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Transcriptional Gene Silencing of the Autism-Associated Long Noncoding RNA *MSNP1AS* in Human Neural Progenitor Cells

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Abstract

The long noncoding RNA *MSNP1AS* (moesin pseudogene 1, antisense) is a functional element that was previously associated with autism spectrum disorder (ASD) with genome-wide significance. Expression of *MSNP1AS* was increased 12-fold in the cerebral cortex of individuals with ASD and 22-fold in individuals with a genome-wide significantly associated ASD genetic marker on chromosome 5p14.1. Overexpression of *MSNP1AS* in human neuronal cells caused decreased expression of moesin protein, which is involved in neuronal process stability. In this study, we hypothesize that *MSNP1AS* knockdown impacts global transcriptome levels. We transfected the human neural progenitor cell line SK-N-SH with constructs that caused a 50% suppression of *MSNP1AS* expression. After 24 h, cells were harvested for total RNA isolation. Strand-specific RNA sequencing analysis indicated altered expression of 1,352 genes, including altered expression of 318 genes following correction for multiple comparisons. Expression of the *OAS2* gene was increased >150-fold, a result that was validated by quantitative PCR. Gene ontology analysis of the 318 genes with altered expression following correction for multiple comparisons indicated that upregulated genes were significantly enriched for genes involved in immune response, and down-regulated genes were significantly enriched for genes involved in chromatin remodeling. These data indicate multiple transcriptional and translational functions of *MSNP1AS* that impact ASD-relevant biological processes. Chromatin remodeling and immune response are biological processes implicated by genes with rare mutations associated with ASD. Our data suggest that the functional elements implicated by association of common genetic variants impact the same biological processes, suggesting a possible shared common molecular pathway of ASD.

Keywords

RNA sequencing; Autism spectrum disorders; Noncoding RNA; Long noncoding RNA; Small interfering RNA; Small noncoding antisense RNA; Antisense therapy

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Introduction

Autism spectrum disorder (ASD) is a neurodevelopmental condition characterized by deficits in social communication and behavioral flexibility [1, 2]. Recent advances in genomics have begun to identify the genetic variants that contribute to ASD risk [3–5]. The important steps are to translate these genetic findings to a biological understanding of ASD pathogenesis and to the development of biologically based treatments [6]. Recently, we identified a long noncoding RNA (lncRNA) that contributes to ASD risk [7]. A genome-wide association study (GWAS) identified a single peak on chromosome 5p14.1 for ASD, indicating a significant association ($p = 10^{-10}$) for rs4307059 [3]. The same rs4307059 allele was also associated with social communication phenotypes in a general population sample [8]. Directly under the chromosome 5p14.1 ASD GWAS peak, we identified an lncRNA that was almost 4 kb [7]. This transcript is 94% identical and antisense to the X chromosome transcript, *MSN*, which encodes a neuronal architecture protein and is on the opposite (antisense) strand of moesin pseudogene 1 *(MSNP1)* [7]. Therefore, we named the newly discovered gene *MSNP1AS* (moesin pseudogene 1, antisense) [7]. Expression of *MSNP1AS* was detected in 5 brain regions (frontal cerebral cortex, temporal cerebral cortex, occipital cerebral cortex, cerebellum and spinal cord) of the adult and in the fetal frontal cerebral cortex [7]. In peripheral tissues, *MSNP1AS* was expressed at high levels in the adult peripheral blood and fetal heart [7]. These data indicate that *MSNP1AS* is expressed at high levels in both the nervous and circulatory systems. Here we use publicly available databases of gene expression to provide additional details of *MSNP1AS* expression. *MSNP1AS* expression is increased 12.7 times in the postmortem temporal cerebral cortex of individuals with ASD compared to controls and increased 22 times in individuals with the ASD-associated rs4307059 allele [7]. Therefore, the highly significant GWAS findings led to the identification of a functional lncRNA that contributes to ASD risk [7].

Jiang et al. [9] have recently reported rescue of cellular phenotypes of Down syndrome by transgenic expression of another lncRNA, *XIST* (X inactive specific transcript). When *XIST*, which normally targets the “extra” X chromosome of females for inactivation, was expressed on chromosome 21 in cells derived from Down syndrome patients, the extra copy of chromosome 21 was inactivated [9]. These studies provide a clear path toward translation of manipulating lncRNA expression to rescue disorder phenotypes. Because *MSNP1AS* is overexpressed in the postmortem cerebral cortex of individuals with ASD [7], we used transcriptional gene silencing (TGS) in human neuronal progenitor cells to examine the potential for rescue. By targeting the *MSNP1AS* gene promoter, we were able to reduce the expression of the *MSNP1AS* transcript to half of its normal expression and mimic a potential therapeutic for ASD and social communication deficits. RNA sequencing was used as an unbiased approach to identify genes with altered expression following knockdown of *MSNP1AS*.

Materials and Methods

Cell Culture

SK-N-SH neuroblastoma neural progenitor cells were demonstrated previously to express levels of *MSNP1AS, MSN*, and moesin protein consistent with those of developing human

brain [7]. The human neural progenitor cell line SK-N-SH (American Type Culture Collection; Manassas, VA, USA) was cultured in minimum essential medium supplemented with 10% heat-inactivated fetal bovine serum, 1% penicillin/streptomycin, nonessential amino acids, and 1.5 g/L sodium bicarbonate in a 75-cm^2 flask at 37 ° C and 5% CO_2. When cells were 75% confluent, the human neuronal cells were subcultured to a density of 1×10^6 cells/well in a 6-well plate for harvests 24 h after transfection.

Design of Antisense RNA to the MSNP1AS Proximal Gene Promoters

Small noncoding antisense RNAs (sasRNA) to silence gene transcription were designed with proprietary software [10–13]. The program was used to identify the best sasRNA to silence the lncRNA *MSNP1AS* gene transcript (Fig. 1). We cloned sasRNAs directed to the putative gene promoter into the pCDNA3-U6M2 expression vector with the following target sequence for *MSNP1AS* knockdown: AATTCTAGAAATGTGCCAACA (Fig. 1).

Transfection of sasRNAs

Targets for TGS of *MSNP1AS* were defined in human neuronal cell lines. Eight sasRNAs were designed directed to *MSNP1AS* TGS targets. The TGS targets were screened for effectiveness in U87 or Lan 6 cells. The TGS sasRNAs were screened for efficacy, and 3 were chosen for use in SK-N-SH cells. Each transfection set was considered an independent experiment. The 3 sasRNAs were tested at 5 different vector concentrations (online suppl. Table 1; see www.karger.com/doi/10.1159/000453258 for all online suppl. material). Cell harvest occurred 24 h after transfection. Based on optimization experiments, we used 1 μg of sasRNA/million cells to achieve ~50% suppression of *MSNP1AS* in further studies.

Cells were transfected using Amaxa Nucleofector (Lonza; Walkersville, MD, USA) technology and subcultured into 6-well plates. One milliliter of fresh prewarmed medium was added to each well, and the cells were centrifuged twice at 130 *g* for 10 min for the SK-N-SH cells with PBS washes of 7 and 4 mL, respectively. The cell pellet was resuspended in Nucleofector solution containing supplement using 1×10^6 cells/well. One hundred microliters of cell/nucleofector solution were added to a new cuvette and placed in the Amaxa Nucleofector using the T-16 program. Five hundred microliters of fresh prewarmed medium were added to the cuvette, mixed, and transferred to the well with a disposable pipet. Two milliliters of culture medium were added to the transfected cells in the 6-well plate. The cells were incubated at 37 °C in 5% CO_2 until harvest. Each experiment was repeated 4 times.

Neural Progenitor Cell Harvest

After 24 h, the culture medium was removed and discarded. The cells were washed with 1 mL of PBS wash, and the PBS wash was aspirated. One milliliter of trypsin/EDTA solution was added to each well, and the cells were incubated for 4 min. Five milliliters of cell type-specific medium were added, and the cells were triturated and transferred to 15-mL conical tubes. SK-N-SH cells were centrifuged at 130 *g* for 10 min. The supernatant was aspirated and 5 mL of PBS added. The cell pellet was triturated and centrifuged at 130 *g* for 10 min. The PBS wash was aspirated, and 1 mL of fresh PBS was added. Half of the solution was

transferred to each of two 1.5-mL Eppendorf tubes. The tubes were centrifuged at 4 °C at 130 *g* for 10 min. The supernatant was decanted, and the cell pellets were frozen at −80 °C.

RNA Purification

The Qiagen RNEasy kit was used to isolate total RNA using vacuum technology according to the manufacturer's protocol (Qiagen; Valencia, CA, USA). The RNA was eluted with 35 μL of RNAse-free water and quantified using the NanoDrop ND-1000 spectrophotometer (v3.1.2; Thermo Fisher Scientific, Waltham, MA, USA). The RNA was stored at −80 °C.

Quantitative RT-PCR to Confirm MSNP1AS Knockdown

To confirm knockdown of *MSNP1AS*, cDNA was synthesized using the SuperScript III First-Strand Synthesis System for qRT-PCR protocol (Invitrogen/Thermo Fisher Scientific, Waltham, MA, USA). Five hundred nanograms of RNA were used to make one and a half reaction volumes (35 μL) of cDNA. The cDNA was stored at −20 °C. The qRT-PCR protocol described in Kerin et al. [7] was used to validate knockdown of *MSNP1AS*.

Construction of Strand-Specific, Ribosomal RNA-Depleted RNA Sequencing Libraries

Directional RNA sequencing libraries were prepared for Illumina HiSeq 2000 sequencing using the Stranded Total TruSeq RNA Sample Preparation kit with Ribo-Zero Gold (Illumina) using the manufacturer's protocol using the Hamilton Starlet Liquid Handling robot. One nanogram of RNA was used for each sample. Ribo-Zero was used to deplete cytoplasmic and mitochondrial ribosomal RNA from total RNA. The depleted RNA was fragmented and primed with random hexamers to synthesize first-strand cDNA using Superscript II (Life Technologies). Next, the second strand was synthesized, incorporating dUTP in place of dTTP. A single "A" base was added to the 3 ′-ends of the fragments, and the indexed adaptors were ligated to the ends of the double-stranded cDNA to prepare them for hybridization onto the flow cell. PCR was used to selectively enrich the fragments with ligated adaptors and to amplify the amount of DNA in the library. The libraries were produced in a 96-well format and quality controlled using the Agilent Technologies 2200 TapeStation Instrument. Libraries were pooled (4 samples/lane) and sequenced on Illumina HiSeq 2000 to a targeted depth generating an average of 20 million paired-end 50-cycle reads for each sample (online suppl. Table 2).

Data Analysis

Data analysis was performed using TopHat (version 2.0.10) [14] to align the Illumina short reads against the reference human genome ENSEMBL GrCH38 version 81. Sequence alignments were generated as BAM files [15], and then Cuffdiff (version 2.2.1) [16] was used to summarize the gene expression values as FPKM measures. The gene expression of samples with *MSNP1AS* knocked down was compared to the gene expression of the negative control experiment samples to find other differentially expressed genes. Cuffdiff was also used to calculate the expression *n*-fold change, *p* values and false discovery rate (FDR) values. Genes with FDR $q < 0.05$ were used as the input for DAVID (version 6.7) functional annotation [17, 18].

Quantitative PCR to Confirm Altered Gene Expression Observed in RNA Sequencing

Ten RNAs with $q < 0.05$ in the RNA sequencing results were selected to validate by quantitative PCR (qPCR) using Taqman gene expression assays from Life Technologies: ABCA1 (assay ID Hs01059118_m1), ANGPTL4 (assay ID Hs01101127_m1), ATF3 (assay ID Hs00231069_m1), CXCL8 (assay ID H s00174103_m1), GADD45B (assay ID Hs04188837_g1), ID1 (assay ID Hs03676575_s1), INSM2 (assay ID Hs00261625_s1), MIR320A (assay ID Hs04233529_s1), OAS2 (assay ID Hs00942643_m1), and TMEM215 (assay ID Hs01942498_s1).

Analysis of BrainSpan Data

Normalized expression data representing reads per kilobase per million mapped reads (RPKM) were downloaded from the BrainSpan Atlas of the Developing Human Brain database. Details on the tissues, materials, and sequencing protocols are available on the BrainSpan website (www.brainspan.org). Data used in this analysis were restricted to neocortical brain tissues given the importance of these regions in ASD pathology as well as the altered expression of *MSNP1AS* in these regions of the brain (primary auditory cortex, dorsolateral prefrontal cortex, inferior parietal cortex, inferolateral temporal cortex, primary motor cortex, medial prefrontal cortex, orbital frontal cortex, primary somatosensory cortex, superior temporal cortex, primary visual cortex, ventrolateral prefrontal cortex). The data were also partitioned into 10 age groups to identify the expression pattern of *MSNP1AS* across age. Only genes showing a RPKM >1 in at least 1 sample were used in this analysis to reduce the amount of noise generated from genes showing extremely low expression values in all samples. All RPKM values were then transformed to the log2 + 1 as suggested when constructing a weighted gene coexpression network.

Results

Expression of the ASD-associated lncRNA *MSNP1AS* is increased 12 times in the cerebral cortex of individuals with ASD compared to controls [7]. We used TGS to knock down *MSNP1AS* and thus mimic a potential therapeutic for ASD. To quantitatively model the consequences of *MSNP1AS* knockdown, sasRNA-mediated knockdown of *MSNP1AS* was performed in the human neural progenitor cell line SK-N-SH. The knockdown efficiency of *MSNP1AS* transcript levels was confirmed via qPCR (Fig. 2).

MSNP1AS Knockdown Results in Genome-Wide Transcriptional Changes

Changes in the transcriptome caused by *MSNP1AS* knockdown may be able to reveal the impact of biologically based therapeutic approaches on human neural progenitor cell differentiation. The transcriptional changes resulting from the knockdown were evaluated using genome-wide transcriptome profiling of human neuronal progenitor cells SK-N-SH, via RNA sequencing. Differential gene expression analysis using the Ensembl GrCH38 genome annotation revealed 1,352 differentially expressed genes ($p < 0.05$) (online suppl. Table 3). When a more stringent test for multiple comparisons was applied, a significance level of $q < 0.05$ showed 318 differentially expressed genes (Table 1, Fig. 3), with 65% of the genes downregulated. Subsequent qPCR validation was performed for genes with large changes in expression following *MSNP1AS* knockdown. Comparison of gene expression

levels measured by RNA sequencing and qPCR indicated a highly significant Pearson correlation coefficient (Fig. 4), validating the magnitude of gene expression changes identified by RNA sequencing.

Transcriptional Consequences of MSNP1AS Knockdown

Gene ontology analysis using the DAVID web server of the *MSNP1AS* knockdown revealed functions important in chromatin assembly and nucleosome organizaton (Fig. 5). We also performed gene ontology analysis grouping the differentially expressed genes ($q < 0.05$) by their direction of differential expression. Genes that are upregulated by *MSNP1AS* knockdown were enriched for immune response (Fig. 6a). Genes that are downregulated by *MSNP1AS* knockdown were enriched for nucleosome assembly and chromatin assembly (Fig. 6b). DAVID analysis indicates that genes encoding proteins involved in protein synthesis and chromatin regulation were altered in expression following *MSNP1AS* transcriptional gene silencing. There is no evidence of enrichment of genes in the moesin pathway, suggesting that inhibition of moesin protein translation is independent of transcriptional regulation. Comparison of genes with altered expression following *MSNP1AS* TGS did not reveal enrichment of genes previously implicated in ASD [19].

Expression of MSNP1AS in Developing Human Brain

Analysis of BrainSpan data indicate high levels of *MSNP1AS* in the cerebral cortex during early embryogenesis and again immediately after birth (Fig. 7). GTex indicates levels of expression of *MSNP1AS* at low to undetectable levels in 12 regions of the adult brain. However, GTex indicates high levels of *MSNP1AS* expression in peripheral blood, consistent with our previous report [7].

Discussion

Our findings indicate that *MSNP1AS* knockdown disrupted the expression of 318 genes ($q < 0.05$), many of which are involved in chromatin organization and immune response. *MSNP1AS* is the functional element revealed by genome-wide significant association with ASD [3, 7] and is increased 12.7-fold in the postmortem cerebral cortex of individuals with ASD [7]. We test the effect of a rescue of this phenotype by TGS of *MSNP1AS* in human neurons. After knocking down the lncRNA in SK-N-SH cells, we evaluated changes in the transcriptome using RNA sequencing to elucidate the consequences of suppressing *MSNP1AS* expression. These experiments seek to define the role of *MSNP1AS* in the pathways that lead to ASD pathogenesis and suggest that *MSNP1AS* acts at the transcriptional level to affect immune response and chromatin organization.

The biological function of the *MSNP1AS* lncRNA revealed by these data, chromatin organization, is similar to that of genes with rare de novo mutations associated with ASD [20, 21]. The etiology of ASD seems to be affected by both rare and common variants in similar ways. lncRNAs are regulated through spliceosome machinery and post-translational modifications, much like protein-coding transcripts [22–25]. However, lncRNAs are more loosely regulated, are produced in relatively lower abundance, and have fewer exons than protein-coding genes. In spite of this, lncRNA expression is extremely specific to cell type

and tissues [22, 25, 26]. lncRNAs can regulate proteins in a variety of ways, including as a framework or scaffold to facilitate interaction with other proteins or DNA, as enhancers or silencers to regulate transcription, and as decoys to bind proteins or other RNA [24, 27–35]. In addition, lncRNAs are being evaluated for epigenetic modifications and chromatin accessibility to reveal their contribution to psychiatric disease etiology [36].

Accumulating data support the role of the immune system in the pathogenesis of ASD [37, 38]. Our previously published data showed that *MSNP1AS* binds moesin protein, which is involved in neuronal process stability and immune response [7]. Our data show that after *MSNP1AS* has been knocked down, *MSN* transcript levels remain unchanged (online suppl. Table 4). This lack of expression change suggests that, while *MSNP1AS* regulates moesin protein, suppression of *MSNP1AS* does not alter *MSN* transcription. The lack of *MSN* suppression, combined with the decrease in moesin protein expression shown [7], suggests that *MSNP1AS* binds to the *MSN* transcript to prevent translation. Further experiments will be necessary to determine the mechanisms of moesin protein regulation. The biological function revealed by genes upregulated after *MSNP1AS* knockdown points to the immune response as well. Since *MSN* transcript levels are not perturbed by *MSNP1AS* knockdown, these immune response genes may be acting independently of *MSN*. Our results indicate a 150-fold increase in *OAS2* expression in SK-N-SH human neural progenitor cells following *MSNP1AS* transcriptional gene silencing. *OAS2* is 1 of 3 genes that make up the OAS gene cluster. These genes regulate viral infection resistance and are involved in cell growth, differentiation and death. The role of the OAS gene cluster as an ASD susceptibility locus is currently under investigation. Genetic variants in *OAS2* are associated with another neurodevelopmental disorder, attention deficit hyperactivity disorder [39].

Because the ASD phenotype is highly heterogeneous, a network of many genes is likely contributing to abnormal regulation. A variety of genes may contribute to several different ASD phenotypes; however, strong evidence suggests that many of the pathways involved may overlap to form an interconnected network. The results presented here give some insight into the interconnected network and possible molecular pathology of ASD. Translation of these findings to patients will be a challenge due to technical delivery concerns. However, these first steps are important to identify potential biomarkers and treatment targets.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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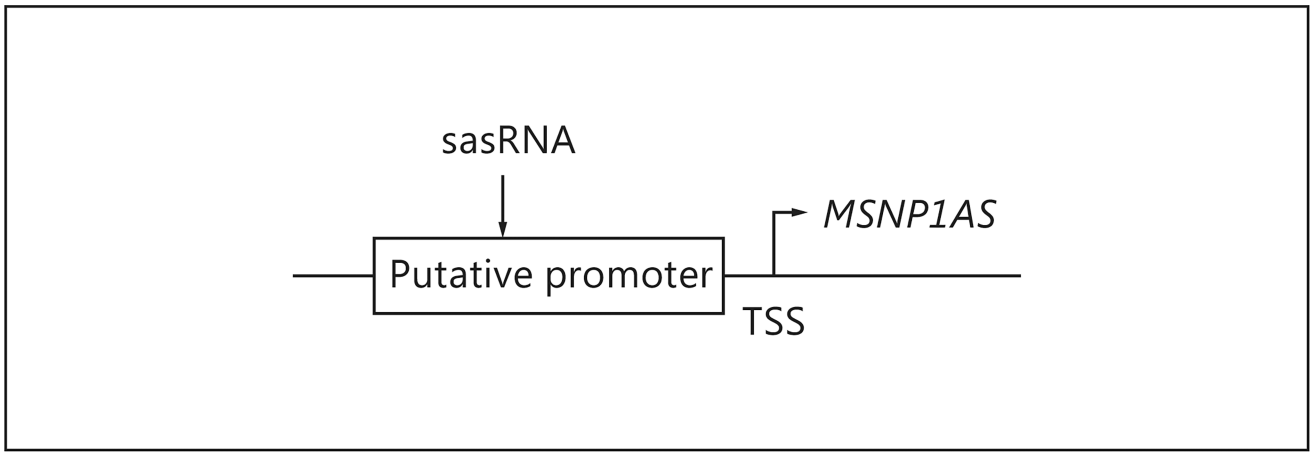

Fig. 1.
sasRNAs were designed to silence transcription by targeting the *MSNP1AS* gene promoter.

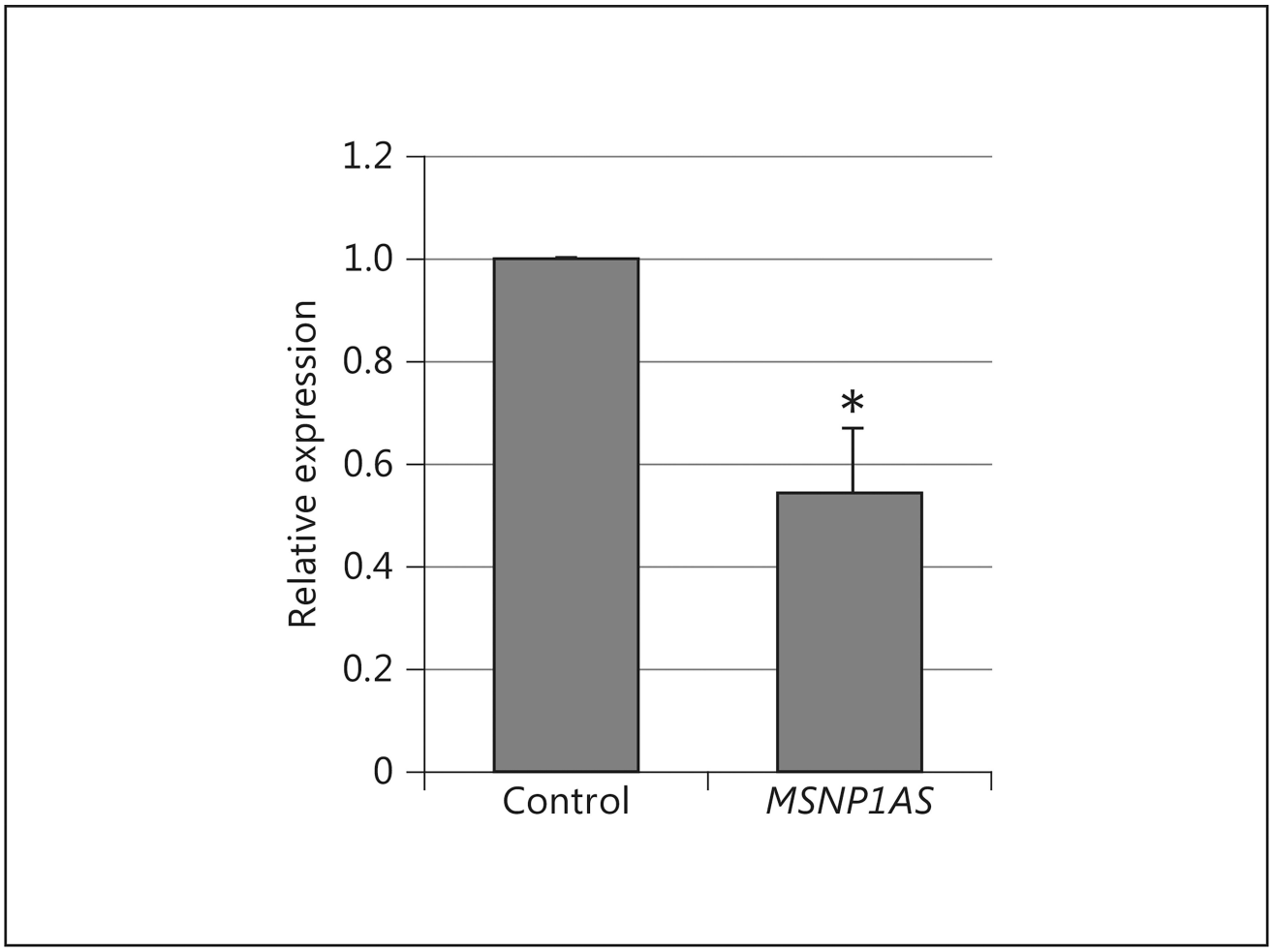

Fig. 2.

Quantitation of *M SNP1AS* knockdown. Quantitative PCR showed that a 1-μg transfection of *MSNP1AS* sasRNA resulted in a ~45% reduction of *MSNP1AS* transcript (* $p < 0.05$ by the Student *t* test).

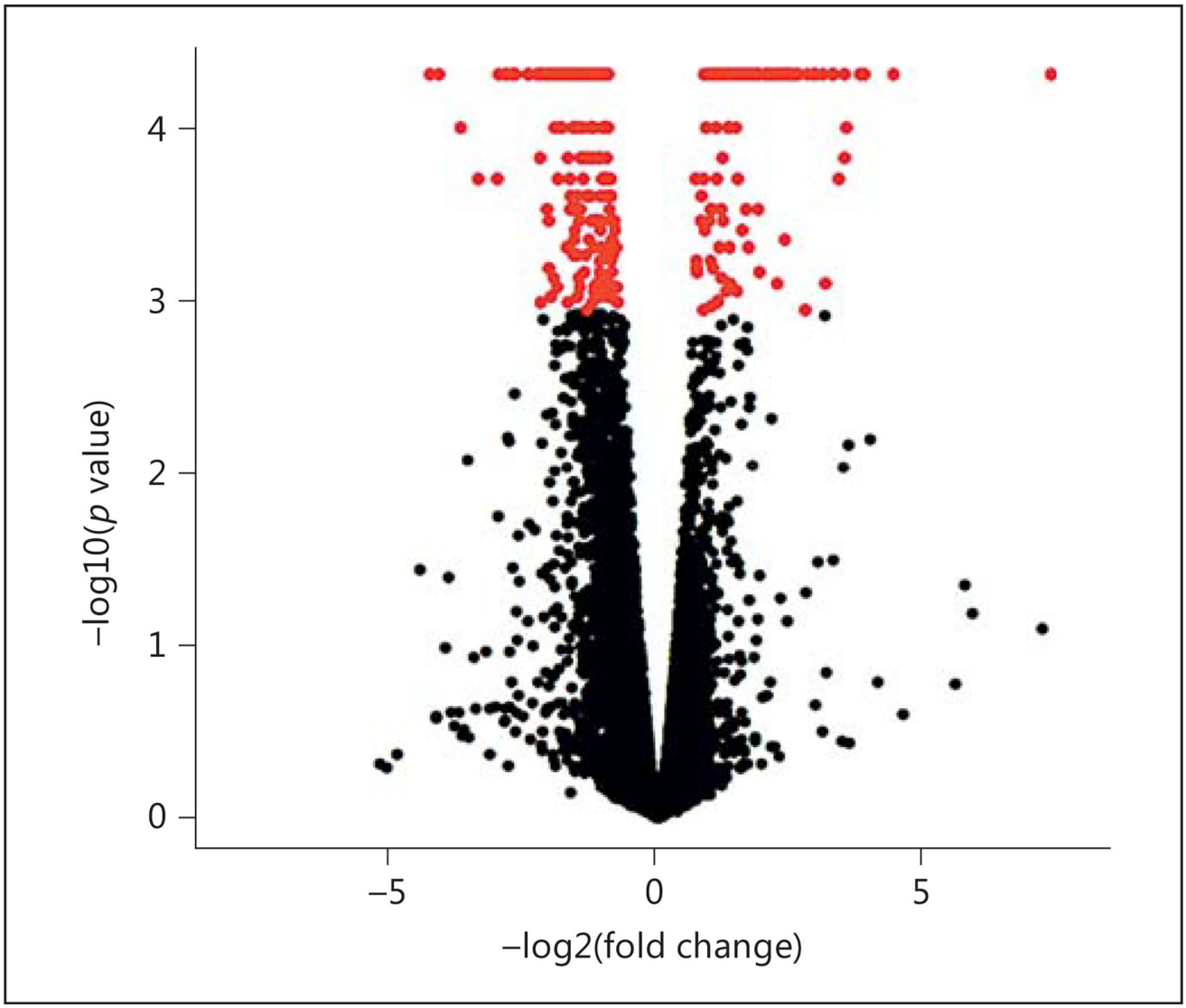

Fig. 3.

Volcano plot generated to show the differentially expressed genes resulting from the *MSNP1AS* knockdown. Each dot represents a change in gene expression, with genes above the FDR-corrected significance threshold shown in red (color in the online version only).

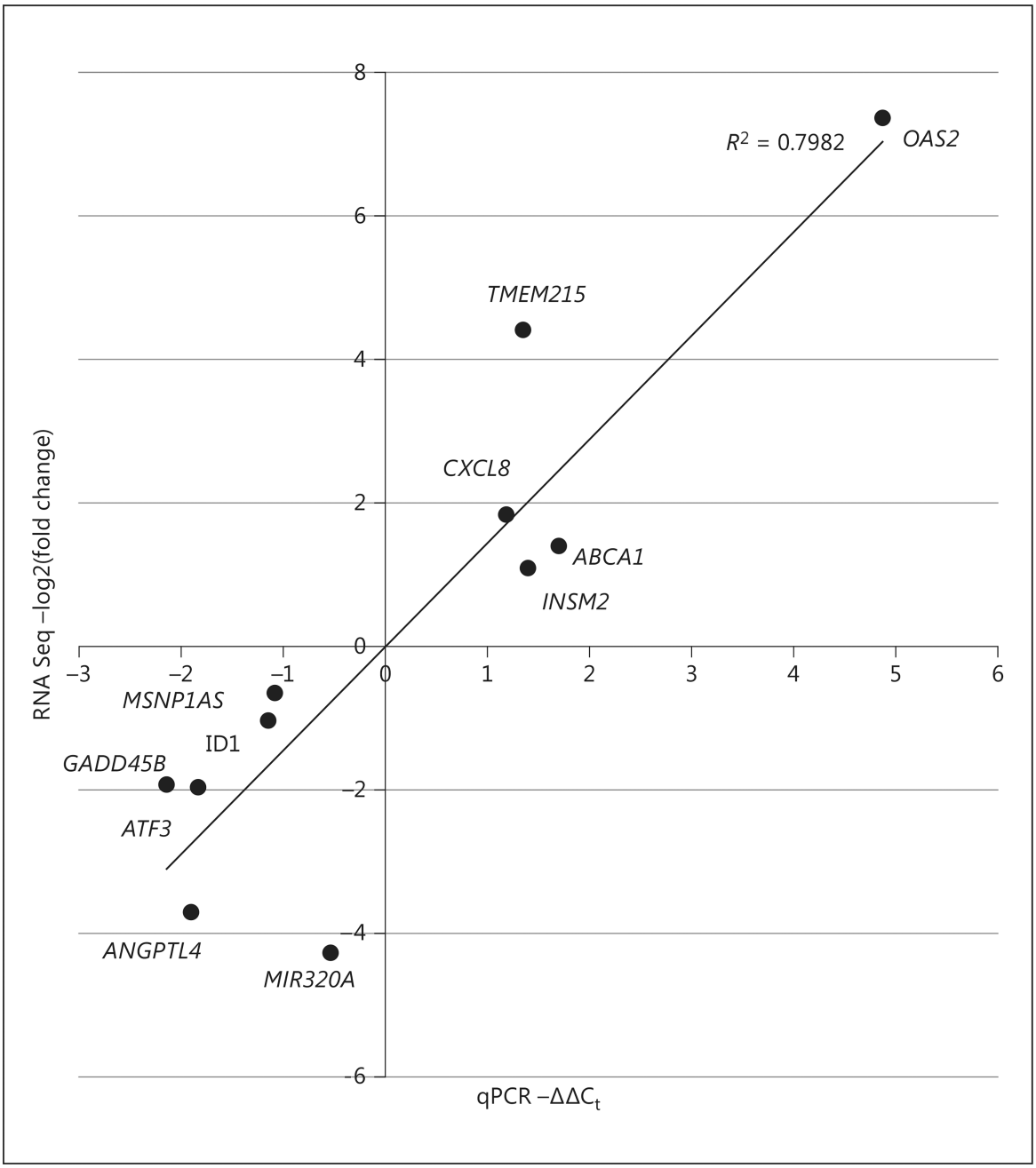

Fig. 4.

Confirmation of altered expression of genes identified by RNA sequencing (RNA Seq). Ten genes with large changes in gene expression following *MSNP1AS* knockdown were assayed via qPCR to establish the validity of the RNA sequencing differential expression analysis. Knockdown of *MSNP1AS* was also validated. qPCR and RNA sequencing values showed a statistically significant correlation with both Pearson ($p = 0.0002$) and Spearman ($p = 0.009$) methods.

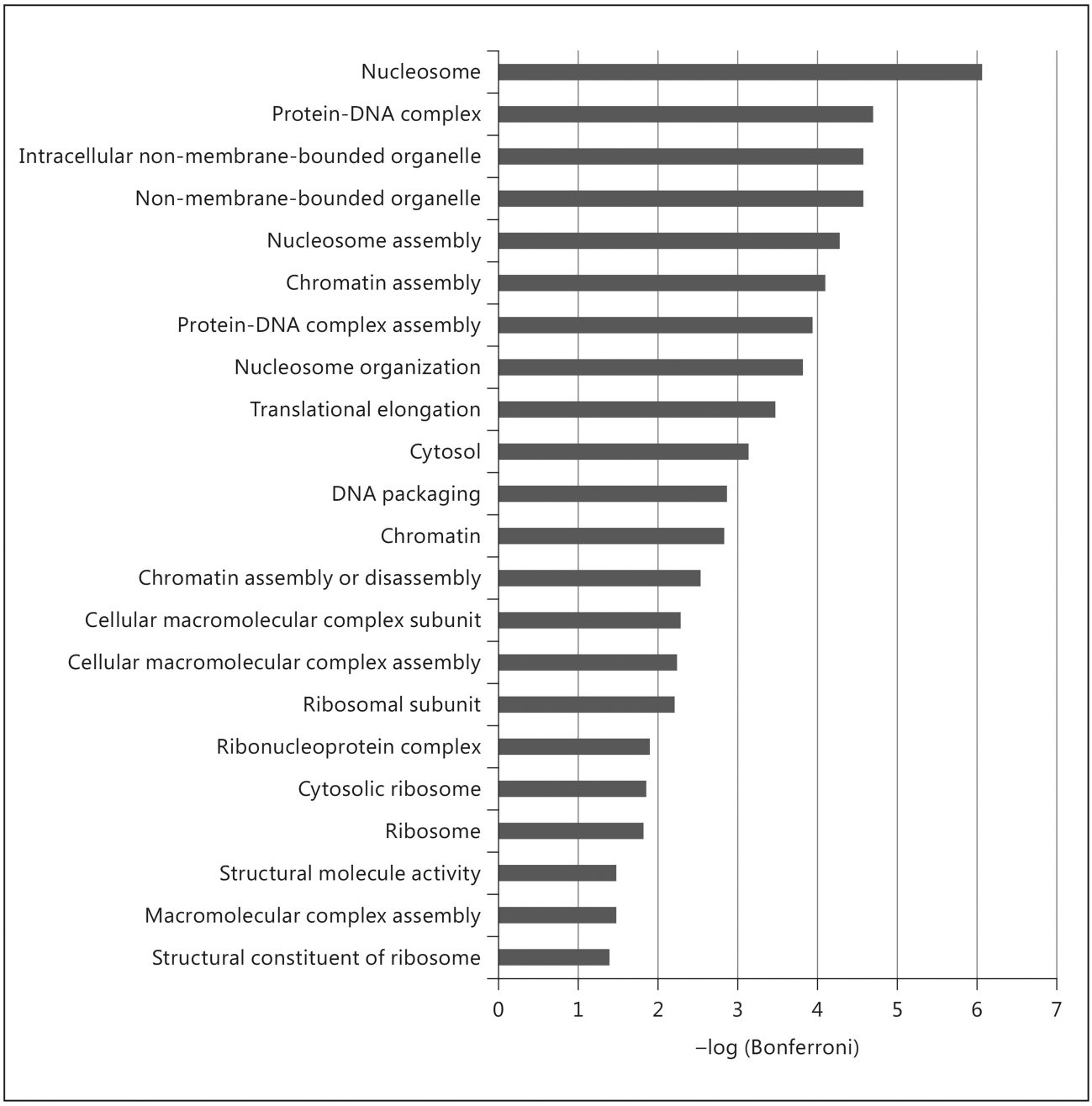

Fig. 5.

Gene ontology enrichment analysis of differentially expressed genes ($q < 0.05$) in SK-N-SH cells after 24 h of *MSNP1AS* knockdown revealed changes in chromatin assembly.

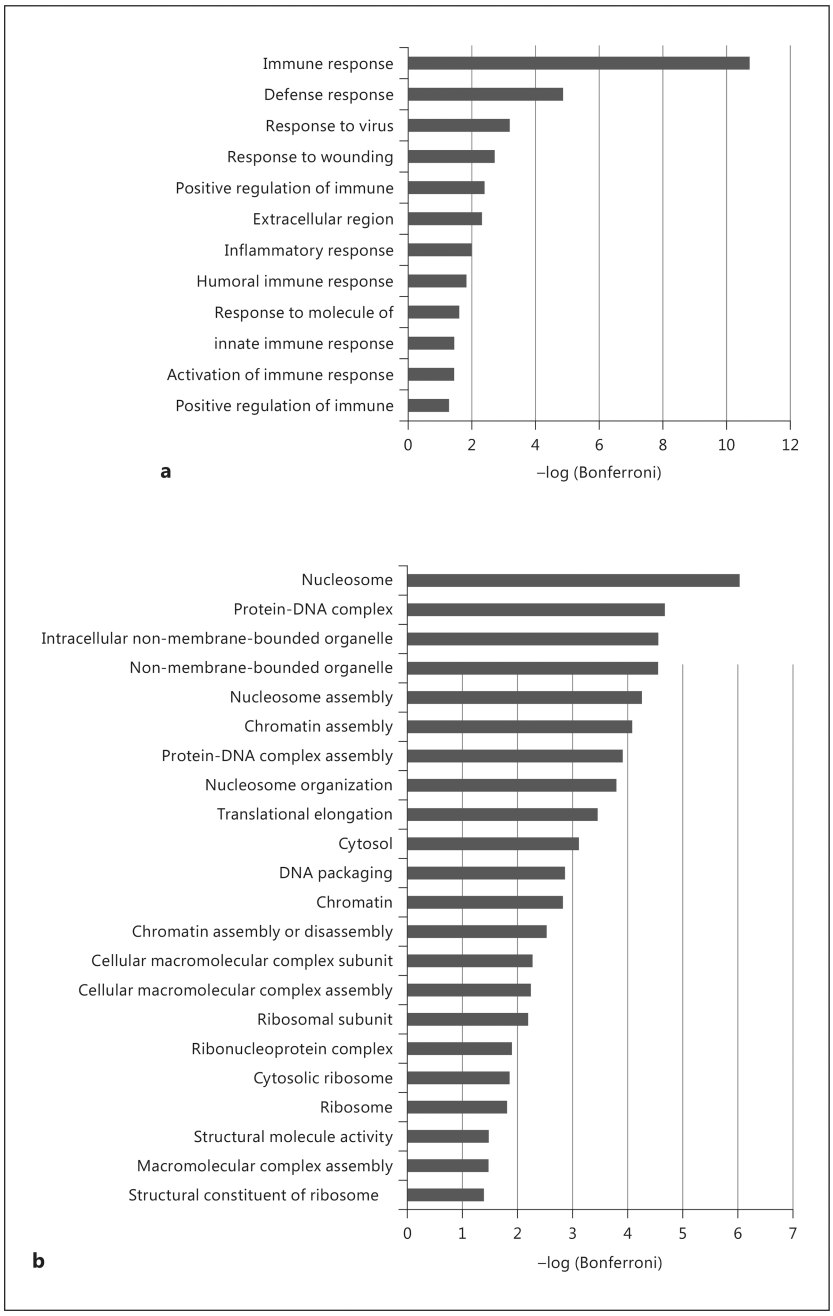

Fig. 6.

Gene ontology enrichment analysis of differentially expressed genes ($q < 0.05$) in SK-N-SH cells after 24 h of *MSNP1AS* knockdown. **a** Analysis of upregulated genes revealed enrichment of genes involved in immune response following knockdown of *MSNP1AS*. **b** Analysis of downregulated genes revealed changes in chromatin and nucleosome assembly following knockdown of *MSNP1AS*.

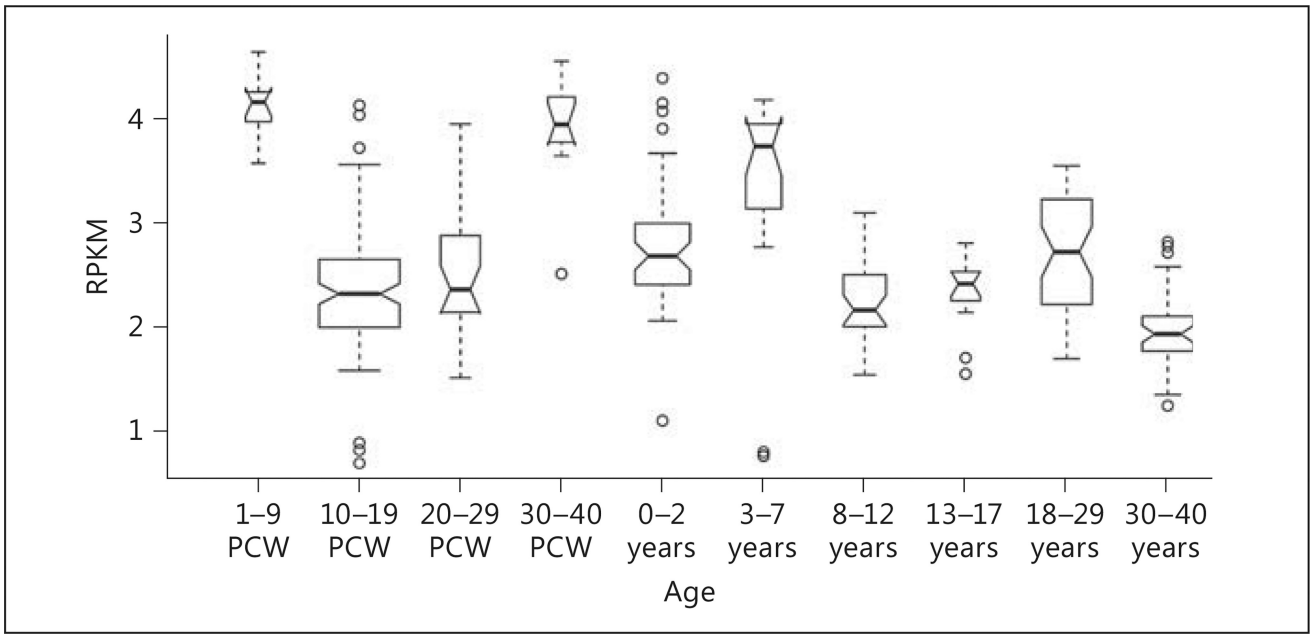

Fig. 7.

Boxplot representing the expression of *MSNP1AS* through human cerebral cortex development. *MSNP1AS* exhibits a dynamic expression pattern across development with the highest expression being evident during early and late prenatal development. PCW, postconception weeks.

Table 1

Top ten differentially expressed genes following knockdown of *MSNP1AS*

Gene	Locus	*n*-fold change	Protein or noncoding	*p* value	*q* value
OAS2	12:112906776 – 113017751	164.90	protein coding	5.00E–05	4.12E–03
TMEM215	9:32783498 – 32787399	21.24	protein coding	5.00E–05	4.12E–03
IFI44L	1:78619921 – 78646145	14.59	protein coding	5.00E–05	4.12E–03
LRRC55	11:57181746 – 57191717	13.67	protein coding	5.00E–05	4.12E–03
LAMP3	3:183122212 – 183163839	11.53	protein coding	1.00E–04	7.33E–03
ITIH4	3:52812974 – 52897596	−8.11	protein coding	2.00E–04	1.28E–02
CDC42EP1	22:37560446 – 37569405	−10.38	protein coding	2.00E–04	1.28E–02
ANGPTL4	19:7958578 – 8489114	−13.10	protein coding	1.00E–04	7.33E–03
HIST1H4J	6:27824107 – 27824480	−17.32	protein coding	5.00E–05	4.12E–03
MIR320A	8:22244961 – 22245043	−19.42	noncoding	5.00E–05	4.12E–03

Gene and locus refer to ENSEMBL human genome GrCH38 build 81.