MeCP2-dependent repression of an imprinted miR-184 released by depolarization

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Both fragile X syndrome and Rett syndrome are commonly associated with autism spectrum disorders and involve defects in synaptic plasticity. MicroRNA is implicated in synaptic plasticity because fragile X mental retardation protein was recently linked to the microRNA pathway. DNA methylation is also involved in synaptic plasticity since methyl CpG-binding protein 2 (MeCP2) is mutated in patients with Rett syndrome. Here we report that expression of miR-184, a brain-specific microRNA repressed by the binding of MeCP2 to its promoter, is upregulated by the release of MeCP2 after depolarization. The restricted release of MeCP2 from the paternal allele results in paternal allele-specific expression of miR-184. Our finding provides a clue to the link between the microRNA and DNA methylation pathways.

INTRODUCTION

The microRNA pathway plays a crucial role in the early development of the brain (1). Several studies support a role for microRNAs in the later stages of neural maturation and synapse development (2–4). The ability of miR-124a, a neuron-specific microRNA, to suppress expression of non-neuronal genes in an in vitro cell system suggests that microRNAs play an important role in the regulation of neuronal differentiation (5). A recent paper reported that conditional Purkinje cell-specific ablation of Dicer, the key microRNA-generating enzyme, leads to Purkinje cell death (6). Fragile X syndrome is characterized by moderate-to-severe mental retardation, macro-orchidism and distinct facial features, including a long face, large ears and prominent jaw, and also carries a high risk of autism. This syndrome is caused by the loss of fragile X mental retardation protein (FMRP), an RNA-binding protein (7). FMRP is thought to be involved in synaptic plasticity through the regulation of mRNA transport and local protein synthesis at synapses (7). This is supported by the study of abnormal dendritic spines in the brains of fragile X patients and mouse models (8–10). FMRP is associated with an argonaute family protein and Dicer activity, both of which are important for microRNA processing, which suggests that it might be involved in the processing of microRNA precursors (11–13). A recent paper reports that FMRP can act as a microRNA acceptor protein for the ribonuclease Dicer and facilitate the assembly of microRNAs on specific target RNA sequences (14).

DNA methylation also plays an important role in synaptic plasticity. Rett syndrome is characterized by severe mental retardation, stereotypic hand movements, jerky truncal ataxia and autism. This syndrome, an important phenotype of which is a defect in synaptic plasticity, is caused by methyl CpG-binding protein 2 (MeCP2) mutations (15). MeCP2 represses gene expression by binding to methylated CpG sites (16). This protein is a nuclear protein dynamically expressed during postnatal mammalian brain development and is a marker for neuronal maturity (17–20). It is hypothesized that elevated MeCP2 expression is required for neuronal differentiation by the regulation of multiple target genes (21). The involvement of MeCP2 in methylation-specific transcriptional repression (22,23) suggests that MeCP2 deficiency in Rett syndrome would result in widespread gene dysregulation. This hypothesis was previously tested using a gene expression microarray analysis with MeCP2-deficient mouse brain (24), Rett patient cell lines

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(25) and post-mortem brain tissue (26). Subtle and non-overlapping transcriptional changes were observed in each of these studies, indicating that MeCP2 deficiency does not result in obviously high levels of genome-wide transcriptional dysregulation. Interestingly, MeCP2 has been shown to bind to the methylated brain-derived neurotrophic factor (BDNF) promoter and is released from it after depolarization, resulting in upregulated gene expression (27,28). It is also interesting that the expression of some imprinted genes is regulated by MeCP2, and these imprinted genes show dysregulated expression in Rett syndrome (29,30). Therefore, it is possible that the brain-specific imprinted genes regulated by MeCP2 are activity dependent.

We describe one such microRNA, called miR-184 in this paper. This microRNA can link between the microRNA and DNA methylation pathways and also may explain the common clinical features of fragile X syndrome and Rett syndrome, such as autism and mental retardation.

RESULTS
Isolation of transcripts around miR-184 locus and analysis of their allelic expression

We concentrated on an imprinted locus on mouse chromosome 9 because all imprinted genes at this locus are brain-specific (31), and miR-184, a microRNA, is located 55 kb from one of the genes. The primary transcript of miR-184 (pri-miR-184) was identified by RT–PCR analysis using several primers around the mature miR-184 coding sequence (Fig. 1A). An analysis of the primary transcript of miR-184 (pri-miR-184) in several mouse tissues revealed specific expression in brain and testis (Fig. 1B). A quantification of the mature miR-184 by TaqMan MicroRNA assays also gave similar results (Fig. 1C). In addition, we identified at least five non-coding alternative antisense transcripts with a poly-adenylation signal in each, as summarized in Figure 1A (AS1a, AS1b, AS2, AS3, AS4). All transcripts were expressed in testis but not in brain, except for AS4.

To analyze whether pri-miR-184 and the antisense alternative splicing transcripts are imprinted in brain and testis, RT–PCR and subsequent restriction fragment length polymorphism (RFLP) analysis were performed with RNA from the adult brain and testis of C57BL/6 (B6), CBA and the reciprocal F1 animals of these parental strains. Sequencing identified single-nucleotide polymorphisms (SNPs) between the B6 and CBA alleles on each transcript. In brain tissue from CBF1 (offspring of CBA females and B6 males) animals, pri-miR-184 was found to be exclusively expressed from the paternal B6 allele, whereas in BCF1 (offspring of B6 females and CBA males) animals, it was exclusively expressed from the paternal CBA allele (Fig. 1D). AS4 also showed exclusive expression from the paternal allele in brain (Fig. 1E). On the other hand, both transcripts showed bi-allelic expression in testis (Fig. 1D and E). AS1a, AS1b, AS2 and AS3 were not expressed in brain (Fig. 1H; data not shown) but in testis. AS1a, AS1b and AS3 showed bi-allelic expression, and AS2 was exclusively transcribed from the B6 allele independent of parental origin (Fig. 1F, G and H). As summarized in Figure 1A, pri-miR-184 and the AS4 transcript were imprinted and expressed in brain exclusively from the paternal allele while none of the transcripts expressed from this genomic region were imprinted in testis. All previously reported imprinted genes at this locus (Rasgrfl1 and A19) also show a paternal-allele-specific expression only in brain (31).

Methylation analysis of miR-184 locus

Some imprinted genes are related to a differentially methylated region (DMR) near the genes. There is no canonical CpG island around the miR-184 gene, but a relatively CpG-rich (CpG-R) region (indicated by the box in Figure 2) is located downstream of the AS4 transcription start site. To determine whether the methylation of CpG di-nucleotides around the miR-184 gene is associated with the imprinted expression, we performed bisulfite sequencing to analyze the methylation status of 29 CpGs, numbered 1–29 (Fig. 2), around miR-184. The relatively CpG-R region numbered 23–29 was found to be hypermethylated in brain. However, CpGs 12–22 between pri-miR-184 and AS4 were relatively unmethylated. Most CpGs were hypomethylated throughout testis, except CpG sites 1–5 (Fig. 2). No difference in methylation was observed between the parental alleles in either organ. Bearing in mind that RT–PCR showed higher expression levels of pri-miR-184 in testis and lower levels in brain (Fig. 1B), these observations suggest that the methylation status would not affect imprinted expression but would control potential transcriptional activity in a tissue-specific manner. A DMR located about 30 kb upstream of the Rasgrfl promoter (Fig. 1A) is known to be required for the expression of imprinted Rasgrfl (32). This DMR could regulate the imprinted expression of pri-miR-184 and AS4, since a single DMR usually regulates several imprinted genes located nearby (33).

Activity-dependent expression of the transcript encoding miR-184

We examined whether the microRNA encoding the transcript is activity dependent or not by analyzing expression before and after depolarization of cultured cortical neurons prepared from mouse embryo at gestational day 16. We found that treatment with 50 mM KCl upregulated the expression of pri-miR-184 similar to Bdnf in cortical neurons (Fig. 3A). Another imprinted transcript in brain, AS4, was also upregulated in 6 day but not 8 day cultured cortical neurons (Fig. 3A). Digestion with RT–PCR product with the polymorphic restriction enzyme revealed that upregulated transcript is derived from the paternal chromosome (Fig. 3B).

Activity-dependent regulation by MeCP2

MeCP2, a methylated CpG-binding protein, is involved in the activity-dependent regulation of BDNF mRNA (27,28). Membrane depolarization causes phosphorylation of MeCP2 proteins and the release of phosphorylated MeCP2 from methylated CpG sites on the specific Bdnf exon IV promoter. This results in the activation of the specific promoter. Likewise, we hypothesized that MeCP2 would bind to the relatively CpG-R region (Fig. 2) and that membrane
depolarization would cause phosphorylation of MeCP2, followed by release from only the paternal allele, thereby resulting in paternal-specific induction. Chromatin immunoprecipitation (ChIP) analysis was performed to test this hypothesis. Mouse cortical neurons were fixed with formaldehyde before and after treatment with 50 mM KCl. MeCP2-bound DNA fragments were immunoprecipitated with anti-MeCP2 antibody. The region containing the CpG-R was amplified and analyzed with RFLP to distinguish its parental origin. We found that the amount of paternal

**Figure 1.** Brain-specific imprinting of the transcript encoding miR-184. (A) A map of around pri-miR-184 on mouse chromosome 9. The structures of the primary transcript of miR-184 and five alternative antisense transcripts are shown magnified. Brain-specific paternally expressed genes are indicated as blue boxes. (B) Expression of pri-miR-184 in several tissues. All RNAs were derived from the C57BL/6 strain. Lanes: Br, brain; Ht, heart; Kd, kidney; Lv, liver; Lg, lung; Sm, skeletal muscles; Ov, ovary; Si, small intestine; Sp, spleen; St, stomach; Ts, testis; Ty, thymus; W, water. (C) Expression of mature miR-184. All RNAs were obtained from C57BL/6 strain and were analyzed by TaqMan microRNA assays. Results shown are for the average of three experimental replicates and represented as fold changes relative to the expression in brain. Data are presented as mean ± SEM. (D) The paternal allele-specific expression of pri-miR-184 in brain and bi-allelic expression in testis. RT–PCR followed by RFLP analysis was performed for pri-miR-184 in adult brain and testis RNA from C57BL/6 (B6), CBA and reciprocally crossed F1 animals of these parental strains. RT–PCR products were treated with NciI and resolved by 12% PAGE. Only the CBA allele was cleaved with NciI. (E) The paternal allele-specific expression of AS4 in brain and bi-allelic expression in testis. RT–PCR was followed by digestion with BsaAI. Only the CBA allele was cleaved with BsaAI. (F) Bi-allelic expression of AS1ab in testis. RT–PCR simultaneously amplified both AS1a/b and AS2. Digestion with NciI distinguished the CBA allele of AS1a/b from the B6 allele of AS1a/b and AS2. Asterisks indicate the cleaved products of AS1a/b and AS2. (G) Strain-specific expression of AS2. Direct sequencing of AS2 was performed with the gel-purified RT–PCR products of CBF1 and BCF1. The sequences from genomic DNA of C57BL/6 (B6) and CBA are shown as controls. The polymorphic site is indicated by arrows. (H) Bi-allelic expression of AS3 in testis. RT–PCR followed by digestion with NciI. Only the CBA allele can be cleaved with NciI.
MeCP2-bound fragments decreased after 6 h of KCl treatment (Fig. 4A). As reported previously, the region containing the Bdnf exon IV promoter bound to MeCP2 also decreased after 6 h of KCl treatment (Fig. 4A). These results suggested that MeCP2 protein associated with the CpG-R on both alleles before KCl treatment was released exclusively from the CpG-R of the paternal allele, although it remained bound at the methylated CpG sites on the maternal allele.

Activity-dependent demethylation of CpG sites was observed in Bdnf promoter (28). To assess whether CpG methylation patterns within the miR-184 locus are changed upon depolarization, we examined the methylation status within the CpG-R by bisulfite sequencing method. Unlike the case of Bdnf, CpG methylation patterns were not changed upon depolarization (Fig. 4B).

Expression of miR-184 in Mecp2-deficient mouse brain
To determine the expression levels of mature miR-184 in Mecp2-deficient mouse brain, TaqMan MicroRNA assays were performed on RNA samples obtained from Mecp2−/− and Mecp2+/− mice brains. On the basis of the results given earlier, one would predict that Mecp2-deficient mouse brain should express more miR-184 than wild-type mouse brain.
A recent paper expressed from the maternal allele in another imprinted (Fig. 1D). Seitz imprinted and exclusively expressed from the paternal allele rather than fragile X syndrome. Our results suggest that MeCP2 protein, binding to both alleles of the pri-miR-184 upstream region before depolarization, is released exclusively from the paternal allele after depolarization. At the same time, it remained bound at the methylated CpG sites on the maternal allele (Fig. 4A). Since no allelic differences in methylation were observed in CpGs in and around this gene (Fig. 2). This is not surprising since many imprinted genes do not have DMRs nearby and are regulated by DMRs situated a long distance from the genes. For example, the paternal allele-specific expression of Rasgrf1, located 110 kb from miR-184 locus, is regulated by a DMR located 30 kb upstream of Rasgrf1 (32) (Fig. 1A). It is possible then that the paternal allele-specific expression of the miR-184-coding transcript is also regulated by the same DMR.

Our results suggest that MeCP2 protein, binding to both alleles of the pri-miR-184 upstream region before depolarization, is released exclusively from the paternal allele after depolarization. At the same time, it remained bound at the methylated CpG sites on the maternal allele (Fig. 4A). Since no allelic differences in methylation were observed in this region (Fig. 2), it is reasonable to suggest that MeCP2 binds to both alleles. The paternal allele-specific release of MeCP2 may be regulated by the DMR located 80 kb upstream of pri-miR-184 (30 kb upstream of Rasgrf1, Fig. 1A) through mechanisms explained by the insulator (50) or looping models (51). The paternal allele could have open chromatin by either of these models, whereas the
maternal allele could not. Therefore, only the MeCP2 protein binding to the paternal allele could be accessed and attacked by the kinase which can release the MeCP2 protein.

On the basis of our finding of MeCP2-dependent repression of miR-184, one would predict that Mecp2-deficient mouse brain should express more miR-184 than wild-type mouse brain. However, this prediction is made under the assumption that the level of neuronal activity is the same in the wild-type brain and Mecp2-deficient brain. Recent report showed reduced cortical activity in the Mecp2-deficient brain (52). In accordance with this, BDNF protein, which also shows activity-dependent release of MeCP2-dependent repression, was found to be reduced in Mecp2-deficient mouse brain to the similar extent as miR-184 (53). Therefore, our finding of reduced miR-184 in Mecp2-deficient brain (Fig. 5) is also in accordance with the reduced cortical activity.

To examine whether miR-184 can induce morphological changes in neuron, we transfected the plasmid which expresses miR-184 to the cortical neuron culture cells. The construct encoding pri-miR-184 and an enhanced green fluorescence protein was introduced into 5 day cultured cortical neurons. However, we did not observe morphological changes such as dendritic growth (data not shown). FXYD1, another MeCP2 protein target, has been reported to be overexpressed in the brains of Rett syndrome patients and Mecp2-deficient mice recently (54). Forced expression of Fxyd1 in neurons reduced the growth of dendritic trees (54). Therefore, the morphological phenotype of Mecp2-deficient mouse neuron could be caused by Fxyd1, and other target genes including miR-184 could be responsible for other phenotypes. Further study of miR-184 function by knockout or transgenic mouse may help the identification of genetic cause of autism.

MeCP2 protein levels are significantly greater in central nervous system (CNS) tissues compared with non-CNS tissues, addressing a major paradox in the pathogenesis of Rett syndrome regarding how mutations in ubiquitously transcribed MECP2 result in a phenotype specific to the CNS (21,55). Several studies show that elevated MeCP2 expression is acquired during postnatal brain development, which suggests that MeCP2 might play an important role during neuronal maturation and synaptogenesis (17,19,21). Furthermore, activity-dependent release of MeCP2 protein by phosphorylation also suggests the important role it plays in the brain (27,28). In accordance with these findings, brain-specific phosphorylation of MeCP2 protein was recently reported (56). Our finding of the activity-dependent induction of a microRNA by MeCP2 release presents a new possibility for the role of microRNAs in neurons and additionally may implicate their involvement in neuronal diseases.

MATERIALS AND METHODS

Analysis of RT–PCR products with RFLPs

RT–PCR products were analyzed with RFLPs to distinguish the parental origin of pri-miR-184 and the antisense

Figure 4. The paternal allele-specific activity-dependent release of MeCP2. (A) ChIP assays were performed using BCF1 cortical neurons cultured with or without 50 mM KCl for 2 or 6 h. The miR-184 CpG-R and the Bdnf/exon IV promoter were amplified from the immunoprecipitate with anti-MeCP2 antibody or control IgG. Mat indicates the maternal allele and Pat indicates the paternal allele. (B) CpG methylation patterns within the miR-184 locus with or without 50 mM KCl for 2 or 6 h. CpG methylation in miR-184 CpG-R was examined by bisulfite sequencing method.

Figure 5. Expression of miR-184 in Mecp2-deficient mouse. TaqMan microRNA assays were performed on RNA samples obtained from 28-day-old Mecp2+/y and Mecp2−/y mice brains. Results shown are for the average of three experimental replicates of six Mecp2+/y and four Mecp2−/y mice. Expression is represented as fold changes relative to the expression in wild-type mice. Data are presented as mean ± SEM.
transcripts. For amplification of pri-miR-184, cDNA was prepared with M-MLV reverse transcriptase (Takara) at 55°C using a gene-specific primer, miR184-R3. Pri-miR-184 was amplified using the primers miR184-F5 and miR184-R4 and treated with a restriction enzyme, NciI, and then resolved on 12% TBE polyacrylamide gels. The cDNA was prepared with random hexamers for all other antisense transcripts. AS1a/b and AS2 transcripts were amplified using primers m-miR184-CpG1-F1 and AS-R3 and treated with the restriction enzyme NciI. AS3 was amplified with primers AS-F2 and AS-R3 and treated with NciI. AS4 was amplified with AS-F2 and AS-R2 and treated with BsaAI. Digested PCR products were resolved on 3% agarose gels and stained with ethidium bromide (Supplementary Material).

**TaqMan microRNA assays**

TaqMan microRNA assays which can only quantify mature microRNA by using stem-loop RT primers were performed as described using the comparative CT method (56). Normalization was done with value obtained with 5S ribosomal RNA TaqMan assays (57).

**Bisulfite sequencing**

Genomic DNA was isolated from mouse brain and testis and treated with sodium bisulfite to analyze methylation status using a CpGenome DNA modification kit, according to the manufacturer’s instructions (CHEMICON). The modified DNA was amplified using the following conditions: 94°C for 3 min followed by 94°C for 20 s, 55°C for 30 s and 72°C for 30 s for 35 cycles, and a final extension at 72°C for 7 min. The PCR product was cloned and sequenced (Supplementary Material).

**Culture of cortical neurons**

Mouse cortical neurons were isolated from mouse embryos at gestational day 16. Cells were plated on culture dishes at a density of 7 × 10^4 cells/cm² and maintained in serum-free medium [Neurobasal medium (Invitrogen), supplemented with 0.5 mM glutamine and B27 supplement (Invitrogen)] at 37°C in a humidified 10% CO₂ atmosphere.

**Chromatin immunoprecipitation**

Cortical neurons (2 × 10^6 per 60 mm dish) were prepared from fetal brains of BCF1 animals and treated with or without an elevated level of KCl (50 mM) for 2 and 6 h. Cultured neurons were subjected to a ChIP assay, according to the Upstate Biotechnology ChIP Kit’s protocol. Shared chromatin by sonication was immunoprecipitated with anti-MeCP2 antibody, or non-specific rabbit IgG (Sigma) as a negative control. CpG-R was amplified using primers AS-F1 and AS-R2 and treated with NciI to distinguish the parental origin. The PCR product derived from the CBA allele was completely digested with NciI. The mouse Bdnf exon IV promoter region served as a positive control to assess the effect of membrane depolarization (Supplementary Material).

**SUPPLEMENTARY MATERIAL**

Supplementary Material is available at HMG Online.

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Conflict of Interest statement. None declared.

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