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

Tasuku Nomura¹, Mika Kimura¹, Takuro Horii¹, Sumiyo Morita¹, Hidenobu Soejima², Shinichi Kudo³ and Izuho Hatada¹,*

¹Laboratory of Genome Science, Biosignal Genome Resource Center, Institute for Molecular and Cellular Regulation, Gunma University, 3-39-15 Showa-machi, Maebashi 371-8512, Japan, ²Division of Molecular Biology and Genetics, Department of Biomolecular Sciences, Faculty of Medicine, Saga University, 5-1-1 Nabeshima, Saga 849-8501, Japan and ³Hokkaido Institute of Public Health, Kita-19, Nishi-12, Kita-ku, Sapporo 060-0819, Japan

Received August 21, 2007; Revised November 11, 2007; Accepted January 16, 2008

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

*To whom correspondence should be addressed. Tel: +81 272208057; Fax: +81 27 2208110; Email: ihatada@showa.gunma-u.ac.jp

© The Author 2008. Published by Oxford University Press. All rights reserved. For Permissions, please email: journals.permissions@oxfordjournals.org
(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. Sequencing identified single-nucleotide polymorphisms (SNPs) between the B6 and CBA alleles on each transcript. In brain tissue from CBF1 (offspring of B6 females and CBA males) animals, pri-miR-184 similar to Bdnf was expressed from this genomic region were imprinted in testis. All previously reported imprinted genes at this locus (Rasgrf1 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 Rasgrf1 promoter (Fig. 1A) is known to be required for the expression of imprinted Rasgrf1 (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; Ow, 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 AS1a/b in testis. 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 derived from the CBA allele. These transcripts were not expressed in brain (data not shown). (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 a bar. (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 Mecp2-2/2 and Mecp2+/y 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.

![Figure 2. DNA methylation of miR-184 locus. DNA methylation status around the miR-184 locus. The methylation status of the 29 CpG sites (1–29) within a 1.5 kb region containing putative promoters for pri-miR-184 and antisense transcripts was assayed by bisulfite sequencing. Genomic DNA samples were extracted from adult brain and testis of BCF1 or CBF1 animals and subjected to a bisulfite sequencing analysis. Each clone was distinguished between the parental alleles by SNPs. But no SNPs were available for the amplified region, including the first to fifth CpG sites [shaded box with Pat (paternal allele)/Mat (maternal allele)]. Each row of circles represents the single cloned allele and each circle represents a single CpG site (open circle, non-methylated; filled circle, methylated).]
DISCUSSION

Both fragile X syndrome and Rett syndrome are commonly associated with autism spectrum disorders (ASD) and involve deficits in synaptic plasticity. Fragile X syndrome is caused by loss of FMRP (7), which was recently linked to the microRNA pathway (11–13). Rett syndrome patients have mutations in MeCP2 (15), which repress the expression of methylated genes (16). Thus, both the microRNA and DNA methylation pathways are known to be associated with ASD and are involved in synaptic plasticity. However, to date, there has been no evidence to link these two pathways. We have shown here that miR-184 undergoes activity-dependent expression regulated by MeCP2, which is involved in synaptic plasticity (Figs 3 and 4). Our finding helps to link the microRNA and DNA methylation pathways. Interestingly, a recent paper reported a brain-specific microRNA regulating dendritic spine development which is important for synaptic plasticity (34). It is intriguing to think that activity-dependent microRNAs that are regulated by MeCP2, such as miR-184, are associated with common phenotypes of fragile X and Rett syndromes, for example, autism and mental retardation, and are involved in synaptic plasticity. Further studies are required to assess the potential role of miR-184 in synaptic plasticity.

Bdnf also undergoes activity-dependent expression regulated by MeCP2 (27,28). BDNF plays important roles in neuronal survival (35), development (36) and plasticity (37). It is highly expressed in neurons, and transcription is upregulated dramatically by membrane depolarization in vitro (36,38,39) and by neuronal activity during kindling, induction of long-term potentiation and associative learning (40–42). BDNF might better explain the Rett syndrome-specific phenotype rather than fragile X syndrome. We demonstrated that the miR-184 coding transcript is imprinted and exclusively expressed from the paternal allele (Fig. 1D). Seitz et al. (43) also reported on microRNAs expressed from the maternal allele in another imprint region located on distal chromosome 12. A recent paper reports a high incidence of hepatocellular carcinoma (HCC) in adeno-associated virus-treated mice, with a high rate of integration of the virus genome adjacent to the genes encoding these imprinted microRNA located on distal chromosome 12 (44). The transcripts were upregulated in these HCCs. One of the interesting characteristics of imprinted genes is that many of them have roles in growth and behavior. Therefore, it is intriguing to consider that imprinted miR-184 may have a role in behavior.

The ASD comprise a complex group of behaviorally related disorders that are primarily genetic in origin. Involvement of epigenetic regulatory mechanisms in the pathogenesis of ASD has been suggested by the occurrence of ASD in patients with disorders arising from defects in key epigenetic regulatory factors FMRP (fragile X) and MeCP2 (Rett syndrome). Moreover, several reports offer supporting evidence that imprinted genes are involved in ASD. The most common recurrent cytogenetic abnormalities in ASD involve maternally derived duplication of the imprinted domain on chromosome 15q11–13 (45–47). In addition, parent-of-origin effects on sharing and linkages to imprinted regions on chromosome 15q and 7q are reported in ASD (48). Interestingly, some imprinted genes in these imprinted loci show abnormal expression in Rett syndrome. These imprinted genes are DLX5, located on 7q22 (29), and UBE3A and GABRB3, located on 15q11–13 (30). DLX5 regulates the production of enzymes that synthesize gamma-aminobutyric acid (GABA), and abnormal expression may alter GABAergic neuron activity in individuals with Rett syndrome. GABRA3 encodes the GABA receptor beta 3 subunit, and UBE3A encodes the ubiquitin ligase. Maternal deletion of UBE3A causes Angelman syndrome, which shares overlapping clinical features with Rett syndrome, such as autism (49). It is interesting to think that imprinted microRNA such as miR-184 show dysregulated expression in ASD.

We demonstrated that the miR-184-coding transcript is imprinted and exclusively expressed from the paternal allele (Fig. 1D); however, 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, 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 \( \text{MECP2} \) 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...
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, NcI I, 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 NcI I. AS3 was amplified with primers AS-F2 and AS-R3 and treated with NcI I. 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 NcI I to distinguish the parental origin. The PCR product derived from the CBA allele was completely digested with NcI I. 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.

**ACKNOWLEDGEMENTS**

We thank Dr H. Ohnishi for technical advice on the culture of cortical neurons, and DNA Chip Research Inc. for technical assistance.

**Conflict of Interest statement**. None declared.

**FUNDING**

This work was supported in part by grants from the Japanese Science and Technology Agency (I.H.) and the Ministry of Education, Culture, Sports, Science and Technology of Japan (I.H.) and the Ministry of Health, Labour and Welfare of Japan (I.H.).

**REFERENCES**


