



Obestatin ameliorates water retention in chronic heart failure by downregulating renal aquaporin 2 through GPR39, V2R and PPARG signaling

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ARTICLE INFO

Keywords:

Obestatin
Aquaporin2
GPR39
Heart failure
Collecting duct

ABSTRACT

Aims: Obestatin regulates water metabolism by inhibiting arginine vasopressin (AVP) release and upregulated obestatin has been detected in patients with chronic heart failure (CHF). However, the significance of obestatin in CHF, particularly with regard to water retention and aquaporin 2 (AQP2) expression, remains unknown.

Main methods: Using a CHF rat model, the effects of 2-week exogenous obestatin administration were evaluated. Expression of AQP2 was evaluated by immunoblotting, immunohistochemical staining, and quantitative real-time PCR (qPCR) in CHF rat model and mouse inner medullary collecting duct (mIMCD) 3 cell line. Moreover, the influence of obestatin on the genetic transcription profile in mIMCD3 cells was evaluated by microarray, and the potential regulatory mechanisms of obestatin on AQP2 were evaluated by RNA silencing of vasopressin receptor 2 (V2R), peroxisome proliferator-activated receptor gamma (PPARG), and G protein-coupled receptor 39 (GPR39).

Key findings: Obestatin increased urinary output and improved expression of CHF biomarker without significantly altering cardiac function, plasma electrolyte concentrations, or the plasma AVP concentration. AQP2 expression was significantly reduced. The results of microarray analyses and qPCR indicated that mRNA levels of *Aqp2*, *Pparg*, and *V2r* were significantly decreased. Inhibition of *V2r* and *Pparg* mRNA further reduced the expression of AQP2, while the inhibitory efficacy of obestatin on AQP2 was significantly offset after *Gpr39* knockdown.

Significance: Long-term treatment with obestatin improves water retention in CHF by increasing urinary output through downregulation of AQP2 expression in renal IMCD cells. These effects may be at least partially mediated by regulation of GPR39, V2R and PPARG signaling.

1. Introduction

Chronic heart failure (CHF) is a severe clinical syndrome that occurs in the end stage of various cardiovascular diseases. Water retention is an important pathophysiological characteristic of CHF and contributes to a variety of clinical symptoms, such as breathlessness and edema. Typically, diuretics are used to relieve these symptoms through increased urinary output. However, long-term use of conventional diuretics may expose patients to metabolic disorders and electrolyte imbalance [1–3]. Therefore, development of a novel treatment against water retention that does not significantly influence the electrolyte balance is important for improving the treatment of CHF patients.

The aquaporin (AQP) family consists of transmembrane proteins that function via the transport channel for water and other small molecule solutes, and 13 AQPs have been identified in mammals to date [4,5]. Among them, aquaporin 2 (AQP2), which is located in the luminal plasma membrane and intracellular vesicles in the principle cells of renal collecting ducts (CDs), has been recognized as the key regulator of water reabsorption and urine concentration, likely via regulation of the water permeability of CDs [6]. It has been confirmed that upregulated AQP2 in the luminal plasma membrane and the associated increase in water reabsorption are the most important pathophysiological mechanisms for water retention in patients with CHF [7]. In fact, novel diuretics targeting AQP2, such as tolvaptan, have been shown to be

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promising for patients with diseases related to water retention, including CHF [8,9].

Obestatin is a 23-amino acid polypeptide homologous to ghrelin that has been suggested to exert various biological functions via activation by amidation [10]. Previous studies confirmed that the physiological function of obestatin may include regulation of appetite, body weight, lipid metabolism, and vascular endothelial functions [11–13]. Interestingly, a previous study showed that intracerebroventricular injection of obestatin may regulate water metabolism by acting on the thirst center [14]. More importantly, obestatin reduced renal reabsorption of water by inhibiting arginine vasopressin (AVP)-mediated expression and activation of AQP2 [14]. Moreover, the results of our previous research showed that the level of obestatin in the peripheral circulation was significantly higher in patients with CHF than in healthy controls [15]. Since plasma obestatin is unable to enter the thirst center through the blood–brain barrier and its receptor GPR39 is abundantly expressed in kidney, we hypothesized that the upregulated obestatin in CHF may regulate water metabolism by acting on the renal system. Therefore, in this study, we evaluated the potential influence of exogenous obestatin on water retention and AQP2 expression in the renal CDs. Moreover, the potential mechanisms underlying the regulatory effects of obestatin on AQP2 expression were explored via microarray analysis and gene silencing technique.

2. Materials and methods

2.1. Main materials

The mouse inner medullary CD (mIMCD) 3 cell line was purchased from JENNIO Biotech (Guangzhou, China). Obestatin and NA-obestatin (nonamidated obestatin), were synthesized by GL Biochem (Shanghai, China). Desmopressin (dDAVP) and obestatin anti-serum (obestatin-IgG, 1 mg/ml) were purchased from Phoenix Pharmaceuticals (Burlingame, CA). Mozavaptan (OPC-31260, OPC) was purchased from Selleckchem (Houston, TX, USA). The lentiviral pLKO.1 plasmid vector was kindly shared by the Shanghai institute for Biological Sciences.

2.2. Animal experiments

Pathogen-free male Sprague–Dawley rats (7 weeks old, body weight 180–200 g) were provided by Shanghai SIPPR-BK Laboratory Animal Co. Ltd. [Animal license number: SCXK (Shanghai) 2013-0016]. The rats were weighed every week, fed commercial rat chow, given free access to water, and housed under a 12-h light/12-h dark cycle at a temperature of $25 \pm 2^\circ\text{C}$. All experimental protocols, which were in agreement with laboratory animal management and use regulations, had been approved by the Animal Care and Use Committee of Second Military Medical University before the performance of the study. All rats were acclimatized for 5 days before modeling. CHF was induced by myocardial infarction (MI) via ligation of the left anterior descending branch (LAD) of the coronary artery [16]. For the rats assigned to the sham group, the same procedure was performed without ligation of the LAD. Limb lead electrocardiograms (ECGs) were recorded during the whole process to evaluate the effect of ligation. MI model rats were fed normally for 6 weeks. Then according to transthoracic echocardiography results (Table 1), the surviving rats were screened by the criterion of an ejection fraction (EF) value $\leq 45\%$ [17,18] as the successful CHF model. The selected CHF model rats were randomly assigned to the following seven groups (n = 6 rats per group) according to different treatments (intravenously injected twice daily in 200 μl physiological saline): blank model group (labeled CHF, physiological saline), high-dose obestatin group (labeled ob-H, 200 $\mu\text{g}/\text{kg}$ body weight, BW), low-dose obestatin group (labeled ob-L, 100 $\mu\text{g}/\text{kg}$ BW) [19], NA-obestatin group (labeled NA, 200 $\mu\text{g}/\text{kg}$ BW), dDAVP group (labeled dDAVP, 2 $\mu\text{g}/\text{kg}$ BW) [20], OPC-31260 group (labeled OPC, 1 mg/rat) [21], obestatin-IgG group (labeled ob-IgG, 3 $\mu\text{g}/\text{rat}$) [14]. Along with the rats

Table 1

Ejection Fraction of groups at the 6th week after MI modeling operation.

Groups (n = 6)	LVEF (%)
Sham	73.11 \pm 3.45
CHF	37.50 \pm 3.69***
NA	37.89 \pm 3.62***
Ob-H	36.16 \pm 6.03***
Ob-L	37.05 \pm 4.79***
OPC	36.99 \pm 4.33***
dDAVP	37.76 \pm 3.00***
ob-IgG	37.28 \pm 4.47***

The data are presented as the means \pm SD. LVEF, left ventricular ejection fraction.

*** Denotes $P < 0.001$, compared with sham group.

in the sham group (labeled sham, n = 6) which were given physiological saline similarly, rats in a total of eight groups were treated for 2 weeks. Among the above groups, the CHF and NA groups served as negative control groups, and the OPC31260 (a selective vasopressin receptor 2 [V2R] antagonist) and dDAVP (agonist to V2R) groups served as positive control groups representing the downregulating and upregulating effects on renal AQP2, respectively.

2.3. Transthoracic echocardiography measurements

Transthoracic echocardiography was performed in anesthetized rats to evaluate the morphology, ventricular wall motion, and function of the left ventricle using an iU22 color Doppler ultrasound diagnostic instrument and S5-1 high frequency probe (Philips, Boston, MA, USA). Parameters obtained included left ventricular internal diameter at end-systole (LVIDs), left ventricular internal diameter at end-diastole (LVIDd), left ventricular end-diastolic volume (LVDV), and left ventricular end-systolic volume (LVSV), of which the latter two were calculated by bullet equation [22]. The left ventricular ejection fraction (LVEF) and shortening fraction (LVFS) were calculated by Simpson method [23]. Three cardiac cycles were continuously measured, and the data obtained were averaged for further analysis.

2.4. Collection and processing of urine and plasma samples

Twenty-four-hour urine samples were collected before and after the treatments by placing all rats in individual metabolic cages. At the same time, total volumes of 24-h urine samples were recorded and adjusted for body weight. Serum samples of 1 ml were collected in duplicate from the femoral vein at 6th and 8th week after anesthesia using heparin lithium anticoagulant tubes and EDTA-K2 anticoagulant tubes. After collection, plasma samples were centrifuged (4°C , 3000g/min, 10 min). The medium yellow clear liquid was transferred to a new tube and stored at -80°C until assay performance. The plasma in heparin lithium tubes was used for electrolyte detection, and that in EDTA-K2 tubes was used for brain-type natriuretic peptide (BNP), AVP, and obestatin measurements. Electrolyte levels in plasma samples were measured by a HITACH 7600 Biochemical Analyzer (Hitachi, Tokyo, Japan) automatically according to the manufacturer's instructions. Plasma BNP, AVP, and obestatin levels were measured using enzyme-linked immunosorbent assay (ELISA) kits (Westang, Shanghai, China). The sensitivity limits of the assays were 16 pg/ml, 10 pg/ml, and 14 pg/ml, respectively.

2.5. Preparation of kidney tissue for RNA and protein extraction

At the end of week 8, all rats were anesthetized and euthanized. Kidney samples were rapidly harvested and rinsed with cold physiological saline. The inner medulla was dissected from the right kidney and

divided into two parts. One half was ground in TRIzol Reagent (Invitrogen, CA, USA) for RNA isolation using a glass homogenizer on ice, and the other half was ground in radio immunoprecipitation assay (RIPA) Lysis Buffer containing 0.1 M phenylmethylsulfonyl fluoride (PMSF) for protein extraction.

2.6. Preparation of kidney tissue for immunohistochemical staining

The left kidneys of rats were perfused and fixed in 4% neutral paraformaldehyde for 24 h before dehydration with a gradient of ethanol concentrations. Dehydrated samples were embedded in paraffin wax and then 5- μ m paraffin sections were prepared for immunohistochemical examination. Briefly, endogenous peroxidase was blocked in 3% H₂O₂ for 10 min at room temperature. For antigen retrieval, sections were heated in a microwave oven and then boiled in a mixed retrieval solution (1 mM Tris, pH 9.9 with 0.5 mM EDTA) for 10 min. After cooling naturally, the sections were blocked in phosphate-buffered saline (PBS) containing 3% bovine serum albumin (BSA) and then incubated with AQP2 antibody (1:2000 dilution) in PBS with 0.1% BSA and 0.3% Triton X-100 at 4 °C overnight. After three 5-min washes with PBS, sections were incubated with horseradish peroxidase-conjugated secondary antibody at room temperature for 60 min. Upon rinsing with PBS buffer, the regions of antibody-antigen reaction were visualized with DAB (3-diaminobenzidine) color-substrate solution. Brown stained regions were the target protein-positive areas. Moreover, the nuclei were visualized by hematoxylin staining. Photographs of the stained sections were taken under microscopy (Leica, Wetzlar, Germany) with the same optical parameters. Six different regions in each section were analyzed twice. Protein expression was assessed through the mean density acquired from areas of interest analyzed by Image-Pro plus 6.0 (Media Cybernetics, MD, USA). Two researchers performed the evaluation independently. The mean density was calculated based on the following formula: mean density = integrated optical density (IOD)/area.

2.7. mIMCD3 cell culture and treatments

Cells were cultured in a 1:1 mixture of Dulbecco's Modified Eagle's Medium and Ham's F12 medium (DMEM/F12) supplemented with 10% fetal bovine serum (Gibco, ThermoFisher, MA, USA) in a humidified incubator with a 5% CO₂ atmosphere at 37 °C. Once the mIMCD3 cells reached approximately 70% confluency, the medium was replaced with serum-free DMEM/F12 medium for 12 h. After serum starvation, the cells were treated with obestatin of different concentrations or for different durations to determine the optimal treatment regimen. Once the optimal conditions for obestatin stimulation were determined, the cells were divided into seven groups treated with PBS (Con group), obestatin-IgG (10 μ g/ml) for 1 h prior to obestatin (10⁻⁷ mM, ob + ob-IgG group), dDAVP (10⁻⁷ mM, dDAVP group), NA-obestatin (10⁻⁷ mM, NA group), obestatin (10⁻⁷ mM, ob-H group), obestatin (0.5 \times 10⁻⁷ mM, ob-L group), and OPC31260 (10⁻⁷ mM, OPC group).

2.8. RNA extraction and qPCR analysis

Total RNA was extracted from approximately 50 mg renal inner medullary tissue or 4 \times 10⁵ mIMCD3 cells using TRIzol Reagent. The concentrations and quality of total RNA were measured at an optical density ratio of 260/280 using the Nanodrop ND-1000 spectrophotometer. The integrity of the total RNA obtained was verified on an Agilent 2100 Bioanalyzer using the RNA 6000 Nano LabChip (Agilent, CA, USA). One microgram of total RNA from each sample was decontaminated of genomic DNA and then reverse transcribed to cDNA using the PrimeScript RT reagent Kit with gDNA Eraser (Takara Bio, Otsu, Japan). qPCR was used to evaluate the mRNA expression of the different genes of interest, and performed in a mixture comprising 10 μ l SYBR Master Mix (Toyobo, Osaka, Japan), 0.4 μ l forward primer and

0.4 μ l reverse primer for the corresponding gene, 7.2 μ l of nuclease-free water, and 2 μ l of cDNA sample diluted 1:10, for a final volume of 20 μ l. B-Actin was used as an endogenous control gene. All cDNA samples were amplified through a Rotor-Gene RG-3000A instrument (Corbett Research, Sydney, Australia) according to the following steps: 1 cycle of 95 °C for 10 s, 40 cycles of 95 °C for 5 s, 58–62 °C for 10 s (optimized for each primer pair), and 72 °C for 15 s. The melt curve was completed at the end of the last cycle when the temperature was increased from 60 to 98 °C. The primers used for qPCR (Tables A1 and A2) were synthesized by Sangon Biotech (Shanghai, China). Relative quantification of different gene expression levels was achieved using the 2^{- $\Delta\Delta$ CT} formula and data from three independent experiments [24].

2.9. Immunoblotting analysis

Protein was extracted from approximately 50 mg renal inner medullary tissue or 4 \times 10⁵ mIMCD3 cells using RIPA Lysis Buffer along with 0.1 M PMSF. The concentrations of protein were measured using a BCA test kit (Beyotime Biotech., Haimen, China). Equal amounts (50 μ g) of protein from different groups were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride membranes, which were then incubated with primary antibodies at 4 °C overnight. The following rabbit polyclonal antibodies were used: anti-AQP2 (1:2000, Abcam, Cambridge, UK), anti-V2R (1:500, Biorbyt, Cambridge, UK), anti-PPARG (1:1000, Abcam, Cambridge, UK), anti-B-ACTIN (1:2000, Servicebio, Wuhan, China), and anti-GPR39 (1:500, Novus, Colorado, USA). The membranes were further incubated with horseradish peroxidase-conjugated anti-rabbit IgG (1:2000, Cell Signaling, MA, USA) for 2 h at room temperature. Enhanced chemiluminescent visualization was performed, and the ImageQuant LAS 4000 Gel Imaging System (GE Healthcare, Uppsala, Sweden) was used to capture the luminous protein bands. Three samples in each group were used for protein extraction, and the experiments were performed in triplicate. Image J software (NIH image, Bethesda, MD, USA) was used for image analysis.

2.10. Microarray analysis and qPCR verification

After serum starvation, mIMCD3 cells at about 80% confluency were divided into two groups (n = 3 samples per group), which were treated with obestatin (10⁻⁷ mM) or PBS for 24 h. Then total RNA was extracted using the TRIzol method and verified for concentration, purity, and integrity as described above. One half of the RNA samples were reverse transcribed into cDNA and stored at -80 °C until further qPCR verification, and the other half of the same batch were applied to the following microarray test (GeneChip Mouse Genome 430 2.0 Array, Affymetrix, CA, USA) according to the manufacturer's instructions. Finally, the images and the original data were obtained through scanning with the GeneChip Scanner 3000 (Affymetrix, CA, USA) and decoding with GeneAtlas software. A more in-depth analysis was carried out via a bioinformatics study. The selected genes of interest were further verified by qPCR.

2.11. shRNA silencing of gene expression

A puromycin-resistant lentiviral pLKO.1 plasmid vector was used for RNA silencing. First, the nucleotide sequences of the *V2r*, *Pparg*, and *Gpr39* mRNAs were obtained in GENEBANK database. Two corresponding short hairpin RNA (shRNA) interference target sequences for each gene (Table 2) were selected using the siRNA selection tool (Ambion website, TX, USA). Then, they were cloned into the linearized pLKO.1 plasmid vector, which was achieved by AgeI and EcoRI restriction site cutting to construct the pLKO.1-V2r-shRNA, pLKO.1-Pparg-shRNA, and pLKO.1-Gpr39-shRNA recombinant plasmids. After connection, transformation, and sequencing identification, mIMCD3 cells were transfected with the recombinant plasmids using Lipofectamine

Table 2
Sequences of shRNAs from *muttus V2r*, *Pparg* and *Gpr39* cDNA.

No.	5'	STEMP	Loop	STEMP	3'
shRNA- <i>V2r</i> 1# F:	CCGGTC	GCATGCCCATGGAGTTCTACA	TTCAAGAGA	TGTAGAACTCCATGGGCATGC	TTTTTTgg
shRNA- <i>V2r</i> 1# R:	AATTCCTCAAAAAA	GCATGCCCATGGAGTTCTACA	TCTCTTGAA	TGTAGAACTCCATGGGCATGC	GA
shRNA- <i>V2r</i> 2# F:△	CCGGTC	GCATGCCAACCAAGAGAAAACG	TTCAAGAGA	CGTTTCTCTTGGTTGGCATGC	TTTTTTgg
shRNA- <i>V2r</i> 2# R:△	AATTCCTCAAAAAA	GCATGCCAACCAAGAGAAAACG	TCTCTTGAA	CGTTTCTCTTGGTTGGCATGC	GA
shRNA- <i>Pparg</i> 1# F:	CCGGTC	GGATGTCTCACAATGCCATCA	TTCAAGAGA	TGATGGCATTGTGAGACATCC	TTTTTTgg
shRNA- <i>Pparg</i> 1# R:	AATTCCTCAAAAAA	GGATGTCTCACAATGCCATCA	TCTCTTGAA	TGATGGCATTGTGAGACATCC	GA
shRNA- <i>Pparg</i> 2# F:△	CCGGTC	GCAAGAGATCACAGAGTATGC	TTCAAGAGA	GCATACTCTGTGATCTCTTGC	TTTTTTgg
shRNA- <i>Pparg</i> 2# R:△	AATTCCTCAAAAAA	GCAAGAGATCACAGAGTATGC	TCTCTTGAA	GCATACTCTGTGATCTCTTGC	GA
shRNA- <i>Gpr39</i> 1# F:	CCGGTC	GCATGCCCATGGAGTTCTACA	TTCAAGAGA	TGTAGAACTCCATGGGCATGC	TTTTTTgg
shRNA- <i>Gpr39</i> 1# R:	AATTCCTCAAAAAA	GCATGCCCATGGAGTTCTACA	TCTCTTGAA	TGTAGAACTCCATGGGCATGC	GA
shRNA- <i>Gpr39</i> 2# F:△	CCGGTC	GCATGCCAACCAAGAGAAAACG	TTCAAGAGA	CGTTTCTCTTGGTTGGCATGC	TTTTTTgg
shRNA- <i>Gpr39</i> 2# R:△	AATTCCTCAAAAAA	GCATGCCAACCAAGAGAAAACG	TCTCTTGAA	CGTTTCTCTTGGTTGGCATGC	GA

indicates the different sequence of the same gene. △ indicates the finally selected shRNA sequence according to the silencing efficiency.

2000 (Invitrogen, CA, USA). The pLKO.1-control-shRNA plasmid with a scrambled sequence was used as the control. Cells successfully transfected with the pLKO.1 plasmids were selected with 5 µg/ml puromycin (Merck, Darmstadt, Germany), and stable clones were maintained in 2.5 µg/ml puromycin. Then the silencing efficiencies of the three genes above were determined by qPCR. One of the most efficient silencing clones was selected for each gene, and the constructed mIMCD3 cell lines with stable interference were stored at -80 °C for further research.

2.12. Statistical analysis

All experimental data are presented as mean ± standard deviation (SD) and were analyzed with the statistical software SPSS 20.0 (SPSS Inc., Chicago, IL, USA). Differences between groups or within groups were analyzed by single factor analysis of variance (ANOVA) with Bonferroni testing. For comparisons between only two groups, the independent *t*-test was utilized. *P* < 0.05 was considered statistically significant.

3. Results

3.1. Plasma obestatin level was increased in CHF rat models

To confirm the previous findings in humans with CHF [15], we evaluated the plasma concentration of obestatin in the peripheral circulation of rats with MI-induced CHF at 6 weeks after operation by ELISA. The results showed that the plasma obestatin level was significantly greater in the CHF group compared with the sham group (1923 ± 59.29 pg/ml vs. 1601 ± 44.22 pg/ml, *P* < 0.01, Fig. 1A).

3.2. Obestatin increased urinary output and improved HF biomarkers without influencing cardiac function or plasma electrolyte levels

Urinary output was markedly increased after treatment with high-dose or low-dose obestatin for 2 weeks (75.18 ± 8.51, 63.69 ± 8.91 vs. 40.77 ± 5.49, 41.37 ± 6.56 ml/kg BW, respectively, Fig. 1B). In addition, in the ob-H or ob-L groups, plasma BNP level, which was regarded as a biomarker related to the diagnosis and progression of HF [25,26], were significantly lower than those in the CHF group (1152.61 ± 99.31, 1223.75 ± 95.17 vs. 1504.54 ± 85.62 pg/ml, Fig. 1C). In addition, the NA group showed no differences compared with the CHF group. Obestatin-IgG showed reverse effects compared with obestatin treatment. However, the cardiac structure and function

indexes as well as electrolyte concentrations in plasma did not differ significantly among all seven modeling groups (Tables 3 and 4). Altogether, these results indicate that obestatin has a dose-dependent diuretic effect that may lead to improvement of CHF.

3.3. Obestatin decreased AQP2 expression in renal inner medullary tissue but had no effect on the plasma AVP level

Immunohistochemical examination suggested that AQP2 protein abundance in the inner medulla was significantly higher than that of the outer medulla and the cortex (IOD value 6229.9 ± 994.3, 4069.7 ± 586.8, 825.6 ± 172.4, respectively) in CHF rats, and mostly decreased after high dose obestatin treatment (IOD value 2303.1 ± 467.1, 1528.6 ± 561.3, 351.4 ± 329.8, decent degree 63.0%, 62.4%, 57.4%, in IMCD, OMCD, CCD respectively, Fig. 2A). Thus, we focus on the inner medulla in the different groups with the techniques of qPCR, immunohistochemical staining, and immunoblotting to address whether AQP2 signaling is responsible for the increased urine output after obestatin treatment. As shown in Fig. 2B, *Aqp2* mRNA expression was significantly lower in the ob-H and ob-L groups than in the CHF group (0.46 ± 0.04 fold and 0.64 ± 0.11 fold changes, respectively). In contrast, *Aqp2* mRNA expression was higher in the ob-IgG group than in the CHF group (1.19 ± 0.07 fold change), suggesting ob-IgG could neutralize endogenous obestatin, thereby attenuating its effects on *Aqp2* expression. Consistently, the results of immunohistochemical staining and immunoblotting analyses showed similar differences among the different groups (Fig. 2C and D). AQP2 expression in the upregulated positive control group (dDAVP group) and downregulated positive control group (OPC group) also revealed corresponding changes as expected. Moreover, immunohistochemical staining showed localized expression of labeled AQP2 protein specifically in the IMCDs.

AVP, a potent circulating hormone, is closely related with water retention in CHF. In our study, the plasma AVP level in the CHF group was significantly elevated compared with that in the sham group (12.13 ± 0.74 pg/ml vs. 8.75 ± 0.84 pg/ml) (Fig. 2E). However, the plasma AVP level was not significantly altered by obestatin treatment in either the ob-H or ob-L group. These results suggest that obestatin has no influence on the plasma AVP level.

3.4. Obestatin decreases AQP2 expression in mIMCD3 cell line

In order to establish the optimal concentration for obestatin treatment, we treated mIMCD3 cells with four different concentration

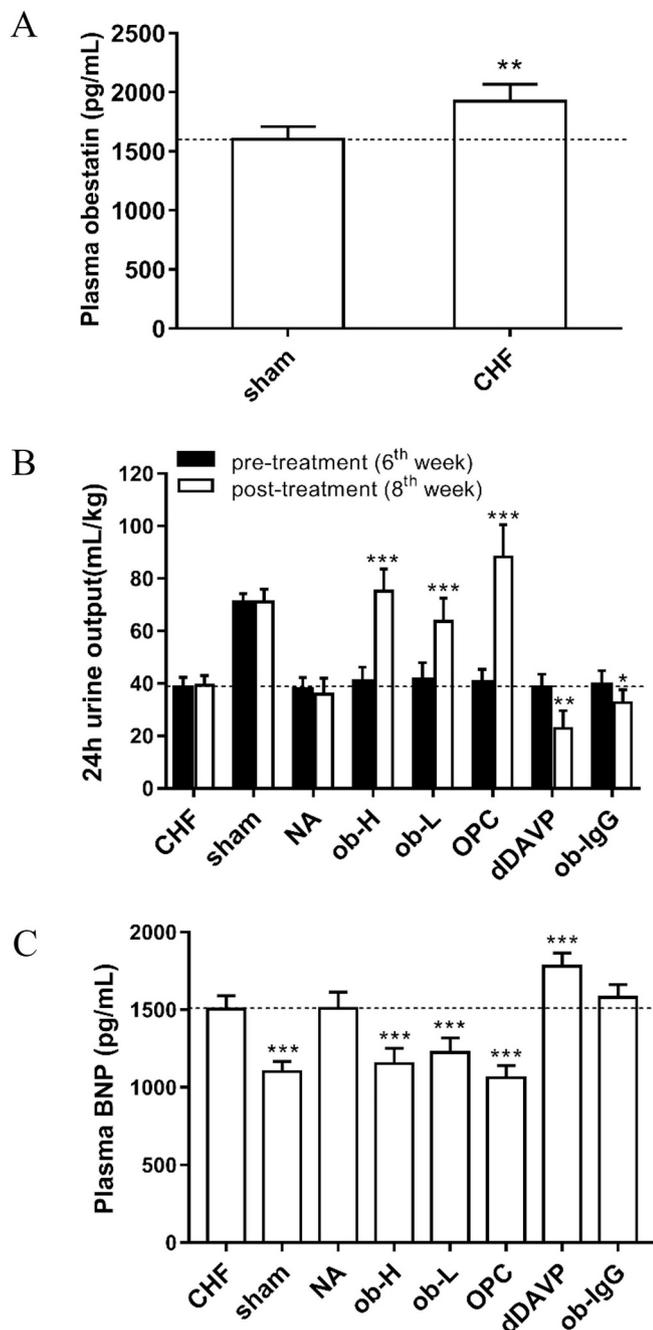


Fig. 1. Plasma level of obestatin and its potential effect on urinary volume and biomarker of CHF in rat models. Groups treated with physiological saline, physiological saline and sham surgery, NA-obestatin (200 μ g/kg), obestatin (200 μ g/kg), obestatin (100 μ g/kg), OPC31260 (1 mg), dDAVP (2 μ g/kg), obestatin-IgG (3 μ g) were labeled as CHF, sham, NA, ob-H, ob-L, OPC, dDAVP, and ob-IgG, respectively. (A) Comparison of peripheral plasma levels of obestatin in the CHF group and sham group before treatment ($n = 6$). ** $P < 0.01$. (B) Urinary output was significantly increased in the rats treated with high-dose or low-dose obestatin. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ compared with pre-treatment values. (C) Plasma BNP was decreased in the rats treated with obestatin at the 8th week compared with the level in the CHF group. *** $P < 0.001$. Data are presented as the means \pm SD.

gradients of obestatin and observed the abundance of AQP2 protein via immunoblotting. We found that 10^{-7} mM obestatin decreased AQP2 protein expression more remarkably than the other three concentrations (by $43 \pm 3\%$ vs. PBS, Fig. 3A).

Furthermore, mIMCD3 cells were stimulated with obestatin for

different time durations, and *Aqp2* gene expression was determined by qPCR. We found that at 6, 12, 24, 36, and 48 h of treatment with 10^{-7} mM obestatin, *Aqp2* expression was significantly decreased compared with that in the control group (0 h). In the range of 6 to 24 h, the downward trend with time was more remarkable, but after 36 h, the downregulated *Aqp2* mRNA expression gradually rebounded. Consistently, immunoblotting of AQP2 protein revealed a similar trend with increasing duration of 10^{-7} mM obestatin treatment (Fig. 3B).

On the other hand, *Aqp2* gene expression was determined by qPCR at 24 h after different interventions. Compared with that in the Con group, the expression levels of the *Aqp2* gene in the ob-H, ob-L, and OPC groups were significantly decreased by $49 \pm 8\%$, $26 \pm 9\%$, and $56 \pm 8\%$, respectively. In contrast, the levels of *Aqp2* gene expression in the ob + ob-IgG and dDAVP group were increased by $11 \pm 6\%$ and $93 \pm 11\%$, respectively; however, the difference between the ob + ob-IgG group and the Con group was not statistically significant ($P > 0.05$). The *Aqp2* expression in the NA group did not differ statistically from that in the Con group. Compared with that in the ob-H group, *Aqp2* expression in the ob + ob-IgG group increased significantly from $51 \pm 8\%$ to $111 \pm 6\%$, indicating that the effect of obestatin on *Aqp2* could be counteracted by ob-IgG.

These changes in *Aqp2* gene expression in mIMCD3 cells exposed to different treatments were further confirmed by the results of immunoblotting for AQP2 protein (Fig. 3C).

3.5. Screening for differentially expressed genes via microarray analysis and validation of these by qPCR in the mIMCD3 cell line

To identify potential action targets for long-term obestatin function, we assessed the changes in the expression of three pairs of mRNAs in mIMCD3 cell line samples through gene chip screening. The cells were treated with obestatin (10^{-7} mM) or PBS for 24 h. Then the potential changes in the genetic transcription profiles in response to obestatin treatment were analyzed using GeneAtlas. Clustering analysis was used to visualize the differential expression in comparison to the PBS control (Fig. 4). The results showed that obestatin treatment resulted in the differential expression of 1260 genes. Six hundred nine among them were downregulated, and 651 were upregulated (Fig. 5A). Seventy-three genes were selected for further validation by qPCR, which included the 69 most differentially expressed genes (60 genes downregulated and 9 upregulated), 3 genes related to renal water metabolism (*Aqp1*, *Aqp3*, and *Aqp4*), and the other gene *Gpr39*, which was previously linked to the function of obestatin [27] (Fig. 5B).

As shown in Fig. 5C, the qPCR results confirmed that obestatin treatment decreased the mRNA levels of *Pparg*, *Aqp2*, and *V2r* by 0.46 ± 0.07 , 0.63 ± 0.06 , 0.75 ± 0.05 fold, respectively (all $P < 0.01$). Unlike the downregulated genes, the upregulated genes detected by microarray showed no significant change upon qPCR validation, as did *Gpr39* and the other three genes.

3.6. Effects of obestatin on AQP2, V2R, PPARG, and GPR39 expression after shRNA interference

First, the efficiency of shRNA on the *V2r*, *Pparg*, and *Gpr39* genes was evaluated by qPCR. Under these conditions, the constructs reduced *V2r* expression by $42 \pm 5\%$, *Pparg* expression by $44 \pm 7\%$, and *Gpr39* expression by $57 \pm 5\%$ (Fig. 6A, B). Second, as shown in Fig. 6A and B, the silencing of *V2r* and *Pparg* together with obestatin treatment decreased the gene expression of *V2r*, *Pparg*, and *Aqp2* to a more remarkable extent than the reductions observed after any single intervention. However, no effects of obestatin on *Gpr39* gene expression with or without the silencing of *Gpr39* were observed, and the silencing of *Gpr39* weakened the effects of obestatin on *Aqp2* gene expression as compared with the results from the shRNA control group ($62 \pm 6\%$ in the shRNA control group vs. $91 \pm 9\%$ in the shRNA *Gpr39* group).

Third, immunoblotting confirmed the effects of obestatin on AQP2

Table 3

Echocardiographic parameters in sham-operated rats and rats with heart failure after receiving different treatments (8th week, n = 6).

Groups	LVIDd (mm)	LVIDs (mm)	LVDV (μ l)	LVSV (μ l)	LVEF%	LVFS%
CHF	8.95 \pm 1.06	7.27 \pm 0.92	448.56 \pm 121.77	282.70 \pm 83.31	37.25 \pm 3.56	18.87 \pm 1.99
Sham	6.47 \pm 0.80***	3.71 \pm 0.50***	217.10 \pm 60.36**	60.02 \pm 19.00***	72.30 \pm 4.24***	42.49 \pm 3.98***
NA	8.82 \pm 0.30	7.16 \pm 0.29	428.55 \pm 32.27	269.15 \pm 24.35	37.23 \pm 1.97	18.84 \pm 1.12
ob-H	9.48 \pm 0.73	7.76 \pm 0.86	504.76 \pm 87.81	326.28 \pm 83.84	36.03 \pm 5.34	18.28 \pm 2.96
ob-L	9.30 \pm 0.87	7.64 \pm 0.82	485.24 \pm 103.09	314.67 \pm 79.88	35.44 \pm 3.76	17.89 \pm 2.09
OPC	8.88 \pm 0.76	7.13 \pm 0.62	437.88 \pm 85.04	268.39 \pm 52.31	38.65 \pm 4.38	19.70 \pm 2.52
dDAVP	9.16 \pm 0.54	7.50 \pm 0.54	466.56 \pm 62.14	300.32 \pm 49.90	35.80 \pm 3.06	18.08 \pm 1.71
ob-IgG	8.98 \pm 0.39	7.30 \pm 0.54	446.66 \pm 43.30	282.22 \pm 49.37	37.12 \pm 2.07	18.83 \pm 2.81

The data are presented as the means \pm SD. **, *** denotes P < 0.01 and P < 0.001 compared with other modeling groups.

Table 4

Plasma electrolyte concentrations in sham-operated rats and rats with heart failure after receiving different treatments (8th week, n = 6).

Groups	Plasma concentrations(mmol/L)		
	Sodium	Potassium	Chloride
CHF	144.50 \pm 1.87	5.13 \pm 0.23	100.00 \pm 2.53
Sham	143.83 \pm 1.72	5.05 \pm 0.19	101.50 \pm 1.05
NA	144.17 \pm 1.94	4.98 \pm 0.25	99.33 \pm 1.86
ob-H	144.00 \pm 1.10	5.05 \pm 0.38	100.83 \pm 0.98
ob-L	142.50 \pm 0.84	5.17 \pm 0.28	101.00 \pm 2.00
OPC	144.33 \pm 1.63	4.87 \pm 0.23	99.00 \pm 2.90
dDAVP	143.33 \pm 2.73	5.10 \pm 0.36	99.67 \pm 3.72
ob-IgG	144.67 \pm 1.63	5.03 \pm 1.97	101.17 \pm 4.67

The data are presented as the means \pm SD.

protein expression in cells with *V2r*, *Pparg*, or *Gpr39* knockdown. The results revealed changes consistent with the results of our qPCR analysis discussed above (Fig. 6C).

Taken together, these results suggest that obestatin downregulated AQP2 expression through the inhibition of V2R and PPARG, which could be regarded as the downstream target of obestatin. Moreover, GPR39 may play a key role in obestatin function.

3.7. Obestatin exerts its effects on V2R and PPARG through GPR39 in mIMCD3 cells

The silencing of GPR39 could potentially weaken the effects of obestatin on AQP2 expression, but whether it can influence V2R and PPARG expression remains unknown. Therefore, we evaluated the expression levels of V2R and PPARG proteins under the condition of GPR39 knockdown.

We prepared a stably GPR39-deficient mIMCD3 cell line as described above. The effect of GPR39 knockdown on V2R and PPARG expression was evaluated with or without obestatin treatment. The results of immunoblotting revealed that GPR39 knockdown reversed the obestatin-activated decreases in V2R and PPARG protein expression from 44 \pm 2% and 71 \pm 4% to 87 \pm 4% and 92 \pm 2%, respectively, observed after treatment with obestatin (10⁻⁷ mM, 24 h, Fig. 7). These results indicate that obestatin exerts its inhibitory effects on V2R and PPARG protein expression through GPR39.

4. Discussion

In this study, we found that increased obestatin in peripheral circulation of CHF may be an important feature of CHF. Moreover, long-term treatment with exogenous obestatin was associated with increased urinary volume without a significant effect on the electrolyte concentration in plasma, along with significantly decreased expression of

the biomarker of CHF. Subsequent studies showed that the benefits of obestatin for diuresis in CHF may be dependent on the interaction of GPR39 and inhibition of V2R and PPARG pathways, which finally lead to the downregulation of renal AQP2 expression. These results suggest that long-term treatment with obestatin improves water retention in CHF by increasing urinary output via downregulation of AQP2 expression in renal IMCDs. These effects may be at least partially mediated by regulation of GPR39, V2R, and PPARG signaling.

Previous studies have confirmed that the regulation of AQP2 is both AVP dependent and independent. The AVP-dependent mechanism [28–30] mainly involves the upregulation of AQP2 expression and enhanced AQP2 phosphorylation resulting from increased release of AVP from the supraoptic and paraventricular nucleus in the hypothalamus [31]. This mechanism responds to the increased plasma osmotic pressure, and increased AVP binds to V2R on the basolateral membrane of principle cells and triggers the classical cAMP-PKA pathway to induce increased water reabsorption via regulation of AQP2. On the other hand, the AVP-independent mechanism [28,29,32] mainly involves other factors involved in the regulation of AQP2 in addition to AVP. Previous studies showed that calcitonin activates its receptor and then activates adenyl cyclase through a pathway similar to the post-AVP signaling pathway. Otherwise, PPARG, a type II nuclear receptor in the kidney that is mainly located in the principal cells of IMCDs [33,34], has a close relationship with the renal water metabolism, and recent research has shown that activation of PPARG by GI262570 or Rosiglitazone increases *Aqp2* mRNA expression, which finally accelerates the transfer of AQP2 vesicles to the luminal membrane, thereby increasing water reabsorption [35,36]. PPARG knockout mice develop a polyuria phenotype without variation in urinary AVP excretion, suggesting PPARG may play a role in directly regulating AQP2 expression apart from the AVP/V2R/cAMP/PKA pathway [37,38]. Even so, the regulatory effect of PPARG on AQP2 remains controversial [39].

In this study, in vivo and in vitro experiments confirmed that obestatin downregulated the expression of AQP2 in the IMCDs of kidney, and the diuretic effect and the improvement of biochemical index of HF was also observed in vivo. However, there were no significant changes in the EF value and heart structure indexes, suggesting that obestatin had little effect on myocardial remodeling. Moreover, no changes in peripheral blood AVP were observed after obestatin treatment, suggesting that the regulation of AQP2 by obestatin occurred independently from the classical AVP-dependent pathway. Bioinformatic analysis of the microarray data and validation by qPCR showed that obestatin downregulated *Aqp2* gene expression through two signaling pathways: *V2r* and *Pparg*. No variation was observed in the expression of additional genes related to renal water metabolism, such as *Aqp1*, *Aqp3*, and *Aqp4*.

Since its discovery in 2005, the reported biological functions of obestatin have varied from a role as a gastrointestinal-central endocrine hormone peptide during development to a systemic multi-organism

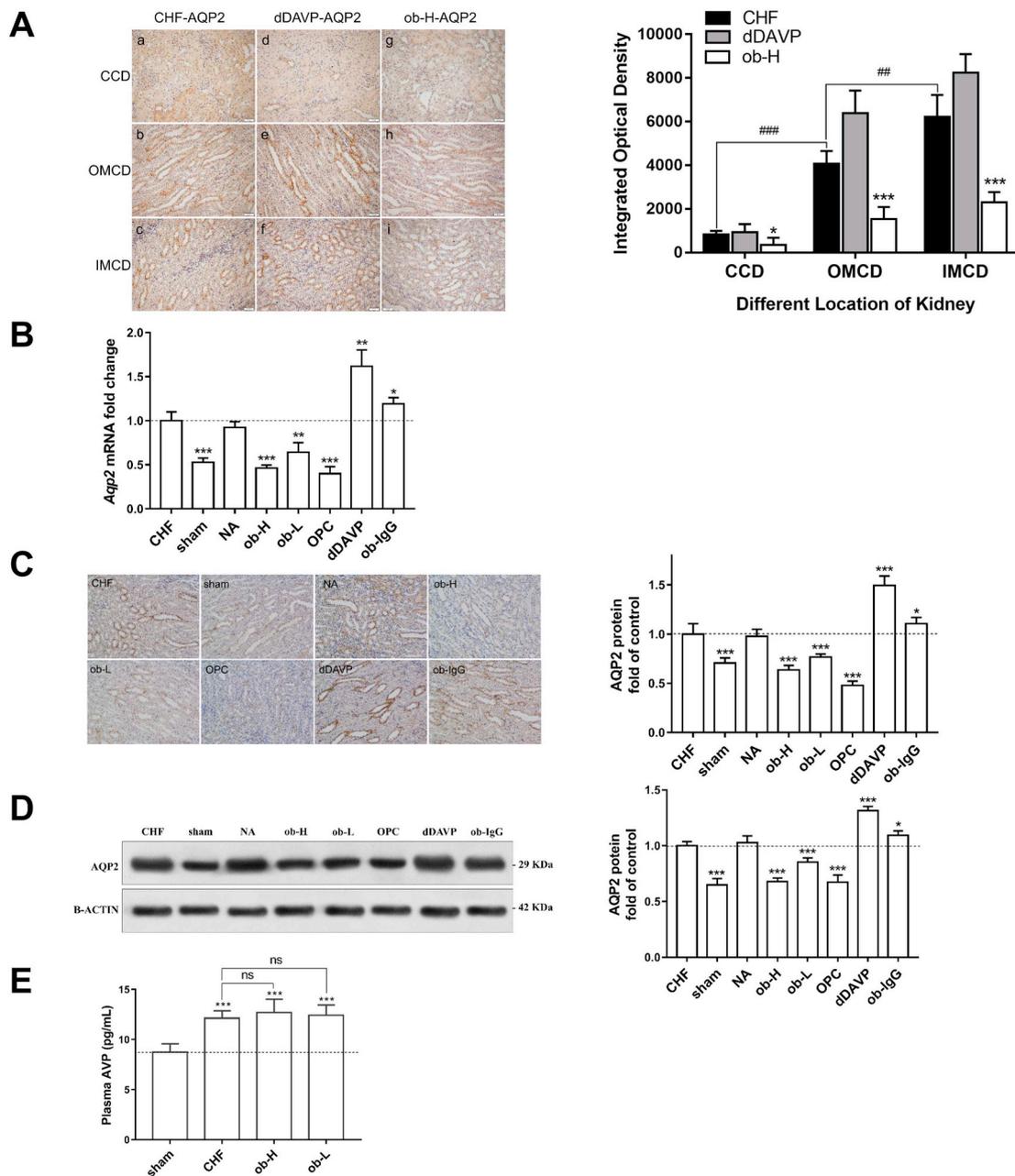


Fig. 2. Chronic effects of obestatin on AQP2 expression in renal collect duct tissue and AVP level in plasma. (A) Immunohistochemical examination of AQP2 protein abundance in the renal CCD, OMCD, IMCD in a tissue section from rats of CHF group (a–c), dDAVP group (d–f) and ob-H group (g–i). Brown staining indicates AQP2 protein expression in the collecting ducts, and blue staining indicates cell nuclei. The AQP2 expression of the inner medulla was significantly higher than that of the outer medulla and the cortex, and decreased mostly after obestatin treatment. Scale bar 50 μ m, Magnification 200 \times . (B) Changes in *Aqp2* mRNA expression in renal inner medulla tissue after 2 weeks of treatment. (C) Immunohistochemical staining for AQP2 expression in different groups at a magnification of 200 \times . AQP2 expression was much stronger in the dDAVP and ob-IgG groups than in the CHF group; in contrast, labeling was much weaker in the sham, ob-H, ob-L, and OPC groups. Densitometric analysis data normalized by CHF group are shown in the bar graph. (D) Immunoblotting of AQP2 protein in the renal inner medulla post-treatment for different groups. Mean data for band densities of AQP2 protein normalized by B-ACTIN are shown in the bar graph. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ above compared with the CHF group. ## and ### above, means $P < 0.01$ and $P < 0.001$. (E) Plasma AVP was elevated in CHF modeling rats, but not influenced by obestatin treatment for 2 weeks. *** $P < 0.001$ compared with the sham group. ns, not significant. The data above are presented as the means \pm SD. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

regulatory peptide. The beneficial effects of obestatin on the cardiovascular system have attracted increasing attention [13,40,41], but studies on the regulation of renal water metabolism and the underlying mechanism are rare. Earlier studies once considered that GPR39 is the only exclusive receptor of obestatin in vivo [42], but subsequent studies have raised more controversy [43,44]. However, the receptor of obestatin in these clinical circumstances remains to be determined.

In this study, we focused on the effect of long-term obestatin treatment on the expression of AQP2 in renal inner medulla through a rat model of CHF and the mIMCD3 cell line. We also explored the possible mechanisms using a high-throughput microarray technique. According to the validated differentially expressed genes, we constructed shRNA vectors that interfered with the expression of three key genes for *V2r*, *Ppar γ* , and *Gpr39* and observed the subsequent regulatory

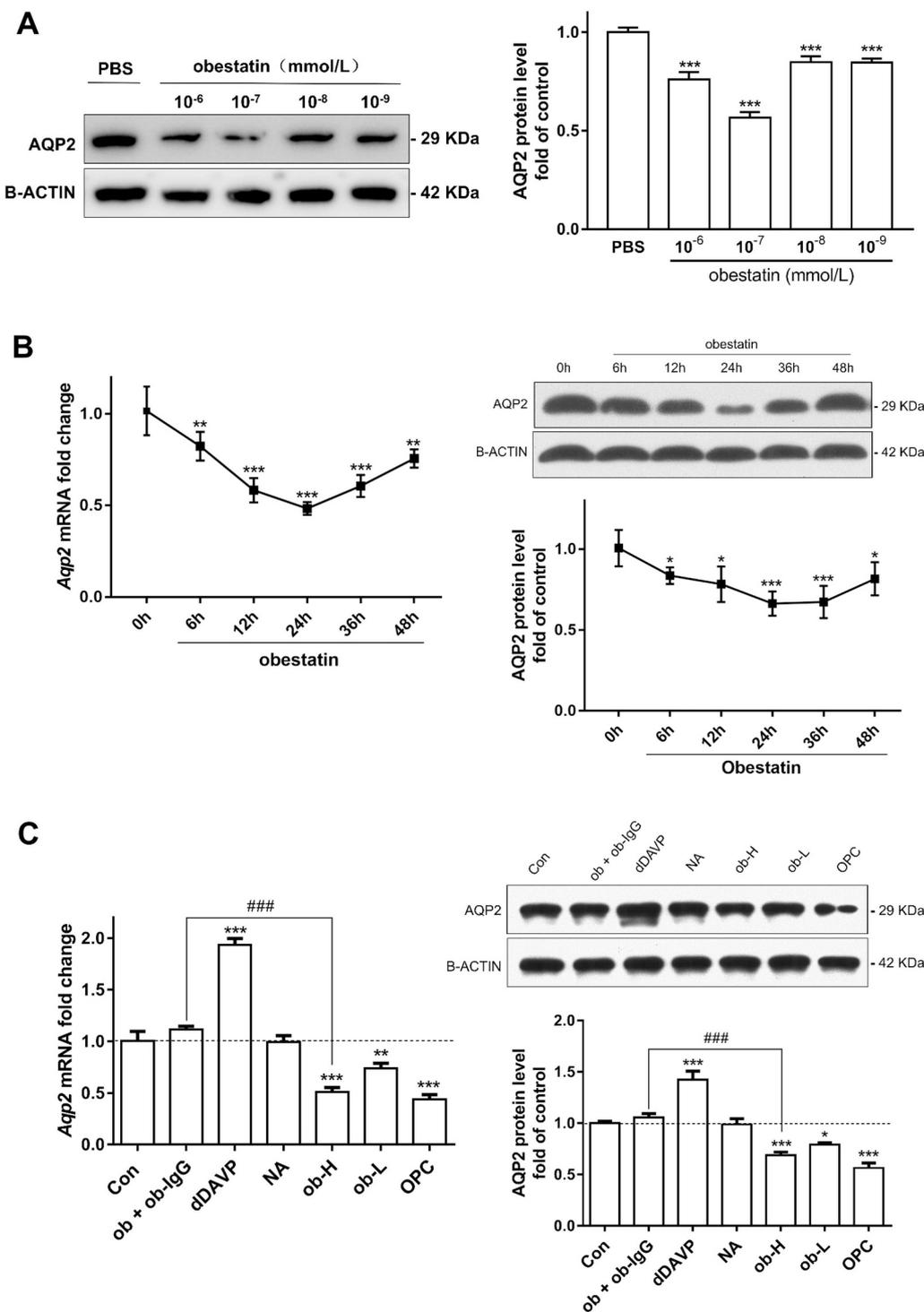


Fig. 3. Obestatin decreased AQP2 expression in the mIMCD3 cell line. (A) Immunoblotting of AQP2 protein in mIMCD3 cells stimulated with obestatin at different concentrations (10^{-6} , 10^{-7} , 10^{-8} , or 10^{-9} mM) for 24 h. Mean data for band densities of AQP2 protein normalized by B-ACTIN are shown in the bar graph. (B) The mIMCD3 cells were stimulated with obestatin (10^{-7} mM) for 6, 12, 24, 36, or 48 h. Changes in AQP2 mRNA and protein expression were observed by qPCR or Immunoblotting. Mean data for AQP2 expression normalized by B-ACTIN were shown in the bar graph. (C) The mIMCD3 cells were subjected to different treatments as follows: PBS, obestatin (10^{-7} mM) plus obestatin-IgG ($10 \mu\text{g/ml}$), dDAVP (10^{-7} mM), NA-obestatin (10^{-7} mM), obestatin (10^{-7} mM), obestatin (0.5×10^{-7} mM), or OPC31260 (10^{-7} mM), and the groups were labeled Con, ob+ob-IgG, dDAVP, NA, ob-H, ob-L, and OPC, respectively. Changes in AQP2 mRNA and protein expression were observed by qPCR or Immunoblotting. Mean data for AQP2 expression normalized by B-ACTIN were shown in the bar graph. The data are presented as the means \pm SD. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ compared with the Con (0 h or PBS) group. ### $P < 0.001$ as compared with the ob-H group.

effects of obestatin on AQP2 expression.

The degree of AQP2 protein downregulation was more remarkable after V2R and *Pparg* mRNA interference, but the inhibitory effects of AQP2, V2R, and PPARG induced with obestatin treatment were significantly attenuated after *Gpr39* knockdown. These results suggest that GPR39 was the upstream target of obestatin through which obestatin may downregulate the expression of AQP2 protein via V2R and PPARG dual-pathway. Furthermore, obestatin has no regulatory effect on GPR39 itself, which suggests that GPR39 may partly serve as the

receptor of obestatin, although this “receptor” role is controversial because of uncertain specificity [45]. Because it has been proven that V2R and GPR39 are both located on the cell membrane, the mechanism of their interaction warrants further investigation.

5. Conclusions

The elevated level of plasma obestatin in CHF may have a beneficial compensatory effect, just like the character of BNP. Long-term

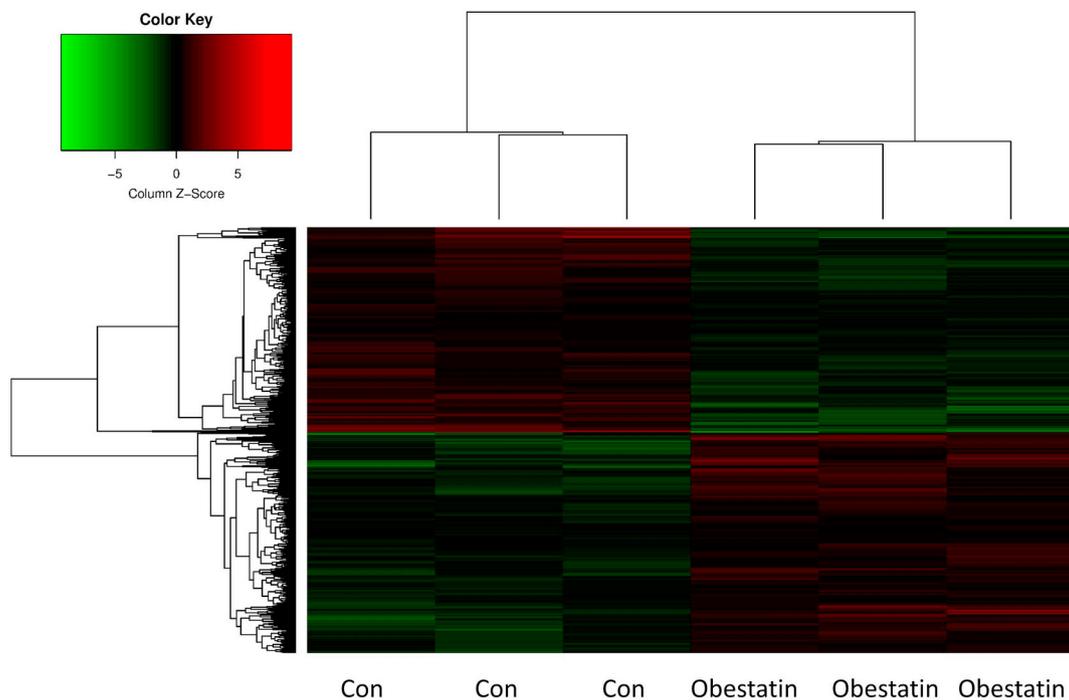


Fig. 4. Cluster diagram of the differentially expressed genes in mIMCD3 cells after treatment with obestatin compared with PBS control.

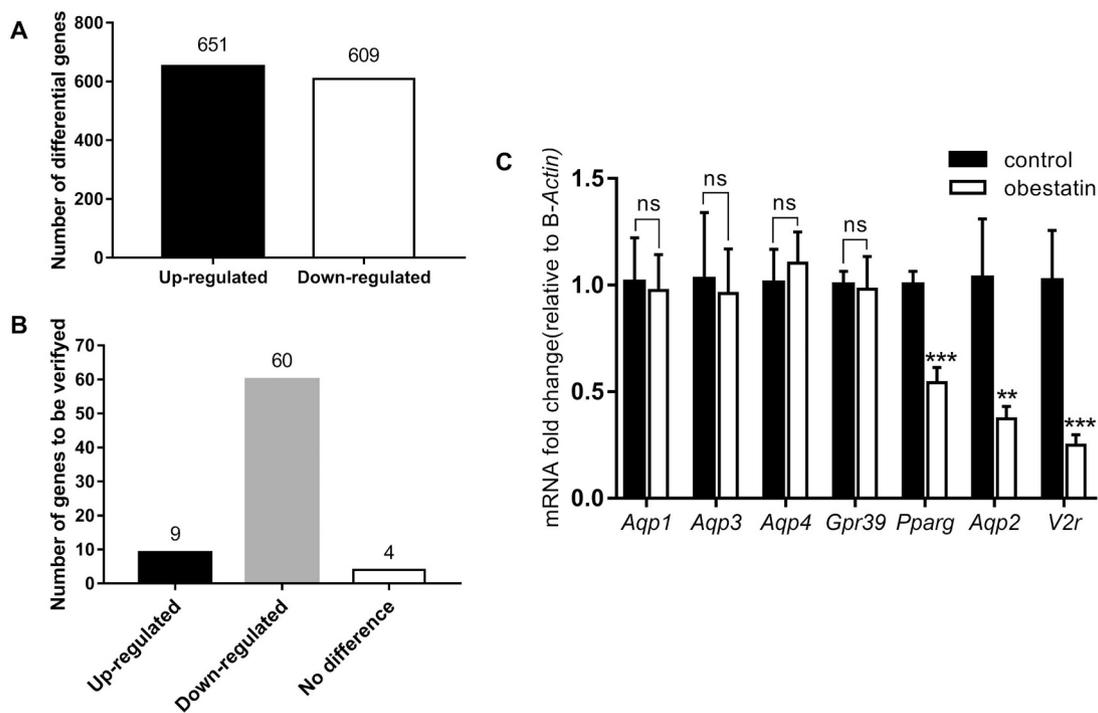


Fig. 5. Major genes of interest identified as differentially regulated upon obestatin treatment using microarray analysis and validation by qPCR. (A) All differentially expressed genes revealed by microarray with a confidence level of $P < 0.05$. (B) Selected genes of interest to be verified. Nine upregulated genes and 60 down-regulated genes were found to be most differentially expressed by microarray. Among the four genes showing no difference, three (*Aqp1*, *Aqp3*, and *Aqp4*) were related to renal water metabolism, and the other gene (*Gpr39*) was considered as a possible receptor of obestatin. (C) Validation of results by qPCR. The data are presented as the means \pm SD. ** $P < 0.01$ and *** $P < 0.001$.

treatment with obestatin improves water retention in CHF by increasing urinary output via the downregulation of AQP2 expression in renal IMCDs. These effects may be at least partially mediated by regulation of GPR39, V2R, and PPARG signaling. Central and peripheral obestatin

play a similar role in regulating water metabolism. Obestatin may be a novel effective diuretic target for CHF that is similarly to V2R antagonists worthy of further study.

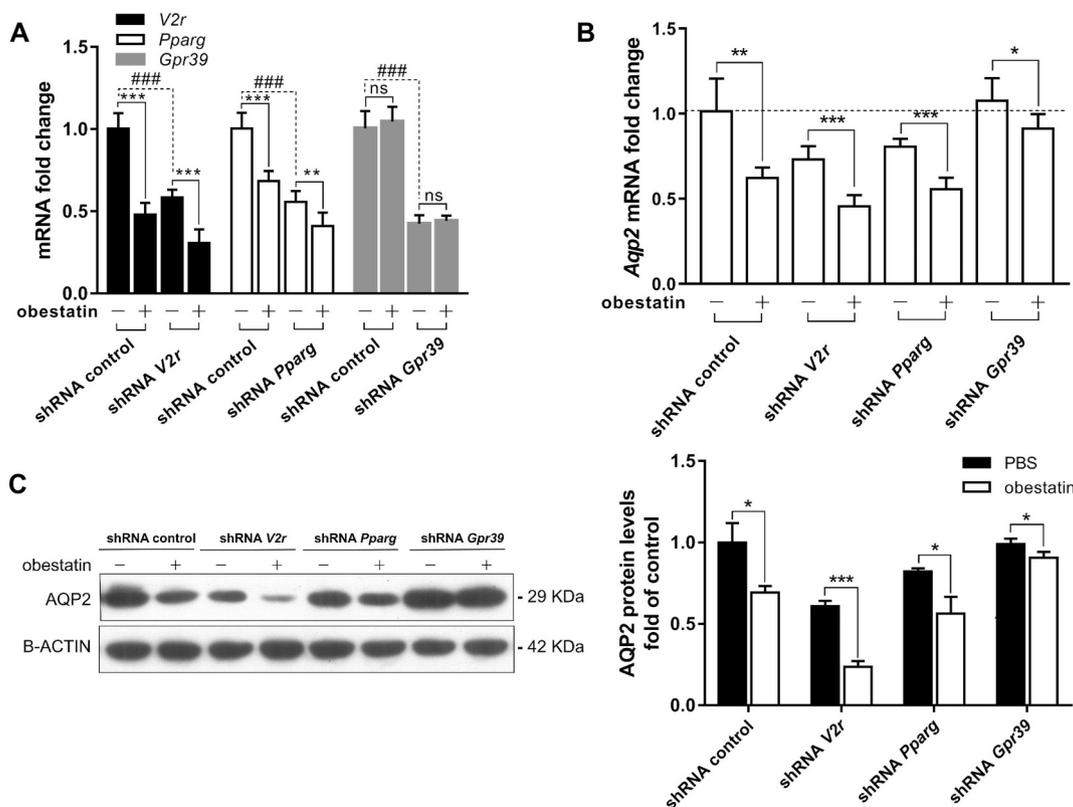


Fig. 6. Effects of obestatin on the expression of AQP2, V2R, PPARG, and GPR39 after shRNA interference. (A) Efficiency of shRNA acting on *V2r*, *Pparg*, and *Gpr39* genes. (B) Changes in *Aqp2* mRNA expression level after shRNA interference of *V2r*, *Pparg*, and *Gpr39* expression. (C) Immunoblotting of AQP2 protein after shRNA interference of *V2r*, *Pparg*, and *Gpr39* expression. Mean data for band densities of proteins normalized by B-ACTIN are shown in the bar graph. The data above are presented as the means \pm SD. *P < 0.05, **P < 0.01, and ***P < 0.001. ###P < 0.001. ns, not significant.

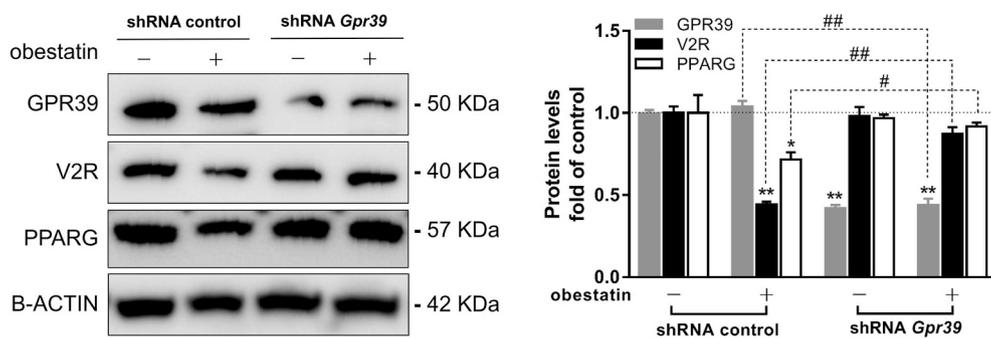


Fig. 7. Effect of *Gpr39* knockdown by shRNA on the expression levels of V2R and PPARG in mIMCD3 cells. The mIMCD3 cells were transfected with shRNA *Gpr39* and subcultured for stable knockdown prior to obestatin treatment (10^{-7} mM, 24 h). The abundances of V2R, PPARG, and GPR39 proteins were denoted as fold change relative to the levels in the shRNA control group. The protein expression was normalized relative to B-ACTIN expression. The data are presented as the means \pm SD. **P < 0.01 as compared with the shRNA control group not treated with obestatin. #P < 0.05 and ##P < 0.01.

Abbreviations

- AVP arginine vasopressin
- CHF chronic heart failure
- AQP aquaporin
- qPCR quantitative real-time PCR
- mIMCD mouse inner medullary collecting duct
- GPR39 G protein-coupled receptor 39
- CDs collecting ducts
- dDAVP desmopressin
- MI myocardial infarction
- LAD left anterior descending branch
- ECG electrocardiogram
- EF ejection fraction
- BW body weight
- V2R vasopressin receptor 2

- LVIDs left ventricular internal diameter at end-systole
- LVIDd left ventricular internal diameter at end-diastole
- LVDV left ventricular end-diastolic volume
- LVSV left ventricular end-systolic volume
- LVEF left ventricular ejection fraction
- LVFS left ventricular shortening fraction
- BNP brain-type natriuretic peptide
- RIPA radio immunoprecipitation assay
- PMSF phenylmethylsulfonyl fluoride
- PBS phosphate-buffered saline
- BSA bovine serum albumin
- IOD integrated optical density
- DMEM Dulbecco's Modified Eagle's Medium
- shRNA short hairpin RNA
- PPARG peroxisome proliferator-activated receptor gamma

Author contributions

Z.-F. G., Q. J. and X. Z. designed this study, obtained the funding and supervised the whole process. L.-Z. B., M. S. and H. Q. performed the most work of experiments and wrote the manuscript. J.-B. S. engaged in rats modeling. T. S. was responsible for the construction of shRNA vector. J.-W. S. took part in the work of cell culture. Z.-K. W. and X.-X. Z. discussed data and reviewed the manuscript.

Funding

This work was supported by the National Natural Science Foundation of China (81470517) and Program of Shanghai Academic/

Technology Research Leader (19XD1423600).

Declaration of Competing Interest

The authors have no financial conflicts of interest.

Acknowledgments

The authors thank Fen Xu for kindly assisting with the echocardiographic studies, and thank the Shanghai institute for Biological Sciences for kindly sharing of the pLKO.1 plasmid vector. We also thank Medjaden (www.medjaden.com) for its linguistic assistance during the preparation of this manuscript.

Appendix A

Table A1

The primer sequences of the rat genes of interest studied.

Genes	Accession no.		Primer sequence(5'-3')	Product size
<i>Aqp2</i>	NM_012909	For	TTGCAGGAACCAGACACTTG	217 bp
		Rev	GCGGAGACGAGCACTTTTAC	
<i>B-Actin</i>	NM_031144	For	GTCAGGTCATCACTATCGGGAATG	147 bp
		Rev	AGAGGTCCTTACGGATGTCAACG	

Table A2

The primer sequences of the mouse genes of interest studied.

Genes	Accession no.		Primer sequence(5'-3')	Product size
<i>Aqp1</i>	NM_007472	For	AGGCTTCAATTACCCACTGGA	99 bp
		Rev	CTTTGGGCCAGAGTAGCGAT	
<i>Aqp2</i>	NM_009699	For	ATGTGGAACTCCGGTCCATA	137 bp
		Rev	ACGGCAATCTGGAGCACAG	
<i>Aqp3</i>	NM_016689	For	CCTTGGCATCTTGGTGGCT	75 bp
		Rev	AGGAAGCACATTGCGAAGGT	
<i>Aqp4</i>	NM_009700	For	ATCAGCATCGCTAAGTCCGTC	88 bp
		Rev	GAGGTGTGACCAGGTAGAGGA	
<i>B-Actin</i>	NM_007393	For	GGCTGTATTCCTCCATCG	154 bp
		Rev	CCAGTTGGTAACAATGCCATGT	
<i>Pparg</i>	NM_00127330	For	AGACCACTGCAATTCCTTTG	155 bp
		Rev	TCGCACTTGTGTATCTTTGG	
<i>V2r</i>	NM_019404	For	TGACCGAGACCCGCTGTTA	111 bp
		Rev	CGACCCCGTCGTATTAGGG	
<i>Fgf23</i>	NM_022657	For	ATGCTAGGGACCTGCCTTAGA	100 bp
		Rev	AGCCAAGCAATGGGGAAAGTG	
<i>Myo1f</i>	NM_053214	For	CTTTCACCTGGCAGAGTCAAA	108 bp
		Rev	ATGAAGCGTTTGGCGAGGTT	
<i>Scnn1g</i>	NM_011326	For	GCACCGACCATTAAGGACCTG	118 bp
		Rev	GCGTGAACGCAATCCACAAC	
<i>Slc8a1</i>	NM_001112798	For	CTCCCTGTTTGTGCTCCTGT	78 bp
		Rev	AGAAGCCCTTTATGTGCCAGTA	
<i>Ahr</i>	NM_013464	For	AGCCGGTGCAGAAAACAGTAA	100 bp
		Rev	AGGCGGTCTAACTCTGTGTTT	
<i>Arid4b</i>	NM_198122	For	ACTGAAGGTAGGAGCTATTGTGG	198 bp
		Rev	AGTGGGAGCTGGTCTAGTGTT	
<i>Bco2</i>	NM_133217	For	TGTCAAAGTTTGAGCCACCTA	195 bp
		Rev	GGTCGTAATGAGGATGTGCAG	
<i>Brca1</i>	NM_009764	For	CGAATCTGAGTCCCCTAAAGAGC	89 bp
		Rev	AAGCAACTTGACCTTGGGGTA	
<i>Cebpg</i>	NM_009884	For	AGCGGCTTACAGCAGGTTT	124 bp
		Rev	GGGGGTATTCTGCTACTATTCC	
<i>Chac1</i>	NM_026929	For	CTGTGGATTTTCGGGTACGG	122 bp
		Rev	CCCCATGGAAGGTGTCTCC	
<i>Col1a2</i>	NM_007743	For	GTAACCTTCGTGCCTAGCAACA	230 bp
		Rev	CCTTTGTGAGAATACTGAGCAGC	
<i>Crebbp</i>	NM_001025432	For	GGCTTCTCCGCAATGACAA	136 bp
		Rev	GTTTGGACGAGCATCTGGA	
<i>Creb1</i>	NM_009952	For	AGCAGCTCATGCAACATCATC	152 bp
		Rev	AGTCCTTACAGGAAGACTGAACT	
<i>Creb3</i>	NM_013497	For	AAGGCTCCGCTGGACTTAGA	170 bp
		Rev	TGTGGAAGGGAGTAGTTGTGA	

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Table A2 (continued)

Genes	Accession no.		Primer sequence(5'-3')	Product size
<i>Creb5</i>	NM_172728	For	AGGATCTTCTGCCGCTCTTGAT	152 bp
		Rev	GCGCAGCCTTCAGTCTCAT	
<i>Crebzf</i>	NM_145151	For	CTGCCCGTCTTAATCGGCTC	155 bp
		Rev	CCGTAGGTAGCGACTCTCCTC	
<i>Cth</i>	NM_145953	For	TTCTGCGCTAGTTTCCAGCAT	124 bp
		Rev	GGAAGTCCTGCTTAAATGGTG	
<i>Cux1</i>	NM_009986	For	TGACCTGAGCGGTCCTTACA	85 bp
		Rev	TGGGGCCATGCCATTACATC	
<i>Cyr61</i>	NM_010516	For	CTGCGCTAAACAACCTCAACGA	109 bp
		Rev	GCAGATCCCTTTCCAGAGCGG	
<i>Cxcl12</i>	NM_001012477	For	TGCATCAGTGACGGTAAACCA	146 bp
		Rev	TTCTTCAGCCGTGCAACAATC	
<i>Eif4g1</i>	NM_001005331	For	AAGACCTCATCTCGCATCCG	143 bp
		Rev	TGTTCTCGGTGCTCTCCATC	
<i>Insr</i>	NM_010568	For	ATGGGCTTCGGGAGAGGAT	121 bp
		Rev	GGATGTCCATACCAGGGCAC	
<i>Nfatc1</i>	NM_001164112	For	GACCCGGAGTTGCGACTTCG	97 bp
		Rev	TGACACTAGGGGACACATAACTG	
<i>Prkar2b</i>	NM_011158	For	CCAGTAAGGGTGCAACTTCG	179 bp
		Rev	GGACTCTGCATCGTCTTCCTC	
<i>Pten</i>	NM_008960	For	TGGATTGCGACTTAGACTTGACCT	180 bp
		Rev	GCGGTGTCAATAATGTCTCTCAG	
<i>Smad4</i>	NM_008540	For	ACACCAACAAGTAACGATGCC	83 bp
		Rev	GCAAAGGTTTCACTTTCCCCA	
<i>Tsc1</i>	NM_022887	For	ATGGCCCAGTTAGCCAACATT	106 bp
		Rev	CAGAATTGAGGGACTCCTTGAAG	
<i>Dusp1</i>	NM_013642	For	GTTGTTGGATTGTCGCTCCTT	129 bp
		Rev	TTGGGCACGATATGCTCCAG	
<i>Efna1</i>	NM_010107	For	CCCCGAGAAGTGTCTGAGA	177 bp
		Rev	ACATGGCCCTGGGGATTATGA	
<i>Enc1</i>	NM_007930	For	CTGTTTCATAAGTCTCCTACGC	169 bp
		Rev	CACCACTGAACATGGCTTCG	
<i>Eri2</i>	NM_027698	For	GAGAAAGTCAGTTACACCAGCAA	96 bp
		Rev	CAGGTAGACTCAAAGTCAACGAC	
<i>Foxc1</i>	NM_008592	For	CCCCGGACAAGAAGATCACTC	109 bp
		Rev	AGGTTGTGCCGTATGCTGTTT	
<i>Gadd45a</i>	NM_007836	For	CGAAAGGATGGACACGGTG	121 bp
		Rev	TTATCGGGGTCTACGTTGAGC	
<i>Gnaq</i>	NM_008139	For	GGTCGGGCTACTCTGACGA	117 bp
		Rev	ACTTGTATGGGATCTTGAGCGT	
<i>Il1rap</i>	NM_008364	For	TGCCTGGGGGAATTGTAC	221 bp
		Rev	CTTAGCCCGCTCAGCTCTTT	
<i>Mga</i>	NM_013720	For	GAGGAGCACCTACCTTCTTTGT	89 bp
		Rev	ACGGGCATCTCGATTAGTAACT	
<i>Mlt10</i>	NM_001252561	For	GTTGGGCTCATGTGGTTTGTG	79 bp
		Rev	AAAACGATTGGCTCCATTGTAGA	
<i>Mthfd2</i>	NM_008638	For	AGTGCGAAATGAAGCCGTTG	149 bp
		Rev	GACTGGCGGGATTGTACC	
<i>Nr2c2</i>	NM_011630	For	GACTCTGCGGTAGCCTCAC	119 bp
		Rev	AGGATGAACTGCTGTTTAGAGGA	
<i>Nr4a2</i>	NM_013613	For	GTGTTTCAGGCGCAGTATGG	153 bp
		Rev	TGGCAGTAATTTCAAGTGTGGT	
<i>Ogt</i>	NM_139144	For	GACGCAACCAAACCTTGCACT	108 bp
		Rev	TCAAGGTTGACAGCCTTTTCA	
<i>Pdlim5</i>	NM_001190857	For	TTCAACATGCCTCTGACAATCTC	80 bp
		Rev	ACCACGTCCCTATTTCTGACA	
<i>Pikfyve</i>	NM_011086	For	TCCCGACACTGGACTCTG	208 bp
		Rev	GGCTGGCCCAACTTGAACCT	
<i>Prdx6</i>	NM_007453	For	CGCCAGAGTTTGCCAAGAG	115 bp
		Rev	TCCGTGGGTGTTTACCATTG	
<i>Rabep1</i>	NM_019400	For	CTTCGTTCTGTCGTGATGCC	277 bp
		Rev	ACTGGTTGTGTTGTTGTCGTT	
<i>Rin1</i>	NM_145495	For	TATGACACACCTGATACCAGAGG	189 bp
		Rev	AGTGTTAGATTTCCGACCAG	
<i>Rtp4</i>	NM_023386	For	TGGGAGCAGACATTTCAAGAAC	179 bp
		Rev	ACCTGAGCAGAGGTCCAACCT	
<i>Steap1</i>	NM_027399	For	GGTCGCCATTACCCTCTTGG	188 bp
		Rev	GGTATGAGAGACTGTAAACAGCG	
<i>Steap2</i>	NM_028734	For	ATGGGAAGCCCTAAGAGCCT	126 bp
		Rev	AAGCCGAATGGTCAGAGACTT	
<i>Tcf4</i>	NM_013685	For	CGAAAAGTTCTCCGGGTTTG	196 bp
		Rev	CGTAGCCGGCTGATTCAT	
<i>Tmx1</i>	NM_028339	For	CACTTGGGGCGTCTTATGGTT	111 bp
		Rev	CCAGTTCTCATCGGTGAGGAC	

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Table A2 (continued)

Genes	Accession no.		Primer sequence(5'-3')	Product size
<i>Pde4b</i>	18578	For	CGCAGGGAGTCGTTCTCTA	263 bp
		Rev	CTCCTGTGGTCGCACACTTG	
<i>Rgs2</i>	NM_009061	For	GAGAAAATGAAGCGGACACTCT	197 bp
		Rev	GCAGCCAGCCCATATTTACTG	
<i>Sgk1</i>	NM_001161849	For	CTGTGCGAAGCACCCCTTACC	175 bp
		Rev	TCCTGAGGATGGGACATTTTCA	
<i>Sorl1</i>	NM_011436	For	AGCAGGAGGAGTCGAGAC	170 bp
		Rev	GTTCCTAGCCGGAGATCCGC	
<i>Vldlr</i>	NM_013703	For	GAGTCTGACTTCGTGTGCAAA	83 bp
		Rev	GAACCGTCTTCGCAATCAGGA	
<i>Klhl30</i>	NM_027551	For	CATCTGCCTTACATGCCCAA	206 bp
		Rev	ACGTCTCGCAGTCCACAC	
<i>Chd9</i>	NM_177224	For	GAGGGCAGTTTGATTGGACAA	178 bp
		Rev	CGTTCTCTGTGATTGGTTGAAT	
<i>Slc26a1</i>	NM_178743	For	AGTGTATGCGGGAACACATGC	141 bp
		Rev	CGAAGGCGTAAGCAATCAGAG	
<i>Fam46c</i>	NM_001142952	For	AACTGGGATCAGGTTAGCCG	212 bp
		Rev	CAACCCAAGCCGTTGTCTT	
<i>Vwce</i>	NM_027913	For	GCCTACATCTTGATCCCGTTG	234 bp
		Rev	GGAAACAGACATTGGGAGCGA	
<i>Scn8a</i>	NM_011323	For	CCCGACAGTTTCAAGCCTTTC	240 bp
		Rev	CGTCAAATAGTACGGGTCAAAGT	
<i>Zc3h7b</i>	NM_001081016	For	GGCAGCCTGCTACTTCACC	98 bp
		Rev	GGAACAGTGCCCGGATACT	
<i>Nrg4</i>	NM_032002	For	CACGCTGCGAAGAGGTTTTTC	104 bp
		Rev	CGCGATGGTAAGAGTGAGGA	
<i>FOXP4</i>	NM_001110824	For	GCTGTACTGCTACCTCGTTT	107 bp
		Rev	CTGTCTCTCCGAGATGTGAGC	

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