs compete for the same miRNA response element and act as a molecular sponge for miRNAs, thereby regulating the de-repression of all target genes of the respective miRNA family. miRNA targets on the mRNA 3' untranslated region and lncRNAs and circRNAs were calculated using the PITA algorithm (<http://genie.weizmann.ac.il/pubs/mir07>) and ceRNA networks were built using Cytoscape software.

Prediction of the coding potential of differentially expressed circRNAs based on open reading frames & N(6)-methyladenosine motifs

In this study, we wanted to explore whether CoCl₂ affected circRNAs with translation potential. According to Yang *et al.* previous report [23], we identified sequences with at least one open reading frame (ORF) and at least one m⁶A motif based on our microarray data. 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 [24].

Figure 1. CoCl₂-induced cytotoxicity in mouse skeletal C2C12 myotubes. (A) Cell apoptosis was measured by flow cytometry. The x- and y-axes indicate Annexin V and 7-amino-actinomycin D staining, respectively. **(B)** Cell viability was assessed using the Cell Counting Kit-8 assay. **(C)** Western blot analysis revealed the expression of Myog, PI3K, p-AKT and AKT. **(D)** Cytoskeleton was stained with phalloidin. **p* < 0.05 compared with the control group.

Statistical analysis

Data are reported as means ± standard error of the mean. Statistical significance was assessed using two-tailed Student's *t*-tests. *p* < 0.05 was taken to indicate statistical significance.

Results

CoCl₂-induced cytotoxicity in mouse skeletal C2C12 myotubes

The cytotoxic effects of CoCl₂ were analyzed by flow cytometry. As shown in Figure 1A, the percentage of apoptotic cells was increased following CoCl₂ treatment. As verified by the CCK-8 assay, CoCl₂ induced toxicity in C2C12 cells in a time-dependent manner at 100 and 200 μM (Figure 1B). Myog is a specific differentiation marker required for the fusion of myoblasts to form myotubes. Inhibition of PI3K-AKT signaling is thought to be important in the induction of muscle atrophy. Our results showed that the expression of Myog, PI3K and p-AKT was dose-dependently decreased following CoCl₂ treatment (Figure 1C). Actin fibers were also significantly reduced in CoCl₂-treated cells (Figure 1D).

Global profiling of circRNAs, lncRNAs & mRNAs in the control & CoCl₂-treated groups

We identified 605 circRNAs (Figure 2A), 4409 lncRNAs (Figure 2B) and 3965 mRNAs (Figure 2C) that were differentially expressed with fold change ≥ 2.0, FDR < 0.05 and *p* < 0.05. Overall, we identified 378 upregulated and 227 downregulated circRNAs (Figure 2D), 2467 upregulated and 1942 downregulated lncRNAs (Figure 2E), and 1412 upregulated and 2553 downregulated mRNAs (Figure 2F).

GO & KEGG pathway analyses

Our data revealed that the differentially regulated transcripts primarily involved in the BP category were metabolic processes, including organic substance and cellular protein metabolic processes. Meanwhile, the significantly enriched GO terms in the MF category revealed that the upregulated mRNAs were closely related to ion binding, metal ion binding, cation binding, nucleic acid binding, histone binding and protein binding, whereas the downregulated mRNAs were associated with glycosaminoglycan binding and catalytic activity (Figure 3A & B).

Figure 2. Microarray profiles of differentially expressed circular RNAs (circRNAs), long noncoding RNAs (lncRNAs) and mRNAs. (A–C) Heat maps showing the expression profiles of circRNAs, lncRNAs and mRNAs. **(D–F)** Volcano plots presenting differences in the expression of circRNAs, lncRNAs and mRNAs between the control and CoCl₂ groups. Values plotted on the x- and y-axes represent the averaged normalized signal values of each group (log₂-scaled).

KEGG pathway analyses identified the significantly differentially expressed mRNAs that contributed to these pathways and molecular interactions. Figure 3C shows that the systemic lupus erythematosus and viral carcinogenesis pathways were the top pathways associated with the upregulated mRNAs, whereas the top two enriched KEGG pathways associated with downregulated mRNAs were focal adhesion and pathways in cancer.

To explore whether circRNAs regulate parental gene transcription, GO analysis of the genes producing differently expressed circRNAs was performed, and the top 10, 10 and 8 terms of BP, CC and MF categories, respectively, were classified according to the number of linear counterparts of differentially expressed circRNAs. Compared with the control group, the linear counterparts of differentially upregulated circRNAs in the CoCl₂-treated group were mainly involved in catabolic processes, mRNA processing, reproduction, chromosome segregation and cell division, and closely related GO terms were RNA, DNA, enzyme and ubiquitin-like protein binding; the genes producing downregulated circRNAs in the CoCl₂-treated group favored the circulatory system, locomotion, cell adhesion, anatomical structure development and cell motility. MF analysis showed that the linear counterparts of downregulated circRNAs participated in signal transducer activity, transmembrane transporter activity, cytoskeletal protein binding and hydrolase activity and acted on carbon–nitrogen bonds (Figure 3E).

To further understand the pathways involved in CoCl₂-induced cytotoxicity based on the differentially expressed circRNAs, we performed KEGG pathway analysis to analyze genes that produced differentially expressed circRNAs. According to the results, 22 pathways were identified. The top three related pathways in which the genes resulted in upregulated circRNAs were protein processing in the endoplasmic reticulum, adherens junctions and endocrine resistance, whereas the downregulated circRNAs were involved in the AGE-RAGE signaling pathway in diabetic complications and the relaxin and estrogen signaling pathways (Figure 3F).

Figure 3. Gene Ontology and Kyoto Encyclopedia of Genes and Genomes pathway analyses. (A & B) Gene Ontology annotations of up- and downregulated mRNAs with the top ten enrichment scores covering the biological process, cellular component and molecular function categories. **(C & D)** Kyoto Encyclopedia of Genes and Genomes pathway enrichment analysis of up- and downregulated mRNAs with the top ten enrichment scores. **(E)** Gene Ontology annotations of the linear counterparts of differentially expressed circRNAs in the cellular component, molecular function and biological process categories. **(F)** Kyoto Encyclopedia of Genes and Genomes pathway enrichment analysis of the genes that resulted in up- and downregulated circRNAs.

Construction of co-expression networks

The differentially expressed lncRNA-mRNA co-expression networks consisted of nine protein-coding transcripts, 590 lncRNAs and 594 edges and were built according to the degree of correlation. Among them, the downregulated lncRNAs, 2900056M20Rik was positively correlated with *Mef2a*, *Myog* and *Egfr*, while XLOC.006822 and XLOC.009311 were positively correlated with *Igf1* and *Egfr*, respectively, which have been linked with myogenesis and metastasis (Figure 4).

Cis- & trans-regulation prediction of lncRNAs

A nearby gene located less than 300 kb upstream or downstream from a lncRNA may represent a potential target regulated by that lncRNA in a *cis* manner. Our data revealed the top 10 up- and downregulated lncRNAs and the distance between nearby coding genes. The upregulated lncRNA Lonrf3 had the maximum number, six, of adjacent coding genes, whereas the downregulated lncRNA Cxcl14 had five adjacent coding genes. These networks provided valuable clues about these lncRNAs and nearby coding genes (Figure 5A).

Figure 3. Gene Ontology and Kyoto Encyclopedia of Genes and Genomes pathway analyses (cont.). (A & B) Gene Ontology annotations of up- and downregulated mRNAs with the top ten enrichment scores covering the biological process, cellular component and molecular function categories. **(C & D)** Kyoto Encyclopedia of Genes and Genomes pathway enrichment analysis of up- and downregulated mRNAs with the top ten enrichment scores. **(E)** Gene Ontology annotations of the linear counterparts of differentially expressed circRNAs in the cellular component, molecular function and biological process categories. **(F)** Kyoto Encyclopedia of Genes and Genomes pathway enrichment analysis of the genes that resulted in up- and downregulated circRNAs.

Figure 4. lncRNA-mRNA co-expression network. Red and green diamonds represent up- and downregulated lncRNAs, respectively. Purple and yellow arrows indicate up- and downregulated mRNAs, respectively.

Our results also indicated that the upregulated lncRNA/TF network consisted of 38 TFs and was correlated with the top ten upregulated lncRNAs, which were connected by 123 edges (Figure 5B). The downregulated lncRNA/TF network consisted of 37 TFs and was correlated with the top ten downregulated lncRNAs, which were connected by 138 edges (Figure 5C).

Construction of ceRNA networks

In this study, we constructed lncRNA-miRNA-circRNA-mRNA ceRNA networks based on our microarray data. The left pattern represented the ceRNA network that covered 104 miRNAs connected with four mRNAs (up), ten lncRNAs (up) and 30 circRNAs (up) through 761 edges (Figure 6A), whereas the right pattern covered 117 miRNAs connected with six mRNAs (down), ten lncRNAs (down) and 30 circRNAs (down) through 832 edges (Figure 6B).

Prediction of coding potential & presumed encoded polypeptide functions of differentially expressed circRNAs

It has been reported that consensus m⁶A motifs are enriched in circRNAs and that m⁶A-driven translation requires the initiation factor eIF4G2 and the m⁶A reader YTHDF3. In this study, we explored whether circRNAs have translation potential. We examined the relative mRNA expression levels of *eIF4G2* and *YTHDF3* and showed that the levels of both were decreased in the CoCl₂-treated group (Figure 7A).

We also identified 242 circRNAs with coding potential with an ORF and at least one m⁶A motif in the sequence. According to the expression levels of dysregulated circRNAs with translation potential, hierarchical clustering was performed to identify the top 20 upregulated and top 20 downregulated circRNAs (Figure 7B).

Figure 5. *Cis*- and *trans*-regulation prediction of lncRNAs. (A) Distances between lncRNAs and their *cis*-regulated genes. Red and green columns indicate up- and downregulated lncRNAs, respectively. The left vertical axis shows the sequence name of the coding genes, and the right vertical axis indicates the gene symbol of the sequence. **(B & C)** Up- and downregulated lncRNA/transcription factor networks. Yellow nodes indicate transcription factors, and red and green diamonds indicate up- and downregulated lncRNAs, respectively.

Figure 6. Competing endogenous RNA network construction. (A) Competing endogenous RNA network based on the interaction of microRNAs (miRNAs) and upregulated lncRNAs, circRNAs and mRNAs. **(B)** Competing endogenous RNA network based on the interaction of miRNAs and downregulated lncRNAs, circRNAs and mRNAs.

The 242 circRNAs with coding potential were classified into three distinct types: 93.802% were exonic (227), 2.479% were intronic (6) and 3.719% were sense-overlapping (9). Next, we counted the number of ORFs: 62 circRNAs had one ORF, 47 circRNAs had two ORFs, 27 circRNAs had three ORFs and 106 circRNAs had four or more ORFs. We also determined the number of m⁶A motifs, which are required for circRNAs to encode polypeptides. In total, 44 circRNAs had one m⁶A motif, 35 circRNAs had two m⁶A motifs and 163 circRNAs had three or more m⁶A motifs (Figure 7C).

According to the screening of circRNAs with coding potential with known functional domains and more than 30 amino acids, we identified 71 circRNAs and performed GO and KEGG analyses based on the linear counterparts of these circRNAs. We found that the linear counterparts of the 71 dysregulated circRNAs favored cellular and metabolic processes. At the same time, the GO terms of the MF category were closely related to binding and catalytic activity (Figure 7D). KEGG analysis revealed that the 71 identified circRNAs were related to cancer, focal adhesion, PI3K-AKT, mammalian target of rapamycin (mTOR), FoxO, Ras and mitogen-activated protein kinase (MAPK) pathways (Figure 7E).

Discussion

Severe poisoning of skeletal muscle has been reported in orthopedic joint replacements in humans, especially in metal-on-metal bearings in hip joint arthroplasty, which are composed of cobalt-chrome alloys [5]. The release of soluble metal ions and metal debris on polyethylene has been shown to result in the destruction of bone and soft tissue [1]. It is tempting to speculate that ionized cobalt is the primary toxic form *in vivo*. Based on this assumption, C2C12 cells were treated with different concentrations of CoCl₂ as an *in vitro* toxic trauma model, which seems to reside primarily with ionized cobalt. Our results confirmed that the toxic effects of cobalt were notable, resulting in the induction of apoptosis and inhibition of myotube differentiation. The toxic effects were obvious and accumulated with long-term exposure.

ncRNAs are rapidly being recognized as crucial regulators of gene expression in development and disease [11,12]. However, studies on ncRNAs in sarcos toxicity induced by CoCl₂ are rare. Microarray analysis of circRNA and lncRNA expression demonstrated the potential roles of ncRNAs in the CoCl₂-induced cytotoxicity of myotubes, revealing 378 upregulated and 227 downregulated circRNAs and 2467 upregulated and 1942 downregulated

Figure 7. Prediction of the coding potential of differentially expressed circRNAs. (A) Expression of eukaryotic translation initiation factor 4 gamma 2 and YTH domain family protein 3 was detected by quantitative real-time polymerase chain reaction. (B) Heat map of the top 20 upregulated and top 20 downregulated circRNAs among the 242 differentially expressed circRNAs with coding potential. (C) Number of types, open reading frames and m⁶A motifs of the 242 circRNAs with coding potential. (D) Gene Ontology analysis of the genes related to the circRNAs with coding potential with known functional domains and more than 30 amino acids. The y-axis on the left indicates the number of genes in a category and the y-axis on the right indicates the percentage of a specific category of genes in the main category. (E) Kyoto Encyclopedia of Genes and Genomes pathway enrichment analysis of the genes that produced circRNAs with coding potential with known functional domains and more than 30 amino acids. ORF: Open reading frame.

lncRNAs. According to our analysis, the types and chromosomal distributions of differential circRNAs and lncRNAs differed widely (Supplementary Figure 1), which suggested that each chromosome was related to the varying degrees of abnormality in the *CoCl₂*-treated group.

GO analysis revealed that both upregulated and downregulated mRNAs were involved in terms in the MF category, including ion binding, metal ion binding, cation binding, nucleic acid binding, protein binding and histone binding. Interestingly, the top pathways of KEGG pathway analysis was systemic lupus erythematosus and viral carcinogenesis pathways. The common genes driving the significance of systemic lupus erythematosus and viral carcinogenesis pathways comprise the genes of histone cluster: HIST1-H2A, -H3A, -H3C, -H3F, -

Figure 7. Prediction of the coding potential of differentially expressed circRNAs (cont.). (A) Expression of eukaryotic translation initiation factor 4 gamma 2 and YTH domain family protein 3 was detected by quantitative real-time polymerase chain reaction. (B) Heat map of the top 20 upregulated and top 20 downregulated circRNAs among the 242 differentially expressed circRNAs with coding potential. (C) Number of types, open reading frames and m⁶A motifs of the 242 circRNAs with coding potential. (D) Gene Ontology analysis of the genes related to the circRNAs with coding potential with known functional domains and more than 30 amino acids. The y-axis on the left indicates the number of genes in a category and the y-axis on the right indicates the percentage of a specific category of genes in the main category. (E) Kyoto Encyclopedia of Genes and Genomes pathway enrichment analysis of the genes that produced circRNAs with coding potential with known functional domains and more than 30 amino acids. ORF: Open reading frame.

H4K and so on, which played an important role in DNA repair, cell cycle or cell development [25]. Doynova *et al.* [26], showed differential and ongoing expression of replicative histone variants within the HIST1 locus during muscle cell development. Another publication reported that several histone H1 genes were downregulated during differentiation [27]. It was also reported that replication-dependent histones, which were comprised of the H2A, H2B, H3 and H4 histone core, were consumed upon cell differentiation due to newly synthesized DNA [28,29]. CoCl₂ treatment suppressed C2C12 differentiation with histone cluster decreasing, suggesting a possible instrument of loose and nonspiral chromatin was observed during CoCl₂ treatment. The downregulated enriched signaling pathway, PI3K-AKT, has been implicated in the induction of muscle atrophy. The TF FoxO3a is activated when AKT activity is inhibited and FoxO3a has been shown to negatively regulate autophagy, which is followed by the induction of expression of the ubiquitin ligases, Murf1 and Atrogin1 [30].

Analysis of the genes that produced differentially expressed circRNAs was performed to explore the potential enriched GO terms of the differentially expressed circRNAs. Of note, some GO terms of the BP and MF categories of the linear counterparts of the differentially regulated circRNAs were related to protein degradation and structure development. Based on the enrichment score of the KEGG analysis, we noticed the FoxO pathway was one of the top ten pathways in which the genes produced upregulated circRNAs. We also noted another potent autophagy-related pathway, the AMP-activated protein kinase pathway, which has been shown to stimulate autophagy through the suppression of mTOR phosphorylation in C2C12 myotubes following CoCl₂ treatment [10].

Co-expression experiments have effectively shown that genes with similar functional properties tend to interact or exhibit similar profiles in networks [31]. We built a co-expression network to predict lncRNA functions and identified candidate lncRNAs correlated with the cytotoxicity of CoCl₂ by regulating co-expression genes. Both *Myog* and *Mef2a* are necessary and sufficient to drive the transcription required for myogenic differentiation [32], whereas *Egfr* is a tyrosine kinase that has been implicated in cell growth, survival, migration [33,34] and differentiation [33]. Our results revealed that the downregulated lncRNA 2900056M20Rik was positively correlated with *Myog*, *Mef2a* and *Egfr*, suggesting that CoCl₂ may affect cell growth, survival, migration or differentiation by regulating 2900056M20Rik. Two other lncRNAs, XLOC.006822 and XLOC.009311, were associated with *Igf1* and *Egfr*. It has been conclusively shown that attenuated Igf-1R/Egfr crosstalk and altered levels of cell cycle proteins favor reduced epidermal proliferation [35]. Amplification and mutations in genes are associated with many types of cancer [36,37].

lncRNAs may impact the transcription of neighboring or distant genes by *cis*- and/or *trans*-regulatory mechanisms [38]. We delineated a network of the top ten up- and downregulated lncRNAs with their adjacent coding genes. The upregulated lncRNA Lonrf3 had a maximum number of six adjacent coding genes. Among them, Nxf2 encodes a member of a family of nuclear RNA export proteins involved in the maintenance of genome integrity [39], whereas *Armxc2* is involved in development, maintenance of tissue integrity and tumorigenesis [40,41]. The downregulated lncRNA Cxcl14 had five adjacent coding genes. H2afy was linked with nucleosome remodeling and the movement of neighboring DNA segments [42]. This network provided valuable clues about these lncRNAs and nearby coding genes.

Several lncRNAs are regulated by TFs. We constructed a lncRNA/TF network in this study to further study the lncRNAs involved in the toxic effects of CoCl₂ on myotubes. Because *Myod1* and *Myog* are vital for driving the transcription required for myogenic differentiation, we realized the upregulated lncRNAs 1700001K23Rik, AK035437 and 2310034O05Rik and the downregulated lncRNAs AK020242, AK161362 and Fam20a were correlated with *Myod1* and *Myog*. Therefore, these lncRNAs may play important roles in CoCl₂-dependent inhibition of differentiation.

Both circRNAs and lncRNAs have been confirmed to act as ‘miRNA sponges’, regulating gene expression at the transcriptional or post-transcriptional level [11,16]. Here, we chose ten verified mRNAs (Supplementary Figures 2 & 3) to construct lncRNA-miRNA-circRNA-mRNA ceRNA networks to further reveal a novel regulatory network of the noxious effects of CoCl₂ on myotubes. For instance, we focused on miRNA-29b as a promoter atrophic-miRNA. circRNA_018676, circRNA_38400, circRNA_38398 and AK035437 could regulate the expression of hexokinase 2 and Atrogin1 via the competing miRNA response elements of miRNA-29b. We also focused on miRNA-320 as a promoter differentiated-miRNA. circRNA_21401, circRNA_21401, AK035437, AK037505 and AK046686 could regulate the expression of *Egfr*, *Igf1* and *Myog* through competing miRNA-320.

Many circRNAs are considered noncoding, although several reports have recently subverted this paradigm [18,23,43]. A recent study reported that m⁶A motifs are responsible for circRNA translation through eIF4G2 and YTHDF [23]. The depletion of eIF4G2 and YTHDF3 significantly reduced protein synthesis from

circRNAs, whereas overexpression of eIF4G2 and YTHDF3 increased protein translation from circRNAs. Our results demonstrated that CoCl₂ treatment decreased *eIF4G2* and *YTHDF3* expression, suggesting CoCl₂ may interfere with protein translation from circRNAs by suppressing the expression of eIF4G2 and YTHDF3. Further, we identified 242 circRNAs with coding potential with an ORF and at least one m⁶A motif in the sequence. These candidate circRNAs were categorized based on their type or the number of m⁶A modifications and ORFs. Hierarchical clustering revealed the top 20 up- and downregulated candidate-encoded peptide circRNAs. Finally, according to the screening of circRNAs with coding potential with known functional domains and more than 30 amino acids [24], we identified 71 circRNAs, which were used to perform GO and KEGG analyses based on the linear counterparts of the circRNAs. The GO terms of the linear counterparts of the circRNAs were correlated with the metabolic process, response to the stimulus process and biological regulation. It is worth noting that the PI3K-AKT, mTOR, FoxO and MAPK pathways were significantly enriched pathways closely related to muscle atrophy.

Conclusion

This study is the first report that lncRNAs and circRNAs are differentially expressed in CoCl₂-induced toxicity. Furthermore, we constructed correlated co-expression, ceRNA and *cis/trans* regulation networks with bioinformatics methods to filter ncRNAs correlated with growth, differentiation, or apoptosis. Most importantly, we revealed 71 candidate circRNAs with coding potential, whose linear counterparts exhibited principal functions closely related to muscle atrophy. Our results may reveal novel regulatory mechanisms underlying the noxious effects of CoCl₂ in skeletal muscle. However, this study was limited to cultured cells, and knockout or overexpression of key lncRNAs or circRNAs in toxicity of CoCl₂ need to be done in further studies.

Future perspective

The development of high-throughput microarray allows us to define the dysregulated ncRNAs and the application of bioinformatics provides new insights into understanding of the underlying molecular mechanisms in severe poisoning of skeletal muscle in orthopedic joint replacements. Currently, there are several studies confirming that some miRNAs can be used as diagnostic and prognostic biomarkers for diseases. As such, the improved knowledge of lncRNAs and circRNAs also lead to their use as diagnostic, prognostic and therapeutic biomarkers in the imminent future.

Summary points

- We used CCK8, flow cytometry, western blot and immunocytochemistry to confirm the toxic effects of Co (II) on C2C12 myotubes.
- We identified 605 differentially expressed circRNAs, 4409 lncRNAs and 3962 mRNAs in C2C12 after CoCl₂ treatment.
- Gene ontology and Kyoto Encyclopedia of Genes and Genomes analyses showed the principal functions of the significantly dysregulated genes were binding, protein degradation and structure development.
- Co-expression indicated several lncRNAs correlated with growth, differentiation or apoptosis-related genes.
- The upregulated lncRNA *Lonrf3* had maximum number of six adjacent coding genes, whereas the downregulated lncRNA *Cxcl14* had five adjacent coding genes.
- lncRNA/TF network revealed 6 lncRNAs correlated with *Myod1* and *Myog*, involved in myogenic differentiation.
- The ceRNA networks were conducted to further reveal a novel regulatory network in noxious effects of CoCl₂ on myotubes.
- We filtered 71 candidate circRNAs with coding potential and performed bioinformatics analysis based on the linear counterparts of the circRNAs, which displayed the chief functions were closely related to muscle atrophy.

Author contributions

R Chen conceived and designed the experiments; R Chen, Y She, Q Fu and X Chen performed the experiments and analyzed the data regarding the circRNA expression profiles; R Chen, H Shi, S Lei and S Zhou analyzed the data regarding the lncRNA expression profiles; J Ou and Y Liu performed the experiments to generate circRNA and lncRNA expression profiles data; R Chen and Y She wrote the manuscript. All authors read and approved the final manuscript.

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