Chapter

6

In preparation

MicroRNA-132 regulates diuresis through vasopressin- and prostaglandin-dependent alteration of Aquaporin-2 localization

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Abstract

The collecting duct (CD) principal cells of our kidneys are critical in the maintenance of blood water levels, as binding of vasopressin (AVP) to its V2-receptor and the subsequent translocation of aquaporin-2 (AQP2) water channels to the apical membrane fine-tunes water balance. Cyclooxygenase-2 (Cox2) produces prostaglandins such as PGE2 that counteract renal AVP action by inducing internalization and lysosomal degradation of AQP2. A role for microRNAs (miRNAs) in the regulation of water and electrolyte balance remains unexplored. We identified miR-132 as a potential post-transcriptional regulator of Cox2 expression and investigated the role of this miR132 in diuresis *in vitro* and *in vivo*. Silencing of miR-132 in cultured collecting duct cells as well as in fibroblast cells resulted in the upregulation of Cox2 expression and altered AQP2 expression in collecting duct cells. Silencing of miR-132 in mice caused severe weight loss as a result of acute diuresis characterized by increased plasma osmolality and decreased urine osmolality. In addition, urinary PGE2 levels were elevated and hypothalamic AVP mRNA expression and blood AVP levels were not increased, despite the increased blood osmolality. This resulted in less translocation of AQP2 to the apical membrane in CD cells._Taken together, Silencing of miR-132 causes acute diuresis. Our data indicate that this is the result of a Cox2-dependent decrease in AVP synthesis/release and increase in renal PGE2 counteracting renal AVP-stimulated water reabsorption through AQP2. This is the first demonstration of water balance regulation by a miRNA.

Introduction

For our body, replenishment of water losses by an equivalent intake is essential to maintain our volume and osmo balance, as excessive water retention leads to acute or chronic hyponatremia coinciding with brain edema or osteoporosis, whereas overhydration and dehydration are fundamental to hypertension and hypotension, respectively. The collecting duct principal cells of our kidneys are critical in these regulatory processes. In states of hypovolemia or hypernatremia, the anti-diuretic hormone vasopressin is produced within the hypothalamic paraventricular (PVN) and supraoptic (SON) neurons[1]. AVP is then processed, stored, and secreted into the circulation by the posterior pituitary. Upon binding to its type-2 receptor (V2R) in renal collecting duct principal cells, AVP induces a cAMP signaling cascade and activation of protein kinase A (PKA), which results in translocation of AQP2 to the apical membrane, rendering these cells water permeable[2-4]. Driven by the transcellular osmotic gradient, water will enter the cells through AQP2 and will leave the cells via AQP3 or AQP4, which are present in the basolateral membrane, resulting in concentrated urine[1]. Upon correction of blood tonicity or volume, blood vasopressin levels will drop and AQP2 is internalized again[5]. Besides this short-term effect, vasopressin regulates AQP2 expression on the long term by increasing its transcription via activation of and promoter binding by the cyclic AMP responsive element binding protein (CREB)[6,7]. Lack of functional V2R or AQP2 results in X-linked or autosomal nephrogenic diabetes insipidus (NDI), respectively, disorders characterized by polyuria and polydipsia due to unresponsive to vasopressin[8], and which reveals the essential role of the V2R and AQP2 in principal collecting duct cells in this process.

AVP dependent regulation of water homeostasis can be modulated by endocrine factors such as ANG II, catecholamines, as well as prostaglandins (PGs), metabolites of the cyclooxygenase (Cox) pathway[9]. Accordingly, renal prostaglandin E_2 (PGE_2) is known to counteract renal vasopressin action[10], by inducing the internalization and lysosomal degradation of AQP2[11]. In addition, NSAIDs transiently enhance urine concentration[12]. On the other hand, recent studies showed that inhibition of Cox by indomethacin reduced renal AQP2 expression in rats, implying that PGs may actually help maintain AQP2 levels[13]. Finally, PGE2 could affect central vasopressin levels, as PGE2 has been reported to reduce central AVP production and release[14].

Several studies have assessed the expression of microRNAs (miRNAs) in the kidney[15,16]. MiRNAs are a class of super-regulatory molecules, which control the expression of sets of genes that are often functionally related at the post-transcriptional level[17]. Following several impressive examples for a pivotal role for miRNAs in cardiovascular development and hematopoiesis[18], there is no doubt that miRNAs will also regulate pivotal functions in renal electrolyte homeostasis. Indeed, functional involvement of miRNAs in kidney regulatory processes was shown by several recent papers. For instance, Cre-mediated deletion of dicer from uretic buds and its descendents, including collecting duct epithelium and urothelium, by using Hox7B-CRE mice resulted in severe hydronephrosis[19]. Importantly, most mice had focal microscopic tubular cysts originating from the collecting duct, indicating that preventing pre-miRNA dicing, and thus regulation by miRNAs in these segments, especially affects the renal collecting duct. Also, based on in vitro analysis, miR-192 has been suggested to have a role in aldosteron-mediated sodium and potassium balance[20] and miR-200b and miR-717 were associated with alterations in renal tonicity [21]. However, experimental *in vivo* analysis of, the role of miRNAs in maintaining the body's water and electrolyte balance has virtually remained unexplored.

In the present study, we aimed to identify a role for miRNAs in prostaglandin dependent regulation of water homeostasis. We found that miR-132 can directly target Cox2 and silencing of miR-132 in mice revealed a severe weight loss, polyuria, and internalization of AQP2 from the plasma membrane, suggesting the first identification of a miRNA regulating principal cell water transport.

Materials and Methods

Mice

Male balb/c mice were used (n=10 per group, age=8 weeks, Charles River Nederland, Maastricht, the Netherlands). 24 hours before i.v. tail injection of 40 mg/kg bodyweight of antagomir-132 or control scramblemir, mice were housed in metabolic cages until day of sacrifice. Standard chow diet and drinking water were provided *ad libitum*. Following antagomir injection, 24-hours urine was collected during the days indicated. Mice were anesthetized with isoflurane and blood was drawn by cardiac puncture. Kidney and hypothalamus were removed and samples for RNA and protein (kidney only) analysis were frozen in liquid N2. The samples for immunocytochemistry were fixed in 10% neutral buffered formalin at 4°C for 12 hours, processed, embedded in paraffin wax and sectioned. All tissues were stored at -80°C. Blood plasma was obtained by centrifugation (15 min at 150 *g* at room temperature, no brake). The animal experiments and protocols were approved by the animal welfare committee of the veterinary authorities of the Leiden University Medical Center.

Cell culture

The cell lines NIH3T3 (3T3) and mIMCD3 (IMCD) were obtained from ATCC (American Type Culture Collection, Manassas, VA). 3T3 cells were maintained in Dulbecco's modified Eagle medium (DMEM, Gibco/ Invitrogen, Breda, the Netherlands), and IMCD cells in DMEM/HAM-F12 medium. Both media were supplemented with 10% fetal calf serum (Bio Whittaker/Cambrex, Verviers, Belgium) and 1x L-glutamin (Invitrogen) at a final concentration of 2 mM. Mouse cortical collecting duct (mpkCCD) cells (clone 11) were grown as previously described[22] in a modified defined medium (DMEM:Ham's F12 1:1 vol/vol; 60 nM sodium selenate, 5 μg/ml transferrin, 2mMglutamine, 50 nM dexamethasone, 1 nM triiodothyronine, 10 ng/ml epidermal growth factor, 5 μg/ml insulin, 20 mM D-glucose, 2% fetal calf serum and 20 mM HEPES (pH 7.4).

Antagomir design

Cholesterol-conjugated RNA analogs, 'antagomirs', (Thermo Scientific, Waltham, MA, USA) were synthesized as previously described[23]. For antagomir-132 the following sequence was used: 5'-$c_s g_s$accauggcuguagacug$_s u_s u_s a_s$-Chol-3'. As a control a 'scramblemir' was used, this RNA analog is constructed from a randomized nucleotide sequence which does not bind to any known microRNAs: 5'-$a_s u_s$gacuaucgcuauucgc$_s a_s u_s g_s$-

Chol-3'. The lower case letters represent 2'-*O*Me-modified nucleotides; subscript 's' represents phosphorothiate linkage; 'Chol' represents a cholesterol-group linked through a hydroxyprolinol linkage.

Plasma and urine assays

Multistix 8 SG (Siemens, country etc) were used to qualitatively determine specific gravity in urine. Plasma and urine osmolality were determined using a freezing point depression osmometer. Urinary AVP levels were determined on 10x diluted 24-hrs urine samples using an enzymatic AVP immunoassay (Cayman Chemical, Ann Arbor, MI, USA), according to the protocol of the manufacturer. Plasma and urine PGE2 levels were determined using an enzymatic PGE immunoassay (Cayman Chemical), according to the protocol of the manufacturer. Blood samples were supplemented with indomethacin directly after drawing blood, to prevent *ex vivo* formation of prostaglandins that have the potential to interfere with the assay.

Cox2 3 'UTR miR-132 reporter assay

A synthetic, double-stranded oligonucleotide spanning a 60 bp region of the murine 3' UTR of Cox2 mRNA containing the putative miR-132 binding site (Supplementary material) was cloned downstream of the firefly luciferase reporter gene in the pMIR-report™ Expression Reporter Vector System (Applied Biosystems, Amsterdam, the Netherlands), thereby generating pMIR-132-report. The same construct lacking the miRNA sequence (pMIR132-reportNC) served as a negative control. Sequence analysis confirmed proper cloning and sequence of the inserts. A renilla luciferase expression plasmid (pRL-SV40, Promega, Leiden, the Netherlands) served as a transfection efficiency control. Antagomir-132 or control scramblemir (5 mg/mL) was added to a near confluent layer of IMCD cells. Twenty-four hours after antagomir treatment, IMCD cells were trypsinized, resuspended in 500 mL serum-free Optimem culture medium (Gibco), and 1.5 μg pMIR-132-report or pMIR-132-reportNC, together with 150 ng pRL-SV40 were added. The cell suspension was chilled for 10 min at 4°C and electroporated using the Gene Pulser II (Bio-Rad Laboratories, Veenendaal, the Netherlands). After 10 min recovery time at room temperature, 1.5×10^5 cells were plated in a 24-wells plate in triplicate. After 24 h, the firefly-luciferase and renilla-luciferase signals were measured using a Dual-Luciferase Assay Reporter System (Promega) in a Lumat LB9507 luminometer (EG&G Berthold, Bundoora, Australia).

Western Blot

Western blot analyses were performed on cellular lysates harvested in lysis buffer (50 mM Tris-HCl pH7.5, 150 mM NaCl, 1% SDS, 0,5% deoxycholate, 0,5%

Triton X-100) containing protease inhibitors (Complete protease inhibitor cocktail, Roche, Basel, Switzerland). Protein equivalents were separated using SDS-polyacrylamide gels (SDS-PAGE) under reducing conditions and transferred to a nitrocellulose membrane (Bio-Rad Laboratories, Veenendaal, The Netherlands). Primary antibodies were utilized to detect β-actin, AQP2 and Cox2 (Abcam, Cambridge, UK), AQP2-pS256 and AQP2-pS261 antibodies were kindly provided by Dr. Jason Hoffert, University of Aarhus, Aarhus, Denmark and Dr. Knepper respectively[24,25]. Bound primary antibody was labeled with HRP-conjugated secondary antibody in blocking solution. Bound fragments were detected with chemiluminescent reagents (West Dura supersignal; ThermoFisher Scientific, Waltham, MA, USA) and exposed on Hyperfilm ECL (Amersham). Quantitative analysis of the protein bands was performed using ImageJ software and normalized to β-actin.

RNA isolation and qRT-PCR analysis

Total RNA was isolated from kidney and hypothalamus using Trizol reagent (Invitrogen). Validation of miR-132 expression was performed using Taqman® miR assays (Applied Biosystems, Foster City, CA, USA) according to manufacturer's instructions. RNU6B was utilized for normalization (sense U6: CTCGCTTCGGCAGCACA and antisense U6: AACGCTTCACGAATTTGCGT). To quantify mRNA levels, 250 ng total RNA was reverse transcribed using oligo(dT) primers and M-MLV First-Strand Synthesis system (Invitrogen) according to the manufacturers protocol. Quantitative PCR of target genes was done using SYBR Green Master Mix (Applied Biosystems). Used primer sequences of target genes were: AVP (sense): CTCTCCGCTTGTTTCCTGAG; AVP (antisense): GCAGATGCTTGGTCCGAAG; NHE3 (sense): TACATGGCCGGGCTTTCGACC; NHE3 (antisense): GAGGACTTTGCTGAGGAACTTCCGG; NKCC2 (sense): GGCTTGATCTTTGCTTTTGC; NKCC2 (antisense): CCATCATTGAATCGCTCTCC; NCC (sense): CTTCGGCCACTGGCATTCTG; NCC (antisense): GATGGCAAGGTAGGAGATGG; ENaC (sense): ACCAGGCCCCCTGCAATCAG; ENaC (antisense): CGCAGTGTCAGGGACAAACCATTG ; GAPDH (sense): ACTCCCACTCTTCCACCTTC; GAPDH (antisense): CACCACCCTGTTGCTGTAG. Levels of expression were normalized to GAPDH and quantified using the delta delta Ct method.

Immunohistochemistry

Paraffin-embedded kidneys were sectioned at 4 μm. After de-paraffinization, they were subjected to heat-induced antigen retrieval method using citrate buffer (10mM, pH 6.0) in a microwave for 20 min. Next, sections were incubated with a specific antibody against murine AQP2 (Abcam) or Cox2 (Abcam) followed by an appropriate secondary antibody labeled with Alexa-568 (Molecular Probes). Images were made using a confocal microscope (Carl Zeiss, Sliedrecht, the Netherlands).

In Situ Hybridization

In situ hybridization for miR-132 was performed by Bioneer (Denmark) as previously described[26].

Results

miR-132 directly targets Cox2

PGE2, produced by Cox2, is known to counteract vasopressin-induced renal water reabsorption in collecting duct by inducing the internalization and lysosomal degradation of AQP2. To potentially identify miRNAs involved in this proces, we used online available algorithms (www.microrna.org) and identified miR-132 as a potential post-transcriptional regulator of Cox2. To confirm functional inhibition of Cox2 mRNA by this microRNA, we cloned part of the 3'UTR of murine COX2 in a luciferase construct and introduced this construct in the mIMCD3 (IMCD) collecting duct cell line. Following transfection with antagomir-132 or the scramblemir control, we found increased luciferase expression in the presence of antagomir-132 as compared to control scramblemir, illustrating that miR-132 can directly bind and regulate Cox2 mRNA expression (Figure 1A). As miRNAs are mainly negative gene regulators, we next investigated whether silencing of miR-132 in collecting duct cells would lead to a de-repression of Cox2 expression. To that end, we used the mpkCCD collecting duct cell line that is known to induce AQP2 expression after dDAVP stimulation[22]. Indeed, both in the absence and presence of dDAVP silencing of miR-132 led to a modest but significant increase in Cox2 expression as compared to scramblemir controls (Figure 1BC).

Next to renal collecting duct cells, renal interstitial cells have also been reported to express Cox2 and may thus be under regulation of miR-132. To test this, we used 3T3 cells as an *in vitro* model for renal interstitial cells and cultured them in the presence of antagomir-132 or scramblemir. Indeed, also in these cells we found a significant 2-fold increase in Cox2 expression in cells treated with antagomir-132 as compared to the scramblemir (Figure 1DE).

Blocking miR-132 results in increased AQP2 expression in vitro

Since miR-132 targets Cox2 and Cox2-produced prostaglandins reduce dDAVP-induced AQP2 abundance, silencing miR-132 was anticipated to decrease AQP2 abundance. To test this, we cultured mpkCCD cells in the presence of dDAVP to induce AQP2 expression, and blocked miR-132 by adding antagomir-132. Surprisingly, subsequent western blot analysis showed increased levels of AQP2 with miR-132 silencing (Figure 1FG). This indicates that in this collecting duct cell line, miR-132 dependent AQP2 regulation does not involve direct Cox2 mediated signaling. How miR-132 mediates the increase in AQP2 expression in mpkCCD cells needs further investigation.

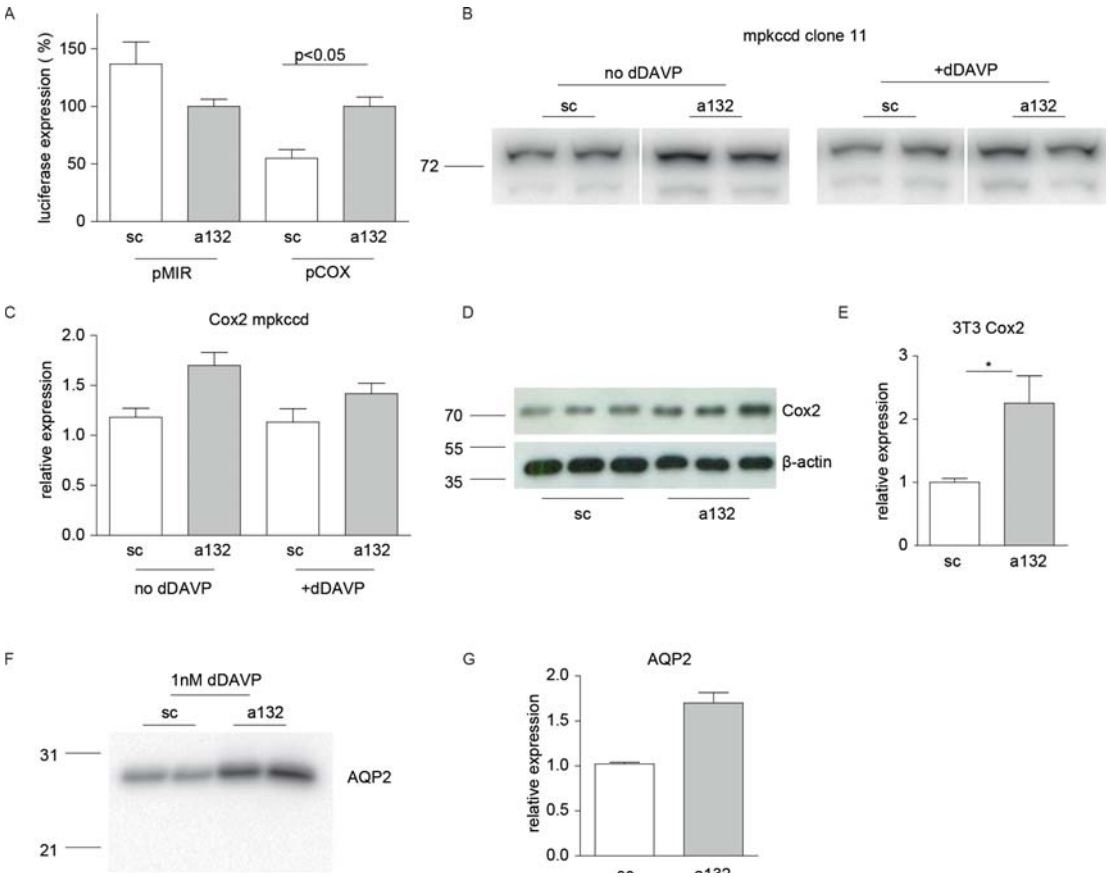


Figure 1. miR-132 targets Cox2 and affects AQP2 expression in vitro. A, luciferase assay confirms functional, 3'UTR-dependent repression of Cox2 expression by miR-132. B-C, representative western blot for Cox2 in mpkccd (B) and 3T3 (D) cells and corresponding quantification (C and E) shows increased Cox2 expression following miR-132 silencing. F and G, representative western blot (F) for AQP2 and quantification (G) in mpkccd cells after dDAVP stimulation illustrates miR-132 dependent regulation.

MiR-132 silencing in mice results in weight loss and diuresis

As, *in vivo,* the Cox2 expressing target cell for miR-132 could be different from the collecting duct cell or, because miR-132 action might be influenced by systemic factors, we next sought to determine the effect of silencing miR-132 on water homeostasis in mice. To keep track of physiological parameters, we housed the mice in metabolic cages to collect 24-hours urine following injection of antagomir-132 or control scramblemir. Mice were sacrificed either 1 day or 3 days after injection, blood was collected and kidneys and hypothalami were isolated. To validate *in vivo* miR-132 silencing we quantified miR-132 expression by qRT-PCR and observed an almost 1000 fold downregulation of miR-132 in total kidney tissue at both 1- and 3 days after administration (Figure 2A). To get insight in the regional expression of

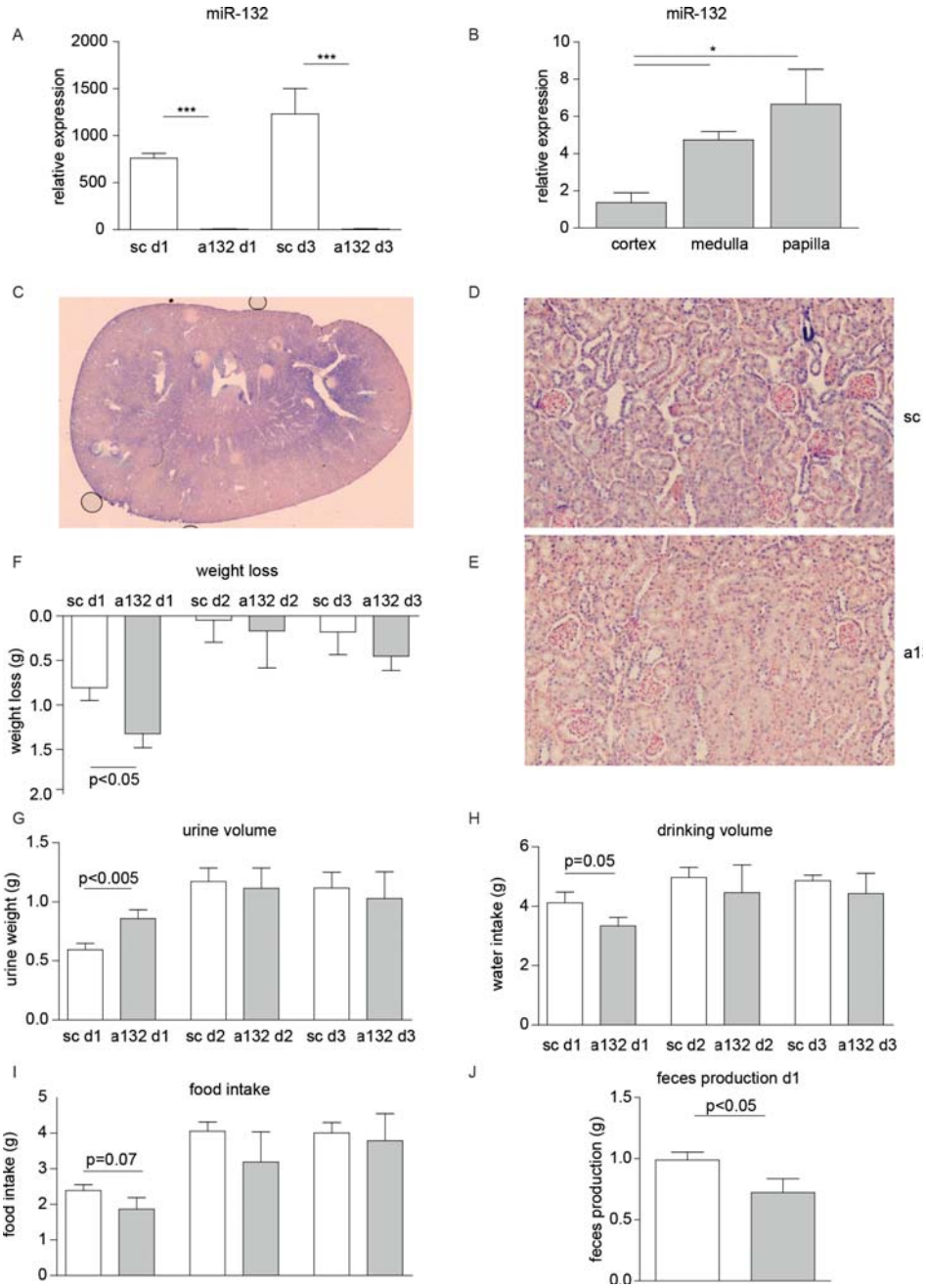


Figure 2. Silencing of miR-132 induces diuresis. A, antagomir-132 successfully knocked down miR-132 in vivo in the kidney determined by qRT-PCR. B, qRT-PCR for miR-132 in dissected kidneys shows higher miR-132 levels in medullary regions. C, representative whole mount image of in situ hybridization (ISH) for miR-132 confirms highest miR-132 expression in medulla. D, representative microscopic images of ISH illustrate predominant tubular epithelial localization of miR-132 and (E) knockdown by antagomir-132. F, miR-132 silencing results in weight loss 1 day after injection as compared to scramblemir control. G, antagomir-132 treatment results in diuresis 1 day after injection. H-J, antagomir-132 treatments results in a trend towards less water (H) and food (I) intake 1 day after injection, as well as reduced feces production (J).

miR-132 in the untreated kidney we dissected cortex, medulla and papilla and subsequent qRT-PCR analysis demonstrated that miR-132 expression displays a regional increase from cortex to papilla (Figure 2B). Although expression was found throughout the entire kidney, *in situ* hybridization (ISH) confirmed abundant medullary expression of miR-132 (Figure 2C) with highest levels in distal tubular epithelial cells (Figure 2D). Also some vascular smooth muscle cells clearly stained positive (Supplementary Figure 1). ISH of kidneys of antagomir-132 treated mice validated miR-132 silencing as miR-132 staining was markedly reduced (Figure 2DE).

Physiological parameters also revealed striking effects of miR-132 silencing. As early as 24 hours after injection, mice injected with antagomir-132 lost 0.5 gram more body weight than scramblemir-injected mice, which corresponds to 2-2.5% of their total body weight (Figure 2F).

Importantly, and in line with the significant weight loss, at 24-hours post-injection, urine and drinking volumes of mice treated with antagomir-132 were significantly higher and lower, respectively, as compared to the volumes of control mice (Figure 2GH). This suggests miR-132 silencing affects thirst sensation which, in combination with polyuria, resulted in even more dehydration explaining the observed weight loss. Antagomir-132 treated mice showed a trend towards decreased food intake, which corresponded to a reduced feces production (Figure 2IJ).

As we observed antagomir-132 induced polyuria (Figure 3A), we sought to determine if urine and plasma osmolality were changed. As shown in Figure 3B we found an increase in plasma osmolality associated with a decrease in urine osmolality. In concordance, we observed a decrease in specific gravity of these urines, assessed qualitatively using urine strips (data not shown). As sodium is considered to be the main determinant of plasma osmolality, we measured sodium levels in plasma but, surprisingly did not find an increase in sodium concentration (Figure 3C). Urinary sodium did show a trend towards increased levels (figure 3D), suggesting miR-132 silencing partly hampers active sodium reabsorption.

Silencing of miR-132 abrogates apical localization of AQP2 in collecting duct cells

Given the increased fractional excretion of water in the antagomir-132 treated mice, we next quantified AQP2 protein expression by western blot. However, we found no significant reduction in total AQP2 expression in mice that were injected with antagomir-132 compared to the scramblemir treated controls (Figure 4AB). In contrast, when we investigated the cellular localization of AQP2 using immunofluorescent staining we observed a striking difference

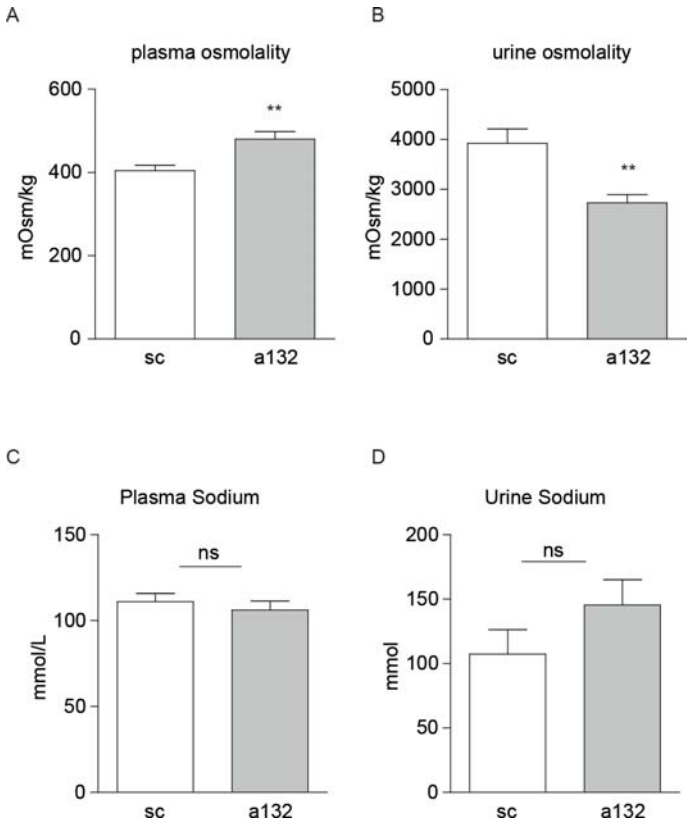


Figure 3. Blocking miR-132 results in a strong change in osmolality while not in sodium levels. A and B, plasma osmolality (A) increased after miR-132 silencing, while urine osmolality decreased (B). C and D, plasma (C) and urine (D) sodium levels were not significantly altered, although urinary sodium showed a trend towards increased levels.

between both treatment groups. In kidney sections from control mice, a clear apical staining is observed whereas in antagomir-132 treated mice however, AQP2 is mainly located intra-cellularly (Figure 4C). Subsequently, we sought to determine if S256 and S261 phosphorylation of AQP2 had been affected as this has been described to be involved in the localization of AQP2[27]. As shown in Figure 4C-E, no differences were observed.

MiR-132 silencing suppresses sodium-hydrogen exchanger 3 (NHE3) gene expression

Unexpectedly, despite the increased osmolality we did not measure a difference in plasma sodium levels. However, urinary sodium showed a trend towards increased levels, suggesting antagomir-132 partly inhibits active sodium reabsorption. Therefore we investigated gene expression of four main sodium transporters along the tubular system, sodium-hydrogen exchanger 3 (NHE3), sodium-chloride symporter (NCC), Na-K-Cl cotransporter

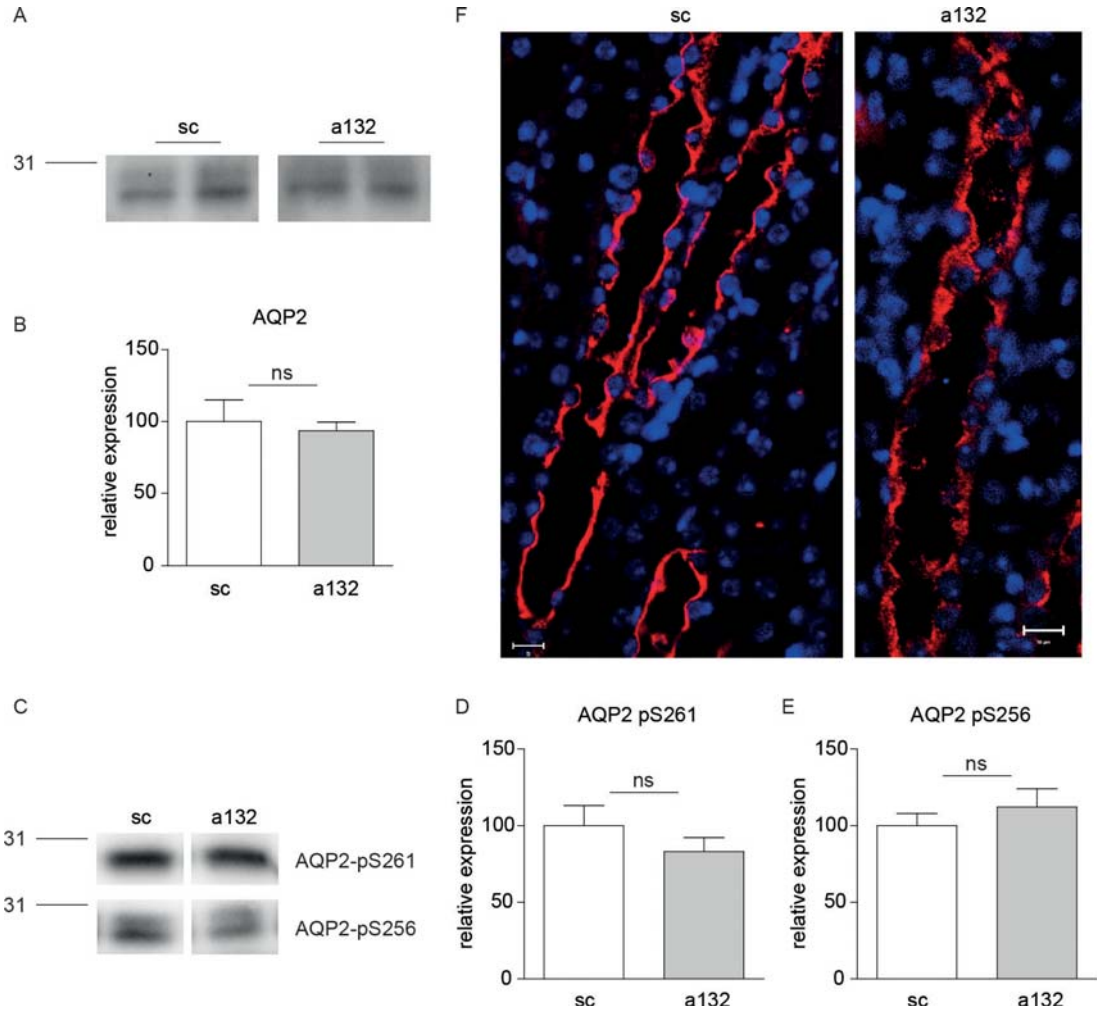


Figure 4. miR-132 silencing results in altered AQP2 localization. A and B, representative western blot for AQP2 (A) and quantification (B) shows no differences in expression. C-E, representative western blots for phosporylated AQP2 variants AQP2-pS261 and AQP2-pS256 (C) and qunatification (D and E) indicate no differences in phosporylation. F, representative microscopic images of AQP2 staining reveals different localization of AQP2. Blocking miR-132 results in less apical AQP2 staining.

(NKCC2) and the amiloride-sensitive epithelial sodium channel (ENaC). Interestingly, a striking decrease in NHE3 was found (Supplementary Figure 2).

Silencing of miR-132 increases urinary PGE levels

Since we demonstrated that miR-132 could directly target Cox2, we hypothesized that silencing of miR-132 should result in a de-repression of Cox2 expression and increased production of PGE2. Therefore we determined renal prostaglandin production (which is also used as a measure

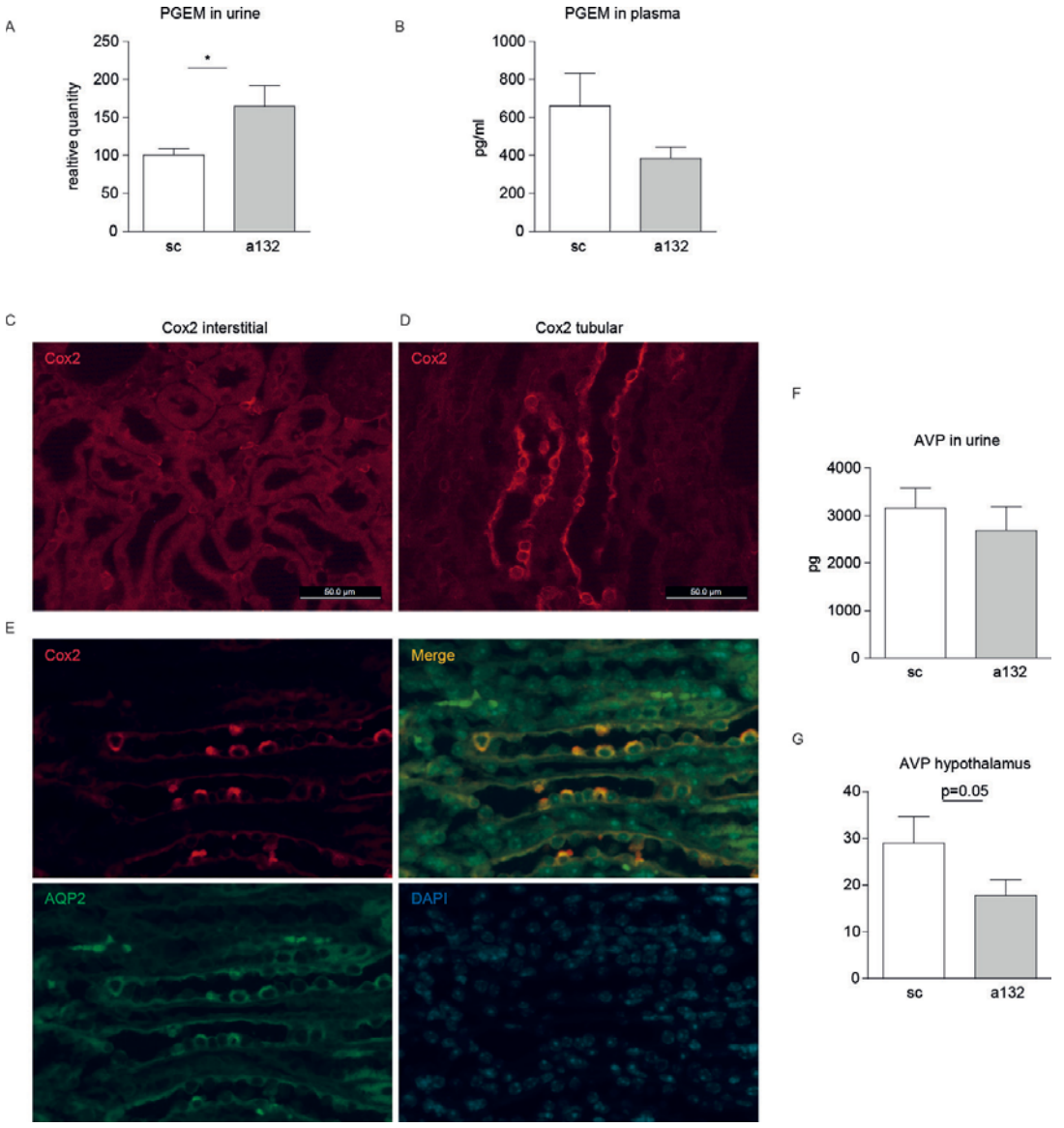


Figure 5. Increased renal PGE2 production while no increase in AVP. A, urinary PGE2 levels increased due to miR-132 silencing. B, Plasma PGE2 levels were not significantly altered but showed a trend towards reduced levels. C and D, representative microscopic images of renal Cox2 shows interstitial (C) and tubular epithelial (D) cell positive staining. E, representative microscopic images of double staining for Cox2 and AQP2 shows co-localization in collecting ducts. F, AVP levels in urine, reflecting plasma AVP, did not alter due to miR-132 silencing, despite increases plasma osmolality. G, pituitary AVP gene expression is decreased due to blocking miR-132.

for Cox2 activity) by measuring this in the urine, as urinary PGE2 excretion is mainly derived from medullary synthesis[28]. Indeed, we found significantly increased PGE2 levels in urine of mice treated with antagomir-132 (Figure 5A). In contrast, circulating PGE2 levels had a tendency to be decreased

(Figure 5B). To determine where miR-132 could affect Cox2 we performed a staining to assess where Cox2 is expressed. Although some controversy exists about the location of constitutive Cox2 expression, we found it to be expressed in interstitial cells, cortical tubular structures (based on morphology predominantly distal, data not shown) and in specific medullary tubular epithelial structures (Figure 5C-D). The latter we identified to be collecting ducts as determined by co-staining with AQP2 (Figure 5E). This pattern highly corresponds to miR-132 localization, consistent with a functional link between the miR and Cox2.

MiR-132 blocking attenuates hypertonicity-induced increase in blood AVP levels

In normal physiology increased plasma osmolality stimulates drinking and secretion of AVP, resulting in increased renal water reabsorption, more concentrated urine, and less concentrated blood plasma. We assessed vasopressin levels in the urine, which reflects AVP levels in the blood[29], and surpisingly found no increase in AVP levels (Figure 5F). In fact, we see a trend towards decreased levels of vasopressin. Alternatively, or additionally, the observed effect of increased diuresis, may be due to reduced central vasopressin production and release, possible through PGE2 as well, as PGE2 has been implied in central AVP production and release[14]. To assess this hypothesis we excised the hypothalamus of the animals and determined AVP gene expression by qPCR. We found decreased levels of AVP gene expression in the hypothalamus in mice that received antagomir-132 (Figure 5G). These data suggest that silencing of miR-132 has a central effect on AVP production providing an additional explanation for the (trend towards) lower urinary AVP levels.

Discussion

In this study we identified a novel regulatory role for miR-132 in diuresis by affecting the translocaton of AQP2 to the apical membrane in principal collecting duct cells. Silencing of miR-132 results in less AQP2 mediated water reabsorption and therefore in polyuria. This finding is accompanied by increased plasma osmolality and decreased urine osmolality. The effects we observed seem to be acute, as they are present within 24 hours after injection of antagomir-132, while 48 hours after injection, no further weight loss or polyuria is found. This suggests the body responds to this interference in homeostatic conditions by a feedback mechanism, which seems appropriate as regulation of water and electrolyte balance is tightly controlled. Antagomir-132 treated mice showed a trend towards decreased food intake that corresponded with reduced feces production which is therefore no contributor to weight loss. However, these observations suggest the mice display signs of malaise.

We identified Cox2 to be a novel direct target of miR-132 and show in collecting duct (mpkccd) cells as well as in interstitial (fibroblast) cells (NIH3T3) that blocking miR-132 results in increased Cox2 levels. *In vivo*, this is illustrated by increased renal PGE2 production as demonstrated by PGE2 levels in the urine. However, plasma PGE2 levels are decreased, suggesting that miR-132 can have ambiguous effects, depending on the site of action.

We found miR-132 expression predominantly in tubular structures, with highest levels in the renal medulla and more distant regions of the tubule. In addition, we observed most Cox2 expression in distant regions of the tubule, mainly in collecting ducts, although contradictory findings have been reported on this subject[30,31]. As we demonstrated that Cox2 is a direct target of miR-132, this suggests its regulation could be mainly taking place here. This would mean that PGE2 is produced in the same cells as they target. However, also (cortical) interstitial cells are expressing Cox2, and as we have shown in 3T3 cells, silencing miR-132 in interstitial cells resulted in a firm increase in Cox2 expression. To elucidate the exact site of action of miR-132 and its target(s) involved in diuresis further investigations are necessary.

The increased renal PGE2 levels can explain the decrease in AQP2 translocation and subsequent increased diuresis as it is known to counteract AVP induced AQP2 translocation[10,11]. In addition, AVP levels in the urine, reflecting AVP levels in the circulation[29] were slightly decreased, possibly due to decreased AVP production in the hypothalamus as we show that

hypothalamic AVP gene expression is decreased as a results of antagonizing miR-132. These decreased levels could again be explained by an alteration in PGE2 levels[14] although the exact mechanism needs further investigation. In addition, to clarify the role of central AVP production on the observed phenomena in this study, one needs to investigate the effect of antagomir-132 while also providing synthetic dDAVP to the mice, which could rescue the phenotype if it was completely due to central AVP interference.

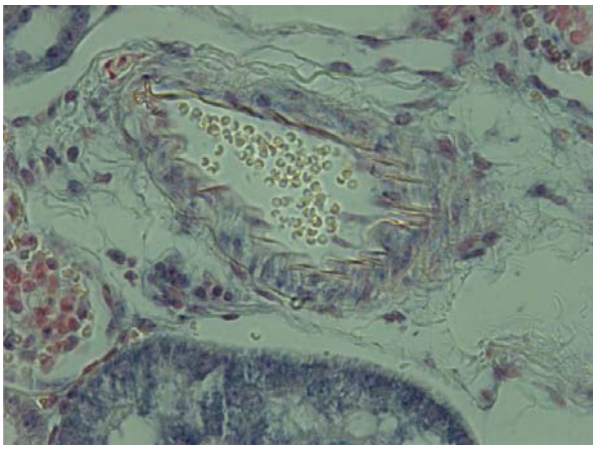
It has been well established that AVP also has long term effects in principal collecting duct cells by activating transcription of APQ2 via activation of and promoter binding by the cyclic AMP responsive element binding protein (CREB)[6,7]. CREB itself is known to regulate transcription of miR-132[32], providing a potential feedback loop in signaling. Furthermore, miR-132 can target p300[33], which is a transcriptional co-activator that can act in concert with CREB. In addition, promoter activity of Cox2 is dependent on CREB/p300 transcription factors[34,35]. These factors are adding additional layers of possible autoregulation within water maintenance. Besides, this might explain the *in vitro* discrepancies we found with AQP2 expression. On the other hand, it has been shown that inhibition of Cox2 by indomethacin reduced renal AQP2 expression in rats, implying that prostaglandins may actually help maintain AQP2 levels[13], which might also explain our *in vitro* observations.

We observed an increase in plasma osmolality suggesting the mice are hypovolemic. This would normally be accompanied by decreased blood pressure and subsequent activation of the RAAS pathway. Angiotensin II, that will be increased due to RAAS activation, is known to exert a dipsogenic effect[36]. The dipsogenic action of angiotensin II is thought to be mediated by the subfornical organ (SFO), because the SFO contains a high concentration of the angiotensin AT1 receptor[37] and ablation of the SFO completely abolished the dipsogenic response to angiotensin II[38]. However, we surprisingly found the polyuric mice to drink less. It could be expected therefore that angiotensin II signaling is disturbed. Indeed, a crosstalk between miR-132 and angiotensin II has been described[39]. Furthermore it was shown that miR-132 can directly target Angiotensin II type 1 Receptor (AT1R)[40]. These data suggest that also angiotensin II signaling is disturbed in these mice by silencing miR-132. Interestingly, NHE3 is also known to be regulated by angiotensin II[41], so disturbed angiotensin II signaling could also reduce NHE3 expression, which is what we observed.
Some neurons in the above mentioned SFO are osmoreceptors, being sensitive to the osmotic pressure of the blood. These neurons project to

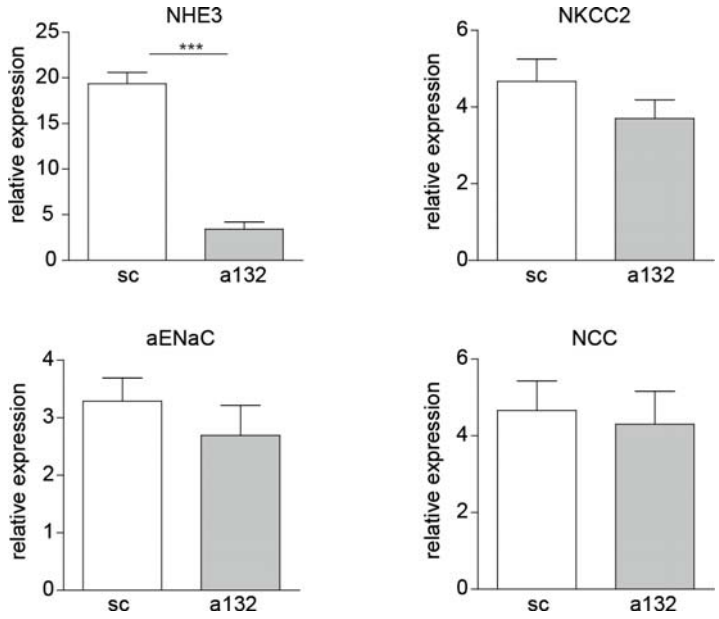
the supraoptic nucleus and paraventricular nucleus to regulate the activity of vasopressin-secreting neurons. AVP levels are known to be subject to circadian regulations. Also CREB mediated miR-132 expression is circadian-regulated, in the suprachiasmatic nucleus (SCN), where it is important for proper clock-resetting responses to light[42]. Furthermore, AVP plays an important role in nocturnal enuresis. It is therefore that DDAVP is used as a drug to treat nocturnal enuresis, although this phenomenon is not mediated by the V2R and does not involve AQP2[43,44]. It would be plausible however for miR-132 to play a role in this regulation as it is involved in diuresis. Interestingly, a comphrehensive miRNA sequencing study indicated miR-132 to be highly enriched in the pituitary[15], suggesting antagomir-132 could strongly affect the above mentioned processes at this site.

Taken together, it is evident that miR-132 plays a central role in physiology, specifically in water and volume balance regulation. It is therefore likely that miR-132 expression is crucial to associated disorders like hypertension, edema, Diabetes Insipidus and syndrome of inappropriate antidiuretic hormone hypersecretion (SIADH) .This makes miR-132 a very attractive candidate for future therapeutics and further investigation in physiological processes.

Supplementary Files



Supplementary figure 1. In situ hybridization (ISH) shows miR-132 is present in vascular smooth muscle cells.



Supplementary figure 2. qRT-PCR of 4 main sodium transporters along the tubular system reveals a strong decrease in NHE3 expression due to miR-132 silencing.

References

1. Sinke, A.P. & Deen, P.M. The physiological implication of novel proteins in systemic osmoregulation. FASEB journal : official publication of the Federation of American Societies for Experimental Biology 25, 3279-3289 (2011).
2. Nielsen, S., et al. Vasopressin increases water permeability of kidney collecting duct by inducing translocation of aquaporin-CD water channels to plasma membrane. Proceedings of the National Academy of Sciences of the United States of America 92, 1013-1017 (1995).
3. Nielsen, S., DiGiovanni, S.R., Christensen, E.I., Knepper, M.A. & Harris, H.W. Cellular and subcellular immunolocalization of vasopressin-regulated water channel in rat kidney. Proceedings of the National Academy of Sciences of the United States of America 90, 11663-11667 (1993).
4. Fushimi, K., et al. Cloning and expression of apical membrane water channel of rat kidney collecting tubule. Nature 361, 549-552 (1993).
5. Nonoguchi, H., et al. Immunohistochemical localization of V2 vasopressin receptor along the nephron and functional role of luminal V2 receptor in terminal inner medullary collecting ducts. The Journal of clinical investigation 96, 1768-1778 (1995).
6. Matsumura, Y., Uchida, S., Rai, T., Sasaki, S. & Marumo, F. Transcriptional regulation of aquaporin-2 water channel gene by cAMP. Journal of the American Society of Nephrology : JASN 8, 861-867 (1997).
7. Yasui, M., Zelenin, S.M., Celsi, G. & Aperia, A. Adenylate cyclase-coupled vasopressin receptor activates AQP2 promoter via a dual effect on CRE and AP1 elements. The American journal of physiology 272, F443-450 (1997).
8. Robben, J.H., Knoers, N.V. & Deen, P.M. Cell biological aspects of the vasopressin type-2 receptor and aquaporin 2 water channel in nephrogenic diabetes insipidus. American journal of physiology. Renal physiology 291, F257-270 (2006).
9. Antunes-Rodrigues, J., de Castro, M., Elias, L.L., Valenca, M.M. & McCann, S.M. Neuroendocrine control of body fluid metabolism. Physiological reviews 84, 169-208 (2004).
10. Hebert, R.L., Jacobson, H.R. & Breyer, M.D. PGE2 inhibits AVP-induced water flow in cortical collecting ducts by protein kinase C activation. The American journal of physiology 259, F318-325 (1990).
11. Breyer, M.D. & Breyer, R.M. Prostaglandin E receptors and the kidney. American journal of physiology. Renal physiology 279, F12-23 (2000).
12. Anderson, R.J., Berl, T., McDonald, K.D. & Schrier, R.W. Evidence for an in vivo antagonism between vasopressin and prostaglandin in the mammalian kidney. The Journal of clinical investigation 56, 420-426 (1975).
13. Kim, S.W., et al. Indomethacin enhances shuttling of aquaporin-2 despite decreased abundance in rat kidney. Journal of the American Society of Nephrology : JASN 15, 2998-3005 (2004).
14. Kennedy, C.R., et al. Urine concentrating defect in prostaglandin EP1-deficient mice. American journal of physiology. Renal physiology 292, F868-875 (2007).
15. Landgraf, P., et al. A mammalian microRNA expression atlas based on small RNA library sequencing. Cell 129, 1401-1414 (2007).
16. Chandrasekaran, K., et al. Role of microRNAs in kidney homeostasis and disease. Kidney international 81, 617-627 (2012).
17. Xie, X., et al. Systematic discovery of regulatory motifs in human promoters and 3' UTRs by comparison of several mammals. Nature 434, 338-345 (2005).
18. Small, E.M. & Olson, E.N. Pervasive roles of microRNAs in cardiovascular biology. Nature 469, 336-342 (2011).

19. Pastorelli, L.M., et al. Genetic analyses reveal a requirement for Dicer1 in the mouse urogenital tract. Mammalian genome : official journal of the International Mammalian Genome Society 20, 140-151 (2009).
20. Elvira-Matelot, E., et al. Regulation of WNK1 expression by miR-192 and aldosterone. Journal of the American Society of Nephrology : JASN 21, 1724-1731 (2010).
21. Huang, W., et al. Tonicity-responsive microRNAs contribute to the maximal induction of osmoregulatory transcription factor OREBP in response to high-NaCl hypertonicity. Nucleic acids research 39, 475-485 (2011).
22. Boone, M., Kortenoeven, M., Robben, J.H. & Deen, P.M. Effect of the cGMP pathway on AQP2 expression and translocation: potential implications for nephrogenic diabetes insipidus. Nephrol Dial Transplant 25, 48-54 (2010).
23. Krutzfeldt, J., et al. Silencing of microRNAs in vivo with 'antagomirs'. Nature 438, 685-689 (2005).
24. Hoffert, J.D., et al. Dynamics of aquaporin-2 serine-261 phosphorylation in response to short-term vasopressin treatment in collecting duct. American journal of physiology. Renal physiology 292, F691-700 (2007).
25. Nishimoto, G., et al. Arginine vasopressin stimulates phosphorylation of aquaporin-2 in rat renal tissue. The American journal of physiology 276, F254-259 (1999).
26. Nielsen, B.S., et al. High levels of microRNA-21 in the stroma of colorectal cancers predict short disease-free survival in stage II colon cancer patients. Clinical & experimental metastasis 28, 27-38 (2011).
27. Tamma, G., Robben, J.H., Trimpert, C., Boone, M. & Deen, P.M. Regulation of AQP2 localization by S256 and S261 phosphorylation and ubiquitination. Am J Physiol Cell Physiol 300, C636-646 (2011).
28. Lote, C.J. & Haylor, J. Eicosanoids in renal function. Prostaglandins, leukotrienes, and essential fatty acids 36, 203-217 (1989).
29. Moses, A.M. & Steciak, E. Urinary and metabolic clearances of arginine vasopressin in normal subjects. The American journal of physiology 251, R365-370 (1986).
30. Breyer, M.D. & Harris, R.C. Cyclooxygenase 2 and the kidney. Current opinion in nephrology and hypertension 10, 89-98 (2001).
31. Yang, T., et al. Regulation of cyclooxygenase expression in the kidney by dietary salt intake. The American journal of physiology 274, F481-489 (1998).
32. Vo, N., et al. A cAMP-response element binding protein-induced microRNA regulates neuronal morphogenesis. Proceedings of the National Academy of Sciences of the United States of America 102, 16426-16431 (2005).
33. Lagos, D., et al. miR-132 regulates antiviral innate immunity through suppression of the p300 transcriptional co-activator. Nature cell biology 12, 513-519 (2010).
34. Deng, W.G., Zhu, Y. & Wu, K.K. Role of p300 and PCAF in regulating cyclooxygenase-2 promoter activation by inflammatory mediators. Blood 103, 2135-2142 (2004).
35. Ramsay, R.G., Ciznadija, D., Vanevski, M. & Mantamadiotis, T. Transcriptional regulation of cyclo-oxygenase expression: three pillars of control. International journal of immunopathology and pharmacology 16, 59-67 (2003).
36. Fitzsimons, J.T. Angiotensin, thirst, and sodium appetite. Physiological reviews 78, 583-686 (1998).
37. Allen, A.M., et al. Angiotensin receptors in the nervous system. Brain research bulletin 47, 17-28 (1998).
38. Simpson, J.B. & Routtenberg, A. Subfornical organ: a dipsogenic site of action of angiotensin II. Science 201, 379-381 (1978).

39. Jin, W., et al. Small RNA sequencing reveals microRNAs that modulate angiotensin II effects in vascular smooth muscle cells. The Journal of biological chemistry 287, 15672-15683 (2012).
40. Elton, T.S., et al. MiR-132 Regulates Angiotensin II Type 1 Receptor Expression Through a Protein Coding Region Binding Site. Circulation 118, S_513 (2008).
41. Houillier, P., et al. Signaling pathways in the biphasic effect of angiotensin II on apical Na/H antiport activity in proximal tubule. Kidney international 50, 1496-1505 (1996).
42. Cheng, H.Y., et al. microRNA modulation of circadian-clock period and entrainment. Neuron 54, 813-829 (2007).
43. Muller, D., Marr, N., Ankermann, T., Eggert, P. & Deen, P.M. Desmopressin for nocturnal enuresis in nephrogenic diabetes insipidus. Lancet 359, 495-497 (2002).
44. Robben, J.H., et al. Relief of nocturnal enuresis by desmopressin is kidney and vasopressin type 2 receptor independent. Journal of the American Society of Nephrology : JASN 18, 1534-1539 (2007).