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Inhibition of miR-15a promotes BDNF expression and rescues dendritic maturation deficits in MeCP2-deficient neurons

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Abstract

In both the embryonic and adult brain, a critical step in neurogenesis is neuronal maturation. Deficiency of MeCP2 leads to Rett syndrome, a severe neurodevelopmental disorder. We have previously shown that MeCP2 plays critical roles in the maturation step of new neurons during neurogenesis. MeCP2 is known to regulate the expression of brain-derived neurotrophic factor (BDNF), a potent neurotrophic factor for neuronal maturation. Nevertheless, how MeCP2 regulates BDNF expression and how MeCP2 deficiency leads to reduced BDNF expression remain unclear. Here we show that MeCP2 regulates the expression of a microRNA, miR-15a. We find that miR-15a plays a significant role in the regulation of neuronal maturation. Overexpression of miR-15a inhibits dendritic morphogenesis in immature neurons. On the other hand, a reduction in miR-15a has the opposite effect. We further show that miR-15a regulates expression levels of BDNF, and exogenous BDNF could partially rescue the neuronal maturation deficits resulting from miR-15a overexpression. Finally, inhibition of miR-15a could rescue neuronal maturation deficits in MeCP2-deficient adult-born new neurons. These results demonstrate a novel role for miR-15a in neuronal development and provide a missing link in the regulation of BDNF by MeCP2.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

INTRODUCTION

In both the embryonic and adult brain, a critical step in neural stem cell differentiation and neurogenesis is neuronal maturation, which includes dendritic arborization, axonal growth, dendritic spine development, synaptogenesis, and neural circuitry integration. The seminal discovery of *MECP2* as the mutated gene behind most cases of Rett syndrome (RTT) brought epigenetic regulation center stage in neurodevelopmental research [1]. We and others have shown that MeCP2 plays important roles in the development of newborn neurons, particularly during neuronal maturation [2–6]. However, precisely how MeCP2 regulates neuronal maturation is not fully clear.

There have been great efforts to identify MeCP2 downstream effectors that mediate neuronal maturation. One known target of MeCP2 is BDNF. Extensive studies have shown that BDNF is a potent neurotrophic factor, and BDNF levels directly impact neuronal maturation [7]. However, although early data suggested that MeCP2 binds to the *Bdnf* gene promoter and represses BDNF expression [8, 9], both MeCP2-deficient mice and RTT patients, who also have reduced MeCP2, have lower BDNF protein levels; moreover, enhancing BDNF levels can alleviate neurological symptoms associated with MeCP2 deficiency [8–14]. That said, how MeCP2 regulates BDNF expression and how MeCP2 deficiency leads to reduced BDNF expression remain unclear.

MicroRNAs (miRNAs) are a large family of 20–22-nucleotide non-coding RNAs that are involved in numerous cellular processes [15, 16]. About 70% of detectable miRNAs are expressed in the brain, where half of them are either brain-specific or -enriched [16]. Many miRNAs can act locally at the neuronal dendritic spines and regulate dendritic patterning, spine morphogenesis, and synaptic plasticity. A widely known function of microRNAs is translational repression by targeting mRNA, which results in either reduced translation efficiency or cleavage of the target mRNAs [15, 17, 18]. In fact, MeCP2 is regulated by miR-132 and miR-483 [12, 19]. The expression of miRNAs is regulated by complex mechanisms, including epigenetic regulation [16]. We and others have found that MeCP2 deficiency leads to both increased and decreased expression levels of miRNAs [20, 21], including miR-137, a miRNA important for neuronal maturation [6]. The functions of most MeCP2-regulated miRNAs remain largely unexplored.

Here we show that miR-15a, a miRNA upregulated in MeCP2-deficient neural stem cells and neurons, is a regulator of BDNF expression and neuronal maturation. High levels of miR-15a inhibit neuronal maturation by repressing BDNF. Inhibition of miR-15a using either a sequence-specific inhibitor (anti-miR) or sponge rescues neuronal maturation deficits in MeCP2-deficient neurons. Our work demonstrates a novel role for miR-15a in regulating neuronal differentiation, providing new mechanistic insight into the regulation of BDNF by MeCP2 in the context of RTT.

MATERIALS AND METHODS

More detailed methods are provided in the supplemental file.

Animals

All animal procedures were performed according to protocols approved by the University of Wisconsin Animal Care and Use Committee. Only male mice were used for experiments. Wild-type C57/B6 mice, MeCP2-floxed (*Mecp2^{f/y}* or *Mecp2^{f/f}*), and MeCP2 FLAG tag knock-in (*Mecp2^{FLAG}*) mice were used for *in vivo* and *in vitro* studies. The MeCP2-floxed mice used in this study were created previously [22]. The *Mecp2^{FLAG}* mice used for ChIP were published elsewhere [23].

Target prediction of miRNAs

We used an open access miRNA target prediction program (www.microRNA.org; August 2010 update) maintained by the Computational Biology Center at Memorial Sloan-Kettering Cancer Center. According to the program developer, "Target predictions are based on a development of the miRanda algorithm which incorporates current biological knowledge on target rules and on the use of an up-to-date compendium of mammalian microRNAs. The target sites predicted by miRanda are scored for likelihood of mRNA downregulation using mirSVR, a regression model that is trained on sequence and contextual features of the predicted miRNA::mRNA duplex. Expression profiles are derived from a comprehensive sequencing project of a large set of mammalian tissues and cell lines of normal and disease origin." We asked the program to show conserved miRNAs predicted to target human or mouse *Bdnf* mRNA. We set the filter for at least 7-mer seed sequence complementarity. We found 28 miRNAs targeting human *Bdnf* and 46 miRNAs targeting mouse *Bdnf*. Among them, 15 miRNAs were predicted to target both human and mouse *Bdnf*. We then compared this list of 15 miRNAs with published MeCP2-regulated miRNAs [20, 21, 24].

Chromatin immunoprecipitation and gene promoter-specific real-time PCR

ChIP was performed as described [23]. Briefly, cortex was dissected from 9–12-week-old *Mecp2^{FLAG}* mice and their WT littermates, diced into small pieces, and cross-linked in 1% formaldehyde, washed with ice-cold PBS, sonicated with Misonix Sonicator 3000 in lysis buffer (1% SDS, 10 mM EDTA, 20 mM Tris-HCl, pH 8.1, Roche protease inhibitor cocktail), and cleared by centrifugation to generate sheared chromatin. Dynabeads (Invitrogen) were pre-washed with PBS/BSA (0.5% BSA in PBS) and incubated with anti-FLAG antibody (Sigma) to form the bead/antibody complex. For immunoprecipitation, sheared chromatin was diluted in dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl, pH 8.1, 167 mM NaCl) and incubated with bead/antibody complex overnight on a Nutator at 4°C. The next day, the bead/antibody/chromatin complex was washed with washing buffer (50 mM Hepes, pH 7.6, 1 mM EDTA, 0.7% DOC, 1% NP-40, 0.5 M LiCl) and TBS. The immunoprecipitated chromatin was then eluted from the beads in elution buffer (1% SDS, 10 mM EDTA, 20 mM Tris-HCl, pH 8.1) and reverse cross-linked. Sheared chromatin not incubated with bead/antibody complex was processed the same way to generate input DNA. Both ChIP DNA and input DNA were treated with RNase A (Thermo Scientific) and proteinase K (Promega), purified by ethanol precipitation, and dissolved in water (Promega). Equal volumes of ChIP DNA and input DNA were used for each quantitative real-time PCR. Real-time PCR was performed on a StepOne Plus Real-Time PCR System (Applied Biosystems) using iQ SYBR Green Supermix (Bio-Rad). The

ChIP DNA level was normalized to the input DNA level using the $2^{-\Delta C_t}$ method. All ChIP primers were designed based on genomic DNA sequence and sequences of the primer pairs are provided in the Supplemental Methods.

Isolation of primary neurons and transfection of cultured neurons

Hippocampal or cortical neurons were isolated from wild-type and MeCP2-floxed P0 male mice and grown as described previously [6]. Hippocampal or cortical neurons were transfected on day 4 as they were undergoing dendritic and axonal morphogenesis during this time. Transfection was performed as described.

Bisulfite sequencing

DNA methylation analysis using bisulfite sequencing was performed as described [25]. Bisulfite conversion of the non-methylated cytosine (C) to uracil (U) nucleotides was performed by using EZ DNA Methylation™ Kit (Zymo Research), according to the manufacturer's instructions. The primers used for amplification of converted DNA are provided in the Supplemental Methods.

miR-15a promoter activity assays

Promoter activity analysis using luciferase reporter was performed as described [25, 26]. Briefly, A ~600 base pair genomic fragment upstream of miR-15a region was PCR amplified using Phusion High-Fidelity DNA Polymerase (NEB) and cloned into pISO firefly luciferase (Fluc) vector (Addgene) using In-Fusion® HD Cloning Kit (Clontech) according to the manufacturer's recommendations. To determine the impact of MeCP2 levels on miR-15a promoter activities, miR-15a promoter-Fluc vector together with renilla luciferase (Rluc) vector (serving as transfection efficiency control) were then co-transfected shMeCP2 or PCDH-MeCP2, as well as corresponding controls into Neuro2A cells using Lipofectamine 2000 (Life Technologies). Luciferase activities were analyzed at 48 hours after transfection using dual luciferase assays (Promega) and miR-15a promoter activities were calculated by Fluc readings normalized to Rluc readings. The detailed cloning procedure, primers used, and transfection methods are provided in the Supplemental Methods.

Production of lentivirus and infection of cortical neurons

Lentivirus were produced as described previously [27, 28]. Lentiviral infection of cortical neurons was carried out as described previously [6]. Briefly, cortical neurons from one pup were plated into 4 wells of a 6-well plate. On day 4 *in vitro* (4 DIV), MeCP2 shRNA lentivirus and shRNA-negative control lentivirus were added to the culture media. At 7 DIV, lentivirus-infected primary cortical neurons were washed with PBS and lysed in 1 mL TRIzol (Ambion, Life Technologies). RNA extraction was performed according to the manufacturer's instructions. To study the effects of miR-15a inhibitor on BDNF protein level in cultured MeCP2-deficient neurons, 2 μ l of lentivirus Cre-GFP or lentivirus dCre-GFP were added to the MeCP2-floxed cortical neurons (DIV4) cultured in Neurobasal A medium (Invitrogen) supplemented with 25 nM glutamate, 0.5 mM L-glutamine, and 1% antibiotics one day after plating. After 24 h, the medium was replaced with 2 μ l virus and

medium described above and incubated for an additional 24 h. Infected neurons were transfected with miR-15a inhibitor or miR-C inhibitor as control; 48 h later the cortical neurons were collected in cell lysis buffer for ELISA assay.

Pri-miRNA and mature miRNA expression analysis

Real-time PCR was carried out using our published method [29]. Design and sequences of PCR primers are provided in Supplemental Methods.

Nucleic acid and expression constructs

Control miRNA (miR-Con), miR-15a, anti-miR-15a, and anti-miRNA control (anti-miR-Con) were purchased from GenePharma (Shanghai, China). Lenti-Cre and Lenti-dCre vectors were provided by Dr. Lu Chen (Stanford University) [30]. Mammalian expression vector (pCDNA3) expressing mouse MeCP2 coding sequence was a gift from Dr Qiang Chang (University of Wisconsin-Madison). SureSilencing shRNA vectors expressing shRNA against Mecp2 as well as GFP were purchased from Qiagen.

Morphological analysis of transfected neurons

Low transfection efficiencies (1–2% neurons) permit imaging and quantification of single GFP-expressing neurons. Transfected neurons were fixed with 4% paraformaldehyde for 30 min, then washed with Dulbecco's Phosphate-Buffered Saline, pH 7.4 (DPBS) for 30 min for morphological analysis. GFP-expressing neurons were imaged with an Olympus BX51 microscope with 20x lens, a motorized stage, and digital camera. Dendritic traces were performed in real time using Neurolucida (MicroBrightField, Inc.) image analysis software. Data were extracted for Scholl analysis and total dendritic length of each GFP+ neuron.

BDNF ELISA assay

BDNF protein levels were determined using a Promega BDNF ELISA kit (cat # G7610) according to the manufacturer's instructions. Data from each experiment were first normalized to the average of all data points before comparing among different experimental conditions.

Viral vectors

Retro-GFP-2A-Cre and Retro-GFP-2A-dCre were cloned by using Retro-CAG-RFP [31] as backbone. RFP coding sequence was removed, and two new restriction sites, SmaI and MluI, were added using NotI and BamHI restriction sites. GFP-2A-Cre and GFP-2A-deltaCre were generated separately by using overlapping PCR, and then were inserted into the backbone through SmaI and MluI restriction sites. Both the constructs were confirmed by sequencing.

Retro-miR-15a-sponge-RFP and Retro-miR-15a-sponge-RFP control were cloned by using Retro-CAG-RFP as the backbone. The miR-15a sponge was designed using a bulge design method based on a published paper [32, 33]. Briefly, we constructed miR-15a-sponge into CAG-RFP vector by inserting 6 tandem arrayed miR-195 binding sites into the NotI site located in the immediate 3'UTR of RFP. Binding sites for miRNA-15a were complementary in the seed region, with a bulge at positions 9–12 to prevent RNA interference-type cleavage

and degradation of the sponge RNA. The construct with reversely inserted miR-15a sponge was used as a sponge control (Retro-sponge Control). The sequences of created constructs were confirmed by sequencing.

***In vivo* retroviral grafting and rescue of MeCP2 deficiency**

Retrovirus production was performed as described previously [26–29, 32, 34]. *In vivo* retroviral grafting was performed based on published methods with modifications [28, 29, 32, 34]. Confocal imaging analysis was carried out as described [28, 29, 32, 34]. 200- μ m thick floating brain sections containing eGFP+ or RFP+ cells were selected. For dendritic branching analysis, fluorescent protein (FP)-positive (GFP+ or RFP+) cells were imaged on an ApoTome confocal with a 20x objective. The dendrites and the cell body of single expression of FP+ neurons were analyzed by Neurolucida software (MicroBrightField, Inc.). Roughly 30–50 neurons per DG were traced. Data were extracted for Scholl analysis, total dendritic length for each GFP+ neuron, and both GFP+ and RFP+ neurons.

Statistical analysis

Statistical analyses between two groups were performed using unpaired, two-tailed Student's t-test (when there was no normalization) or paired t-test (when experimental groups were normalized). Statistical analyses among multiple groups were carried out using one-way ANOVA with either post-hoc Bonferroni or post-hoc Dunnett corrections. The data bars and error bars indicate mean $\pm$ standard error of the mean. (s.e.m). One-way ANOVA and t-test analysis were carried out using GraphPad software. Scholl analysis was carried out using a multivariate analysis of variance (MANOVA) using SPSS statistical software (SPSS version 17, SPSS Inc., Chicago, Ill, USA).

RESULTS

MeCP2 regulates miR-15a, a microRNA predicted to target BDNF

MeCP2 is known to repress the expression of BDNF by directly binding to its promoter [8, 9]; however, reduced expression of BDNF protein in the brain is a characteristic of both MeCP2-deficient mice and RTT patients [11]. How MeCP2 deficiency leads to reduced BDNF levels remains unclear. The 3' untranslated region (3'UTR) of both human and mouse *Bdnf* mRNAs contains sequences predicted to be targeted by miRNAs. Using open access miRNA target prediction tools (mircoRNA.org), we discovered 15 miRNAs predicted to target *Bdnf* mRNA in both species, so we named them "*Bdnf*-targeting miRNAs" (Figure S1). We and others have shown that MeCP2 regulates the expression of miRNAs [20, 21, 24] and among them there are three *Bdnf*-targeting miRNAs: miR-206, miR-495, and miR-15a. It has been shown that miR-206 regulates BDNF [35–38], whereas the functions of miR-495 and miR-15a are much less clear. Since the miR-15a family miRNAs have been implicated in human diseases [16, 39], we decided to focus on miR-15a as a potential MeCP2-regulated miRNA capable of modulating BDNF expression.

We next proceeded to explore whether MeCP2 directly regulates the expression of miR-15a. The biogenesis of miRNAs starts with transcription of primary miRNAs (pri-miRNAs) from the genome [16]. The primary miR-15a (pri-miRNA) is expressed from a single-copy

genomic sequence located on mouse chromosome 14, which is 20 kb away from the nearest protein-coding gene, *Kcnrg*, but within the intron of a non-coding RNA gene, *Dleu2* [40]. The genomic region immediately surrounding miR-15a has a number of CpG dinucleotides with a nearby “AT-hook” sequence motif (A/T 4) that can facilitate MeCP2 binding [41]. To test whether MeCP2 interacts directly with genomic regions proximal to miR-15a, we employed chromatin immunoprecipitation (ChIP) using an anti-FLAG tag antibody on chromatin prepared from the cortex of *Mecp2^{FLAG}* knock-in mice [23] to evaluate the binding of MeCP2 from -5 kb upstream to +2 kb downstream [Figure 1A, Region 1 (R1)] of the miR-15a gene. Quantitative PCR of immunoprecipitated (IP) DNA demonstrated that MeCP2 was enriched on the genomic sites surrounding miR-15a in *Mecp2^{FLAG}* knock-in mice compared to WT mice without FLAG (Figure 1A and 1B). Since miR-15a was found to be co-regulated with *Dleu2* transcription in leukemia cells, we also performed ChIP on the upstream genomic region [Figure 1A, Region 2 (R2), -956 to -6492 kb] of *Dleu2* and found that MeCP2 also bound to this region, although at lower levels than the region flanking miR-15a (Figure S2).

Since MeCP2 is known to specifically bind methylated CpG sites (CpGs), we performed bisulfite sequencing analysis to assess the CpG methylation status in four selected genomic regions (Figure 1B, R1–R4) enriched with MeCP2. We found that CpGs in these regions are mostly methylated (Figure 1C), permissive for MeCP2 binding.

To further determine whether MeCP2 regulates the expression of pri-miR-15a, we performed both gain of function and loss of function assays. We found that acute knockdown of MeCP2 in mouse neurons using lentivirus expressing shRNA (Figures 1D, Figure S2A) led to increased pri-miR-15a expression (Figure 1E) while having no effect on the RNA levels of *Dleu2*, *Kcnrg2*, as well as *Bdnf* transcripts (Figure S2). On the other hand, overexpression of MeCP2 using lentivirus expression mouse MeCP2 coding sequence led to reduced pri-miR-15a expression (Figure 1F). We then cloned around 600 base pair sequence at the immediate 5' region of miR-15a (termed “miR-15a promoter” for simplicity) into a luciferase reporter and performed a classic promoter activity luciferase assay. We found that acute knockdown of MeCP2 led to enhanced miR-15a promoter activity whereas overexpression of MeCP2 led to reduced miR-15a promoter activity (Figure S2). Taken together, our data suggest that MeCP2 regulates miR-15a expression in developing neurons.

We next determined whether miR-15a regulates BDNF protein expression. Because miR-15a had a strong repressive effect on both firefly and *Renilla* luciferase independently of *Bdnf* sequence (data not shown), we chose to determine BDNF protein levels in primary neurons using enzyme-linked immunosorbent assay (ELISA) (Figure 2A). We found that overexpression of miR-15a led to reduced BDNF protein expression (Figure 2B, $n = 3$, $p < 0.01$), whereas inhibiting endogenous miR-15a led to enhanced BDNF protein expression (Figure 2C, $n = 3$, $p < 0.01$). Therefore, MeCP2-regulated miR-15a has a direct impact on BDNF expression in developing neurons.

MiR-15a regulates neuronal dendritic development

We next used both gain of function and loss of function approaches to investigate the impact of miR-15a levels on neuronal dendritic maturation in developing hippocampal neurons. To

overexpress miR-15a, we transfected neurons with synthetic miR-15a mimic RNA, together with an eGFP expression plasmid. Concurrently, we performed a loss-of-function assay by knocking down endogenous miR-15a using an anti-miR-15a 2'-O-methyl oligonucleotide. At 48 h post-transfection, transfected GFP⁺ neurons had clearly identifiable dendrites and axons (Figures 3A and 3B, Figure S3). We found that neurons transfected with synthetic miR-15a showed a 24.7% reduction in dendritic length (Figure 3C, $p = 0.028$) and reduced dendritic complexity (Figure 3D, $F_{1,100} = 10.797$, $p = 0.001$) compared with miR-Control-transfected neurons. On the other hand, neurons transfected with a specific inhibitor of miR-15a (anti-15a, Figure 3E and 3F, Figure S3) had a 25.3% increase in total dendritic length (Fig 3G, $p = 0.0076$) and dendritic complexity (Figure 3H, $F_{1,94} = 8.868$, $p = 0.004$) compared with control (anti-C)-transfected neurons. The total number of dendritic ends and nodes showed a similar trend of a reduction in neurons transfected with miR-15a and an increase in anti-15a-transfected neurons, although these differences did not reach statistical significance (data not shown). Therefore, high levels of miR-15a inhibit neuronal dendritic development.

Inhibition of miR-15a rescues MeCP2 deficiency-induced dendritic developmental deficits in hippocampal neurons

We have previously shown that MeCP2 mutant mice exhibit impaired maturation of developing neurons [4]. We further validated this finding by using a small inhibitory RNA (shMeCP2) to acutely knock down MeCP2 in developing hippocampal neurons. As expected, neurons transfected with shMeCP2 exhibit significantly reduced dendritic length and complexity compared with neurons transfected with control shRNA (shNC; Figure S4A and S4B). On the other hand, increased MeCP2 expression led to enhanced dendritic branching and complexity compared to controls (Figure S4C and S4D), consistent with published literature [42, 43].

Since MeCP2 deficiency leads to increased miR-15a, and a high level of miR-15a inhibits BDNF expression and neuronal maturation, we wanted to investigate whether inhibition of miR-15a could rescue MeCP2 deficiency-induced neuronal deficits in hippocampal neurons. To this end, we isolated primary hippocampal neurons from MeCP2-floxed neonate mice (*Mecp2^{f/y}* or *Mecp2^{f/f}*) [22] and transfected DNA vectors expressing either Cre recombinase to delete MeCP2 or inactive Cre (dCre) as a control. We confirmed that infection of lentivirus expressing Cre led to deletion of MeCP2 in these neurons (Figure S5), consistent with the literature [22]. We also co-transfected these neurons with either a miR-15a inhibitor (Anti-15a) or an inhibitor control (Anti-C, Figure 4). We first determined whether inhibition of miR-15a could rescue the BDNF levels in MeCP2-deficient neurons using ELISA analysis. We found that acute knockdown of MeCP2 led to reduced BDNF protein levels (Figure 4C, dCre+Anti-C vs. Cre+Anti-C $p < 0.05$), which is also consistent with the literature [11, 44]. Inhibition of miR-15a leads to a significant increase in BDNF levels in both control (Figure 4C, dCre+Anti-15a vs. dCre+Anti-C, $p < 0.01$) and MeCP2-deficient (Figure 4C, Cre+Anti-15a vs. Cre+Anti-C, $p < 0.01$) neurons. Most interestingly, inhibition of miR-15a in MeCP2-deficient neurons rescues BDNF protein levels (Figure 4C, dCre +Anti-C versus Cre+Anti-15a). Therefore, inhibition of miR-15a rescues BDNF levels in MeCP2-deficient neurons.

Next we determined whether inhibition of miR-15a could rescue MeCP2 deficiency-induced neuronal maturation deficits. Consistent with our previous results and the literature [4] and Figure S4, neurons with MeCP2 deletion (Cre+Anti-C) exhibit significantly reduced dendritic length and complexity compared to controls (dCre+Anti-C) (Figure 4D and 4E, Figure S6). Although miR-15a inhibition had no significant effect on control neurons (dCre +Anti-15a vs dCre+Anti-C), inhibition of miR-15a enhanced the neuronal dendritic length and complexity of MeCP2-deficient neurons to levels similar to wild-type controls (Cre +Anti-C vs. Cre+Anti-15a; $p < 0.05$). Therefore, inhibition of miR-15a rescues the neuronal maturation deficits of MeCP2-deficient neurons.

Inhibition of miR-15a rescues MeCP2 deficiency-induced dendritic developmental deficits in adult-born new neurons

In adult brains, new neurons undergo a neuronal developmental process that recapitulates the one during early development [45]. Ample evidence demonstrates that the molecular mechanisms regulating the generation and maturation of new neurons are highly conserved between embryonic and adult neurogenesis, and disruptions of these molecular pathways lead to neuronal developmental deficits that are characteristic of human disorders. We have shown before that MeCP2 deficiency leads to reduced maturation of developing neurons in the adult hippocampus [4], so we chose to investigate whether inhibition of miR-15a inhibitor could rescue MeCP2 deficiency-induced neuronal deficits in hippocampal neurons *in vivo*. We stereotactically injected a retrovirus expressing Cre recombinase and GFP (Retro-Cre-2A-GFP) together with a retrovirus expressing either *miR-15a-sponge* (and RFP; Retro-RFP-sponge) or sponge *control* (and RFP; Retro-RFP-control) into the dentate gyrus (DG) of MeCP2 floxed (*Mecp2^{f/y}*) adult male mice (Figure 5A). The retrovirus labeled only dividing neural progenitors and their differentiated progenies [4]. RFP marked neurons with miR-15a inhibition (miR-15a-sponge), whereas GFP indicated neurons with MeCP2 deletion (Figure 5B). A retrovirus expressing inactive Cre recombinase (dCre) and GFP (Retro-dCre-2A-GFP) was used as a control for MeCP2 deletion. At 4 weeks post-viral injection, neuroprogenitors infected by retroviruses had differentiated into mature neurons (Figure 5B). Quantitative assessment showed that neurons with MeCP2 deficiency exhibit reduced dendritic length (Figure 5C) and complexity (Figure 5D) compared to controls (Figure S7), and inhibition of miR-15a by sponge rescues the dendritic length (Figure 5E) and complexity (Figure 5F) of MeCP2-deleted neurons (Figure S7). Therefore, inhibition of miR-15a rescues the neuronal maturation deficits of MeCP2-deficient neurons *in vivo*.

BDNF rescues miR-15a-induced dendritic maturation deficits

We next determined whether exogenous BDNF could rescue dendritic developmental deficits resulting from miR-15a overexpression. We transfected primary hippocampal neurons with either miR-15a or miR-C and treated these neurons with BDNF. As expected, BDNF treatment led to enhanced dendritic length and complexity in both control (miR-C-transfected) and experimental (miR-15a-transfected) neurons (Figure 6 and Figure S8). More importantly, BDNF treatment led to significant increases in both dendritic length (Figure 6C, $p < .05$) and dendritic complexity (Figure 6D, $F_{1, 58} = 67.564$; $P < 0.001$) in miR-15a-overexpressing neurons versus vehicle treatment. Therefore, BDNF can rescue the neuronal maturation deficits that result from miR-15a overexpression.

Taken together, our data suggest that MeCP2 regulation of miR-15a expression plays an important role in the modulation of BDNF levels in developing neurons, which has a profound impact on neuronal maturation (Figure 7). Inhibition of miR-15a may therefore make a plausible therapy for MeCP2 deficiency.

DISCUSSION

Previous studies have implicated both MeCP2 and BDNF in neurogenesis and neuronal maturation. Although MeCP2 is known to regulate BDNF expression, the mechanism behind this is not fully clear. Here we demonstrate that MeCP2 regulation of miR-15a has a significant impact on BDNF expression and neuronal maturation, which means miRNAs could be the missing link between MeCP2 regulation of BDNF levels in developing neurons during the process of neuronal development.

MeCP2 regulation of miRNA

Extensive studies have shown that miRNAs are involved in numerous cellular processes, including stem cell proliferation and differentiation. Recent scientific advances point to the important roles of small non-coding RNAs, particularly miRNAs, in neuronal development [15, 16]. About 70% of detectable miRNAs are expressed in the brain, where half of them are either brain-specific or -enriched [16]. Many miRNAs, for example, miR-134, miR-138, miR-218, miR-9, and miR-132, can act locally at the neuronal dendritic spines and regulate dendritic patterning, spine morphogenesis, and synaptic plasticity. One widely known function of miRNAs is translational repression by targeting mRNA, resulting in either reduced translation efficiency or cleavage of the target mRNAs [15, 17, 18, 46–48]. In fact, MeCP2 itself is regulated by miR-132, miR-483, and miR-181 [12, 19, 49]. miRNAs can also enhance protein translation under certain conditions [50]. We have found that MeCP2 deficiency leads to both increased and decreased expression levels of miRNAs in neural progenitors undergoing neuronal differentiation [20]. One of these miRNAs, miR-137, indeed regulates several aspects of neuronal maturation [6].

Some microRNAs are known to be embedded within coding or non-coding genes and can be co-expressed and co-regulated with their host genes [51]. Although MeCP2 binds to upstream genomic regions of both miR-15a and its host *Dleu2*, a non-coding RNA with tumor suppressor potential, MeCP2 seems to only regulate the expression of miR-15a, not *Dleu2*, in developing neurons. While our finding seems to contradict what has been found in human leukemia cells, where these two genes are co-expressed (human 13q14) and co-regulated [40], we did not assess whether these two genes can be co-repressed or activated by other genes or stimuli. It is also possible that the regulation of miRNAs and their hosts is cell type-dependent.

Mechanisms regulating BDNF levels

Although BDNF is a known target of MeCP2 and MeCP2 was found to bind *Bdnf* gene promoter [8, 9], how MeCP2 regulates BDNF has not been clear. Our observations that MeCP2 deficiency led to reduced BDNF proteins expression without affect *Bdnf* mRNA levels underscores the importance of post-transcriptional regulation in modulating BDNF

levels in developing neurons. In addition to miR-15a, a number of other miRNAs also show sequence complementary to *Bdnf* mRNA and could regulate BDNF expression in the brain. Among these, miR-206 and miR-495 also exhibit altered expression in MeCP2 mutant neural tissues. miR-206 is known to regulate BDNF in several tissues, and upregulation of miR-206 is proposed as a mechanism that represses BDNF in a transgenic Alzheimer's model [35–38]; the function of miR-495 is less clear. BDNF can also regulate miRNA expression. For example, BDNF promotes miR-132 expression, which downregulates p250GAP, leading to axonal branching [52]. The fact that the effect of Anti-15a on mutant *Bdnf* 3'UTR was not abolished in our study (Figure 5E) suggests that other miRNAs may also regulate BDNF expression. It will be interesting to determine any synergistic effects on BDNF expression from multiple miRNAs in MeCP2 mutant neurons.

Extensive studies have shown that BDNF is a potent neurotrophic factor for neuronal maturation [7]. BDNF plays important roles in neuronal maturation in both cultured hippocampal neurons and adult new neurons [53, 54]. Reduced expression of BDNF protein in the brain is a characteristic of MeCP2-deficient mice and RTT patients, and exogenous or enhanced endogenous BDNF can alleviate neurological symptoms associated with MeCP2 deficiency (see recent review [55]). Thus, methods that can increase BDNF signaling in the brain could possibly be used as treatments for RTT. Unfortunately, the therapeutic utility of BDNF is hampered by its poor efficiency at crossing the blood-brain barrier. Several TrkB agonists have been tested in mouse models for this purpose [44] [56]; however, these small molecules are not BDNF itself, so their *in vivo* actions may not be the same as BDNF's. To date, we have lacked an effective method of elevating BDNF protein levels in the brain. Fortunately, there are a number of microRNAs known to regulate BDNF [57] [58], and our findings suggest that the manipulation of small miRNA levels could be a potential method for elevating BDNF levels in neurological conditions that involve reduced BDNF, such as RTT and Alzheimer's disease, among others.

Network for regulating the late stage of neurogenesis

In both developing and adult brains, new neurons must undergo the critical maturation process, which includes dendritic arborization, axonal growth, dendritic spine development, synaptogenesis, and neural circuitry integration. The formation of an appropriate neural network is a prerequisite for normal brain functions. Even seemingly mild alterations in neuronal maturation can exert a profound impact on brain development and function [59]. Therefore, this final stage of new neuronal production is regulated by complex molecular mechanisms, and impaired neuronal maturation is a shared pathology among many neurodevelopmental disorders, including autism and schizophrenia [6]. In adult brains, new neurons undergo a neuronal developmental process that recapitulates the one during early development [45]. Ample evidence shows that the molecular mechanisms regulating the generation and maturation of new neurons are highly conserved between embryonic and adult neurogenesis, and disruptions in these molecular pathways lead to neuronal developmental deficits characteristic of human disorders. For example, deficiency in schizophrenia-related DISC1 is found to impair the neuronal dendritic maturation and functional integration of adult-born new neurons [60, 61]. We have also demonstrated that MeCP2 deficiency leads to impaired neuronal maturation, dendritic complexity, and

dendritic spine density of adult-born new neurons [4–6]. Interestingly, deficiency in either MeCP2 or DISC1 seems to have mild effects on neuronal maturation; however, the impact on neuronal network integration and brain function is profound. It is likely that many genes responsible for postnatal neurodevelopmental disorders, such as autism, are involved in the fine-tuning of neuronal maturation and integration.

Taken together, our data suggest that MeCP2 and miRNA form an important regulatory network that controls the levels of BDNF in new neurons. Deciphering such a mechanism represents a step towards unraveling the regulatory network that underlies brain plasticity, which brings us closer to an eventual therapy for brain disorders.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

MeCP2 regulates miR-15a expression. (A) A schematic drawing of the genomic region on mouse chromosome 14 containing miR-15a and its host non-coding gene, *Dleu2*, as well the nearest coding gene, *Kcnrg*. The *Kcnrg* gene overlaps with the *Dleu2* gene, but *Kcnrg* mRNA is transcribed in an opposite direction to *Dleu2* and miR-15. Black arrows indicate genomic regions encoding RNA transcripts and their directions of transcription from the genome. Arrows indicate the beginnings of pri-miR-15a (red) and *Dleu2* (yellow) transcripts. The 8-kb region proximal to the miR-15a gene (Region A) and the 7-kb region

proximal to the start of the *Dleu2* gene (Region B) were assayed in chromatin immunoprecipitation (ChIP). The locations of each set of primers for ChIP-qPCR analysis are indicated. **(B)** The enrichment of MeCP2 protein at genomic sequence between 5 kilobases (kb) upstream and 2 kb downstream of the miR-15a locus in *Mecp2^{FLAG}* mouse brains, as assessed by ChIP using an anti-FLAG tag antibody, followed by qPCR. ChIP of wild-type (WT) mice (without FLAG) were used as negative control. Relative enrichment of IP in either FLAG knock-in mice or WT control samples was calculated relative to their corresponding input (n = 3). Red arrow indicates the beginning of pri-miR-15a transcript. The green bars indicated the 4 genomic regions (R1–R4) subjected to bisulfite sequencing analyses in C. **(C)** Results of bisulfite sequencing analyses showing all methylated CpGs (black circles) and unmethylated CpGs (open circles) in the 4 genomic regions analyzed. Each line represents one DNA clone analyzed for that genomic region. There are total 11 CpGs in the genomic region analyzed (R1–R4). **(D)** Sample images showing primary cortical neurons infected with lentivirus expressing a small hairpin inhibitory RNA against MeCP2 (lenti-shMeCP2), as well as GFP. Scale bar = 250 μ m. **(E)** Acute knockdown of MeCP2 by lenti-shMeCP2 in cortical neurons resulted in increased expression of primary transcript for miR-15a (pri-miR-15a); (n = 3, p = 0.023, Student's t-test). **(F)** Acute overexpression of MeCP2 by lenti-MeCP2 in cortical neurons resulted in increased expression of primary transcript for miR-15a (pri-miR-15a). (n = 6, p = 0.036, Student's t-test).

Figure 2.

miR-15a regulates BDNF protein levels in neurons. (A) Schematic drawing of a timeline of the experiment. Primary cortical neurons transfected with either miR-15a mimic (miR-15a) or control (miR-C) at day 4 *in vitro* (DIV). Neuron cell lysates were collected on 7DIV, and BDNF levels were determined by enzyme-linked immunosorbent assay (ELISA), (B) miR-15a overexpression led to reduced BDNF protein expression in neurons ($n = 3$, $p < 0.01$, student t-test). (C) Inhibition of endogenous miR-15a led to increased BDNF protein expression in neurons ($n = 3$, $p < 0.01$, student t-test). **, $p < 0.01$.

Figure 3.

MiR-15a regulates neuronal maturation. (A) Representative images of primary hippocampal neurons transfected with either miR-15a mimic (miR-15a) or control (miR-C). An expression plasmid for GFP was co-transfected for visualization. Scale bar = 50 μm (B) Sample traces of GFP-expressing neuron shown in (A). (C) Neurons overexpressing miR-15a had reduced total dendritic length ($n = 5$, $p = 0.028$, Student t-test). (D) Sholl analysis showing neuronal dendritic complexity (Number of Intersections: $F_{1,100} = 10.797$, $p = 0.001$, MANOVA). (E) Representative images of primary hippocampal neurons transfected with either miR inhibitor for miR-15a (Anti-15a) or control (Anti-C). An expression plasmid for GFP was co-transfected for visualization. Scale bar = 50 μm . (F) Sample traces of GFP-expressing neurons shown in (E). (G) Neurons with miR-15a inhibition had increased total dendritic length ($n = 5$, $p = 0.0076$, Student t-test). (H) Sholl analysis showing neurons with miR-15 inhibition had greater dendritic complexity; $F_{1,94} = 8.868$, $p = 0.004$, MANOVA.

Figure 4.

Inhibition of miR-15a rescues neuronal development caused by Mecp2 mutation *in vitro*.

(A) Representative images of primary hippocampal neurons isolated from MeCP2-floxed mice co-transfected with either Cre-2A-GFP plasmid or dCre-2A-GFP, together with either anti-miR control (Anti-C) or anti-miR-15a (Anti-15a). Scale bar = 50 μm (B) Sample traces of GFP-expressing neurons shown in (A). (C) MeCP2-deficient neurons (Cre-transfected) had reduced BDNF protein levels compared to control (dCre+Anti-C v.s Cre+Anti-C, $p < 0.05$, $n = 4$, one-way ANOVA with Dunnett's multiple comparison test), but this difference was rescued by inhibition of miR-15a (dCre+Anti-C v.s Cre+Anti-15a, $p < 0.01$, $n = 4$, one-way ANOVA with Dunnett's multiple comparison test). In MeCP2-deficient neurons, inhibition of miR-15 rescued BDNF protein levels (Cre + Anti-C vs. Cre + Anti-15a, $p < 0.001$, $n = 4$, one-way ANOVA with Bonferroni's post hoc test). (D) MeCP2-deficient neurons had reduced total dendritic length compared to control (dCre+Anti-C v.s Cre+Anti-C, $n = 5$, $p < 0.05$, one-way ANOVA with Bonferroni's post hoc test), but this difference was rescued by inhibition of miR-15a (dCre+Anti-C v.s Cre+Anti-15a, $p > 0.05$, ns. $n = 5$, one-way ANOVA with Bonferroni's post hoc test). In MeCP2-deficient neurons, inhibition of miR-15 rescued BDNF protein levels (Cre + Anti-C vs. Cre + Anti-15a, $p < 0.05$, $n = 5$,

one-way ANOVA with Bonferroni's post hoc analysis). **(E)** Sholl analysis showing neurons with miR-15a inhibition had enhanced dendritic complexity compared to control (Cre + Anti-C v.s. Cre + Anti- 15a, $F_{1,76} = 18.555$, $p < 0.001$. MANOVA). *, $p < 0.05$).

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Figure 5.

Inhibition of miR-15a rescues neuronal development in MeCP2-deficient neurons *in vivo*. (A) A schematic illustration of retroviral vectors expressing Cre recombinase (or control inactive Cre or dCre) as well as GFP (Retro-Cre-2A-GFP) and retroviral vectors expressing miR-15a sponge (or sponge control) as well as RFP (Retro-RFP-sponge). A timeline of the *in vivo* labeling of newborn neurons in the DG experiment is shown. (B) Representative confocal images of retroviral infected new neurons in the adult DG at 4 weeks post-viral injection. Scale bar = 50 μ m. (C) MeCP2-deficient (Cre-infected) neurons show reduced

dendritic length compared to controls (dCre infected); (n = 25 neurons/condition, p= 0.048, t-test). **(D)** MeCP2-deficient (Cre-infected) neurons show reduced dendritic complexity compared to controls (dCre infected) (# of Intersections, dCre vs. Cre, $F_{1,50} = 5.111$, p= 0.028, MANOVA). **(E)** Inhibition of endogenous miR-15a by overexpression of miR-15-sponge (sponge) rescued the dendritic length deficit resulting from MeCP2 deficiency (Cre + Control, n = 45 neurons vs. Cre + Sponge, n = 21 neurons, p = 0.0383, t-test). **(F)** Inhibition of endogenous miR-15a by overexpression of miR-15-sponge (sponge) rescued the dendritic complexity deficit that resulted from MeCP2 deficiency (Cre+ Control vs. Cre +Sponge, $F_{1,53} = 5.691$, P = 0.021, MANOVA). *, p < 0.05).

Figure 6.

Exogenous BDNF rescues neuronal development deficits caused by miR-15a overexpression. **(A)** Representative images of primary hippocampal neurons transfected with either miR-15a mimic (miR-15a) or control (miR-C), and treated with vehicle (Veh) or BDNF. An expression plasmid for GFP was co-transfected for visualization. Scale bar = 50 μm **(B)** Sample traces of GFP-expressing neuron shown in (A). **(C)** Exogenous BDNF rescued total dendritic length resulting from miR-15a overexpression ($n = 4$, one-way ANOVA with Dunnett's multiple comparison test). In miR-15a-overexpressing neurons, BDNF rescued the dendritic length deficit (miR-15a + Veh vs. miR-15a + BDNF, $p = 0.013$, $n = 4$, one-way ANOVA with Bonferroni's correction. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). **(D)** Exogenous BDNF rescued the dendritic complexity resulting from miR-15a overexpression (miR-15a+Veh vs. miR-15a+BDNF, $F_{1, 58} = 67.564$, $p < 0.001$, MANOVA).

Figure 7.

Model for the role of miR-15a in mediating MeCP2 regulation of BDNF in neuronal maturation. MeCP2 regulates the expression of miR-15a in neurons. MeCP2 deficiency leads to increased expression of miR-15a, which inhibits BDNF expression and represses neuronal dendritic development. On the other hand, inhibition of endogenous miR-15a levels promotes BDNF expression and can rescue MeCP2 deficiency-induced neuronal maturation deficits.[9]