Role of miR-132/methyl-CpG-binding protein 2 in the regulation of neural stem cell differentiation

Abstract

Methyl-CpG-binding protein 2 (MeCP2) is a well-known transcription repressor, and mutations in MECP2 cause serious neurological disorders. Many studies have suggested that MeCP2 is involved in neural maturation only, and have not reported its role in neural stem cell differentiation. In the present study, we investigated this possible role of MeCP2 in neural stem cells. We used two different differentiation methods to explore how MeCP2 influences neural stem cell differentiation. When we transfected MeCP2-overexpressing lentivirus into neural stem cells, astrocytic differentiation was impaired. This impaired astrocytic differentiation occurred even in conditions of 20% fetal bovine serum, which favored astrocytic differentiation. In addition, miR-132 had the largest expression change after differentiation among several central nervous system related miRNAs. A luciferase assay confirmed that miR-132 directly targeted MeCP2, and that miR-132 was able to reduce MeCP2 expression at both the RNA and protein levels. The upregulation of miR-132 by miRNA mimics promoted astrocytic differentiation, which was fully recovered by MeCP2 overexpression. These results indicate that miR-132 regulates cell lineage differentiation by reducing MeCP2. The study was approved by the Ethics Committee of Shanghai Tenth People’s Hospital of Tongji University, China (approval No. SHDSYY-2018-4748) on March 10, 2018.

Key Words: astrocytes; factor; model; pathways; stem cell

Introduction

During the early stages of central nervous system (CNS) development, quiescent neural stem cells (NSCs) are activated to proliferate, and the stem cell pool constantly grows. In the later stages, the proliferative signals are reduced, and neurogenic and astrogliogenic signals are turned on. Driven by cues that remain elusive, NSCs start to differentiate into neurons, astrocytes, and oligodendrocytes; these cells are generated sequentially and are temporally orchestrated (Sun et al., 2017; Wang et al., 2017). This transition (or cell fate determination) is co-regulated by both cell niche and intrinsic cues (Juliandi et al., 2010; Vieira et al., 2018). The cell niche, where the cell resides, is composed of extracellular matrix, adhesions, cytokines, blood vessels, and signaling pathways (for example, Janus kinase, signal transducer, and transcription activator pathways). The intrinsic cues mainly include epigenetic regulation (Honda et al., 2018).

Methyl-CpG-binding protein 2 (MeCP2) belongs to the methyl-CpG-binding domain protein family, and normally acts as a repressor of transcription by binding methylated DNA (Cheng and Qiu, 2014). MeCP2 is predominantly expressed in the CNS, especially in neurons, and is involved in neural development. Mutations in MeCP2 cause serious neurological disorders, such as Rett syndrome (Liu et al., 2017). Most studies have proposed that the main function of MeCP2 is to regulate neural maturation, rather than cell specification (Kishi and Macklis, 2004; Smrt et al., 2007; Du et al., 2016). However, the role of MeCP2 in neural precursors has not yet been fully studied. In the present study, we investigated how MeCP2 influences NSC lineage specifications and explored its upstream regulator, miR-132.

Materials and Methods

Cell culture and differentiation

Primary NSCs were extracted from the spinal cord of female C57BL/6 mice (specific-pathogen-free level, provided by Shanghai Tongji Biomedical Technology Co., Ltd., Shanghai, China, license No. SYXX (Hu) 2018-0034) on embryonic 14 day (E14d). The embryonic mice protocols and all operations were approved by the Ethics Committee of Shanghai Tenth People’s Hospital of Tongji University, China (approval No. SHDSYY-2018-4748) on March 10, 2018.
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Involving animals in our research were approved by the Ethics Committee of Shanghai Tenth People’s Hospital of Tongji University, China (approval No. SHDSYY-2018-4748) on March 10, 2018. The procedure of NSC extraction was as follows. The fetal mouse spinal cord was quickly removed and placed in pre-cooled D-Hanks fluid. The meninges were stripped and the spinal cord was cut into pieces using ophthalmic scissors. The tissue was then transferred to a centrifuge tube and centrifuged at 1000 r/min for 5 minutes. Next, the supernatant was removed and added to NSC growth medium containing Dulbecco’s modified Eagle medium/nutrient mixture F-12, 1% B27, and 20 ng/mL basic fibroblast growth factor and epidermal growth factor, at 37°C in 5% CO2. The NSCs were adjusted to a density of 5 × 10^6/mL and were grown to shape a neurosphere on day 5. On day 7, the cells reached their highest density, and passaging was required. The identification of NSCs was performed using immunofluorescence staining with Nestin.

The spheres were then mechanically isolated to prepare single-cell suspensions, and were incubated in differentiation medium without basic fibroblast growth factor or epidermal growth factor. Neural differentiation was induced in the cells using 1% fetal bovine serum (FBS), while astrocytic differentiation was induced using 20% FBS. The differentiated cells were identified using immunofluorescence staining (class III beta-tubulin (TuJ1) for neuronal cells, Olig2 for oligodendrocytes, and glial fibrillary acidic protein (GFAP) for astrocytic cells).

Cell transfection

The miR-132 mimics (50 nM) and mimic controls (25 nM) were purchased from Life Technologies (Carlsbad, CA, USA). The transfection procedures that were used followed the Lipo3000 protocol (Thermo Fisher Scientific, Inc., Waltham, MA, USA). At 6 hours after transfection, the Dulbecco’s modified Eagle medium/nutrient mixture F-12 containing 1% B27 was replaced to reduce the cytotoxicity of the Lipo3000 reagent (Thermo Fisher Scientific, Inc.). Quantitative polymerase chain reaction was performed to check the transfection efficiency.

Construction of the MeCP2 overexpression lentivirus

The MeCP2 overexpression plasmid was purchased from Genechem (Shanghai, China). The lentivirus packaging procedures were as follows. For the MeCP2 group, we added 1 μg MeCP2 plasmid (1 μg/μL), 0.75 μg psPAX2, and 0.25 μg pMD2.G (Promega Corporation, Madison, WI, USA) to OPTI-MEM (Thermo Fisher Scientific, Inc.) (total volume 20 μL). For the transfection reagent master, we added 6 μL FuGENE®6 (Promega Corporation) to OPTI-MEM (total volume 20 μL). We then added the FuGENE®6 master to the plasmid solution and incubated the solution for 20 minutes. Next, we added the solution to 293T cells (Central Laboratory of Shanghai Tenth People’s Hospital, Shanghai, China) and incubated the mixture at 37°C for 48 hours. For the control group, we made a transfection efficiency.

Western blot assay

The protein samples were extracted from the control and MeCP2 groups using a radioimmunoprecipitation assay buffer, and were quantified using bichinchoninic acid protein assays (Sigma-Aldrich, St. Louis, MO, USA) (Diamandis and Christopoulos, 1991). Usually, 20 μg protein was mixed with the loading buffer to a total volume of 15 μL. The 10% gels with 10 wells were made according to standard protocol. The samples and 5 μL marker were loaded and separated using 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis and blotted onto polyvinylidene fluoride membranes (100 V for 2 hours). The polyvinylidene fluoride was blocked with 5% fat-free milk for 1 hour at room temperature. The membranes were incubated with primary antibody (rabbit anti-MeCP2, rabbit anti-GFP, and rabbit anti-B-actin; all 1:1000; Abcam, Cambridge, UK) solution overnight at 4°C. Next, the membranes were incubated at room temperature for 1 hour with goat anti-rabbit IgG (1:5000; Jackson Immuno Research Laboratories, West Grove, PA, USA). The membranes were then washed before being immersed in enhanced chemiluminescence solution (A + B; Sigma-Aldrich) for 5 minutes. The optical densities of the protein bands were analyzed using Image Lab software 3.0 (Bio-Rad Laboratories, Hercules, CA, USA).

Immunofluorescence staining

The cells were mechanically separated to single-cell suspensions. Cell densities were adjusted to 1 × 10^6/mL, and 1 mL cell suspension and 1 mL medium were added to 24-well plates with pre-installed glass and incubated for 3 days. The cells were fixed at room temperature for 30 minutes with 4% paraformaldehyde. 0.5% Triton X-100 was used for membrane penetration. The cells were blocked for 1 hour with 10% bovine serum albumin. Next, cells were incubated with primary antibody (rabbit anti-Nestin, rabbit anti-B-actin, rabbit anti-beta III tubulin, rabbit anti-GFP, and rabbit anti-Olig2; all 1:1000; Abcam) solution at room temperature for 6 hours, and were then blocked in 10% bovine serum albumin for 1 hour. Goat anti-rabbit secondary antibody (1:1000; Abcam) solution was then added, and cells were incubated for 1 hour at room temperature. Cell nuclei were stained with 4′,6-diamidino-2-phenylindole (Abcam) at room temperature for 30 minutes. The wells were then sealed with glass for fluorescent microscopic observation. Images were quantified using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Real-time polymerase chain reaction

Total RNA was extracted following the Trizol protocol (Behera et al., 2002). RNA quantification was performed using a NanoDrop™ 2000 Spectrophotometer (Thermo Fisher Scientific, Inc.). RNA reverse transcription was performed using TaqMan MicroRNA Assays (Thermo Fisher Scientific, Inc.). The cDNA product was mixed with TaqMan® Universal Master Mix II and into the matched TaqMan® MicroRNA Assay (Thermo Fisher Scientific, Inc.) to run the quantitative polymerase chain reactions. The primer sequences were as follows: miR-132 PCR primers, sense: 5′-GGG GTA ACA GTC TAC AGC C-3′, antisense: 5′-CAG GTG TGT TCG TGA GT-3′; miR-124 PCR primers, sense: 5′-AAG CTC CGG AGG GCA CGC GGT GA-3′, antisense: 5′-TTC CTG GTT GGT GGA GTC G-3′; miR-128 PCR primers, sense: 5′-TGA GCT GTT GGT GGA TTC TCG ACC-3′, antisense: 5′-GGG ACA GCA AGG TCA TAC TCA TAC GGT G-3′; miR-381 PCR primers, sense: 5′-AGT CCA TAC ATG GGC AAG CTC TC-3′, antisense: 5′-GCA AAT AAC CAT TCA TAC ATG G-3′; miR-9 PCR primers, sense: 5′-CTC CGG AGG GCA TGG GGA TCC CAG-3′, antisense: 5′-GC CTC CTT GAT CCA GTA AA-3′; U6 PCR primers, sense: 5′-CGT TCG GCA GCA GCA AA-3′, antisense: 5′-AAC GCT TGA ATG TGC GT-3′. U6 was used as the internal reference gene for normalization using the 2−ΔΔCt method (Livak and Schmittgen, 2001).

Statistical analysis

Data are presented as the mean ± standard deviation (SD)
significant difference.

Results

NSC proliferation and cell specification induced by FBS

NSCs isolated from E14d mouse spinal cords were grown in growth medium and gradually formed neurospheres. These cells expressed Nestin, which was identified using immunofluorescence. Ki67 and Nestin double-stained NSCs represented the active proliferating cells (Figure 1A). After withdrawing basic fibroblast growth factor and epidermal growth factor, NSC differentiation was induced by different concentration of FBS (1% or 20%). The immunofluorescence results demonstrate that 1% FBS generated more neurons (Figure 1B), while 20% FBS produced more astrocytes (Figure 1C). There was no clear difference in oligodendrocyte differentiation between the 1% and 20% FBS treatments (Figure 1D).

miR-132/MeCP2 regulate astrocytic differentiation

To investigate whether MeCP2 was involved in cell specification in NSCs, we first used 20% FBS to induce astrocytic differentiation. MeCP2 gradually decreased when cells were induced to differentiate (Figure 2A), and NSCs that expressed high levels of MeCP2 gave rise to fewer GFAP-positive astrocytes ($P < 0.01$; Figure 2B and C), suggesting that MeCP2 blocks astrocytic differentiation. MeCP2 is a transcription repressor, and may therefore inhibit astrocyte-specific gene transcription to interfere with this process. To investigate how MeCP2 might be regulated, we explored its upstream regulator. We evaluated several CNS-related miRNA profiles following the 20% FBS treatment, and miR-132 had the largest change ($P < 0.05$; Figure 2D). miR-132 was increased during astrocytic differentiation and reached its highest levels at in vitro day 7 (Figure 2E). Even when 1% FBS was added to induce neuronal generation, NSCs transfected with miR-132 mimics were more likely to differentiate into astrocytes compared with control NSCs ($P < 0.001$; Figure 2F).

miR-132 is able to directly target the 3′ untranslated region of MeCP2 to regulate its expression

The findings reported in the previous section demonstrate that miR-132 and MeCP2 have opposite expression trends and functions in astrocytic differentiation. We next investigated the correlations between these two important regulators. miR-132 reduced the fluorescence intensity of MeCP2 ($P < 0.05$; Figure 3A) and lowered MeCP2 expression both at the mRNA and protein levels ($P < 0.05$; Figure 3B). A luciferase assay confirmed that complementary sequences exist between miR-132 and the 3′ untranslated region of MeCP2, because the relative luciferase activity changed in wild-type MeCP2 compared with its mutant counterpart (Figure 3C). GFAP was used as a marker of astrocytes (Raponi et al., 2007), and its expression reflected astrocyte numbers. Our results showed that miR-132-induced elevations in GFAP were able to be recovered by the co-transfection of miR-132 and MeCP2 ($P < 0.05$; Figure 3D). Furthermore, miR-132 increased the number of GFAP-positive astrocytes, and this increase was balanced by MeCP2 overexpression. These changes were confirmed by immunofluorescence ($P < 0.01$; Figure 3E). Together, these results demonstrate that miR-132 can directly target MeCP2 to regulate astrocytic differentiation.

Discussion

Cell fate determination is a complex process that involves both intrinsic and extrinsic factors (Jiao et al., 2017). MeCP2 is a well-known repressor of transcription, and functions as part of epigenetic regulation. Mutations in MECP2 are often found in patients diagnosed with Rett syndrome. These patients display mental retardation and have a progressive regression of acquired skills, suggesting that MeCP2 is vital for brain development (Liao et al., 2012; Zhang et al., 2016). However, the role of MeCP2 in spinal cord neuronal differentiation during the embryonic stages remains largely elusive. In the present study, we demonstrated that MeCP2 blocked GFAP expression and inhibited astrocytic differentiation. Moreover, MeCP2 was regulated by miR-132 in our study. Previous investigations have reached contradictory conclusions. Some studies have reported that MeCP2 is mainly involved in neural maturation and maintenance but has no effect on cell proliferation or differentiation because neural precursors can differentiate into functional neurons and glial cells to form neural networks in MeCP2 mutants (Samaco et al., 2009; Kifayathullah et al., 2010; Bertulat et al., 2012; Ballinger et al., 2019; Sun et al., 2019). However, other studies have demonstrated that ectopic MeCP2 expression in brain-derived neural precursor cells blocks astrocytic differentiation in mice (Tsujimura et al., 2009; Delépine et al., 2015). In addition, MeCP2 has been reported to bind to highly methylated regions of GFAP and suppress its translation. A loss of MeCP2 promotes astrocytic differentiation, with elevated GFAP.
miR-132+MeCP2

0 d  1 d   3 d  5 d   7 d

expression (Forbes-Lorman et al., 2014), and even NSCs derived from patients with Rett syndrome are inclined to differentiate toward an astrocytic lineage (Andoh-Noda et al., 2015). These previous findings are consistent with our findings in mouse E14d spinal-cord-derived NSCs.

To explore the upstream regulation of MeCP2, we investigated the expression changes of several CNS-related miRNAs before and after differentiation, including miR-124, miR-132, miR-128, miR-381, and miR-9. The change in miR-132 expression was the most obvious. Moreover, the trend of increased miR-132 expression after inducing astrocytic differentiation was in direct contrast with that of MeCP2. Furthermore, elevated miR-132 levels promoted astrocytic differentiation even under conditions that favored the induction of neuronal differentiation. A luciferase assay confirmed the complementary sequences between MeCP2 and miR-132, and miR-132 decreased MeCP2 levels and facilitated astrocytic differentiation. Some studies have proposed that the miR-132/MeCP2 pathway might be involved in numerous physiological and pathological processes, including neuronal maturation, cognitive dysfunction in chronic cerebral hypoperfusion, pain transmission, and bladder outlet obstruction (Sadegh et al., 2015; Zhang et al., 2015; Yao et al., 2017; Xie et al., 2019).

miR-132 is enriched in the CNS and is regarded as a neural-specific miRNA. miR-132 has been reported to promote neurite outgrowth in developing neurons, but negatively regulates postsynaptic protein expression (Yoshimura et al., 2016). One study revealed that miR-132 combines with MeCP2 and brain-derived neurotrophic factor to form a regulation loop, in which MeCP2 upregulates brain-derived neurotrophic factor, thus inducing miR-132 overexpression, which in turn lowers MeCP2 levels (Klein et al., 2007). In the current study, we found that miR-132 was involved in the specification of NPCs, and that a high expression of miR-132 reduced MeCP2 levels and facilitated astrocytic differentiation. In a previous study, the miR-132/C-terminal-binding protein 2 circuit was reported to regulate glial progenitor fate choice via Notch signals (Salta et al., 2014). In the current study, we confirmed that miR-132/MeCP2 reduced GFAP expression, leading to decreased astrocytic differentiation.

In conclusion, we have revealed a novel function of miR-132 and MeCP2 in neural precursor cells. As well as promoting the neuronal maturation of developing neurons, the miR-132/MeCP2 pathway is also involved in stem cell lineage specification. A drawback of our study is that it only explored astrocytic differentiation; therefore, whether miR-132/MeCP2 influences neuronal differentiation and the mechanisms by which this might occur require further investigation. The present study also lacked an in vivo study to investigate whether miR-132- or MeCP2-modified NSCs transplanted into an animal model of spinal cord injury might promote the recovery of neuronal function. However, our findings suggest that miR-132/MeCP2 are potential therapeutic targets for spinal cord injury and other related disorders. Transplanting MeCP2- or miR-132-modified NSCs into the injured spinal cord may produce more differentiated neurons and enhance neural regeneration.

Author contributions: Study design: DC, SHL; data collection and manuscript editing: ZW; statistical analysis: JL; manuscript writing: DC. All authors approved the final version of the manuscript.
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