Published online 17 September 2010 Nucleic Acids Research, 2011, Vol. 39, No. 2 475–485
doi:10.1093/nar/gkq818

Tonicity-responsive microRNAs contribute to the maximal induction of osmoregulatory transcription factor OREBP in response to high-NaCl hypertonicity

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Received May 25, 2010; Revised August 13, 2010; Accepted August 31, 2010

ABSTRACT

Osmotic response element binding protein (OREBP) is a Rel-like transcription factor critical for cellular osmoresponses. Previous studies suggest that hypertonicity-induced accumulation of OREBP protein might be mediated by transcription activation as well as posttranscriptional mRNA stabilization or increased translation. However, the underlying mechanisms remain incompletely elucidated. Here, we report that microRNAs (miRNAs) play critical regulatory roles in hypertonicity-induced induction of OREBP. In renal medullary epithelial mIMCD3 cells, hypertonicity greatly stimulates the activity of the 3′-untranslated region of *OREBP* (*OREBP-3′UTR*). Furthermore, overexpression of *OREBP-3′UTR* or depletion of miRNAs by knocking-down *Dicer* greatly increases OREBP protein expression. On the other hand, significant alterations in miRNA expression occur rapidly in response to high NaCl exposure, with miR-200b and miR-717 being most significantly down-regulated. Moreover, increased miR-200b or miR-717 causes significant down-regulation of mRNA, protein and transcription activity of OREBP, whereas inhibition of miRNAs or disruption of the miRNA–3′UTR interactions abrogates the silencing effects. *In vivo* in mouse renal medulla, miR-200b and miR-717 are found to function to tune *OREBP* in response to renal tonicity alterations. Together, our results support the notion that miRNAs contribute to the maximal induction of *OREBP* to participate in cellular responses to osmotic stress in mammalian renal cells.

INTRODUCTION

Osmotic response element binding protein (OREBP), also called tonicity-responsive element binding protein (TonEBP) or nuclear factor of the activated T cells-5 (NFAT5), is a Rel-like transcription factor that serves as a master regulator for cellular osmoregulation in the kidney and in T lymphocytes (1–4). Recent studies indicate that it also controls a number of other processes such as embryogenesis (5), cancer invasion (6), HIV replication (7) and myogenesis (8). It is established that the p38 MAPK pathway transduces the environmental signals to activate the transcription of *OREBP* (9–11). The transcription activation is followed by several posttranscriptional mechanisms, which include stabilization of mRNA and increased protein synthesis (12,13), protein phosphorylation (9,14–16) and nuclear localization (17–19), to maximally up-regulate OREBP activity. High NaCl has been shown to be capable of increasing the stability of *OREBP* mRNA and its translation (12–14), indicating that these levels of regulation are of great importance in the induction of OREBP. The underlying mechanisms and the regulators involved in these posttranscriptional regulations, however, have remained unclear.

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The authors wish it to be known that, in their opinion, the first three authors should be regarded as joint First Authors.

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miRNAs are noncoding small RNAs that serve as important regulators of gene expression by targeting the 3′-UTR of mRNA to induce mRNA cleavage and/or translational repression (20,21). Emerging data show that miRNAs are implicated in cellular response to various types of stresses including oxidative stress, radiation and UV and serum or nutrient deprivation (22,23). Recently, it has been demonstrated that the miR-8 family miRNAs are important for osmoresponses in embryonic zebrafish. In particular, miR-200b was found to be involved in osmoregulation in the inocytes cells through targeting Na/H exchange regulatory factor-1 (*Nherf1*), a regulator of apical trafficking of trans-membrane ion transporter (24). Because zebrafish inocytes are believed to be functionally equivalent to the renal intercalated cells in the mammalian kidney nephron and the collecting tubules, the findings from zebrafish raised an important question of whether similar systems function in osmoregulation in the mammalian cells and the kidneys. In our current investigation, we found that miRNA-mediated gene silencing plays important roles in the regulation of *OREBP* expression in mouse renal cells and kidneys. We demonstrated that miR-200b and miR-717 are highly tonicity sensitive and able to function to regulate the stability of *OREBP* mRNA and the accumulation of OREBP protein *in vitro* and *in vivo*. Together, our results suggest that these two miRNAs are important endogenous regulators of osmoregulation and osmoadaptation in mammals.

MATERIALS AND METHODS

Plasmid construction

Chimeric *OREBP-3′UTR-luciferase* reporter plasmids and a chimeric aldose reductase (*AR) promoter-luciferase reporter* plasmid (pAR-ORE-luc) were prepared as described in Supplementary Figure S1 and Supplementary Table S1. miRNA overexpression plasmids were generated as described in Supplementary Table S2.

Cell culture, tonicity treatments and transient transfections

Mouse collecting duct epithelial mIMCD3 cells were obtained from ATCC and normally cultured in isotonic medium (300 mOsmol/kg H_2O) as described (12). Plasmid DNA, small interfering RNAs (siRNA) and miRNA mimics and miRNA inhibitor (anti-sense oligonucleotides) transfections were performed with Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's protocol. For hypertonic treatment, hypertonic medium was prepared by adding NaCl to the isotonic medium to adjust the osmolarity to the level of 550 mOsmol/kg H_2O. Following transfections and/or tonicity treatments, cells were harvested at specified time points for analyses.

For transient miRNA precursor and miRNA-inhibitor transfections, mIMCD3 cells were co-transfected with either phOREBP-3′UTR-luc or pOREBP-3′UTR-luc-1 or pOREBP-3′UTR-luc-2 and pSV40-β-galactosidase (3:1), together with 30 nM miRNA precursor or 30 nM of inhibitor or controls under isotonic condition and grown for 24 h. miRNA precursors are custom-prepared RNAs and the inhibitors are anti-sense oligonucleotides (Supplementary Table S3).

For miRNA overexpression, cells were co-transfected with a miRNA-overexpressing plasmid, a luciferase reporter plasmid (pOREBP-3′UTR-luc-1 or pOREBP-3′UTR-luc-2 or pAR-ORE-luc) together with pSV40-β-galactosidase (4:3:1) under isotonic condition and grown for 24 h. For miRNA recognition element (MRE) site-mutant transfection, cells were co-transfected with one of the four luciferase reporters (p200b-WT-luc, p200b-MT-luc, p717-WT-luc and p717-MT-luc), one of the three miRNA overexpressing plasmids (pFlag-CMV, pmiR-200b and pmiR-717), together with pSV40-β-galactosidase at the ratio of 4:3:1 under isotonic condition and grown for 24 h. Following 24 h of incubation, cells were subjected to either isotonic or hypertonic treatments for 8 h and then harvested for the luciferase reporter assays and β-galactosidase activity assay.

Time-course expression of OREBP mRNA and protein in mIMCD3 cells

mIMCD3 cells were replated on a 6-well plate and incubated in isotonic media for 24 h and until ~80% confluency. Tonicity treatments were started by replacing old media with fresh isotonic media, hypertonic media in the presence or absence of cycoloheximde (CHX, Beyotime, Nanjing, 10 mg/ml) or actinomycin (ActD, Beyotime, Nanjing, 5 mg/ml) respectively. Cell samples were collected at 0-, 2-, 4-, 6- and 8-h time points for mRNA and protein analyses.

siRNA knocking-down of *Dicer*

All siRNAs were purchased from GenePharma. The sense sequences for three *Dicer* siRNAs and control siRNA are 5′-CUUUGGACAUUGACUUUAATT-3′ (siDicer-1), 5′-AGUGAGGUUUCAACGGAUCTT-3′ (siDicer-2), 5′-UUCUAACGUGCGAUUGUAGTT-3′ (siDicer-3) and 5′-UUCUCCGAACGUGUCACGUTT-3′ (Control) respectively. To knock-down *Dicer* (NM_148948), mIMCD3 cells were transfected with *Dicer* siRNA and control siRNA at the final concentration of 50 nM as previously described (23) and incubated for a further 48 h. Cells were then subjected to the isotonic and hypertonic treatments for 4 h and harvested for analyses.

Real-time RT–PCR analyses of mRNAs and miRNAs

For real-time RT–PCR mRNA quantification, reverse transcription was performed with TRIzol (Invitrogen)-extracted total RNAs using a High Fidelity primeScript^{TM} RT-PCR Kit as instructed (Takara, Dalian). Real-time RT–PCR was performed by using the SYBR Green Real-time PCR Master Mix (TOYOBO, Shanghai) and the StepOne Real-time PCR system (Applied Biosystems Inc., Foster City, CA) according to the manufacturer's protocol and using the following primer pairs: *OREBP* (NM_133957) (Forward: 5′-CTCC TCAGATCCAGTTGGTTCA-3′, Reverse: 5′-GCTGCA TGTCTGGTTGGTTTAT-3′); *18S rRNA* (NR_003286)

(Forward: 5′-GGTCATAAGCTTGCGTTGATTAAG-3′, Reverse: 5′-CTACGGAAACCTTGTTACGACTTT-3′).

For miRNAs, real-time RT–PCR was performed with the stem-loop primers as reported (25). U6 RNA served as an internal control. The miRNA-specific reverse transcription primers, the universal primer and the miRNA-specific reverse LNA-primers were all purchased from (Sangon, Shanghai). miR-200b (MIMAT0000233) primers: 5′-GT CGTATCCAGTGCGTGTCGTGGAGTCGGCAATTG CACTGGATACGACTCATCAT-3′ (RT), 5′-GGGGTA ATACTGCCTGGT-3′ (forward), 5′-TGCGTGTCGTG GAGTC-3′ (reverse). miR-717 (MIMAT0003510) primers: 5′-GTCGTATCCAGTGCGTGTCGTGGAGT CGGC-AATTGCACTGGATACGACTAGAGAA-3′ (RT), 5′-GGGGCTCAGACAGAGATA-3′ (forward), 5′-TGCGTGTCGTGGAGTC-3′ (reverse). U6 primers: 5′-CGCTTCACGAATTTGCGTGTCAT-3′ (RT), 5′-G CTTCGGCAGCACATATACTAAAAT-3′ (forward), 5′-CGCTTCACGAATTTGCGTGTCAT-3′ (reverse).

Luciferase reporter assays

Luciferase reporter activities were determined using a Luciferase Reporter Gene Assay System (Promega) as instructed. For all luciferase assays, β-galactosidase activities were determined to calibrate for the transfection efficiency. The calibrated value for a proper control was used to normalize all other values to obtain the normalized relative luciferase units (RLU) representing the activities of *OREBP-3′UTR* and *AR-ORE*.

Western blot analyses

Western blots were performed according to standard protocols. The detection was achieved using the Immobilon Western Chemiluminescent HRP Substrate kit (Millipore). Primary antibodies are: OREBP (Santa Cruz Biotechnology, Inc., sc-13035, 1:5000) and AR (Santa Cruz Biotechnology, Inc., sc-17735, 1:5000).

Mouse experiments

Animal experiments were conducted according to protocols and guidelines that were approved by the Xiamen University Institutional Animal Care and Use Committee. Wild-type (WT) and *AR*-deficient ($AR^{-/-}$) male C57BL/6 mice were maintained as described previously (26). Three groups of 6- to 8-week mice were prepared (three per group), i.e. two for WT mice and one for $AR^{-/-}$. For the treatments, one WT group was injected intraperitoneally with 0.5 mg/kg of furosemide once while the control group (euhydration) and the $AR^{-/-}$ mice were injected intraperitoneally with the same volume of vehicle saline solution. Four hours after the injections, urine samples were collected and the mice were then were sacrificed together by cervical dislocation. Kidney medulla samples were dissected to prepare total RNA for analyses and miRNA Northern hybridization. Urine osmolarity was assayed with a STY-1 Osmometer (Tianda, Tianjin).

miRNA Northern hybridization

The digoxigenin-labeled LNA probe for miR-200b was purchased from Exiqon. Northern Hybridization was performed with digoxigenin-labeled probes as described (27).

Bioinformatics, data acquisition, image processing and statistical analyses

Mature and pre-miRNA sequences were based on MiRbase (http://microrna.sanger.ac.uk). miRNA target predictions were performed with the MiRanda (http://www.microrna.org) or Targetscan (http://www.targetscan.org) algorithms. Western and Northern images were captured by Biosense SC8108 Gel Documentation System with GeneScope V1.73 software (Shanghai BioTech). Gel images were imported into Photoshop for orientation and cropping. The digital density values were acquired by Image-Pro Plus software (Media Cybernetics) and analyzed by Prism 5.0 (Graphpad). Data are the means ± SEM. One-way analysis of variance with Bonferonni's post-test was used for multiple comparisons and the Student's *t*-test (two-tailed) for pair-wise comparisons.

RESULTS

Enhanced transcription and mRNA stability and increased translation contribute significantly to hypertonicity-induced accumulation of OREBP protein

To determine how high NaCl concentration might affect the expression of *OREBP* mRNA and protein in a time-dependent manner, we subjected mIMCD3 to isotonic (Iso, 300 mOsmol/kg H_2O) or hypertonic (Hyper, 550 mOsmol/kg H_2O) media. Because the first 8 h are the most critical period for osmoregulation and the induction of osmoprotective genes including *OREBP* (4,12,28), we assayed the expression of *OREBP* mRNA and protein at 2-h intervals up to 8 h. Consistent with previous studies by other groups (12,18), *OREBP* mRNA increased gradually in response to hypertonic exposure and peaked at 4 h and fell subsequently, whereas its level in cells under isotonic condition remained virtually constant (Figure 1A). Furthermore, mRNA degradation in actinomycin (ActD)-treated cells under hypertonic condition was slower than that of the cells under isotonic condition, indicating that hypertonicity tended to stabilize *OREBP* mRNA.

Also consistent with previous reports (12,28), significant inductions of OREBP protein became apparent at 2 h and reached peak levels by 6–8 h (Figure 1B and C) after high NaCl exposure. Although mRNA stabilization had been shown to contribute to hypertonicity-induced accumulation of *OREBP* mRNA and protein (12) in mIMCD3 cells, it was not clear what the underlying mechanism was and whether other posttranscriptional mechanisms might also be involved. To investigate the contribution of protein stabilization, we utilized cycloheximide (CHX) to inhibit protein biosynthesis. Following the inhibition of translation, OREBP protein decreased time-dependently

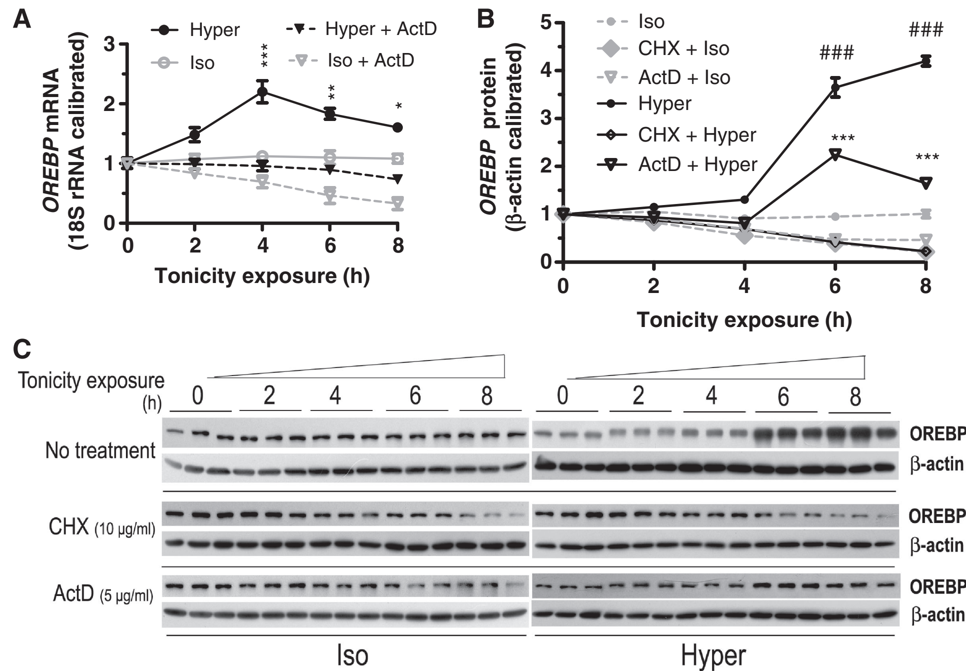

Figure 1. Enhanced transcription and mRNA stability and increased translation contribute significantly to maximal induction of *OREBP* induced by hypertonicity. Hyper, high-NaCl hypertonicity (550 mOsmol/kg H_2O); Iso, isotonicity (300 mOsmol/kg H_2O); ActD, actinomycin D (5 µg/ml); CHX, cycloheximide (10 µg/ml) (**A**) Time-dependent induction of *OREBP* mRNA in mIMCD3 by high NaCl exposure. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$; all compared with isotonic controls (Iso) (n = 3) (**B**) Time-dependent expression of OREBP protein in mIMCD3 cells under isotonic and hypertonic condition as determined by western blots (n = 4). All values were calibrated with the expression of β-actin and normalized with the β-actin-calibrated values at 0 h for the control cells, the CHX-treated cells or the ActD-treated cells, respectively (n = 4). $^{###}P < 0.001$, Hyper versus Iso, $***P < 0.001$, (ActD + Hyper) versus (ActD + Iso). (**C**) Typical western blot analyses of OREBP protein expression in mIMCD3 cells under isotonic and hypertonic conditions (n = 3).

for cells under either isotonic or hypertonic condition respectively. No apparent difference in the rate of degradation of OREBP protein, however, was observed between cells grown under different tonicity conditions (Figure 1B and C). This is consistent with previous pulse-chase OREBP analyses showing that hypertonicity did not significantly affect OREBP protein stability in MDCK cells (18) and it suggests that protein stabilization is not the major underlying mechanism for OREBP accumulation in mIMCD3 cells, at least during the observed period. To determine how hypertonicity might affect OREBP accumulation in the absence of *de novo* transcription, we further utilized ActD to stop transcription in mIMCD3 cells. Interestingly, following ActD treatment, cells grown under isotonic condition appeared to have small decreases in OREBP protein expression. In contrast, the level of OREBP protein was relatively stable for the first 4 h for cells under hypertonic condition despite the inhibition of transcription, with a big surge appeared 6 h after the tonicity exposure and then fell slightly (Figure 1B and C). Taking the absence of *de novo* transcription and the lack of increased protein stability into account, the surges at 6 h of OREBP protein in ActD-treated cells grown under hypertonic condition suggests important contributions from translational de-repression or increased translational efficiency of OREBP. Together, these results indicate that enhanced translation might be one of the most important factors contributing to hypertonicity-induced accumulation of OREBP protein.

miRNA-mediated silencing plays critical regulatory roles in the maximal induction of OREBP by hypertonicity

Since the 3′UTR of mRNA plays very important regulatory roles in gene expression (29), we constructed a few chimeric luciferase reporters for mouse *OREBP-3′UTR* and one for human *OREBP-3′UTR* and used them for transfections in mIMCD3 cells. Plasmid phOREBP-3′UTR-luc and pOREBP-3′UTR-luc-1 contains full-length sequences for human and mouse *OREBP-3′UTR*, respectively, while pOREBP-3′UTR-luc-2 contains a truncated region of mouse *OREBP-3′UTR* (Supplementary Figure S1 and Table S1). Consistent with the results from a previous report (12), in mouse mIMCD3 cells transfected with phOREBP-3′UTR-luc, high NaCl exposure resulted in substantial decreases in the reporter activity, suggesting that hypertonicity tended to suppress the activity of human *OREBP-3′UTR* (Figure 2A). When similar assays were performed with pOREBP-3′UTR-luc-1, however, a very different pattern of time-course reporter activities was obtained. The luciferase activity reflecting the stability of the chimeric mRNA and its translation was virtually not different between the isotonic and hypertonic treatment groups for the first 4 h of the tonicity exposure (Figure 2B). There were, however, sudden surges in the activity, commencing from the 6-h time

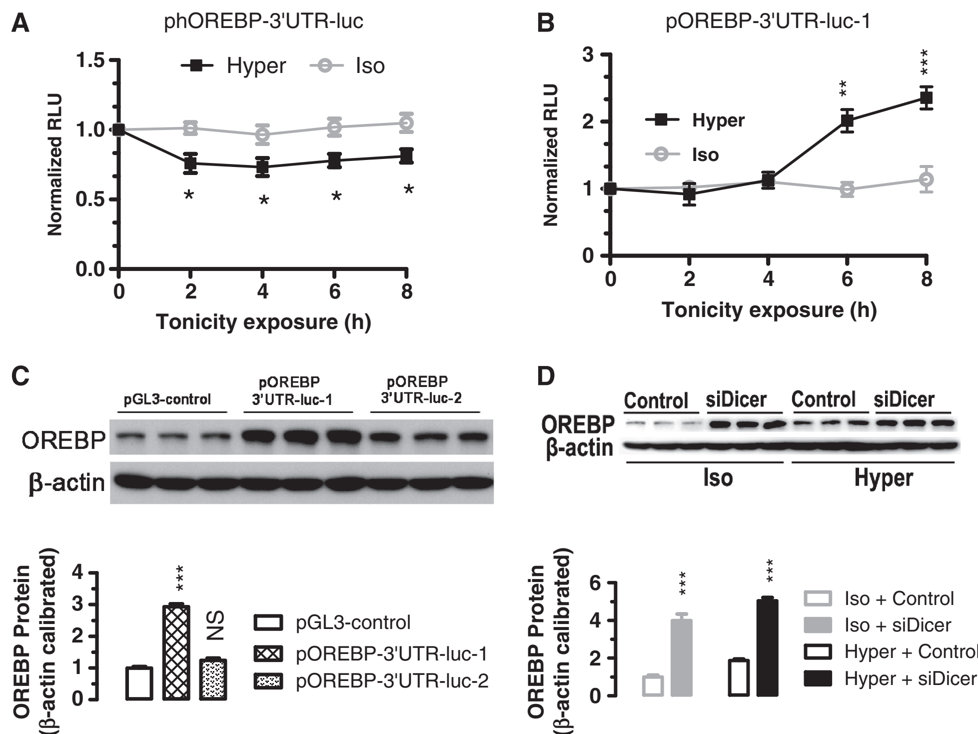

Figure 2. miRNA-mediated mechanisms are involved in hypertonicity-induced induction of *OREBP*. NS, not significant; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$; all compared with tonicity-matched controls. Experiments were performed with at least three separate samples analyzed in triplicates ($n = 3$). (**A**) Time-course effects of high NaCl hypertonicity on the activity of human *OREBP-3′UTR*. (**B**) High NaCl hypertonicity time-dependently increased the activity of mouse *OREBP-3′UTR*. (**C**) Overexpression of chimeric *OREBP-3′UTR-luciferase* mRNAs by pOREBP-3′UTR-luc-1 resulted in a significant elevation in OREBP protein while a small but insignificant elevation was observed in cells transfected with pOREBP-3′UTR-luc-2. mIMCD3 cells were grown and transfected as described under isotonic condition. Thirty-two hours after transfection, cells were harvested for protein analyses. (**D**) Depletion of *Dicer* significantly up-regulated OREBP protein expression. mIMCD3 cells were grown and siRNA-transfected under isotonic condition to knock-down *Dicer* (Supplementary Figure S2). Following subsequent treatment in isotonic or hypertonic media for 4 h, cells were harvested for Western blot analyses. siDicer, siRNA for *Dicer*; Control, control siRNA. Gel image represents data obtained from the use of siDicer-2. The bar chart represents statistical data obtained from the use of three siRNAs, namely siDicer-1, siDicer-2 and siDicer-3.

point for the hypertonic treatment group while that of the control remained virtually constant. The big increases in the 3′UTR activity at 6 and 8 h correlated well with the temporal surges of mouse OREBP protein under hypertonicity (Figure 1B, upper), suggesting that hypertonicity greatly stimulated the activity of mouse *OREBP-3′UTR* and this stimulation might play important roles in the maximal induction of *OREBP*. The fact that high NaCl induced a great induction of luciferase reporter activity in mIMCD3 cells with mouse *OREBP-3′UTR* but not that of human *OREBP-3′UTR* suggests that the 3′UTR-mediated mechanisms are highly sequence specific.

Since the 3′UTR of a gene potentially contains regulatory sequence elements recognized by miRNAs and RNA-binding proteins to regulate mRNA stability and protein translation (20,30), we reasoned that the overexpression of such 3′UTR sequences would compete for the binding of cognate miRNAs and/or RNA-binding proteins to affect the expression of the endogenous gene. Interestingly, in mIMCD3 cells overexpressing the chimeric mouse *OREBP-3′UTR-luciferase* mRNA (pOREBP-3′UTR-luc-1) under isotonic conditions, endogenous OREBP protein expression was increased by ~3-fold ($P < 0.001$; Figure 2C). In contrast, transfection with pOREBP-3′UTR-luc-2, which contains only a truncated region of the *OREBP-3′UTR*, resulted in only a small but insignificant increase. These results suggest that *OREBP-3′UTR*-mediated mechanisms might be responsible for the maximal induction of *OREBP*.

To explore the regulatory roles of miRNAs in the induction of *OREBP*, we first utilized siRNAs to knock-down the expression of *Dicer* (23), one of the most critical components responsible for the biogenesis of miRNAs (20,23,31,32). Three different siRNAs were designed and used for transfections in mIMCD3 cells. Significantly, in cells transfected with any of the three siRNAs, *Dicer*-depletion caused great up-regulations in OREBP protein under both the isotonic and hypertonic conditions (e.g. 2.6-folds for hypertonic condition, $P < 0.001$) (Figure 2D), indicating that *OREBP* expression could be tightly regulated by miRNAs.

High NaCl exposure induces significant alterations in miRNA expression

In an attempt to identify potential miRNAs that might respond to osmotic stress, we profiled miRNA expression in mIMCD3 cells subjected to isotonic and hypertonic treatments for 2 and 8 h, utilizing the miRCURY LNA array as instructed. Following the tonicity treatment for 2 h, there were totally 57 miRNAs that were found to be

differentially expressed between the mIMCD3 cells under hypertonicity and isotonicity, with a minimum fold-change of 1.5 in either the up-regulated or down-regulated direction ($P < 0.05$; Supplementary Table S4). Among 18 down-regulated miRNAs, miR-200b, miR-143 and miR-717 were down-regulated by ~10-, 7- and 5-fold (Iso/Hyper), respectively. Interestingly, being one of the most significantly down-regulated miRNAs from our array analyses, miR-200b turned out to be a member of the miR-8 family miRNAs that were found to play important roles in osmoregulation in the zebrafish inocytes recently (24). This lent credence to the validity of our dataset. In contrast to the significant alterations in miRNA expression following a 2-h tonicity exposure, only very few miRNAs were detected to be differentially expressed between cells under isotonic and hypertonic conditions following an 8-h tonicity exposure (Supplementary Table S5). This suggests that hypertonicity-induced alterations in miRNA are not sustained for long.

Of the three most significantly down-regulated miRNAs, bioinformatics analyses indicated that miR-200b and miR-717 might be *OREBP*-targeting whereas miR-143 is not (Supplementary Figure S3). Real-time RT–PCR was performed to determine the time-course expression for miR-200b and miR-717 in response to hypertonicity. The results indicated that following high NaCl exposure, miR-200b and miR-717 were quickly and significantly down-regulated. Within 2 h of hypertonic exposure, the levels of miR-200b and miR-717 were reduced by ~92% and 80%, respectively (Figure 3), which largely confirmed the results from the microarray analyses. The levels of these two miRNAs, however, were gradually restored thereafter such that both were returned to the levels close to that of the isotonic controls by 8 h. Intriguingly, the expression of miR-200b primary and precursor sequences were largely consistent with the trend of the expression of the mature sequences that was strongly down-regulated time-dependently (Supplementary Figure S5). In contrast, while the expression of miR-717 precursor sequences was also consistent with the trend of the expression of the mature sequences, the expression of miR-717 primary sequences was largely

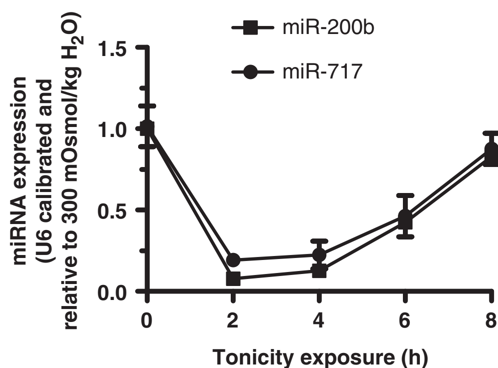

Figure 3. miR-200b and miR-717 are rapidly and significantly down-regulated by hypertonicity. Results were obtained from three separate samples analyzed in triplicates ($n = 3$).

constant and not affected by hypertonicity (Supplementary Figure S5). miR-200b and miR-717 thus appeared to be affected by hypertonicity at different stages of miRNA biogenesis or maturation. In spite of these, the above results established that miR-200b and miR-717 are highly tonicity-sensitive and tonicty-responsive, suggesting strongly that these two miRNAs might serve as tonicity-responsive factors that contribute to the regulation of *OREBP*.

Overexpression of miR-200b and miR-717 silences the expression of *OREBP* and its transcriptional activity in mIMCD3 cells

To determine whether miR-200b and miR-717 regulate *OREBP* expression, we constructed three miRNA expression plasmids (pmiR-143, pmiR-200b and pmiR-717; Supplementary Table S2) to overexpress miR-200b, miR-717 and miR-143, respectively, in mIMCD3 cells (Figure 4A). Interestingly, in cells overexpressing miR-200b and miR-717, respectively, the levels of *OREBP* mRNA were down-regulated by ~24–39% under either isotonic or hypertonic condition ($P < 0.001$, Figure 4B). Paralleling the trend of mRNA down-regulation, OREBP protein exhibited significant reduction in cells overexpressing either miR-200b or miR-717 (Figure 4C). Additionally, in cells overexpressing both miR-200b and miR-717 simultaneously, the reduction of OREBP protein was greater than that of the single-plasmid transfected. Moreover, with the down-regulation of OREBP protein, the protein level of AR, which is under the transcription control of OREBP (1), was also significantly reduced in the miRNA-overexpressing cells. The functional deficits of OREBP in the miRNA-overexpressing cells were further analyzed using a chimeric pAR-ORE-luc containing three osmotic response elements (OREs) (33). While overexpression of miR-143, which is not predicted to target *OREBP* by either the MiRanda or Targetscan algorithms, did not cause any change in transactivation activity of OREBP, overexpression of miR-200b or miR-717 resulted in significant reductions in its transactivation activity (Figure 4D). In cells overexpressing miR-200b and miR-717 simultaneously, the transcriptional activity of OREBP was reduced by ~65% under hypertonic condition ($P < 0.001$; Figure 4D), suggesting strong regulation by these two miRNAs. Together, these results indicate that miR-200b and miR-717 are capable of modulating *OREBP* expression.

miR-200b and miR-717 control OREBP expression through targeting its 3′UTR

To determine whether miR-200b and miR-717 function to regulate *OREBP* through interactions with *OREBP-3′UTR*, we first co-transfected chemically synthesized miRNA mimics (pre-miRNAs) and miRNA inhibitors (anti-sense oligonucleotides) with the luciferase reporter pOREBP-3′UTR-luc-1 into mIMCD3 cells. Transfections of the precursors for either miR-200b or miR-717 (Supplementary Table S3) significantly reduced *OREBP-3′UTR* activity under both isotonic and hypertonic conditions ($P < 0.05$; Figure 5A). Conversely,

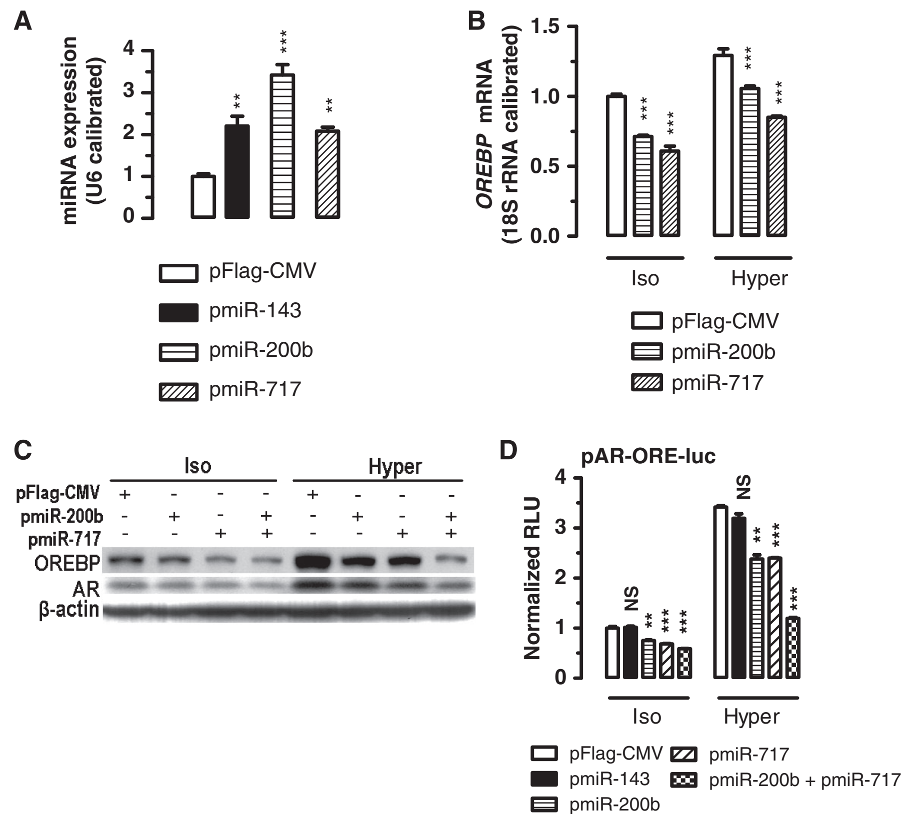

Figure 4. miR-200b and miR-717 function to silence OREBP in mIMCD3 cells. Cells were transfected with pmiR-200b or pmiR-717, respectively, under isotonic condition. Twenty-four hours after the transfection, cells were fed with either isotonic or hypertonic media and harvested 8 h later for mRNA, protein and luciferase reporter assay analyses. NS, not significant; $^{**}P < 0.01$; $^{***}P < 0.001$; all compared with tonicity-matched controls ($n \geq 3$). (**A**) Transfection of miRNA-overexpressing plasmids significantly increased miR-143, miR-200b and miR-717. (**B**) Overexpression of miR-200b and miR-717 significantly down-regulated the mRNA expression of *OREBP*. (**C**) Overexpression of miR-200b and miR-717 significantly down-regulated the protein expression of OREBP and AR. (**D**) Overexpression of miR-200b and miR-717 significantly attenuated the transcriptional transactivation of AR by OREBP.

inhibition of both miR-200b and miR-717 resulted in small yet significant increases in *OREBP-3′UTR* activity under both isotonic and hypertonic condition ($P < 0.05$), except that the inhibition of miR-717 did not result in a significant change in the reporter activity (Figure 5B). These results suggest that miR-200b and miR-717 might exert their silencing effects on *OREBP* through miRNA–3′UTR interactions.

To further test the miRNA–*OREBP-3′UTR* interactions, we also co-transfected mIMCD3 cells with miRNA-overexpressing plasmids and the luciferase reporter pOREBP-3′UTR-luc-1. Consistent with the results from the miRNA precursor transfection, overexpression of both miR-200b and miR-717 significantly decreased the activities of *OREBP-3′UTR* under both isotonic and hypertonic conditions (Figure 5C). In contrast, overexpression of miR-143 did not result in any significant change in *OREBP-3′UTR* reporter activity. In another series of transfection where miRNA-overexpressing plasmids were co-transfected with pOREBP-3′UTR-luc-2, which contains no MRE for either miR-200b or miR-717 or miR-143, no significant effects were observed (Figure 5D). These results suggest that the interactions between the putative MREs on *OREBP-3′UTR* and miR-200b or miR-717 were specific. To further verify the specificity of the interactions, we mutated the MRE sites for miR-200b and miR-717 on the *OREBP-3′UTR* and created corresponding luciferase reporters (Supplementary Figures S1 and S4 and Supplementary Table S1). Using mutant and control reporters, we demonstrated that the mutations of the MREs for miR-200b and miR-717 on both the truncated versions of *OREBP-3′UTR* (p200b-MT-luc and p717-MT-luc; Figure 5E and F) or the full-length *OREBP-3′UTR* (pOREBP-3′UTR-200MT-luc and pOREBP-3′UTR-717MT-luc; Supplementary Figure S4) largely abrogated the regulatory effects by the corresponding miRNAs or miRNA inhibitors, confirming that the disruption of the putative miRNA–MRE interactions abolished the silencing effects of miRNA on *OREBP*. Together, these results indicate that miR-200b and miR-717 regulate *OREBP* by targeting *OREBP-3′UTR* to destabilize the mRNA and/or suppress protein translation.

miR-200b and miR-717 are linked physiologically with urine osmolarity to regulate *OREBP in vivo*

To determine whether miR-200b and miR-717 function to regulate *OREBP* expression *in vivo*, we investigated the effects of the alterations in the tonicity condition on the

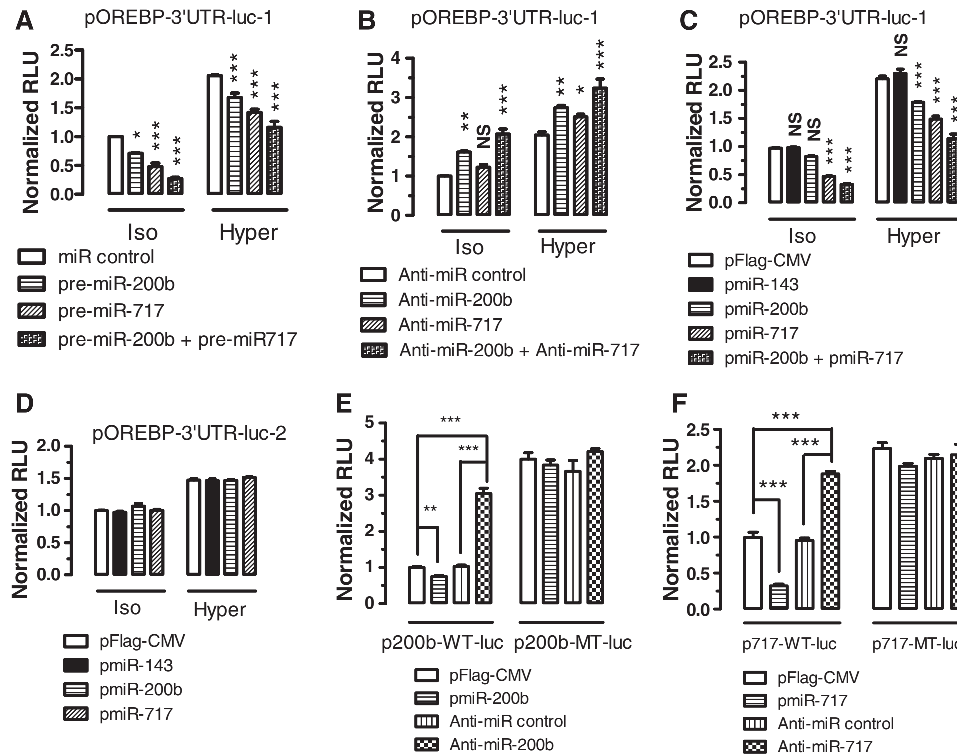

Figure 5. miR-200b and miR-717 suppress *OREBP* expression by targeting *OREBP-3′UTR*. mIMCD3 cells were co-transfected with a luciferase reporter together with miRNA precursors, miRNA inhibitors or miRNA-overexpressing plasmids, respectively, under isotonic condition for 24 h. Transfected cells were subsequently fed with either isotonic or hypertonic media and harvested 8 h later for the luciferase reporter assays as described. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$, all compared with tonicity-matched controls ($n \geq 3$). (**A**) Co-transfection of miRNA precursors for miR-200b and miR-717 with pOREBP-3′UTRluc-1 significantly reduced *OREBP-3′UTR* activities. (**B**) Inhibition of miR-200b and miR-717 significantly increased *OREBP-3′UTR* activities. (**C**) Overexpression of miR-200b and miR-717 significantly reduced the reporter activity of pOREBP-3′UTR-luc-1. (**D**) Overexpression of miR-200b and miR-717 did not cause significant changes in the reporter activity of pOREBP-3′UTR-luc-2. (**E**) MRE site-mutation abolished the silencing effects of miR-200b under isotonic condition. (**F**) MRE site-mutation abolished the silencing effects of miR-717 under isotonic condition.

expression of miRNAs and *OREBP* in the renal medulla, a region constantly exposed to hypertonicity under normal circumstances. We used the diuretic furosemide to induce hypo-osmolality in the kidneys of the WT C57BL/6 mice. In addition, we also included a line of knockout mice deficient in AR ($AR^{-/-}$, also in C57BL/5 background) that develop mild diabetes insipidus and accumulate hypotonic urine in the medulla due to defects in urine-concentrating mechanisms (26). As a consequence of AR deficiency or diuretics-treatment, the urine osmolarity in the $AR^{-/-}$ mice and the furosemide-treated mice was found to be ~35 and 17% of that of the age-and gender-matched WT mice, respectively ($P < 0.001$; Figure 6A). In line with the significant alterations in the tonicity environment, the renal medullary expression of *OREBP* mRNA in $AR^{-/-}$ mice and furosemide-treated mice was greatly down-regulated (Figure 6B). On the other hand, the expression of both miR-200b and miR-717 was greatly up-regulated in $AR^{-/-}$ mice and furosemide–treated mice (Figure 6C and D). Since the $AR^{-/-}$ mice are genetically manipulated models, the similar trends of down-regulation of miR-200b and miR-717 between the $AR^{-/-}$ mice and furosemide-treated mice ruled out the possible side effects on renal miRNA by furosemide. These results therefore established the inverse correlations between the renal tonicity, the expression of tonicity-responsive miR-200b and miR-717 and the expression of osmoregulatory transcription factor *OREBP*. Together, they strongly suggest that miR-200b and miR-717 play important roles in osmoregulation/ osmoadaptation by regulating the expression of *OREBP*.

DISCUSSION

The stability of mammalian mRNA is determined by various *cis*- and *trans*-acting factors (29,30). A previous study in mIMCD3 cells suggested that hypertonicity increased *OREBP* mRNA and protein by stabilizing *OREBP* mRNA (12). Using luciferase reporter assays and the UTRs of human *OREBP*, however, the same study suggested that hypertonicity tended to stabilize *OREBP*-5′UTR but destabilized the 3′-UTR mRNA. This result was perplexing since the 3′-UTR is usually much longer and contains more *cis*-regulatory elements than the 5′-UTR to offer much more robust regulatory control on mRNA (29). To further clarify the issue, we first verified in this current study that hypertonicity indeed tended to destabilize human *OREBP-3′UTR* in mIMCD3 cells (Figure 2A). However, using a 3′-UTR from mouse *OREBP*, we demonstrated clearly that *OREBP-3′UTR* was strongly stabilized rather than destabilized

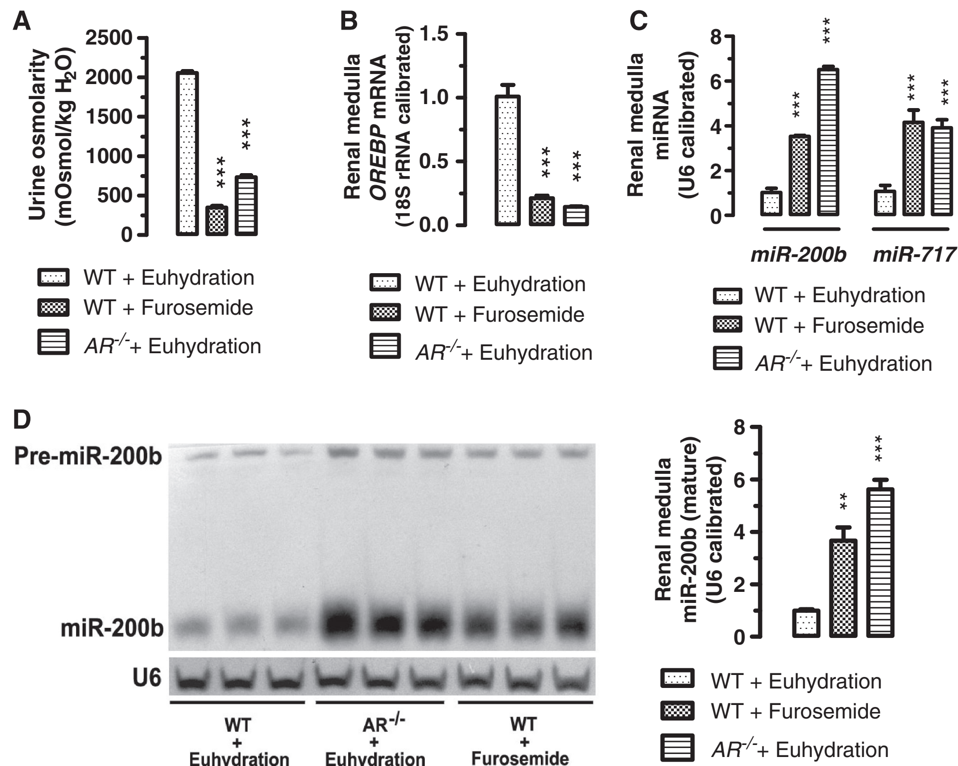

Figure 6. Renal medullary expression of miR-200b and miR-717 correlates inversely with the expression of *OREBP in vivo* in mouse renal medulla. Six- to 8-week-old male WT and $AR^{-/-}$ C57BL/6 mice were provided with drinking water *ad libitum* and with or without furosemide injection as described. Four hours after the furosemide treatment, urine samples were collected and urine osmolarity was assayed. Kidney medulla samples were dissected and total RNA was extracted for real-time RT–PCR or northern blot analyses as described. $^{**}P < 0.01$; $^{***}P < 0.001$; all compared with the control sample from the euhydrated WT mice ($n = 3$) (**A**) Urine osmolarity was significantly reduced in furosemide-treated or AR-deficient mice. (**B**) *OREBP* mRNA expression was significantly down-regulated in furosemide-treated or AR-deficient mice. (**C**) Furosemide treatment and AR deficiency significantly up-regulated miR-200b and miR-717 expression in the renal medullas analyzed by real-time RT–PCR. (**D**) Up-regulation of miR-200b in the renal medulla in AR-deficient mice or furosemide-injected WT mice as demonstrated by northern blot analysis.

(Figure 2***B***). Bioinformatics analysis revealed substantial RNA sequence differences between human and mouse *OREBP-3′UTR*, with sequence identity being only 66.9%. Apparently, the opposite trends of responses to hypertonicity between human and mouse *OREBP-3′UTR* in mIMCD3 cells arose from the sequence variations between these two RNA sequences. When a human *OREBP-3′UTR* is expressed in mouse cells, normal interactions between *OREBP-3′UTR* and RNA-binding proteins or miRNAs might be greatly compromised due to the sequence variations in the *cis*-regulatory elements. Our data thus firmly established that *OREBP-3′UTR* is strongly stabilized by hypertonicity to contribute critically to the induction of *OREBP* in the early phase of osmoregulation.

The most important posttranscriptional regulatory mechanisms that act through interacting with the 3′-UTR of mRNA include the interactions between 3′-UTR and RNA-binding proteins as well as miRNAs (20,30). Mammalian mRNA 3′-UTRs normally contain multiple *cis*-elements such as the adenylate/uridylate rich elements (AREs) to be recognized by RNA-binding proteins. Interactions between the 3′-UTR and a variety of RNA-binding proteins through AREs affect primarily mRNA stability, e.g. the binding of HuR with AREs tends to stabilize (34,35) whereas AUF1 tends to destabilize mRNA (35,36). Bioinformatics analyses indicate that human *OREBP-3′UTR* contains 30 AREs whereas mouse *OREBP-3′UTR* has 26. The interactions between *OREBP-3′UTR* AREs and RNA-binding proteins and their contributions to the induction of *OREBP* are not clear and are yet to be investigated further.

Long mammalian mRNA 3′-UTRs usually contain many MREs to be recognized by cognate miRNAs. In contrast to the ARE–RNA-binding protein interactions, the miRNA–MRE interactions regulate both mRNA stability and protein translation and are of great importance in gene regulation. In our current study, we investigated the regulatory roles of miRNAs in the expression of *OREBP* in response to environmental tonicity alterations. We demonstrated that: (i) overexpression of *OREBP-3′UTR* or knocking-down of *Dicer* greatly increases the expression of OREBP protein (Figure 2B, C and D); (ii) the expression of miRNAs are highly responsive to environmental tonicity alterations (Supplementary Table S4 and Figure 3). For example, hypertonicity down-regulates the expression of miR-200b and miR-717 rapidly and significantly (Figure 3); (iii) overexpression of miR-200b and miR-717 significantly reduces mRNA and protein expression of OREBP and

its transcriptional activity (Figure 4); (iv) overexpression of miR-200b and miR-717 or transfection of chemically synthesized miRNA precursors greatly reduces the stability of *OREBP-3′UTR* and its translation, whereas the inhibition with anti-sense oligonucleotides or disruption of the miRNA–MRE interactions by mutating putative miRNA-binding sites abrogates the suppressive effects of these two miRNAs. Moreover, the silencing effects of individual miRNAs are additive (Figure 5); (v) in the renal medulla, the expression of miR-200b and miR-717 correlates negatively with urine osmolarity and the expression of *OREBP* (Figure 6). Together, these results provide strong evidence that miRNAs are important for the maximal induction of *OREBP* in response to hypertonicity exposure. In particular, miR-200b and miR-717 are two important regulators that are significantly down-regulated in response to hypertonicity to contribute to the maximal induction of *OREBP* through interacting with its 3′-UTR. Together, these findings reveal a novel layer of regulations for cellular osmosignaling and osmoregulation that will help with the better understanding of osmoresponses under physiological and pathophysiological conditions. Furthermore, miR-200b and miR-717 are two novel osmoregulators playing important regulatory roles in mammalian osmosignaling and osmoregulation.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

ACKNOWLEDGEMENTS

The authors thank Prof. Z.M. Yang of XMU for helpful discussions and technical assistance and Dr Dongyan Jin of HKU for critical reading.

FUNDING

This work was supported in part by grants from the National Science Foundation of China (#30970649); 973 Program of Ministry of Science and Technology of China (#2009CB941601); 111 Project of Ministry of Education and Bureau of Foreign Experts of China (#B06016) and the Science Planning Program of Fujian Province (#2009J1010). Funding for open access charge: National Science Foundation of China (#30970649).

Conflict of interest statement. None declared.

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