Reciprocal regulation of autism-related genes MeCP2 and PTEN via microRNAs

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MeCP2 encodes a methyl-CpG-binding protein that plays a critical role in repressing gene expression, mutations of which lead to Rett syndrome and autism. PTEN is a critical tumor suppressor gene that is frequently mutated in human cancers and autism spectrum disorders. Various studies have shown that both MeCP2 and PTEN proteins play important roles in brain development. Here we find that MeCP2 and PTEN reciprocally regulate expression of each other via microRNAs. Knockdown of MeCP2 leads to upregulation of microRNA-137, which in turn represses expression of PTEN, thus PTEN would be down-regulated when MeCP2 is knockdown. Furthermore, we find that deletion of PTEN leads to phosphorylation of Serine 133 of CREB, then increases the expression of microRNA-132. miR-132 inhibits the expression of MeCP2 by targeting on the 3′UTR of MeCP2 mRNA. Our work shows that two critical disorders-related gene MeCP2 and PTEN reciprocally regulate expression of each other by distinct mechanisms, suggesting that rare mutations in various disorders may lead to dysregulation of other critical genes and yield unexpected consequences.

MeCP2 belongs to a family of methyl-CpG-binding proteins that regulate gene expression by DNA methylation via recruitment of histone deacetylases1-2. MeCP2 is indispensable for neural development, for example regulating expression of the brain-derived neurotrophic factor (BDNF) gene3. MeCP2 has demonstrated a key role in synaptic homeostatic plasticity4.5. The Mecp2-null mouse is a mouse model of Rett syndrome6, a severe form of autism-spectrum disorder7.

PTEN is a tumor suppressor gene that negatively regulates the phosphatidylinositol-3-kinase (PI3K)/AKT signaling pathway8, which in turn plays a critical role in regulating cell growth, survival, and proliferation. Abnormalities in PTEN lead to neurological and psychiatric disorders such as brain tumors, autism, macrocephaly, seizures, mental retardation, and schizophrenia9-11.

MicroRNAs (miRNAs) are 20–25-nucleotide-long, noncoding RNAs that modulate gene expression and development by post-transcriptionally targeting RNA-induced silencing complexes12. miRNAs have important regulatory functions in basic biological processes such as development, cellular differentiation, proliferation, apoptosis, and tumorigenesis12-14. The expression of miRNAs was shown to be altered in the brains of Mecp2-null mice15,16. Furthermore numerous miRNAs have been shown to regulate PTEN expression17-22.

In this study, Mecp2 knockdown repressed PTEN expression and increased AKT phosphorylation. Furthermore, the Mecp2-mediated effect on PTEN expression occurs via a mechanism involving miR-137. Interestingly, we also found that MeCP2 expression was down-regulated by PTEN short hairpin RNA. We further found that phosphorylation of Ser-133 of cyclic AMP-response-element-binding-protein (CREB), a substrate of PTEN phosphatase, increased after knocking down PTEN then led to down-regulation of MeCP2 targeted by miR-132. Our work revealed that the two critical genes, Mecp2 and Pten, regulate expression of each other by microRNA targeting and yield further molecular insights for disorders-related mechanisms.

Results

PTEN down-regulated by Mecp2 knockdown. To determine if MeCP2 may affect the expression of PTEN, we cultured primary neurons from the mouse brain, and transfected with lentivirus expressing green fluorescent protein (GFP) (control) or short hairpin MeCP2 (for Mecp2 knockdown). Surprisingly, we found that
PTEN protein expression was significantly reduced after 5 days in neurons transfected with MeCP2 RNAi compared with controls (Fig. 1A,B). Consistently, Pten mRNA expression was also reduced in neurons with MeCP2 knockdown, as shown by quantitative real-time polymerase chain reaction (PCR) assays (Fig. 1C). To determine the effect of MeCP2 knockdown on the activation status of AKT, the well-known downstream of PTEN, we compared the phosphorylation level of AKT at Thr308 in primary cultured neurons transfected with lentivirus expressing GFP, PTEN RNAi (for PTEN knockdown), and MeCP2 RNAi. Remarkably, AKT phosphorylation was consistently up-regulated by PTEN and MeCP2 RNAi, compared with control cells (Fig. 1D). These results suggest that knockdown of MeCP2 photocopy the alternations in signaling pathway similar with knockdown of PTEN.

miR-137 is intermediate in MeCP2 regulation of PTEN. It is previous reported that MeCP2 represses miR-137 expression in neural stem cells. Indeed, we found that miR-137 was up-regulated for over 2.1 fold in Mecp2-knockout mouse cortical neurons, as demonstrated by Solexa-based RNA sequencing (RNA-seq) (Fig. 2A). We also confirmed that pri-miR-137 levels were increased in primary cultured neurons transfected with lentivirus expressing GFP or MeCP2 RNAi compared with control neurons (Fig. 2B). To determine if miR-137 regulated the expression of PTEN, we transduced primary cultured neurons with miR-137 mimic oligonucleotides. Overexpression of miR-137 mimic reduced PTEN expression by about 56% (Figs 2C,D). These results suggest the existence of a regulatory cascade from MeCP2, miR-137, to PTEN (Fig. 2E).

Pten knockdown suppressed MeCP2 expression. Next in an experiment of knocking down PTEN with short hairpin RNA, we surprisingly found that reducing the expression of Pten resulted in a marked decrease in expression of MeCP2 (0.33 ± 0.03 vs. 1.00; P < 0.01) (Figs 1A and 3A,B). Similarly, Mecp2 mRNA expression was downregulated by PTEN RNAi (0.67 ± 0.02 vs. 1.00; P < 0.001) (Fig. 3A). We tested the regulatory effect of PTEN on the MeCP2-target genes Slc2a3, Klhl24, and Prkcb, which were decreased > 2-fold in Mecp2-knockdown cultured neurons, as demonstrated by RNA-seq (data not shown). Slc2a3, Klhl24, and Prkcb were significantly reduced by Pten knockdown, as confirmed by real-time PCR (Fig. 3C), suggesting that knockdown of MeCP2 and knockdown of PTEN may share common downstream targets.

PTEN regulated CREB phosphorylation and miR-132. As PTEN has been found to regulate the de-phosphorylation of CREB, which plays a critical role in regulating microRNA-132. To investigate the molecular mechanism by which PTEN regulates the expression of MeCP2, we examined CREB phosphorylation at
Ser133 in primary cultured neurons with or without PTEN upon KCl stimulation. As shown in Fig. 4A, PTEN deletion together with 50 mM KCl stimulation resulted in a significant increase in CREB phosphorylation. MeCP2 is reportedly regulated by the CREB-induced miRNA, miR-132. To determine if the CREB-induced increase in miR-132 may result from reduced PTEN levels, we examined miR-132 levels in PTEN RNAi neurons stimulated with 10 mM KCl, compared with KCl-stimulated control neurons. miR-132 levels were 50% higher in PTEN RNAi compared with control neurons (2.95 ± 0.175 vs. 4.43 ± 0.34; P < 0.05) (Fig. 4B).

Finally, we transfected mouse primary cortical neurons with GFP and PTEN RNAi followed by depolarization with 10 mM KCl for 2h. Mecp2 expression was dramatically suppressed by KCl-induced PTEN repression (Fig. 4C). Overall, these results indicate that PTEN knockdown suppresses expression of MeCP2 and its target genes in neurons.

Taken together, we therefore proposed that PTEN dephosphorylated CREB at Ser133 and repressed miR-132, resulting in the reciprocal regulation of MeCP2. Thus, knockdown PTEN leads to increased phosphorylation level of CREB Ser-133 site, which in turn promotes expression of miR-132 and then decreases expression of MeCP2 (Fig. 4D).

**Discussion**

Emerging evidence implicates PTEN and MeCP2 as critical regulatory factors in various aspects of the central nervous system. However, the interrelationship between the two genes has proved elusive. Our results suggest that two specific miRNAs act as intermediates in the reciprocal regulation of PTEN and MeCP2. We showed that
inhibition of MeCP2 affected the overexpression of miR-137 and decreased PTEN expression, while overexpres-
sion of miR-137 could also inhibit the expression of PTEN, suggesting that miR-137 is critical to the regulation
of PTEN by MeCP2. We also demonstrated that PTEN regulated MeCP2 via the transcription factor CREB, and
another specific miRNA, miR-132. Previous studies found that CREB acted as a protein substrate of PTEN phos-
phatase in the nucleus23, and miR-132 inhibitors largely blocked the effects of CREB on dendrite maturation24.
MeCP2 translation has also been reportedly regulated by miR-132, and blocking miR-132-mediated repression
increased MeCP2 levels in cultured neurons, and loss of MeCP2 reduced miR-132 levels
in vivo25. Our results
indicated that CREB may be phosphorylated by KCl-induced inhibition of PTEN. In addition, pri-miR-132 levels
were increased by KCl stimulation in PTEN RNAi neurons. These results suggest that CREB and miR-132 may be
involved in the regulation of MeCP2 by PTEN.

In conclusion, we demonstrated that MeCP2 regulates PTEN expression via the specific miRNA, miR-137.
Conversely, PTEN can indirectly regulate MeCP2 via CREB and miR-132. Demonstration of this reciprocal reg-
ulation between PTEN and MeCP2 may provide the basis for more detailed studies of the mechanisms of these
critical genes.

Methods
Primary neuron cultures and transduction, Mice. E15.5 mouse cortical neurons were cultured and
transduced with Amaxa Nucleofector or transfected with lentiviruses (10^8-9 vg/ml) (packaged by Neuron Biotech,
Shanghai). Cells were collected at 5 days. Experiments were performed for over three times. All experimental
procedures were complied with the guidelines and under the approval of the Animal Care and Use Committee of
the Shanghai Institute for Biological Science of the Chinese Academy of Sciences.

Plasmid construction. The RNAi targeting sequences were as follows: PTEN shRNA: 5′- AGA CAA GGC
CAA CCG ATA C-3′; MeCP2 shRNA: 5′- ÀAG TCA GAA GAC CAG GAT C-3′.

Western blotting. Protein samples were separated by sodium dodecyl sulfate-polyacrylamide gel electro-
phoresis and transferred to polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA). Membranes
were processed according to the ECL Western Blotting Protocol. α-MeCP2 (Cell Signaling Technology,
Boston, MA, USA, 1:1000 dilution), α-PTEN (Cell Signaling Technology, 1:1000 dilution), α-CREB (Cell
Signaling Technology, 1:1000 dilution), α-p-CREB (Cell Signaling Technology, 1:1000 dilution), and control
α-glyceraldehyde phosphate dehydrogenase (Abcam, Cambridge, MA, USA, 1:5000 dilution) were used as pri-
mary antibodies. All Western blot quantifications were performed using Image J software.
Real-time PCR relative quantification. Total RNA from cultured neurons was extracted using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). Reverse transcription was carried out with Reverse Transcriptase M-MLV (RNase H-free) (Takara Bio, Otsu, Japan) and quantitative PCR was carried out using a Rotor-Gene Q (Qiagen, Valencia, CA). Primer sequences were as follows: Pten-forward: 5′-TGG ATT CGA CTT AGA CTT GAC CT-3′, Pten-reverse: 5′-TGG CGG TGT CAT AAT GTC TCT-3′, Mecp2-forward: 5′-ACA GCG CTC CAT TAT C-3′, Mecp2-reverse: 5′-CCC AGT TAC CGT GAA GTC AAA A-3′, Slc2a3-forward: 5′-ATG GGG ACA ACG AAG GTG AC-3′, Slc2a3-reverse: 5′-ATG GGG ACA ACG AAG GTG AC-3′, Pten-forward: 5′-ATG AGT TCG TCA CGT TCT CCT-3′, Prkcb-forward: 5′- ATG AGT TCG TCA CGT TCT CCT-3′, Pten-reverse: 5′- CCA TAC AGC GAT CCA CAG-3′, pri-miR-132-forward: 5′-ACC GTG GCT TTC GAT GAC CT-3′, pri-miR-137-forward: 5′-ACT TTC GGT GAC GGG GAA GT-3′, pri-miR-132-reverse: 5′-GCC GAC CAT GGC TGT AGA CT-3′, and pri-miR-137-forward: 5′-GCC GAC CAT GGC TGT AGA CT-3′, and pri-miR-137-reverse: 5′-GCC GAC CAT GGC TGT AGA CT-3′.
RNA-seq Deep Sequencing. One P30 MeCP2 KO mouse and one P30 wide type mouse cortex RNA samples were harvested as described by Kwak et al. (2009) Both samples were subjected to Solexa of BGI Shenzhen.

Statistical analysis. All results are expressed as mean ± SEM and were analyzed using Stata v. 10 software (Stata Corporation, College Station, TX USA). Results were compared using two-sample t-tests with equal variance. A P value < 0.05 was considered statistically significant.

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Author Contributions
J.L., Q.Z. and W.Z. drafted the manuscript, J.L. conceived the study, carried out western blotting, immunofluorescence, cell culture, plasmid construction, real-time PCR, and statistical analysis. B.Y. and T.C. participated in the western blotting. Z.C. conceived of the study and participated in its design. All authors read and approved final manuscript.

Additional Information
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