mRNA and microRNA transcriptomics analyses in a murine model of dystrophin loss and therapeutic restoration

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Duchenne muscular dystrophy (DMD) is a pediatric, X-linked, progressive muscle-wasting disorder caused by loss of function mutations affecting the gene encoding the dystrophin protein. While the primary genetic insult in DMD is well described, many details of the molecular and cellular pathologies that follow dystrophin loss are incompletely understood. To investigate gene expression in dystrophic muscle we have applied mRNA and microRNA (miRNA) microarray technology to the mdx mouse model of DMD. This study was designed to generate a complete description of gene expression changes associated with dystrophic pathology and the response to an experimental therapy which restores dystrophin protein function. These datasets have enabled (1) the determination of gene expression changes associated with dystrophic pathology, (2) identification of differentially expressed genes that are restored towards wild-type levels after therapeutic dystrophin rescue, (3) investigation of the correlation between mRNA and protein expression (determined by parallel mass spectrometry proteomics analysis), and (4) prediction of pathology associated miRNA-target interactions. Here we describe in detail how the data were generated including the basic analysis as contained in the manuscript published in Human Molecular Genetics with PMID 26385637. The data have been deposited in the Gene Expression Omnibus (GEO) with the accession number GSE64420.

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1. Direct link to deposited data

http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE64420.

2. Materials and methods

2.1. Experimental design

Wild-type mice (n = 3) were compared with dystrophic mdx mice (n = 4) and mdx mice treated with Pip6e-PMO (n = 4) an experimental therapy for Duchenne muscular dystrophy (DMD). Pip6e-PMO is a covalent conjugate of an arginine-rich, cell penetrating peptide (Pip6e) with a phosphorodiamidate morpholino oligonucleotide (PMO) which induces exclusion of exon 23 from the Dmd transcript, open reading frame restoration, and therapeutic rescue of dystrophin protein expression [1–3]. All mice used were male and 14 weeks old at the end of experiment. This time point was selected as it represents a period of established pathology in the dystrophic mice.
2.2. Animal procedures

Animal experiments were carried out in accordance to procedures authorized by the UK Home Office in accordance with UK law (i.e. Animals (Scientific Procedures) Act 1986). The C57/B10 or C57/B10ScSn-Dmdmdx/J (mdx) strains were maintained at the Biomedical Sciences Building, University of Oxford. Treated animals were injected with a single 12.5 mg/kg dose of Pip6e-PMO in sterile saline via the tail vein at 12 weeks of age under isoflurane anesthesia. Pip6e peptide: Ac-RXRRBRRXYRFLIRXRBRXRB-OH (where X is aminohexanoyl and B is β-alanine) was synthesized using Fmoc chemistry and purified by reverse-phase HPLC. Pip6e peptide was conjugated to the PMO (5′-GGCCAAACCTCGGCTTACCTGAAAT) (Gene Tools LLC, Philomath, OR) through an amide linkage at the 3′ end terminus. Conjugates were purified by cation exchange HPLC, desalted, and analyzed by MALDI-TOF MS as described previously [1]. Mice were sacrificed by escalating CO2 concentration and death confirmed by cervical dislocation. Mice were then exsanguinated via the jugular vein and blood was collected using Microvette CB300 serum collection tubes (Sarstedt, Leicester, UK). Tibialis anterior (TA) muscles were macerated and snap frozen in liquid nitrogen-cooled isopentane. TA muscles were mounted on cork disks with Tissue-Tek OCT Compound (Sakura Finetek Europe, Leiden, Netherlands) with the muscle arranged so as to be perpendicular to the cork. Tendinous tissue was removed by sectioning. Fifty 8 µm cryosections were prepared from the mid-belly of the muscle and placed microcentrifuge tubes. Mounted muscles and tubes containing sections were stored at −80 °C until ready for analysis.

2.3. RNA isolation

TRIzol reagent (Life Technologies, Paisley, UK) was added to each tube containing muscle sections and samples homogenized using a Precellys 24 (Bertin Technologies, France). The resulting TRIzol solution was collected and RNA extraction continued according to manufacturer’s instructions. Samples were treated with DNase I using the TURBO DNA-free kit (Life Technologies) in order to remove genomic DNA contamination. The RNA samples used for mRNA analysis were further column-purified (RNeasy Mini kit, Qiagen, Manchester, UK). Each RNA sample was analyzed using the Agilent 2100 Bioanalyzer (Agilent Technologies, USA) to assess RNA quality. The resulting RIN (RNA Integrity Number) values ranged from 6.3 to 8.5.

2.4. mRNA and miRNA microarray processing and statistical analysis

Labeling and hybridization were performed according to standard Affymetrix protocols at the Affymetrix Core facility BEA, Bioinformatics and Expression Analysis, at Karolinska Institutet, Novum, Huddinge, Sweden. The processing and data analyses were performed in Affymetrix Expression Console Software.

For mRNA analysis, 100 ng of column-purified total RNA was analyzed for each of the 11 samples using Mouse Gene ST 1.1 Array plates (Affymetrix, Santa Clara, CA). The Mouse Gene ST 1.1 array covers 26,166 RefSeq transcripts. Probes are evenly distributed across the target exons with a median of 27 probes per transcript. The ST1.1 arrays were analyzed using Median polish, RMA background correction and Sketch-Quantile normalization (RMA-Sketch in Expression Console Software). We only analyzed 28,350 probe sets with category “main” (i.e. excluding technical control probe sets), according to Affymetrix annotation. To filter out very low fluorescence signal intensities we used a cutoff of 30. Probe sets with signals below 30 in all three biological groups were removed (15,219 probe sets remained after filtering). Unpaired 2-sided Student’s t-test was performed with the criterion for differentially expressed transcripts set to P < 0.05. False Discovery Rate (FDR) estimations were performed by calculating q values (q-value R package) from the P-value lists.

For miRNA analysis, 500 ng of total RNA was analyzed for each of the 11 samples using Affymetrix GeneChip miRNA 3.0 arrays (Affymetrix, Santa Clara, CA). The miRNA 3.0 array covers 153 organisms and contains 19,724 mature miRNA probe sets; the number of probes for each probe set is 9. For Mus musculus there is 1111 mature miRNA and 855 pre-miRNA probe sets present on the array. The miRNA arrays were analyzed using Robust Multichip Analysis (RMA) and DABG (detected above background). The data were filtered in order to consider Mus musculus miRNAs only (1966 probe sets remaining). Transcripts with absent detection signals in at least three of the four replicates (or two out of the three replicates in the controls) were removed (682 probe sets remained after filtering).

The microarray data discussed in this study have been deposited in NCBI’s Gene Expression Omnibus [3] and are accessible through GEO Series accession number GSE64419 (miRNA data: GSE64418).

3. Discussion

We have described the generation of high quality mRNA and miRNA data in the most commonly used mouse model of DMD. These data have been interrogated in multiple ways, in order to further our understanding of the molecular pathogenesis of DMD, characterize the response to therapeutic dystrophin re-expression, identify novel biomarkers, investigate the relationship between protein and mRNA expression in dystrophic muscle, and predict disease-relevant miRNA-target interactions. The resulting analyses were published in our recent manuscript [4].

Conflict of interest

The authors declare no conflicts of interest.

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