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Original Article

Identification of differentially expressed microRNAs and their target genes in the hippocampal tissues of Fmr1 knockout mice

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Abstract: Fragile X syndrome (FXS) is one of the most common forms of inherited mental retardation; it is usually associated with the transcriptional silencing of the Fmr1 gene and loss of its encoded protein, the fragile X mental retardation protein (FMRP). FMRP is an RNA-binding protein and participates in regulating the development of dendritic spines and synaptic plasticity. To uncover the possible role of microRNAs (miRNAs) in FXS and their relationship with FMRP, we used microarray analysis to investigate the miRNA expression profiles in the hippocampal tissues of Fmr1 knockout (Fmr1-KO) mice and wild type (WT) mice. A total of 75 differentially expressed miRNAs were identified, of which 58 were significantly upregulated and no miRNAs were significantly downregulated in Fmr1-KO mice. Quantitative real-time PCR (qRT-PCR) analysis was applied to validate the expression of 7 upregulated miRNAs; results indicated that the levels of only miR-449a and miR-720 were significantly upregulated. We further used bioinformatics software and databases to predict the target genes of these two miRNAs. The genes were related to dendritic spine development and synaptic plasticity; the qRT-PCR and western blotting results showed that cyclin-dependent kinase 5 (CDK5) and synaptotagmin 1 (SYT1) were differentially expressed in the Fmr1-KO mice and WT mice. In conclusion, this study evidenced diverse changes in the expression of miRNAs, and validated the miRNAs and their targeted genes in Fmr1-KO mice. Although further studies are required to better understand the function of miRNAs in FXS, the present research highlights a potential role of miRNAs in the pathogenesis of FXS.

Keywords: Fragile X syndrome, fragile X mental retardation protein, microarray analysis, microRNAs, synaptic plasticity

Introduction

Fragile X syndrome (FXS) is one of the most common forms of inherited mental retardation; it affects approximately 1 in 4000 males and 1 in 8000 females [1]. FXS results from the abnormal expansion of trinucleotide repeats (CGG) in the 5' untranslated regions of the Fmr1 gene; then, the hypermethylation of CGG leads to the transcriptional silencing of Fmr1 and loss of its encoded protein, the fragile X mental retardation protein (FMRP) [2, 3]. FMRP is an RNA-binding protein; it directly binds to the mRNAs of a large number of genes related to neurodevelopment and inhibits their translation, ultimately affecting the development of dendritic spines and synaptic plasticity [4]. However, the mechanisms underlying the FMRP-mediated regulation of mRNA translation and protein synthesis have not yet been elucidated.

Of the various types of small RNAs, miRNAs play the major role in the regulation of gene activities with regards to a variety of processes such as growth, differentiation, apoptosis, str-

ess response, etc. [5]. miRNAs are small non-coding RNAs (19-25 nucleotides) that reduce protein expression by inhibiting mRNA translation or promoting mRNA degradation [6, 7]. The miRNA database of the Sanger center has shown that about 460 mature miRNAs exist in the human brain [8]; 20-40% of these miRNAs have been reported to be involved in regulating the development of the nervous system [9]. Schratt et al. have found the first miRNA (miRNA-134) that plays a role in dendritic spine development; it is highly expressed in the hippocampal neurons. Its expression has been found to gradually increase after birth and reach a peak on the 13th day after birth [10]. MiRNA-124 has been reported to promote dendritic morphogenesis and enhance the spine density in newborn olfactory bulb neurons [11]. It has been demonstrated that the occurrence of immature dendritic spines is the central pathological change in both FXS patients and Fmr1 knockout (Fmr1-KO) mice [12]. Identifying the miRNAs related to the development of dendritic spines and synapses will be greatly helpful in understanding the pathogenesis of FXS.

FMRP has been reported to be highly expressed in the brain during human development; its mutation could significantly affect the physiological activities of the brain [13, 14]. dFMRP, which is a homolog of FMRP, has been shown to modulate the maturation of miRNAs in Drosophila, and then influence the expression of their target genes [15]. The levels of mature miR-124a have been reported to be partially reduced during the neuronal development in dfmr1 mutants of zebrafish [16]. dFMRP has also been shown to regulate the proliferation and fate of stem cells in *Drosophila* by genetically interacting with bantam miRNA [15]. Further, Warren et al. have found that FMRP participates in miRNA pathways by interacting with Dicer and Argonaute 1 (AGO1), ultimately influencing neuronal synaptogenesis and development [17]. These findings prompted the theory that FMRP deletion may cause changes of miRNAs in Fmr1-KO mice, thereby altering the expression of their target genes that are related to neuronal development.

Although an increasing number of miRNAs have been found in the nervous system of mammals, a few miRNAs and their target genes have been verified and demonstrated to have important functions in vivo. Profiling the expression of miRNAs is useful to address their roles. In the present study, to investigate whether changes in miRNAs and their target genes that are related to neuronal development participated in FXS, we analyzed the miRNA expression profiles in the hippocampal tissues of Fmr1-KO mice and wild type (WT) mice, and confirmed the differentially expressed miRNAs by quantitative real-time PCR (qRT-PCR). Additionally, the target genes of the miRNAs were predicted; these genes are related to dendritic spine development and synapse plasticity. The changes in the expression of these genes were validated by RT-PCR and western blotting analyses.

Materials and methods

Animals

Six-week-old wild-type (n = 9) and Fmr1-knockout ($FMRP^{-/-}$) (n = 17) mice with the FVB.129-P2(B6)-Fmr1tm1Cgr/J background were kind gifts from Dr. Oostra BA (Institute for cell biology and genetics, Erasmus University, the Netherlands) and Dr. Yonghong Yi (the Second Affiliated Hospital of the Guangzhou Medical University). The mice were maintained at the animal facility of the Guangzhou medical school under specific pathogen-free conditions and used to breed new knockout mice. Two animal groups were included: one-week-old knockout mice and the age-matched wild type mice. The genotype of the knockout mice was identified by PCR, and then, the lack or the presence of FMRP was confirmed by western blotting analysis.

All animal experiments were performed according to the Guide for the Care and Use of Medical Laboratory Animals (Ministry of Health, P. R. China, 1998) and the guidelines of the Ethical Committee for the care and use of experimental animals of the Guangzhou Medical University and the Ethical Committee for the care and use of experimental animals of the Jinan University. The mice were euthanized after being anesthetized with sodium pentobarbital; efforts were made to minimize the suffering of the mice. The two aforementioned ethics committees specifically approved this study. The genotype of the knockout mice used in this study was confirmed by tail DNA genotyping.

Microarray analysis of miRNAs

The brains of the anesthetized mice were removed quickly and the hippocampal tissues were dissected and placed on ice. Total RNA from the hippocampal tissues of each animal was isolated using Trizol reagent (Invitrogen), according to the manufacturer's protocols. The purity of each RNA sample was analyzed using an RNA NanoDrop® ND-1000 system; the samples were considered to be pure if they had an OD_{260}/OD_{280} ratio of 1.8-2.1. The integrity of each RNA sample was evaluated by performing 1% agarose gel electrophoresis using the RNA 6000 Nano LabChip Kit (Cat# 5065-4476, Agilent Technologies, Santa Clara, CA, USA) on the Agilent 2100 Bioanalyzer instrument; the appearance of three strips, i.e., bands of 5s rRNA, 18s rRNA, and 28s rRNA, confirmed the integrity of the total RNA. The RNA extracts that showed integrity values > 6 were further analyzed. The extracted RNA was labeled using fluorescent tags according to the procedures described in the miRCURY™ Array Power Labelling kit, and then, the labeled RNA was used for the chip hybridization experiment. The affymetrix miRNA 3.0 array was used for microarray analysis, which was based on the database of the Sanger institute, miRBase V15.0, which included 15,664 mature miRNA sequences of 131 organisms. The array also comprised an additional 2,334 encompassed human snoRNAs and scaRNAs (from the Ensembl database and snoRNABase) and 2,202 probe sets unique to pre-miRNA hairpin sequences. Control targets were also included in the array that contained 95 background probe sets, 22 oligonucleotide spike-in control probe sets, probes for human 5.8s rRNA, and hybridization control probe sets. In the present study, the miRNA expression of six pairs of samples from Fmr1-KO mice and WT mice was examined. The G4470A chip hybridization instrument and Axon GenePix 4000B chip scanner were used for the chip hybridization experiment and chip image acquisition.

Image analysis and the processing of the miRNA data

The software LuxScan 3.0 (The Bioss Biotechnology Company, Beijing, China) was used for analyzing the chip images and converting the images into data. The data were Log2 transformed and median centered based on the genes using the Adjust Data function of the CLUSTER 3.0 software; then, further analysis was performed via average-linkage hierarchical clustering [18]. Threshold values of ≥ 1.5- or ≤ -1.5-fold change and an FDR significance level of < 5% were used to select the differentially expressed genes. Finally, tree visualization was performed by using Java Treeview (Stanford University School of Medicine, Stanford, CA, USA).

qRT-PCR for the validation of the differentially expressed miRNAs

Based on the microarray analysis results, the expression levels of seven upregulated miRNAs were selected for validation using qRT-PCR. Four one-week-old male mice obtained from Fmr1 knockout mice of the FVB strain or age-matched wild type mice (as the controls) were used. First, the animals were intraperitoneally injected with 1% sodium pentobarbital (0.1-0.2 ml) and anesthetized. Their brains were removed and the hippocampal tissues were dissected. The tissues were placed into liquid nitrogen and then ground. The tissue powder was mixed with 1 ml of Trizol reagent (Invitrogen) for RNA extraction. Next, 1 μg of RNA was reverse-transcribed into cDNA using a cDNA synthesis kit with oligo-dT primers (MBI Ferments, St Leon-Roth, Germany). Subsequently, the cDNA was subjected to qRT-PCR, with a total reaction volume of 20 μl; the reaction mixture contained 200 nM each of both the forward and reverse primers and the SYBR Green PCR master mixture. qRT-PCR was performed using the SYBR Green system (Toyobo, Japan). The cDNA targets were quantified using an ABI PRISM® 7500 Sequence Detection System (ABI, USA). The primer efficiency (E) was determined by calculating the slope of the respective amplification curves. The ΔCt values were standardized against an internal control (18S rRNA), and fold increase in the expression levels was calculated as $(1+E)^{-\Delta\Delta Ct}$, relative to an unstimulated control. The primers used for the detection of the miRNAs in the PCR analysis are shown in Supplementary Table 1.

The prediction of the target genes of the selected differentially expressed miRNAs

Target genes of the two miRNAs were predicted based on three target gene databases: the miRBase (http://microrna.sanger.ac.uk/), Target Scan (http://www.targetscan.org/), and PicTar (http://pictar.bio.nyu.edu/.mir). After

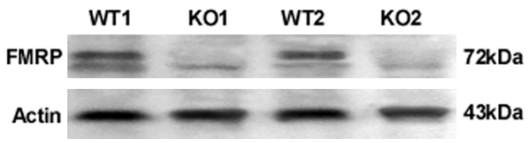

Figure 1. The FMRP protein, which is the expression product of the Fmr1 gene, was absent in the Fmr1 knockout mice but present in the wild type mice. Western blotting was performed to validate the FMRP protein levels in the Fmr1 knockout mice and wild type mice. WT1 and WT2 indicate the wild type animal 1 and wild type animal 2, respectively. KO1 and KO2 indicate the knockout animal 1 and knockout animal 2, respectively.

identifying the miRNA-binding sites, the data obtained after analysis using these three databases were collated and the overlaps were calculated.

qRT-PCR for the validation of the target genes of the miRNAs

The procedures for this analysis were similar to those for the qRT-PCR analysis of the differentially expressed miRNAs. The primers used for the target genes are shown in Supplementary Table 2.

Western blotting

The anesthetized mice were subjected to heart perfusion using saline to wash away the blood. Then, their brains were quickly removed, and the hippocampal tissues were separated and placed on ice. About 100 mg of the tissues were cut into pieces and homogenized in 500 μl RIPA lysis buffer (cat# P0013C, Beyotime Biotechnology, Shanghai, China) with cocktails of protease and phosphatase inhibitors (cat# ST506, Beyotime Biotechnology). Then, the homogenates were centrifuged at 14,000 rpm for 10 min at 4°C. The supernatants were collected in centrifuge tubes, and about 10 μl of each sample was used for protein quantitation by using the Pierce™ Rapid Gold BCA Protein Assay Kit (cat# A53225, Thermo Fisher Scientific, *Waltham,* MA, *USA*). Then, 60 μg of each protein (in a loading volume of 20 μl) was loaded into each well of 10% SDS-PAGE gels; the proteins were then separated by electrophoresis. The resultant protein bands were transferred from the gels onto PVDF membranes (pore size, 0.45 μm). The membranes were rinsed in 0.1% TBST and blocked with 5% skim milk in 0.1% TBST for 2 h at room temperature. The membranes were rinsed again and incubated with the primary antibodies at 4°C overnight. The following primary antibodies were used: antibodies against FMRP (1:1000, cat# ab17722, Abcam, Cambridge, MA, USA), actin (1:1000, cat# bs-10966R, Bioss, Beijing, China), CDK5 (1:1000, cat# ab40773, Abcam), synaptotagmin (SYT1; 1:500, cat# ab131551, Abcam), and GAPDH (1:500, cat# ab8245, Abcam). The membranes were then rinsed in 0.1% TBST and incubated with the secondary antibodies (in a solution containing 5% BSA and 0.1% TBST) for 2 h at room temperature. The bands of the target proteins on the membranes were detected using an ECL Western blotting Detection Kit (BeyoECL Plus Western Blotting Detection Kit, cat# P0018S, Beyotime, Biotechnology) and developed using a gel imaging system (ChemiDoc XRS, Bio-rad, Hercules, CA, USA). The gray values of the protein bands were analyzed using the NIH ImageJ analysis software. Each group contained four animals, and the experiments were repeated four times.

Statistical analysis

All results from the RT-PCR and western blotting analyses are presented as the mean ± standard error (SE). A paired t-test was performed using the SPSS v. 13.0 software to compare the differences between the groups. *P*-values < 0.05 were considered statistically significant.

Results

Identification of the genotype of the experimental animals

In 1994, Bakker et al. inserted neomycin fragments into the Fmr1 gene so that the gene could not express FMRP, and a mouse model of FXS was successfully established [19]. In our present study, the Fmr1-KO mice were hyperactive, irritable, and prone to epilepsy; they often fought with other mice in the same litter. The male Fmr1-KO mice had large testicles and showed poor fertility. The mice were genotyped, and the lack or presence of the Fmr1 gene has been confirmed by PCR in our previous studies [20]. The FMRP protein expression in the Fmr1-KO mice and WT mice is shown in **Figure 1**. A 72 kDa band was found in case of the wild type mice, but not the Fmr1 knockout mice.

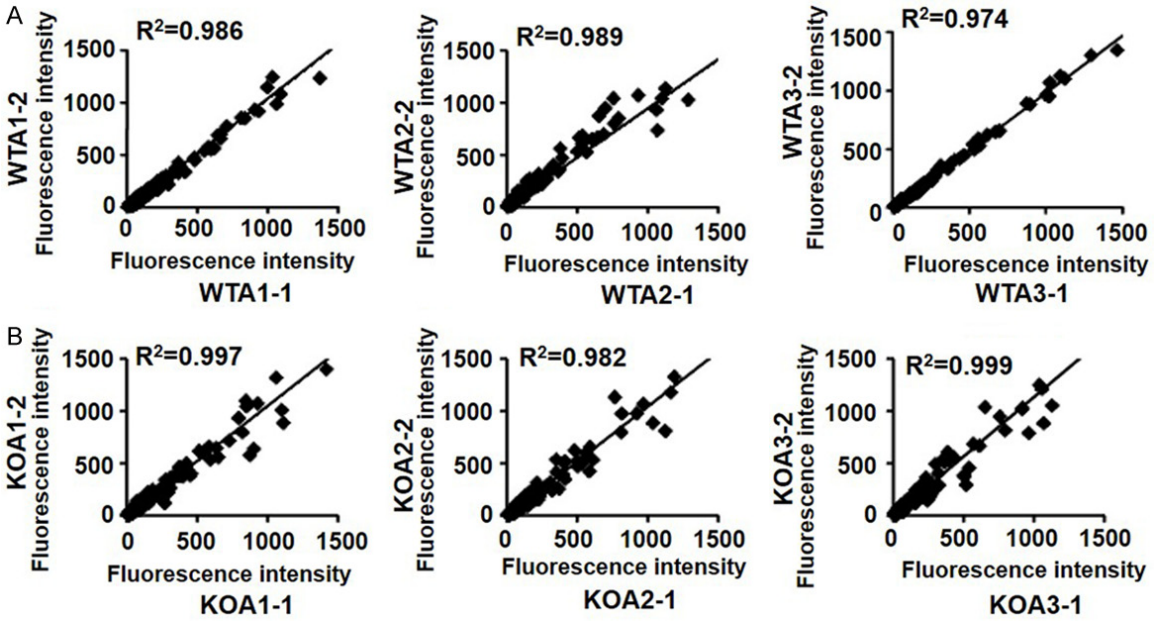

Figure 2. The experimental repeatability of the microarray analysis was between 0.974 and 0.999. WTA1, WTA2, and WTA3 indicate the wild type animal 1, wild type animal 2, and wild type animal 3, respectively. KOA1, KOA2, and KOA3 indicate the knockout animal 1, knockout animal 2, and knockout animal 3, respectively.

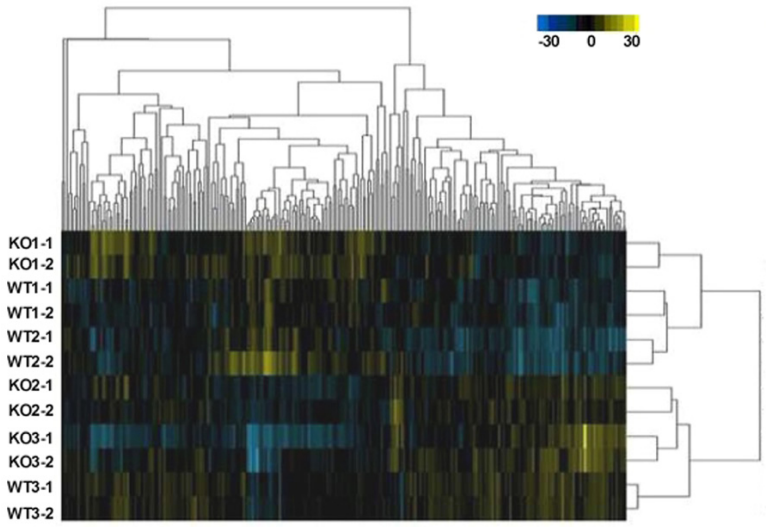

Figure 3. Changes of the miRNA expression in the hippocampal tissues of the Fmr1 knockout mice and wild-type mice. The heatmap shows the hierarchical clustering of miRNAs in each animal from the Fmr1 knockout and wild type groups. The yellow color indicates that the miRNA expression levels in the samples were upregulated, and the blue color indicates that the miRNA expression levels in the samples were downregulated. All differentially expressed miRNAs are listed in the Supplementary Tables 3 and 4.

Differentially expressed miRNAs

The correlation coefficient for the repeatability of microarray analysis in the present study was between 0.974 and 0.999; this indicates the accuracy of the microarray analysis (**Figure 2**).

Our results showed that 251 known miRNAs were identified. A total of 75 miRNAs were observed to show differential expression (58 upregulated and 18 downregulated) among the Fmr1-knockout mice and wild-type mice (**Figure 3**). The total fold changes for the upregulated miRNAs ranged from 1.5-3.7, and those for the downregulated miRNAs ranged from 0.33-0.65, as shown in the Supplementary Tables 3 and 4. The criteria for selecting the differentially expressed miRNAs were: p values ≤ 0.05 and KO/WT ratios indicating > 1.5-fold change. According to these criteria, 57 miRNAs were found to be upregulated in the Fmr1-knockout mice, compared to the case for the wild-type mice (Supplementary Table 3). No miRNAs were significantly downregulated in the Fmr1-knockout mice, compared to the case for the wild-type mice (Supplementary Table 4). The complete information regarding all the miRNAs is listed in the Supplementary Tables 3, 4.

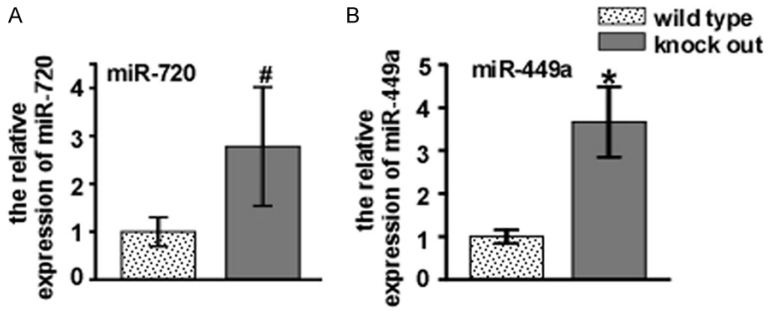

Figure 4. The relative expression of miR-720 and miR-449a in the hippocampal tissues of the Fmr1 knockout mice was higher than that in the hippocampal tissues of the wild type mice. QRT-PCR was performed to validate the miR-720 and miR-449a expression levels in the Fmr1 knockout mice and wild type mice. The miRNA expression levels in the mice from the Fmr1 knockout group were normalized to those in the mice from the wild-type group. *P < 0.01, #P < 0.05.

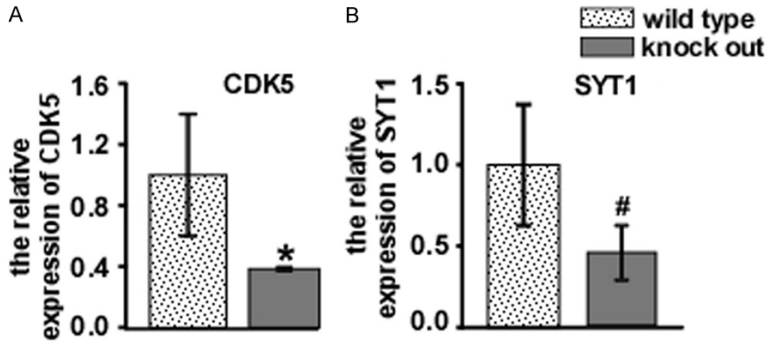

Figure 5. The CDK5 and SYT1 mRNA levels in the hippocampal tissues of the Fmr1 knockout mice were lower than those in the hippocampal tissues of the wild type mice. qRT-PCR was performed to validate the CDK5 and SYT1 mRNA expression levels in the Fmr1 knockout mice and wild type mice. The mRNA expression levels in the mice from the Fmr1 knockout group were normalized to those in the mice from the wild-type group. *P < 0.01, #P < 0.05.

Validation of the identified miRNAs by qRT-PCR

We selected 7 out of the 58 upregulated miRNAs for further validation: miR-9*, miR-15b, miR-30b, miR-376a, miR-410, miR-449a, and miR-720. However, the qRT-PCR results showed that the expression levels of only miR-720 and miR-449a were significantly upregulated in the Fmr1 knockout mice, compared to those in the wild type mice (**Figure 4**). There were no statistical differences between the expression levels of the five other upregulated miRNAs in the Fmr1 knockout and wild type mice. In addition, the relative expression of miR-720 in mice from the Fmr1 knockout group (2.75 ± 1.24) was also significantly higher than that in mice from the wild type group (1.00 ± 0.30, P < 0.05, **Figure 4A**). The relative expression of miR-449a in mice from the Fmr1 knockout group was 3.67 ± 0.82; this was higher than that in mice from the wild type group (1.00 ± 0.16, P < 0.01, **Figure 4B**).

Prediction of the target genes of the two upregulated miRNAs

The identification of the target genes of the two upregulated miRNAs is helpful for understanding their physiological functions. In this study, ThemiRBase, TargetScan, and PicTar were utilized to predict the target genes of the miRNAs. A list of the predicted target genes is included in Supplementary Table 5. The target genes of both miR-720 and miR-449a were related to neuron/dendritic spine development and synaptic plasticity. However, the target genes of miR-720 and miR-449a were completely distinct. In addition, miR-720 and miR-449a could regulate numerous genes. For example, the neuron/dendritic spine development-related target genes of miR-720 were NDEL1, FYN, CDK5, and RELB, and those of miR-449a were MAP1A and ARHGAP1. The synaptic plasticity-related target genes of miR-720 were DLGAP2, EPHB4/EPHB2, PXK, CPNE6, and SNTA1, and that of miR-449a was SYT1.

mRNA analysis for the validation of the targeted genes

Further validation and quantification of the target genes of miR-720 and miR-449a are also important and useful for understanding the fundamental functions of these miRNAs [21]. The qRT-PCR results showed that the expression of CDK5 and SYT1 was significantly downregulated in the Fmr1 knockout mice, compared to the case for the wild type mice (**Figure 5**). There were no statistical differences between the expression levels of the other tar-

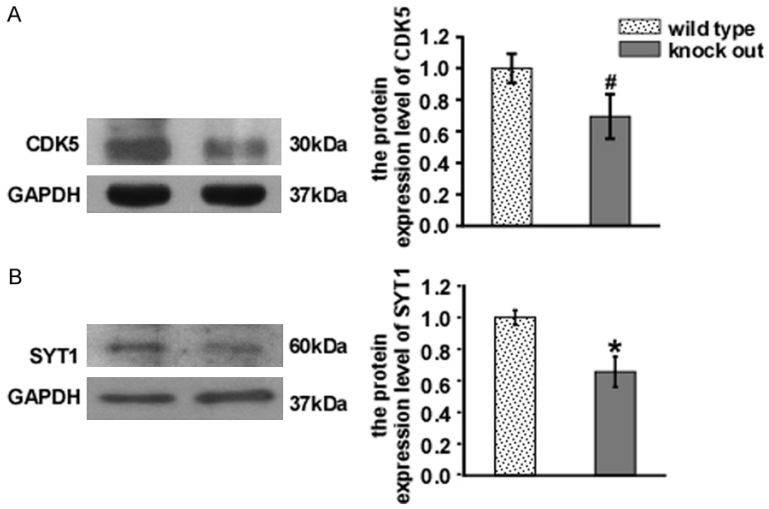

Figure 6. The CDK5 and SYT1 protein levels in the hippocampal tissues of the Fmr1 knockout mice were lower than those in the hippocampal tissues of the wild type mice. Western blotting was performing to validate the CDK5 and SYT1 protein levels in the Fmr1 knockout mice and wild type mice. The protein levels in the mice from the Fmr1 knockout group were normalized to those in the mice from the wild-type group. *P < 0.01, #P < 0.05.

get genes in the two types of mice. The relative expression of CDK5 in mice from the knockout group (0.38 ± 0.01) was significantly higher than that in mice from the wild type group (1.00 ± 0.40, P < 0.01, **Figure 5A**). The relative expression of SYT1 in mice from the knockout group was 0.45 ± 0.17; this was significantly lower than that in mice from the wild type group (1.00 ± 0.37, P < 0.05, **Figure 5B**).

Changes in the protein levels of the target genes in the hippocampal tissues from the Fmr1 knockout mice and wild-type mice

To verify the qRT-PCR results of targeted genes, we used western blotting to detect the protein levels of CDK5 and SYT1 in the Fmr1 knockout mice and wild type mice. All data were normalized to the level of the endogenous control protein (GAPDH; set as 100%). The expression patterns of the CDK5 and SYT1 proteins were similar to those observed in the qRT-PCR analysis (**Figure 6**). The protein level of CDK5 in mice from the knockout group (0.69 ± 0.14) was much lower than that in mice from the wild type group (1.00 ± 0.09, P < 0.05, **Figure 6A**). The protein level of SYT1 in mice from the wild type group was 1.00 ± 0.05; this decreased to 0.66 ± 0.10 in mice from the Fmr1 knockout group (P < 0.01, **Figure 6B**).

Discussion

Our study identified miRNAs expressed differentially in the Frm1-knockout mice and wild-type mice by microarray analysis. A total of 58 miRNAs were found to be upregulated in the knockout mice; no significantly downregulated miRNAs were found. To reveal the potential functions of miRNAs in FXS more clearly, qRT-PCR analysis was performed, and the differential expression of miRNA-449a and miRNA-720 was validated. The target genes of these two miRNAs were predicted; these genes were found to be associated with neuron/dendritic spine development and synaptic plasticity. QRT-PCR and western blotting analyses were used to validate the expression of the target genes; the expression levels of SYT1 and CDK5 were observed to show a decreasing tendency in the Frm1-knockout mice. Our results demonstrated that miRNAs may participate in the pathogenesis of FXS by regulating neuron/dendritic spine development and synaptic plasticity, which provides a basis for further studies and will be helpful in understanding the mechanisms underlying FXS.

According to the standards for differentially expressed miRNAs described under the heading “Image analysis and the data processing of miRNAs” of the “Materials and methods” section and studies related to the roles of miRNAs in brain development, 7 out of the 58 upregulated miRNAs were selected for further validation by RT-PCR: miR-9*, miR-15b, miR-30b, miR-376a, miR-410, miR-449a, and miR-720. MiR-9 has been shown to be involved in the pathway regulating neurite development [22] and the differentiation of neuronal cells [23]. Changes in miR-15b have been reported to influence early neocortical development [24] and neurobehavioral alterations [25]. MiR-30b has been shown to promote the axon outgrowth of retinal ganglion cells [26] and attenuate neuropathic pain [27]. Alterations of miR-376a activity have been reported to influence brain

development [28]. MiR-410 has been shown to play a neuroprotective role in experimental neurodegenerative diseases such as Parkinson's disease (PD) [29] and brain ischemia [30]. The RT-PCR results showed that there were no significant differences between the expression levels of miR-9*, miR-15b, miR-30b, miR-376a, and miR-410 in the mice from the Fmr1-knockout and wild-type groups. These results suggested that these miRNAs may not be involved in the regulation of FXS. Inhibition of miR-449a has been shown to be related to the proliferation of neural stem cells and neuronal survival [31]. Dysregulation of miR-449a has also been reported to be correlated with developmental defects of the brain [32]. The results of our validation experiments showed that the miR-449a expression level was significantly higher in the Fmr1-knockout mice, which suggests that high miR-449a levels may contribute to the occurrence and development of FXS. However, previous studies on miR-720 have mainly focused on its ability to promote the migration and invasion of cancer cells [33, 34]; only a few studies have focused on its function in neurodevelopment. In the present study, we found that the miR-720 expression was significantly higher in the Fmr1-knockout mice, indicating that apart from its known action in cancers, miR-720 may also play an important role in the pathology of FXS.

It has been reported that the length, density, and activity of the dendritic spines of neurons start to change one week after birth [35]. The half-lives of most miRNAs in the brain are not long; to avoid missing the peaks of the miRNA levels in the nervous system, brain samples obtained from the Fmr1-knockout mice at one week following their birth were used for the microarray analysis in this study. We found that 58 miRNAs were upregulated and no miRNAs were significantly downregulated in the hippocampi of knockout mice, compared with those of the wild-type controls. Further qRT-PCR results confirmed that miR-449a and miR-720 were significantly upregulated in the Fmr1-knockout mice, indicating that the perturbed expression of these miRNAs may contribute to the occurrence and development of FXS. Clinical trials have shown that the serum miR-424 expression levels in fragile X-associated tremor/ataxia syndrome patients are higher than those in subjects from the control population [36]. MiRNAs have also been shown to play an essential role in many neurodegenerative diseases such as Alzheimer's disease (AD), PD, and Huntington's disease (HD) [37-39]. The expression levels of miR-34a, miR-141, and miR-9 have been found to be upregulated in an *in vitro* model of PD [40]. The hippocampal miR-128 expression has also been found to be higher in AD patients than in control subjects [41, 42]. However, the expression levels of some other miRNAs have been found to decrease in pathological conditions, and increasing their expression has been reported to play a neuroprotective role. The serum miR-574-3p levels have been reported to be downregulated in fragile X-associated tremor/ataxia syndrome patients [36]; the levels of miR-124, which is the most abundant miRNA in neurons, have been reported to decrease significantly in AD, PD, and HD [43-45]. In addition, the upregulation of miR-128 expression has been shown to alleviate neuronal apoptosis following cerebral ischemia and neuronal excitability following epileptic seizures [37, 44]. MiR-9 has been shown to be downregulated in brain cancers [38], and the restoration of miR-9 expression has been reported to decrease the tumorigenicity [46]. It has been demonstrated that the miRNA expression levels differed based on the measurement time, tissue localization, genetic background, and different pathological conditions [47]. Based on the present results, we put forth the theory that the increase in the expression of miR-449a and miR-720 in Fmr1-knockout mice at a critical period of development (1 week after birth) may be harmful for mental development.

CDK5 and its activator, p35, are localized at neuronal synapses [48]. Toshio et al. have found that CDK5 is essential for the development and maintenance of dendritic spines in the mouse brain by *in vivo* and *in vitro* experiments [48, 49]. Deletion of CDK5/p35 has been shown to result in the impairment of synaptic plasticity and spatial learning [50, 51]. Reduction of CDK5 activity has also been reported to cause neurological disorders such as mental retardation, schizophrenia, epilepsy, etc. [52-54]. In the present study, CDK5 was identified as a target gene of miR-720; its expression was decreased in Fmr1-knockout mice. These results demonstrate that the specific molecular mechanisms underlying the par-

ticipation of miR-720 in FXS possibly involve the reduction of CDK5 activity.

SYT1, which is a synaptic vesicle protein, has been shown to play an important role in mediating Ca^{2+}-triggered neurotransmitter release. Throughout development, SYT1 mRNA has been shown to be widely expressed in the brain; the SYT1 gene expression has been reported to keep increasing during development and remain constant during adulthood. Therefore, its dysregulation may result in neurodevelopmental and neurological disorders [55]. SYT1 has been shown to be crucial for the neurite outgrowth of PC12 cells [56]. Yoo et al. have found that the SYT1 expression is significantly reduced in the temporal cortex of brains from patients with Down's syndrome and AD [57], and that such changes are closely related to impaired synaptogenesis and neuronal transmission. Studies have reported that the decrease in SYT1 expression in the cortex and hippocampus is associated with the development of cognitive dysfunction following experimental subarachnoid hemorrhage. Gerry et al. have identified SYT1 as one of the target synaptic proteins for miR-34a in $p73^{-/-}$ mice [58]; they found that changes in the miR-34a expression could regulate the neurite outgrowth of neurons by negatively modulating the SYT1 expression [59]. MiR-449a was another miRNA detected in our present study, and one of its targeted genes was SYT1. The miR-449a expression increased significantly in the Fmr1-knockout mice, while the expression of its target gene SYT1 was decreased. Therefore, the above findings and the present results underscore a potential a mechanism underlying the involvement of miR-449a and its synaptic protein target (SYT1) in neuron development and synaptic plasticity.

In summary, we examined the miRNAs expressed differentially in Fmr1-knockout mice and wild-type mice. We confirmed that miR-449a and miR-720 were upregulated in Fmr1-knockout mice; the expression of their target genes (CDK5 and SYT1, respectively) was found to be downregulated in the Fmr1-knockout mice, further indicating that miRNAs play an important role in FXS. Although future studies are needed to substantiate our present results, this study will be helpful in elucidating the pathogenesis of FXS and provides another possible strategy for the intervention and treatment of FXS, i.e., targeting the related miRNAs.

Limitations of the study

Firstly, the microarray analysis was used to detect the known miRNAs associated with FXS; however, we did not detect the novel miRNAs involved in FXS. Other techniques such as high-throughput sequencing will be utilized in our further studies in order to better understand the potential roles of such novel miRNAs. Secondly, among the 58 upregulated miRNAs, we selected 7 miRNAs for further validation based on the selection criteria described in the "Materials and methods" section and previous studies related to brain development; the further validation of the levels of the other upregulated miRNAs may be also meaningful. The related experiments will be included in our future studies.

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Disclosure of conflict of interest

None.

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Supplementary Table 1. Primers for the 7 miRNAs selected among the 58 upregulated miRNAs

MiRNA	Sequence	Product size (bp)
U6	F: 5'CTCGCTTCGGCAGCACA	94
	R: 5'AACGCTTCACGAATTTGCGT	
mmu-miR-9*	F: 5'ACACTCCAGCTGGGATAAAGCTAGATAACCG	72
mmu-miR-15b	F: 5'ACACTCCAGCTGGGTAGCAGCACATCATGGTTTA	72
mmu-miR-30b	F: 5'ACACTCCAGCTGGGTGTAAACATCCTACACT	72
mmu-miR-376a	F: 5'ACACTCCAGCTGGGATCGTAGAGGAAAATC	71
mmu-miR-410	F: 5'ACACTCCAGCTGGGAATATAACACAGATGGC	71
mmu-miR-449a	F: 5'ACACTCCAGCTGGGTGGCAGTGTATTGTTAGC	72
mmu-miR-720	F: 5'ACACTCCAGCTGGGATCTCGCTGGGGCCTCC	68

Supplementary Table 2. Primers for the target genes

Gene name		Sequence	Product size (bp)
18S rRNA	F	CCTGGATACCGCAGCTAGGA	112
	R	GCGGCGCAATACGAATGCCCC	
EFNA1	F	CATGTCAACCCACAGGAGAA	160
	R	CAAGCGTACACCCTCACTGA	
MUSK	F	GAATCCCACAATGTCACCTT	160
	R	GCTGGAGTCTTGAGTCAATCA	
RPS27A	F	CACGCTCGGATCTTCCTTTC	214
	R	AGTGTCCGAGGGTTCAACCT	
CDK5	F	CAGCCGCAACGTGCTACATA	200
	R	TGTCGATGGACGTGGAGTACA	
NDEL1	F	CGGGAACGACAACAGGAAGT	300
	R	GCGGGATGCTTGGTCTTTT	
RELB	F	CATCCTCTCTGAGCCTGTCT	150
	R	ACCACGGATATGTCCTCTTT	
SNTA1	F	TCAGTGCCCTATGATGCAGAA	340
	R	CCCCAAAGTCCAGGAAAAGG	
UBC	F	CAACTCCGTGAGAGAGACGATG	125
	R	TGCCCTCCTTGTCCTGGATCTT	
UBB	F	CTGGGCGGTTTGTGCTTTCAT	245
	R	TGCAGGGTTGACTCCTTCTGGA	
SYT1	F	CCTGTGTGCTTAGACACGTA	237
	R	TGGACTAGTGCACAGTGTTG	
ARHGAP1	F	TGGAAGTGACACAGCAGGTT	150
	R	GGTTAGGGCCGAAGACTACA	
MAP1A	F	GTCTTCACCTACCCCATTCC	180
	R	GCAGGATGGTAGAGGATTGG	
FYN	F	AGACAAGGTGCGAAGTTTCC	150
	R	GTTGTTCATGCCTGGGTATG	
DLGAP2	F	AAGGCTGGTGCAAAGAGAT	200
	R	CCAATAGCCAGCTAGATCCT	
UBA52	F	TGTCAAGGCCAAGATCCAAG	335
	R	CATGCCAGGTATGACCACAG	
PICK1	F	CAGATGCGCAAAGATGTTC	100
	R	TGGACATGGTGGATACGA	
SNCA	F	TGGAAGACATGCCTGTGGAT	226
	R	CCGATCACTGCTGATGGAAG	

Supplementary Table 3. Upregulated miRNAs in the Fmr1 knockout mice

ProbeSet Name	Ratio (KO/WT)	*P*-value
mmu-miR-720_st	3.773997341	0.001630901
mmu-miR-449a_st	3.053889798	0.000331593
mmu-miR-714_st	2.91847246	0.006192241
mmu-miR-9_st	2.774575964	0.013950142
mmu-miR-9-star_st	2.614039962	0.002029597
mmu-miR-18a_st	2.384252138	0.000236279
mmu-miR-15a_st	2.379455285	0.019113108
mmu-miR-30b_st	2.37500326	0.004850401
mmu-miR-30e_st	2.324264039	0.000259467
mmu-miR-20b_st	2.323107096	0.000475024
mmu-miR-143_st	2.313435361	0.007554996
mmu-miR-376a_st	2.29221142	0.017452985
mmu-let-7f_st	2.260799705	0.001667708
mmu-miR-883b-3p_st	2.248573289	0.016605304
mmu-miR-195_st	2.246751183	0.00521596
mmu-miR-16_st	2.234760131	0.007156523
mmu-miR-27b_st	2.222776341	0.000604817
mmu-miR-126-3p_st	2.202148852	0.006347928
mmu-miR-344_st	2.166484253	0.015074644
mmu-let-7g_st	2.123323526	0.002959643
mmu-miR-20a_st	2.094731329	0.002047007
mmu-miR-106b_st	2.084464435	0.013804538
mmu-miR-411_st	2.064554034	0.004875924
mmu-miR-495_st	2.045462668	0.000120757
mmu-miR-29a_st	2.017876	0.001756565
mmu-miR-137_st	1.984861125	0.001598433
mmu-miR-410_st	1.94253198	0.006441488
mmu-miR-434-5p_st	1.927790984	0.007348267
mmu-miR-154_st	1.908365374	0.00011582
mmu-miR-30c_st	1.902936833	0.002909568
mmu-miR-30a_st	1.884123493	0.005241756
mmu-miR-22_st	1.880390286	0.021733109
mmu-miR-17_st	1.858824539	0.002157017
mmu-miR-194_st	1.834639951	0.001824465
mmu-miR-34c_st	1.815611422	0.021081886
mmu-miR-146a_st	1.808480232	0.011139754
mmu-miR-188-5p_st	1.793574311	0.009083602
mmu-miR-551b_st	1.788209638	0.036083259
mmu-let-7i_st	1.783115	0.002358375
mmu-miR-106a_st	1.734393653	0.014698682
mmu-miR-487b_st	1.731954639	0.000540444
mmu-miR-26a_st	1.714376569	0.00490016
mmu-miR-152_st	1.703958346	0.027492405
mmu-miR-335-5p_st	1.671335294	0.003728716
mmu-miR-181a-1-star_st	1.653048687	0.002450559
mmu-miR-379_st	1.636552784	0.032642755
mmu-miR-1192_st	1.63112146	0.018444893
mmu-miR-124_st	1.599921519	0.007239062

mmu-miR-503_st	1.585699085	0.035667718
mmu-miR-146b_st	1.573170829	8.69634E-05
mmu-miR-15b_st	1.558245972	0.015129909
mmu-miR-381_st	1.556095416	0.014308882
mmu-miR-329_st	1.550410621	0.026657166
mmu-miR-130a_st	1.546315409	0.010044816
mmu-miR-218_st	1.541405524	0.01478641
mmu-miR-362-5p_st	1.527285078	0.014713476
mmu-miR-99a_st	1.523450683	0.001999315
mmu-miR-199a-3p_st	1.511913989	0.005278593

Supplementary Table 4. Downregulated miRNAs in the Fmr1 knockout mice

ProbeSet Name	Ratio (KO/WT)	*P*-value
mmu-miR-486_st	0.655678824	0.026944052
mmu-miR-99b_st	0.655666426	0.021386283
mmu-miR-652_st	0.635546094	0.004720488
mmu-miR-193b_st	0.61777921	0.013577924
mmu-miR-346_st	0.616001453	0.010721315
mmu-miR-330-star_st	0.595774882	0.010276025
mmu-miR-711_st	0.585563651	0.001971895
mmu-miR-668_st	0.564880087	0.013774019
mmu-miR-744_st	0.533092375	0.041161955
mmu-miR-494_st	0.51192807	0.041296841
mmu-miR-705_st	0.501360965	0.001096583
mmu-miR-762_st	0.448320876	0.017079633
mmu-miR-1196_st	0.438261082	0.005759448
mmu-miR-685_st	0.434269539	0.034517227
mmu-miR-709_st	0.372419113	0.001181997
mmu-miR-699_st	0.36552878	0.033841637
mmu-miR-1195_st	0.35871524	0.006554734
mmu-miR-1224_st	0.330106712	0.016707354

Supplementary Table 5. Predicted target genes of the miRNAs

MiRNA	Neuron/dendritic spine development-related genes	Synaptic plasticity-related genes
miR-449a	MAP1A, ARHGAP1	SYT1
miR-720	NDEL1, CDK5, RELB, FYN	SNTA1, PXK DLGAP2, SNCA, UBB, RPS27A, MUSK, EFNA1, PICK1

Abbreviations: MAP1A: Microtubule-associated proteins 1A; ARHGAP1: GTPase-activating protein RhoGAP; SYT1: Synaptotagmin-1; NDEL1: Nuclear distribution protein nudE-like 1; CDK5: Cyclin-dependent kinase 5; RELB: V-rel avian reticuloendotheliosis viral oncogene homolog B; FYN: Oncogene related to SRC, FGR, YES; SNTA1: Syntrophin-α1; PXK: PX domain containing serine/threonine kinase; DLGAP2: Discs, large (*Drosophila*) homolog-associated protein 2; SNCA: Synuclein, alpha (non A4 component of amyloid precursor); UBB: Ubiquitin B; RPS27A: Ribosomal protein S27a; MUSK: Muscle, skeletal, receptor tyrosine kinase; EFNA1: Ephrin-A1; PICK1: Protein interacting with PRKCA 1.