MBNL1 Regulates the Expression and Alternative Splicing of Genes Enriched in Cell Adhesion and Apoptosis

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Abstract

Muscleblind Like Splicing Regulator 1 (MBNL1), one canonical RNA binding protein (RBP), plays important roles in the regulation of the alternative splicing (AS) on pre-mRNAs. MBNL1 has traditionally been considered involved in the pathogenesis of myotonic dystrophy. Recent researches point out that MBNL1 has an effect on cancer progress, but the underlying mechanisms are unclear. In this study, we obtained the regulated transcriptome profile of MBNL1 in HeLa cells by RNA-seq analysis. The results showed that the knockdown of MBNL1 promoted cell proliferation while inhibited apoptosis. We found 398 genes were differentially up-regulated and 277 down-regulated by MBNL1 knockdown (KD). The differentially expressed genes (DEGs) regulated by MBNL1-KD were enriched in the signal pathways of homophilic cell adhesion, apoptotic process, extracellular matrix organization and cell migration. Systematical AS analysis revealed 504 MBNL1-regulated AS events. The regulated alternative splicing genes (RASGs) were enriched in the signal pathways of apoptotic signaling pathway, positive regulation of apoptotic process, adherent junction, fatty acid elongation and DNA repair. In summary, our results demonstrate that the knockdown of MBNL1 have significant effects on cell proliferation and apoptosis by regulating the expression and alternative splicing of associated genes, illustrating the possible molecular mechanisms of MBNL1 in cancer pathogenesis and progression and other diseases.

Introduction

RNA binding proteins (RBPs) are widely found in animals, plants and microorganisms, accounting for about 2–8% of eukaryotic gene coding proteins (Lunde et al., 2007), regulating gene expression and alternative splicing. RNAs are usually bound by a variety of specific RBPs, and the fate and function of RNAs are closely regulated by RBPs (Hentze et al., 2018). Therefore, RBPs plays a key role in the regulation of RNA biosynthesis, alternative splicing, modification, transport, translation and degradation (Kafasla et al., 2014). Till now, the amount of discovered RBPs is more than 1000 (Hentze et al., 2018). The expression or dysfunction of RBPs is the molecular basis for various diseases (Chothani et al., 2019). It is of great importance to study the post-transcriptional regulation of RBPs for the disease prevention and treatment.

Alternative splicing (AS) regulation is an important mechanism for the diversity and complexity of the transcriptome and proteome, and widely involved in eukaryotic protein production (Lopez, 1998; Kornblihtt et al., 2013). Muscleblind Like Splicing Regulator 1 (MBNL1) is an RBP, with the function regulating RNA locations, stability, transport and alternative splicing (Ho et al., 2004; Terenzi and Ladd, 2010; Masuda et al., 2012; Wang et al., 2012; Batra et al., 2014). Decreased expression of MBNL1 in myotonic dystrophy may induce aberrant alternative splicing of a subset of pre-mRNAs (Teplova and Patel, 2008). It is a vital gene in regulating alternative splicing events in controlling embryonic cell pluripotency (Han et al., 2013). Analysis in human monocytes and macrophages revealed MBNL1 as a major alternative splicing regulator (Liu et al., 2018a).
The dysfunction of MBNL1 is related to various diseases. Research on MBNL1 mainly focuses on the pathogenesis of myotonic dystrophy, including the regulation of skeletal muscle differentiation and the regulation of myocardial maturation (Fardaei et al., 2002; Warf and Berglund, 2007; Kino et al., 2009). Besides, recent papers showed that MBNL1 participates in other diseases. MBNL1 regulates essential alternative RNA splicing patterns in MLL-rearranged leukemia (Itskovich et al., 2020). And loss of MBNL1 induces RNA misprocessing in the thymus and peripheral blood (Sznajder et al., 2020). Repression of MBNL1-dependent alternative splicing may contribute to the regulation of stem cell fates (Welte et al., 2019). Further, MBNL1 is also reported to be involved in the progress of cancers. It is interesting that different MBNL1 splicing isoforms play different roles in cancer. MBNL1 overall expression was down-regulated, described as a tumor suppressor, while MBNL1 isoform exon 7 proteins are antisurvival factors with a defined tumor suppressive role (Tabaglio et al., 2018). MBNL1 suppresses breast cancer metastatic colonization and stabilizes metastasis suppressor transcripts (Fish et al., 2016). A tumor-associated splice-isoform of MAP2K7 drives dedifferentiation in MBNL1-low cancers via JNK activation (Ray et al., 2020). These studies indicate MBNL1 controls alternative splicing through multiple mechanisms in different organs. However, the mechanism is still poorly understood.

In order to further explore the regulatory mechanisms of MBNL1 in other diseases, such as cancers, we knocked down (KD) the expression of MBNL1 in HeLa cells. Cytological experiments demonstrated that MBNL1-KD could reduce cell apoptosis. RNA sequencing (RNA-seq) was conducted to analyze the regulated gene of MBNL1. Results showed that both differentially expressed genes and regulated alternative splicing genes were enriched in cell adherence and cell apoptosis pathways. In summary, MBNL1 has an important regulation in the cell adherence and cell apoptosis in cancer cells. Our research provides a novelty knowledge of MBNL1-regulated transcriptional profiles.

**Methods**

**RNA library construction and sequencing**

The total sampling RNA of was extracted first. The RQ1 enzyme was employed to digest the DNA in RNA. The integrity was detected by electrophoresis. 5μgof total RNA was used for library construction. Oligo (dT)-conjugated magnetic beads were used to enrich and capture mRNA with polyA tail. cDNA was synthesized by reverse transcription of RT primers. PCR amplification was performed on the cDNA after reverse transcription. Illumina Nextseq 500 system was employed to carry out pair-end sequencing with a read length of 151bp.

**RNA-seq differential expression genes (DEG) analysis**

FastQC (Version 0.9.5) was employed to detect the quality of filtered fragments in fastq format. TopHat2 software was used to match the screened sequences to the human GRCH38 genome. The edgeR software was used to determine the differentially expressed genes for sample comparison. The thresholds were based on the difference multiple (the difference multiple ≥2 or ≤0.5) and the error detection rate (FDR≤0.05). ABLas software was employed to analyze the splicing types of each sample.
The functional clustering analysis of the differentially expressed genes and the genes with alternative splicing was carried out by DAVID, GO and KEGG databases.

**Alternative splicing analysis**

The ABLas was employed to define the alternative splicing events (ASEs). Ten kinds of the ASEs are included, exon skipping (ES), cassette exon (Cassette Exon), alternative 5’ splice site (A5SS), alternative 3’ splice site (A3SS), intron retention (IR), mutually exclusive exons (MXE), mutually exclusive 5’UTRs (5pMXE), mutually exclusive 3’UTRs (3pMXE), A5SS&ES and A3SS&ES. After that, significant P value was calculated based on Fisher’s exact test. The P value less than 0.05 and ration more than 0.2 were defined as RBP-regulated ASEs.

**Cell culture and transfection**

HeLa cells (from the China Center for Type Culture Collection) were cultured under the condition Dulbecco’s modified Eagle’s medium (DMEM), supplemented 10% foetal bovine serum (FBS), 100 µg/ml streptomycin and 100 U/ml penicillin. LipofectAMINE 2000 (Invitrogen, Carlsbad, CA, USA) was employed to perform the shRNA transfection of HeLa cells.

**Evaluation of the knockdown of MBNL1**

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was employed to evaluate the efficient of the knockdown of MBNL1. CDNA was performed and q-RCR was conducted on a Bio-Rad S1000 with Bestar SYBR Green RT-PCR Master Mix (DBI Bioscience, Shanghai, China).

**Analysis of Cell proliferation and apoptosis**

The MTT assay was employed to analyze the cell proliferation. Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) was used to transfected the cells with the vector. 0.15 ml dimethylsulfoxide (DMSO) was used to solubilize the resulting formazan crystal.

For the cell apoptosis analysis, the transfected cells were stained with Annexin V-PE/7-AAD Apoptosis Detection kit (BD Pharmingen Biosciences, San Diego, CA). The Beckman MoFlo XDP was employed to detect the apoptosis. And the Flowjo (TreesStar) software was used in the data analysis.

**Statistical analysis**

Two-tailed t test was conducted to tell the difference between the control and knockdown groups. SPSS 19.0 statistical software was employed for the data analysis. P value less than 0.05 were regarded as the significant difference.

**Real-time qPCR validation for DEGs and alternative splicing**
RT-qPCR was employed to verify the DEGs expression and AS. The gene expression was normalized against GAPDH. It was in the condition of 95°C for 10 minutes for denaturing, and 95°C for 15 seconds of 40 cycles, 60°C for 1 minute for annealing and extension. A boundary-spanning primer and an opposing primer were used to detect gene isoforms.

**Western blot**

Cell proteins were extracted by the kit (Sangon Biotech Co., Ltd). Protein concentration was determined by the SDS-PAGE on 10% (Bio-Rad Laboratories, Inc), and transferred to nitrate fiber film. Coll (Southern Tech, Birmingham, AL), CollIV, FN (Dako, Carpinteria, CA), α-SMA (Sigma, St Louis, MO) and β-Actin primary antibody (Santa Cruz, USA) were added and incubated overnight at 4 °C. Secondary antibodies (Santa Cruz, Biotechnology, Santa, Cruz, CA) were added to incubate at room temperature for 1 hour, and luminescent agent (U.S. Cell Signaling Technology) was added. X-ray film was exposed.

**Results**

**MBNL1 knockdown reduces the cell apoptosis.**

In this article, HeLa cells were used in both cytological experiments and RNA-seq. The knocking down of MBNL1 on HeLa cells was conducted to investigate the influence on cell proliferation and apoptosis. The expression of MBNL1 was tested by RT-qPCR, both the shRNA vector and the empty vector. Results show that the expression of MBNL1 is reduced by approximately 50% (Fig. 1A). Cell proliferation was significantly increased (Fig. 1B), while cell apoptosis was significantly inhibited by the knockdown of MBNL1 (Fig. 1C,D), similar to the previous study (Wu et al., 2018). These results illustrated that the cell proliferation was increased, while apoptosis was inhibited by shMBNL1 in HeLa cells.

**Gene expression profiles regulated by the shMBNL1**

Two replicates for both shMBNAL1 and control groups were conducted to eliminate the experimental bias. Complementary DNA (cDNA) libraries were conducted for RNA-seq. Illumina HiSeq X Ten platform was employed for sequencing. The raw reads are 85.5±6.0 million per sample. After removing the adaptor and low-quality reads, 82.0±6.4 million reads were left. These reads were mapped to the human GRCH38 genome with TopHat2 (Kim et al., 2013). 67.2±5.0 million reads were mapped, with 64.5±4.6 uniquely mapped and 2.7±5.5 multiple mapped (Supplementary Table S1).

The uniquely mapped reads were used for gene expression analysis (Fig. 2A). FPKM (fragments per kilobase of exon model per million fragments mapped) was calculated. There were 25,677 genes detected at the level FPKM>0, and 13,121 genes at the level FPKM>1 (Supplementary Table S2 and S3). The relative expression level by FPKM further confirmed the knockdown of MBNL1 (Fig. 2B), similar to the result by RT-qPCR (Fig. 1A). Pearson’s correlation coefficients were generated by the global gene expression levels between the knockdown and control samples. It is shown that the correlations were high between the shMBNL1 and the control, indicating the similarity between them (Fig. 2C).
EdgeR (Robinson et al., 2010) was employed to investigate the differentially expression genes (DEGs) between the shMBNL1 and control. We found 398 genes were up-regulated and 277 genes were down-regulated (fold change ≥2 or ≤0.5 and false discovery rate<0.05). The up-regulated and down-regulated genes were shown in the volcano plot (Fig. 2D). The up and down-regulated genes were 59% and 41% of the total DEGs, respectively (Fig. 2E). Detailed up and down-regulated genes can be obtained in Supplementary Table S4 and S5. The heatmap shows the significant DEGs between the shMBNL1 and control. The number of up-regulated genes is greater than that of down-regulated. The two repeated experiments showed similar results (Fig. 2F).

**MBNL1 Knockdown regulating the differential expression genes enriched in cell adhesion and apoptosis**

GO and KEGG analyses were employed to analyze the potential biological process of DEGs regulated by the knockdown of MBNL1. For the GO analysis, the up-regulated DEGs are enriched in 33 terms and the down-regulated DEGs in 9 terms (Supplementary Table S5). In the top 10 terms of the up-regulated terms, these DEGs are enriched in extracellular matrix organization, extracellular matrix disassembly, synaptic transmission, homophilic cell adhesion, apoptotic process, etc (Fig. 3A). The down-regulated DEGs are enriched in cell migration, nervous system development, DNA-dependent transcription etc. (Fig. 3A). For the KEGG analysis, the up-regulated DEGs are enriched in adipocytokine signaling pathway and insulin secretion (Fig. 3B), while the down-regulated in neuroactive ligand-receptor interactions and tight junctions (Fig. 3B). Some of them have been reported in previous studies, such as synaptic (Wang et al., 2012), insulin abnormality (Dansithong et al., 2005; Sen et al., 2010), but some have not.

To validate the effect of the knockdown of MBNL1, five DEGs (BMF, EDN2, IFITM1, PCDHB11, MYH13) related to cell adhesion and cell apoptotic signal pathways were randomly selected for RT-qPCR. The results showed that the first 4 genes were higher expressed, while the last one was lower expressed, consistent with the results by RNA-seq results (Fig. 3C).

**MBNL1 Knockdown regulating the alternative splicing genes enriched in cell adhesion and apoptosis**

As studies specified, the main function of MBNL1 is to participate in the alternative splicing of pre-mRNAs. Our results also showed that, among the uniquely mapped reads, more than one half were junction reads (Supplementary Table S1), indicating the potential effects of the alternative splicing function of MBNL1. Among the splicing events, 160,922 were known and 149,039 were novel splice junctions detected by TopHat2 (Supplementary Table S6). ABLas pipeline (Xia et al. 2017) was employed to investigate alternative splicing events (ASE). The result showed 19,727 known and 51,225 novel ASE (Supplementary Table S6).

To select significant MBNL1-regulated alternative splicing events (RASEs), two indexes were employed, including P values and changed AS ratios. The thresholds for these two indexes were ≤0.05 and ≥0.15, respectively. As a result, 504 RASEs were detected in total. The types of alternative splicing include exon skipping(ES) alternative 5’ splice site (A5SS) alternative 3’ splice site (A3SS) intron retention (IntronR), mutually exclusive 5’ UTRs (5pMXE) mutually exclusive 3’ UTRs (3pMXE), Cassette
Exon A3SS & ES and A5SS & ES. Among these, the numbers of A5SS, A3SS and ES are relatively high (Fig. 4A). There were 460 MBNL1-regulated alternatively spliced genes (RASGs), only 2 of them overlapped with the DEGs (Fig. 4B). Most of the DEGs and RASGs are not regulated by MBNL1 simultaneously. Of the 504 RASEs, intron retention (IR) events accounts for 25.4%, and non-intron retention (NIR) events 74.6% (Fig. 4C and Supplementary Table S7).

To discover the function of the RASGs, GO and KEGG analysis were employed. The alternative splicing genes are enriched in the apoptotic signaling pathway, positive regulation of apoptotic process, protein ubiquitination and DNA repair by GO analysis (Fig. 4D, Supplementary Table S8). The KEGG analysis demonstrates that the RASGs are enriched in adherent junctions, fatty acid elongation, metabolic pathways (Fig. 4E, Supplementary Table S9). The results show that the knockdown of MBNL1 regulates the alternative splicing gene related to the cell apoptosis.

RT-qPCR was employed to validate the MBNL1 regulated ASEs. Five genes are randomly selected (FLNB, CASP10, PHLDB2, DIDO1, CTNND1). The validation of these genes is consistent with the RNA-seq results (Fig. 5 and Fig. 6). The ratio in the ASE changed significantly, indicating MBNL1 regulates the alternative splicing of these genes.

**Discussion**

MBNL1 is a kind of RNA binding protein, participating in the regulation of the alternative splicing of pre-mRNAs. It plays an important role in several diseases, especially myotonic dystrophy (Wagner et al., 2016; Hinman et al., 2020). Many scientists have paid much attention to the mechanism of MBNL1 in this disease. Besides myotonic dystrophy, MBNL1 also plays an important role in other diseases, including cancer and cardiac fibrosis (Davis et al., 2015; Tabaglio et al., 2018). To research the MBNL1 regulation profile, cytological experiments and RNA-seq were employed. MBNL1 was knocked down in HeLa cells to study its functions on the gene expression and alternative splicing regulation. It is shown that cell proliferation was increased and cell apoptosis reduced by the silence of MBNL1. The gene expression profile shows that 398 genes were up-regulated, while 277 down-regulated. The genes were enriched in the pathways of hemophilic cell adhesion, apoptosis process, DNA-dependent transcription, cell migration, etc. We also found 460 genes were alternative spliced by the knockdown of MBNL1. These genes were enriched in the cell-cell adhesion, apoptosis signal way, positive regulation of apoptotic processes, DNA-dependent transcription etc. Briefly, the regulated gens were enriched in cell adhesion and apoptosis in both the expression level and alternative splicing.

The cytological experiment shows that MBNL1 enhances the cell apoptosis. RNA-seq confirmed the regulation of MBNL1 on the cell apoptosis signaling pathway. Some research have pointed out that MBNL1 may play an important role in the cell apoptosis (Wu et al., 2018; Sznajder et al., 2020). They pointed out that MBNL1 overexpression significantly decreased the proliferation rate and increased apoptosis (Wu et al., 2018). Our results consist with previous studies on cell proliferation and apoptosis. This research further indicated that MBNL1 regulated the cell adhesion in HeLa cell. Cell apoptosis and
cell adhesion play important functions in the progress of cancers. MBNL1 may participated in the cancer development by regulating the gene expression and alternative splicing.

Despite for myotonic dystrophy and cancers, MBNL1 also plays an important role in cardiac diseases (Kalsotra et al., 2008; Dixon et al., 2015). Further research indicated that overexpression of MBNL1 promoted the transformation of fibroblasts into myofibroblasts, while the absence of MBNL1 destroyed the transformation and weakens the fibrosis of wound healing of myocardial infarction (Davis et al., 2015). In the circulatory system, when the myocardium is damaged by ischemia, inflammation, senescence, etc., local myocardial cell apoptosis occurs, whereas myocardial fibroblasts proliferate and increase of extracellular matrix dominated by collagen fibres, resulting in myocardial fibrosis. However, the molecular mechanism still needs further research. In this research, we found that MBNL1 regulates the genes enriched in the cell apoptosis and adhesion pathways. It is likely that MBNL1 promotes myocardial cell apoptosis by the way of regulating the genes related the cell apoptosis, and promoting the increase in extracellular matrix by cell adhesion, leading to myocardial fibrosis.

The genes enriched in the pathways of cell adhesion and apoptosis include EDN2, SMAD4, EGFR, TCF7L2, LMO7, FITM1, PCDHB11, FLNB, CASP10 etc. It has been reported that several of them are associated with the cardiopathy. SMAD4 is a member of the SMAD family of signal transduction proteins. Studies show that SMAD4 plays an important role in myocardial fibrosis (Huang et al., 2014). Amphiregulin promotes cardiac fibrosis by activating the epidermal growth factor receptor (EGFR) pathway (Liu et al., 2018b). LMO7 is induced by TGF-β, and produce a negative feedback on the TGF-β pathway, thus restraining cardiac fibrosis (Xie et al., 2019). And EDN2 is associated with atrial fibrillation risk for the hypertrophic cardiomyopathy patients (Nagai et al., 2007). MBNL1 may influence myocardial fibrosis by these genes enriched in cell adhere and apoptosis. Our research provides a possible way of the influence of MBNL1 on myocardial fibrosis. However, this hypothesis should be validated in the cardiac cell lines and more work should be done.

In conclusion, the regulation profile of MBNL1 was studied in this research. Results show that MBNL1 regulated the expression and alternative splicing of genes enriched in cell adhesion and apoptosis. Some of these genes are associated with cardiac fibrosis in previous research. Our exploration deepens the understanding of MBNL1 regulation, providing a possible mechanism of MBNL1 on diseases, such as cardiac fibrosis.

**Declarations**

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Conflict of interest

The authors have no conflicts of interest and declare no competing financial interests.

Ethical approval

No animal samples were collected during this study.

Consent to Participate

All authors agree the order of author listing and read the final manuscript.

Consent for Publication

All authors approved submission of the final manuscript.

Availability of Data and Material

All data are available upon request.

Code availability

Codes are available upon request.

Author Contributions

YFS conceived and designed this research. MJL, XJS and LBL performed the analysis. CLS drew the figures. YFS and MJL drafted the manuscript.

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**Figures**

A

B

C

D

Annexin V-PE

Annexin V-PE

Annexin V-PE
**Figure 1**

The knockdown of MBNL1 and the effect on the cell proliferation and apoptosis. (A) The relative expression of MBNL1 in both control and the knockdown. (B) The knockdown of MBNL1 promotes the cell proliferation. (C and D) The knockdown of MBNL1 inhibits the cell apoptosis. Error bars show mean ± SEM.**p < 0.01, *p < 0.05

**Figure 2**

The regulation of MBNL1 by RNA-seq. (A) The statistics of the reads number (discarded reads, does not mapped to genome reads, does not uniquely mapped to genome reads and usable reads). (B) Relative
expression of MBNL1 by FPKM. Error bars shows mean ± SEM. ***p < 0.001. (C) Hierarchically clustered Pearson correlation matrix between the control and knockdown of MBNL1. (D) Volcano plot showing the up-regulated (in red) and down-regulated (in blue) genes. (E) The percentage of up and down-regulated genes by MBNL1. (F) The Hierarchical clustering of the regulated DEGs by the control and knockdown of MBNL1.

Figure 3

The enriched signal pathways and the expression of the DEGs. (A) The top 10 up and down-regulated genes enriched signal pathways by GO analysis. (B) Same as A, but for KEGG analysis. (C) The
expressions of the DEGs, quantified by RAN-seq and qPCR, error bars showing mean ± SEM. ***p < 0.001, **p < 0.01.

**Figure 4**

(A) The alternative splicing events regulated by the knockdown of MBNL1. (B) The overlap of the MBNL1 regulated DEGs and RASG. (C) The percentage of intron retention (IR) and non-intron retention (NIR) in RASE. (D) The MBNL1 regulated alternative splicing genes enriched top 10 signal pathways by GO analysis. (E) Same as C, but for KEGG analysis.
Figure 5

The alternative splicing of different genes regulated by MBNL1, the left panels showing the reads distribution, the right top panels showing the alternative splice site, and the right below panels showing the RNA-seq quantification. Error bars show mean ± SEM. ***p < 0.001, *p < 0.05. (A) FLNB, with the exon skipping (ES) event; (B) CASP10, with the mutually exclusive exons (MXE) event; (C) PHLDB2, with the alternative 5’ splice sites (A5SS) event.
Figure 6

Same as Fig. 5, but for (A) DIDO1, with the alternative 5’ splice sites (A5SS) event; (B) CTNND1, with the alternative 3’ splice sites (A3SS) event.

Supplementary Files

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