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Determination of the impact of splicing abnormalities of the NFIX transcription factor mRNA on the pathomechanism of myotonic dystrophy and development of gene therapy tool for this disease

SUMMARY

Myotonic dystrophy type 1 is a hereditary, autosomal disease caused by expansion of trinucleotide CTG repeats in *DMPK* gene. Expanded CUG repeats in mutant mRNA is a major toxic product of mutant gene. It sequester MBNL splicing factors and due to functional insufficiency of these proteins trigger global changes in alternative splicing in skeletal muscles, heart and brain, which result in symptoms characteristic for DM1. The abnormalities in alternative splicing are crucial molecular feature of DM1. The alternative splicing of many genes engaged in muscle homeostasis and proper functioning is impaired. Although, the molecular consequence of majority of genes with altered splicing pattern is still unknown.

One of the MBNL-dependent exons, which inclusion is significantly higher in skeletal muscles of DM, is exon 7 of *NFIX*, a gene encoding for transcription factor essential for muscle development. First aim of this study was to better understand the pathomechanism of DM in case of abnormalities in the splicing of *NFIX*. The examination of whether the contribution of exon 7 has an impact on NFIX transcriptional activity gave a deeper understanding of DM1 pathomechanism which is studied for a long time. To answer this question two alternative approaches were used. First of them was generation of two stable cell lines with inducible overexpression of NFIX isoforms with and without exon 7 (NFIX+7 or NFIX-7) based on a

HEK cell with functional knockout of endogenous *NFIX* (*NFIX*-KO). Unexpectedly, RNA- seq results did not show significant differences in transcriptional activity of these two isoforms in developed cellular models, perhaps due to not well adjusted level of overexpression of exogenes (too high). The second approach based on manipulation of exon 7 inclusion of endogenous NFIX mRNA using the antisense oligonucleotide (AON) targeting (AON) targeting exon 7/intron 7 boundary. In human skeletal muscle cells, in which NFIX+7 isoform predominates, this AON induces efficient exon 7 skipping and production of NFIX-7 isoform.

This approach gave the possibility to generate a model with an cellular environment where NFIX is engaged in the developmental process, but also in DM1 pathogenesis. The expression level of NFIX is relatively high in these cells and is driven by the native promoter. The results of RNA-seq revealed hundreds of genes which expression was significantly changed after AON treatment, suggesting that both splicing isoforms, NFIX+7 and NFIX-7, differ significantly in transcriptional activity. Gene ontology (GO) analysis showed significant enrichment of groups of genes that are sensitive to NFIX isoforms and abnormally expressed in DM1 patients. Among 5641 genes which expression is affected in DM1 ($P<0.05$), 2070 genes were identified which are also sensitive to treatment with AON modulating splicing pattern of NFIX. The major GO molecular function terms enriched in both comparisons (first: genes sensitive to the level of NFIX and its splicing isoforms; second: genes sensitive to the NFIX splicing isoforms and changed in DM1 tissues) were extracellular matrix structural constituent, and collagen binding. These functions are incredibly important for the proper structure and activity of skeletal muscle, therefore *NFIX* splicing abnormality may be essential trigger of DM1 pathogenesis. Taken together, these results showed that abnormal distribution of NFIX exon 7 caused by sequestration of MBNL proteins on CUG^{exp} affects a subset of gene expression changes occurring in DM1 skeletal muscles, which may be responsible for the development of disease phenotype observed in DM1 patients.

DM is an incurable disease. When the number of repeats reach a higher level clinical symptoms are more severe and the sequestration efficiency, which depends on the size expansion, increase. Previously proposed therapeutic strategies leading to increase of the MBNL1 level via gene therapy tools are associated with many undesirable consequences. Uncontrolled MBNL1 overexpression in mice leads to reduced body weight, increased mortality, or muscle damage, including heart muscle. The gene therapy leading to the increase of MBNL1 level is also associated with many limitations. One of them is heterogeneity in sequestration of MBNLs caused by somatic mosaicism of CTG repeat length in cells of the same patient and between individuals with DM1. To deal with these limitations I designed and generated the autoregulated MBNL1 overexpression construct which enables the production of MBNL1 adjusted to the level of active pool of endogenous MBNLs. It was assumed that this therapeutic strategy gave the possibility to overcome the limitations caused by heterogeneity of CTG repeat expansions and as a consequence different levels of MBNL insufficiency in different cells/myofibers. This construct contains MBNL1-coding sequence separated by the fragment of *ATP2A1* pre-mRNA with MBNL-sensitive alternative exon containing in frame stop codon. The inclusion of this exon leads to the arrangement of the inactive form of the protein but its exclusion, occurring during MBNL insufficiency, gives rise in production of fully active MBNL1. This approach enables the restricted expression of MBNL1 protein only if its level in cell is too low and potentially can be controlled by heterogeneity of CUG^{exp} load. It was shown that expression of MBNL1 assembled from this construct is tunable and has therapeutic potential to correct the alternative splicing abnormalities in DM1 patients-derived cells.