



## Dietary vinegar prevents kidney stone recurrence via epigenetic regulations

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### ABSTRACT

**Background:** Epidemiological evidence of over 9000 people suggests that daily intake of vinegar whose principal bioactive component is acetic acid is associated with a reduced risk of nephrolithiasis. The underlying mechanism, however, remains largely unknown.

**Methods:** We examined the in vitro and in vivo anti-nephrolithiasis effects of vinegar and acetate. A randomized study was performed to confirm the effects of vinegar in humans.

**Findings:** We found individuals with daily consumption of vinegar compared to those without have a higher citrate and a lower calcium excretion in urine, two critical molecules for calcium oxalate (CaOx) kidney stone in humans. We observed that oral administration of vinegar or 5% acetate increased citrate and reduced calcium in urinary excretion, and finally suppressed renal CaOx crystal formation in a rat model. Mechanism dissection suggested that acetate enhanced acetylation of Histone H3 in renal tubular cells and promoted expression of *microRNAs-130a-3p*, *-148b-3p* and *-374b-5p* by increasing H3K9, H3K27 acetylation at their promoter regions. These *miRNAs* can suppress the expression of *Ndc1* and *Cldn14*, thus enhancing urinary citrate excretion and reducing urinary calcium excretion. Significantly these mechanistic findings were confirmed in human kidney tissues, suggesting similar mechanistic relationships exist in humans. Results from a pilot clinical study indicated that daily intake of vinegar reduced stone recurrence, increased citrate and reduced calcium in urinary excretion in CaOx stone formers without adverse side effects.

**Interpretation:** Vinegar prevents renal CaOx crystal formation through influencing urinary citrate and calcium excretion via epigenetic regulations. Vinegar consumption is a promising strategy to prevent CaOx nephrolithiasis occurrence and recurrence.

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### 1. Introduction

Nephrolithiasis is a common condition that affects 5% to 8.8% of the population in the world [1,2]. The consequences of stone disease range from pain, need for hospitalization and surgery as well as loss of work in

the acute setting, to chronic kidney disease in the long term [3]. Once afflicted, nephrolithiasis tends to be recurrent with up to 50% in the majority of cases in 5 years since the first stone event [4]. Calcium oxalate (CaOx) is the most common crystal constituent of kidney stones [5]. While surgical techniques to remove obstructive stones have been improved, few therapeutic advances have been made to prevent stone recurrence [6]. Currently available interventions aimed at preventing the CaOx kidney stone formation include increasing fluid intake and treatment with pharmaceutical compounds that interfere with CaOx crystal formation. However, these measures have their own limitations [7,8]. Thus, the high rate of stone recurrence and the surgical burden place these patients at an increased risk for reduced kidney function and chronic kidney damage.

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### Research in context

#### Evidence before this study

Nephrolithiasis is a common ailment that affects nearly 10% of the population in the world. Once afflicted, nephrolithiasis tends to be recurrent with up to 50% in the majority of cases in 5 years since the first stone event. The high rate of stone recurrence and the surgical burden place these patients at an increased risk for reduced kidney function and chronic kidney damage. Currently available interventions aimed at preventing the kidney stone formation include increasing fluid intake and treatment with pharmaceutical compounds that interfere with calcium oxalate (CaOx) crystal formation. However, these measures have their own limitations. Vinegar is consumed worldwide as a food condiment and preservative. The principal bioactive component of vinegar is acetic acid. In 2014, our center performed a nation-wide epidemiological survey to investigate the prevalence and associated factors of nephrolithiasis among adults in China. From this survey we found that daily intake of vinegar is strongly associated with a lesser risk of nephrolithiasis. However, whether vinegar can inhibit nephrolithiasis and its underlying mechanisms remain largely unknown.

#### Added value of this study

We used multiple *in vitro* assays and *in vivo* rat model to demonstrate that acetate, which is the principle bioactive component, could suppress CaOx crystals formation through influencing urinary calcium and citrate excretion via epigenetic regulations. Significantly these mechanistic findings were confirmed in human kidney tissues, suggesting similar mechanistic relationships exist in humans. More importantly, results from a pilot clinical study indicated that daily intake of vinegar increased citrate and reduced calcium in urinary excretion in CaOx stone formers as well as decreased stone recurrence without adverse side effects.

#### Implications of all the available evidence

Vinegar can prevent renal CaOx crystal formation through influencing urinary citrate and calcium excretion via epigenetic regulations. The conclusions of mechanistic studies are clear and supported by a large-scale epidemiological study and experimental evidences from tissue culture, animal model and clinical studies. Vinegar consumption is a promising strategy to prevent CaOx nephrolithiasis occurrence and recurrence.

Vinegar has a very long history, going back to Babylonia in 5000 BCE. Today, vinegar is consumed worldwide as a food condiment and preservative. Especially in China, vinegar is a very common seasoning in popular foods such as dumpling and noodles. The principal bioactive component of vinegar is acetic acid. The concentration of acetic acid in commercially available vinegar ranges from 4% to 8% [9]. In 2014, our center performed a nation-wide epidemiological survey to investigate the prevalence and associated factors of nephrolithiasis among adults in China [1]. A total of 12,570 individuals were invited and finally 9310 participants completed the investigation. From this survey we found that daily intake of vinegar is strongly associated with a lesser risk of nephrolithiasis (OR = 0.36, 95%CI 0.32–0.41,  $p < .001$ ). However, whether vinegar can inhibit nephrolithiasis and its underlying mechanisms remain largely unknown.

Therefore, in this study, we examined the relationship between vinegar consumption and urine chemistry in human subjects. We also determine the consequence of vinegar intake in an experimental hyperoxaluria rat model, and found that miRNA regulation of

transporters for the kidney stone components is critical for the efficacy of vinegar to reduce CaOx crystal formation. These mechanisms have also been validated in human kidneys and in a prospective clinical study.

## 2. Materials and methods

### 2.1. Animal studies

All of the rat experiments were performed under protocols approved by the Institutional Animal Care and Use Committee of the Guangzhou Medical University.

#### 2.1.1. Development of CaOx crystal rat model

Male Sprague Dawley rats, aged 6–8 weeks, were purchased from Guangdong Medical Laboratory Animal Center. Rats were housed in polypropylene cages, and had access to food and water *ad libitum*. We established the CaOx crystal rat model following the reported protocol [10]. Rats were given free access to food and drinking water that contained 1% (v/v) EG for a period of 4 weeks. At the first three days of the 1st and 3rd week of EG treatment, each rat was given 2 ml daily 1% ammonium chloride by gavage to promote CaOx crystals deposition. The rats were placed in metabolic cages for urine collection 1 day before sacrificing. Approximately 2 mL of blood was collected from the ventricle into heparinized syringes and was immediately placed on ice. Whole blood was transferred to serum separator tubes and centrifuged to isolate the plasma, which was then stored at 4 °C until analysis.

#### 2.1.2. Vinegar/acetic acid treatment

8-week-old rats were divided into four groups. In the control group, animals were given tap water as their drinking water and 2 ml/kg ddH<sub>2</sub>O by gavage for 4 weeks. The EG group animal were exposed to 1% EG in their drinking water and 2 ml/kg ddH<sub>2</sub>O by gavage for 4 weeks. In the vinegar group, rats were orally administered vinegar (2 ml/kg, Ninghuafu®, China), daily for 4 weeks. The concentration of acetic acid in the vinegar was 5%. This group also received tap water during experiment. In the vinegar/acetic acid treatment group, animals are exposed to 1% EG in their drinking water and 2 ml/kg dose of vinegar or 5% acetic acid dissolved with ddH<sub>2</sub>O was administered daily by gavage for 4 weeks.

#### 2.1.3. Antagomir treatment

The miR-130a-3p antagomir (5'-AUGCCUUUUAACAUUGCACUG-3'), miR-148b-3p antagomir (5'-ACAAAGUUCUGUGAUGCACUGA-3'), miR-374b-5p antagomir (5'-CACUUAGCAGGUUGUAUUUAU-3') and negative control antagomir were purchased from Guangzhou RiboBio (RiboBio, China). Each antagomir was dissolved by autoclaved PBS according to the manufacturer's guidelines followed by ip injection to rats at a dose of 18 nmol/kg body wt<sup>-1</sup>.

#### 2.1.4. Agomir treatment

The miR-130a-3p agomir (5'-CAGUGCAAUGUAAAAGGGCAU-3'), miR-148b-3p agomir (5'-UCAGUGCAUCACAGAACUUUGU-3'), miR-374b-5p agomir (5'-AUUAUAACAACCGCUAAGUG-3') and negative control agomir were purchased from RiboBio. Each agomir was dissolved by autoclaved PBS according to the manufacturer's guidelines followed by ip injection to rats at a dose of 14 nmol/kg body wt<sup>-1</sup>.

### 2.2. Analysis of blood sample and 24-h urine sample

Urine oxalate and citrate were measured using ion exchange chromatography (Metrohm, Switzerland). Urine sodium, potassium, chloride, phosphate and creatinine, and serum BUN and creatinine were determined by Unicel DxC 600 synchronic biochemical detecting system. Urine uric acid and magnesium were measured using Beckman coulter AU680 automatic biochemistry analyzer. pH values were

determined with a glass electrode in a calibrated pH meter (Mettler Toledo, Switzerland). All 24-h urinary analyses were performed in the Guangdong Key Laboratory of Urology according to standardized protocols. The urine was analyzed within 24 h of collection.

For urine calcium determinations, the urine sample was separated into supernatant and pellet fractions by centrifugation at 12,000 g for 15 min to isolate the ionized forms of calcium (supernatant) from the crystalline/CaOx form (pellet). Pellets were then resuspended in equal volume PBS, and 5  $\mu$ l of 1 N HCL was added to each sample to acidify the fraction and dissolve the crystals. Calcium was measured spectrophotometrically in both the prepared supernatants and pellets according to a published Arsenazo III method [11].

Approximate estimates of ion activity products of calcium oxalate and calcium phosphate were expressed in terms of AP(CaOx) index<sub>s</sub> and AP(CaP) index<sub>s</sub> according to the formulas given in the following sections [12]. In the calculations, 24-h calcium, oxalate, citrate, magnesium, and phosphate were expressed in millimole and the volume in liters.

$$AP(CaOx)index_s = \frac{1.9 * Calcium^{0.84} * Oxalate}{Citrate^{0.22} * Magnesium^{0.12} * 1.5^{1.03}}$$

$$AP(CaP)index_s = \frac{2.7 * 10^{-3} * Calcium^{1.07} * Phosphate^{0.70} * (7.0-4.5)^{6.8}}{Citrate^{0.20} * 1.5^{1.31}}$$

### 2.3. Antibodies

The antibodies used in this study are listed in Supplementary Table 1.

### 2.4. Immunohistochemistry analysis (IHC)

Tissues were fixed in 10% neutral buffered formalin and embedded in paraffin. Kidney sections were stained with hematoxylin and eosin, and MTS. For IHC, sections were incubated with the primary antibodies for Ndc1, Oat, Ndc3, Cldn14, THP, H3K9ac, and H3K27ac. To enhance antigen exposure, the slides were treated with 10 mM sodium citrate/pH = 6.0 at 98 °C for 20 min for antigen retrieval. The slides were incubated with endogenous peroxidase blocking solution, and then were incubated with the primary antibody at 4 °C overnight. After rinsing with PBS, the slides were incubated for 45 min with biotin-conjugated

secondary antibody, washed, and then incubated with enzyme conjugate horseradish peroxidase (HRP)-streptavidin. Freshly prepared DAB (Zymed, South San Francisco, CA) was used as substrate to detect HRP. Finally, slides were counter-stained with hematoxylin/eosin and mounted with aqueous mounting media. The German immunoreactive score [13] (0–12) was calculated by multiplying the percentage of immunoreactive kidney epithelial cells (0% = 0; 1%–10% = 1, 11%–50% = 2, 51%–80% = 3; and 81%–100% = 4) by staining intensity (negative = 0; weak = 1; moderate = 2; and strong = 3). The H3K9ac or H3K27ac-positive cells were determined using light microscopy. Two hundred cells were counted from each tissue specimen.

### 2.5. Immunofluorescence staining (IF)

The IF assays were performed as previously described [14]. The kidney sections were incubated with xylol and descending concentrations of ethanol. Endogenous peroxidases were removed with 0.3% H<sub>2</sub>O<sub>2</sub>. The Ndc1, Cldn14, H3K9ac, H3K27ac, AQP-1, and THP antibodies was then applied overnight in a humidified chamber after blocking with 3% bovine serum albumin for 1 h at room temperature. The slides were rinsed in PBS and incubated with the second antibodies. After washing three times with PBS, the secondary antibody conjugated to Alexa Fluor 488 or Alexa Fluor 647 was applied to these slides for 1 h at room temperature. The nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI). For quantitative assessments, the images were analyzed by Image J software.

### 2.6. Detection of CaOx crystals in rat kidney

Crystal formation within the kidney tissues was examined using Pizzolato staining, as described previously [15]. Crystal formation was quantitatively assessed using Image J software.

### 2.7. Cell lines

The human proximal tubular cell line HK-2, human embryonic kidney cell line HEK-293 T, rat renal proximal tubular cell line NRK-52, and Madin-Darby canine kidney (MDCK) cell line were purchased from the American Type Culture Collection (ATCC). All the cells were maintained in DMEM media with 10% fetal bovine serum and 1% penicillin/streptomycin.

### 2.8. Synthesis and transfection of miRNA mimics and miRNA inhibitors

miRNA mimics and miRNA inhibitors were designed and synthesized by Guangzhou RiboBio (Ribobio, China). miRNA inhibitors were all nucleotides with 2'-O-methyl modification. 24 h prior to transfection, cells were placed onto a 6-well plate (Greiner, Germany) at 40–60% confluence. Transfection was performed with riboFECT™ CP Reagent (Ribobio, China) according to the manufacturer's protocol. The medium was replaced 24 h after transfection with new culture medium, except in the case of HEK293 cells, for which the medium was not replaced after transfection.

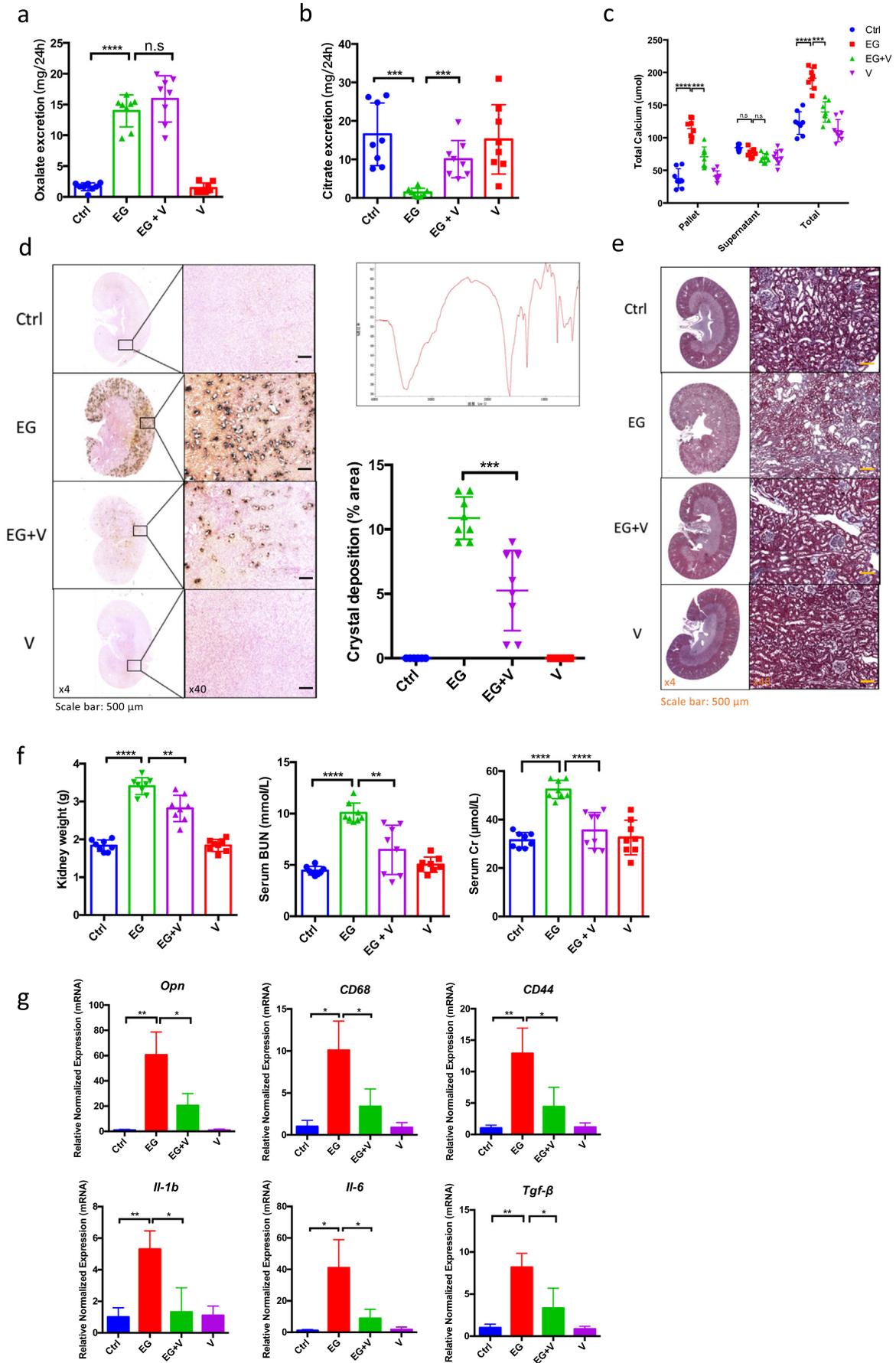
### 2.9. Oxalate, calcium and acetate treatment

Oxalate (Sigma) stock solution (10 mM) in PBS was diluted in medium to achieve final concentration of 0.5 mM. Sodium acetate (Sigma) stock solution (10 mM) in PBS was diluted in medium to achieve final concentration of 2 mM. Calcium chloride (Sigma) stock solution (20 mM) in ddH<sub>2</sub>O was diluted in medium to achieve final concentration of 3 mM. HK-2, NRK-52, or MDCK cells were placed in 6-well culture dishes incubated overnight. The next day, the cell medium was changed to normal medium with 0.5 mM oxalate, and/or 2 mM sodium acetate, and/or 3 mM calcium chloride. After 24 h of treatment, cells were collected for further experiments.

**Table 1**

The 24-h urine profile of adults who do or do not consume vinegar daily.

| Mean $\pm$ SD or n (%)      | Control (n = 61)     | Vinegar (n = 61)     | P value |
|-----------------------------|----------------------|----------------------|---------|
| Age (years)                 | 45.07 $\pm$ 10.87    | 43.56 $\pm$ 10.18    | 0.431   |
| BMI                         | 25.82 $\pm$ 5.74     | 24.95 $\pm$ 3.40     | 0.309   |
| Gender (Male)               | 26 (42.62)           | 24 (39.34)           | 0.713   |
| Hypertension                | 1 (1.64)             | 2 (3.28)             | 1.000   |
| Diabetes                    | 8 (13.11)            | 6 (9.84)             | 0.776   |
| Serum calcium (mM)          | 2.31 $\pm$ 0.47      | 2.34 $\pm$ 0.53      | 0.741   |
| 24 h urine composition      |                      |                      |         |
| Volume (ml)                 | 1440.98 $\pm$ 566.93 | 1669.51 $\pm$ 745.77 | 0.059   |
| Citrate (mg)                | 445.92 $\pm$ 310.78  | 613.31 $\pm$ 328.59  | 0.005   |
| Oxalate (mg)                | 26.74 $\pm$ 13.39    | 24.54 $\pm$ 13.65    | 0.371   |
| Calcium (mmol)              | 5.10 $\pm$ 2.11      | 4.25 $\pm$ 1.81      | 0.019   |
| Phosphate (mmol)            | 18.49 $\pm$ 7.86     | 16.26 $\pm$ 5.19     | 0.068   |
| Creatinine (mmol)           | 10.76 $\pm$ 2.38     | 8.93 $\pm$ 1.84      | <0.001  |
| Uric acid (mmol)            | 3.45 $\pm$ 1.15      | 2.92 $\pm$ 0.74      | 0.003   |
| Sodium (mmol)               | 192.43 $\pm$ 71.45   | 200.19 $\pm$ 69.50   | 0.545   |
| Potassium (mmol)            | 45.10 $\pm$ 20.18    | 44.51 $\pm$ 16.10    | 0.858   |
| Magnesium (mmol)            | 4.30 $\pm$ 1.51      | 4.83 $\pm$ 1.96      | 0.098   |
| Chloride (mmol)             | 182.47 $\pm$ 70.28   | 202.63 $\pm$ 68.44   | 0.111   |
| AP(CaOx) Index <sub>s</sub> | 1.12 $\pm$ 0.75      | 0.75 $\pm$ 0.52      | 0.002   |
| AP(CaP) Index <sub>s</sub>  | 31.95 $\pm$ 19.04    | 21.84 $\pm$ 11.44    | 0.001   |
| pH                          | 5.80 $\pm$ 0.72      | 6.20 $\pm$ 0.35      | <0.001  |



### 2.10. RNA extraction and quantitative real-time PCR (Q-PCR) analysis

Total RNAs were isolated from cells or tissues using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Then 1 µg of total RNAs was subjected to reverse transcription using PrimeScript™ RT reagent Kit (TaKaRa). Q-PCR was conducted using a Bio-Rad CFX96 system with SYBR Green (TaKaRa) to determine the level of mRNA expression of a gene of interest. Expression levels were normalized to the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. All tested primers are listed in Supplementary Table 2.

For miRNA detection, 2 µg of total RNAs was subjected to reverse transcription using All-in-One™ miRNA First-strand cDNA Synthesis Kit. Q-PCR was conducted using a All-in-One™ miRNA qRT-PCR Detection Kits. Expression levels were normalized to the expression of 5S rRNA or U6 snRNA.

### 2.11. Western blot assay

Total protein was extracted by RIPA buffer or SDS lysis buffer containing 1% protease inhibitors (Amresco, Cochran, USA). The following primary antibodies were used: Rabbit anti-Nadcl polyclonal antibody, rabbit anti-Cldn14 polyclonal antibody, mouse anti-GAPDH monoclonal antibody, rabbit anti-Histone H3 polyclonal antibody, rabbit anti-Histone H3 (acetyl K9) polyclonal antibody, rabbit anti-Histone H3 (acetyl K27) polyclonal antibody, rabbit anti-Histone H3 (acetyl K56) polyclonal antibody were used. Standard western blot protocols were adopted. The quantification was carried out by subtracting background from the band intensity of western blots by using Image J software.

### 2.12. Knockdown of Nadcl

RNA interference was performed on HK-2 and NRK-52 cells by using Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions. For silencing Nadcl, siRNA targeting Nadcl (NM\_001145975.1 and NM\_031746.1) and nonspecific control pool (negative control) were purchased from Ribobio, China. The target sequences of siRNA were listed in Supplementary Table 3. siRNA oligos were diluted in RNase-free solution and sorted at -80 °C. HK-2 or NRK-52 cells of 50% to 60% confluency were transfected with the final concentration of 100 nM siRNA or the nonspecific control siRNA pool followed by whole cell lysate preparation 72 h after transfection.

### 2.13. ChIP-qPCR assay

ChIP-qPCR assays were performed using a commercial kit (Pierce™ Agrose ChIP Kit) according to the manufacturer's instructions. Briefly,  $1 \times 10^7$  HK-2 or MDCK cells were cross-linked with 1% paraformaldehyde, lysis and sonicated 13–15 times on ice until chromatin was 100–800 bps in size, with the center being ~300 bp. Solubilized chromatin was immunoprecipitated with ChIP grade antibodies for H3K9 acetyl, H3K27 acetyl or rabbit IgG (negative control). The DNA fragments were detected by qPCR. Histone acetylation marks were mapped at promoter spanning -2 to 2 kb of target genes (miR-130a-3p, miR-148b-3p, miR-374b-5p). Primers spanning the regions with peaks were adopted for ChIP-qPCR analysis. All tested primers targeting miR-130a, miR-148b and miR-374b are list in Supplementary Table 2.

### 2.14. Luciferase reporter assay

Wild-type (WT) human Nadcl 3'UTR and mutated Nadcl 3'UTR (with a mutated sequence on the miR-130a-3p/miR-148b-3p binding site) were amplified from a human cDNA library. WT dog Cldn14 3'UTR and mutated Cldn14 3'UTR (with a mutated sequence on the miR-374b-5p binding site) were amplified from a dog cDNA library. 3'UTRs were then inserted between the *XhoI/NotI* restrictive sites of a firefly/Renilla luciferase reporter pmiR-REPORT™ (RiboBio, Guangzhou, China). HEK293T cells were co-transfected with 25 ng/ml of either the luciferase reporter with WT or mutated 3'UTR, and 100 pmol of either miRNA mimics or miRNA negative control (NC). 48 h after co-transfection, a Dual-Luciferase Reporter Assay (Promega, USA) was carried out according to the manufacturer's protocol.

### 2.15. Transepithelial resistance (TER) measurement

TER measurement was performed on cell monolayers grown on porous filters (Transwell) as previously described [16]. TER was measured using the EVOM Epithelial Volt/Ohm Meter. TER of the confluent monolayer of cells was determined in buffer A (145 mM NaCl, 2 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 10 mM glucose, and 10 mM HEPES, pH = 7.4), and the TER of blank filters was subtracted. Dilution potential were measured when buffer B (80 mM NaCl, 130 mM mannitol, 2 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, and 10 mM HEPES, pH 7.4) replaced buffer A on the apical of basal side of filters.

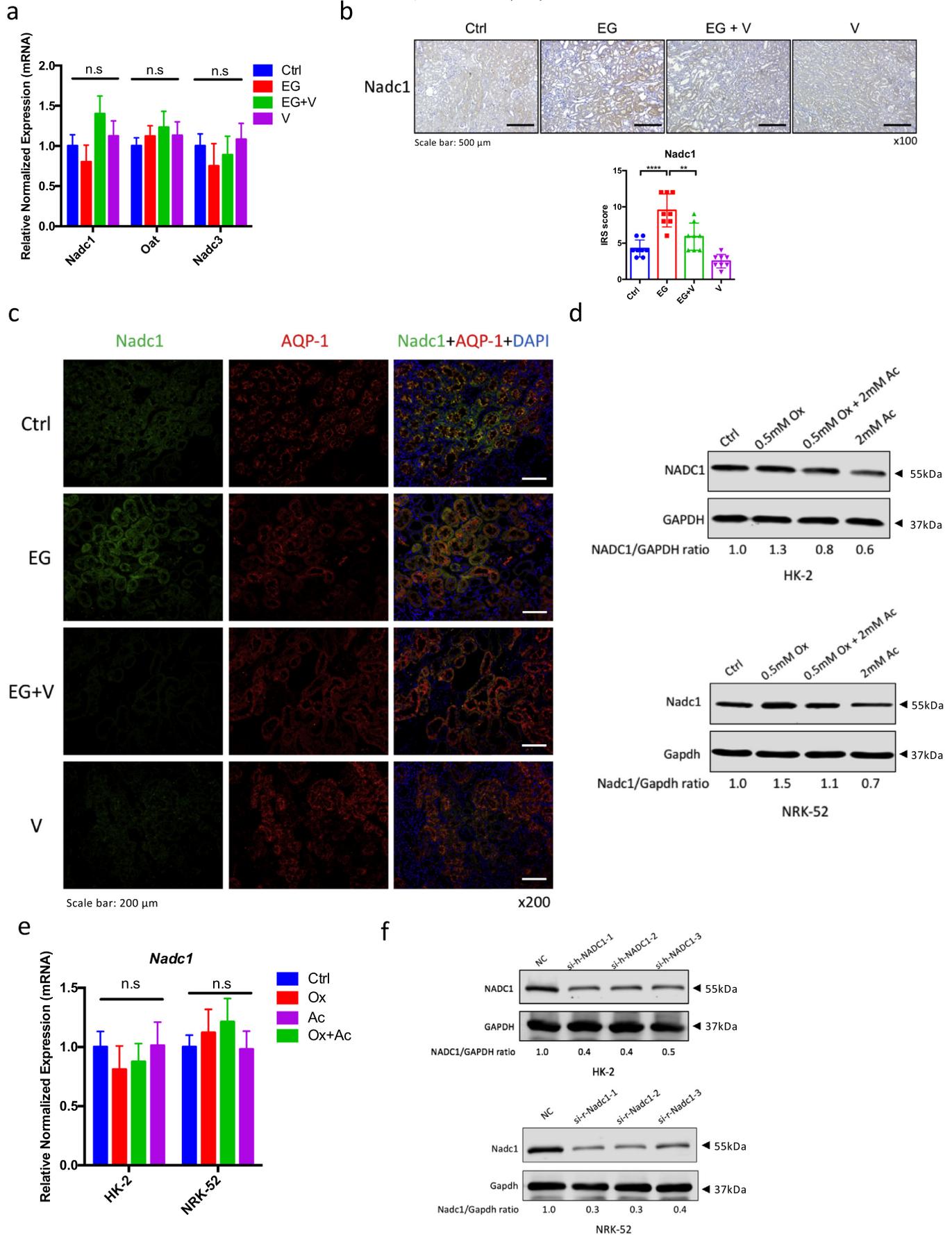
### 2.16. Human kidney tissue samples

Human kidney tissue samples were derived from the areas of normal kidneys tissues of radical nephrectomy for renal cell carcinoma, and obtained from the First Affiliated Hospital of Guangzhou Medical University and People's Hospital of Changzhi, with consents from the patients. The procedure related to human subjects was approved by the Institutional Ethics Committee.

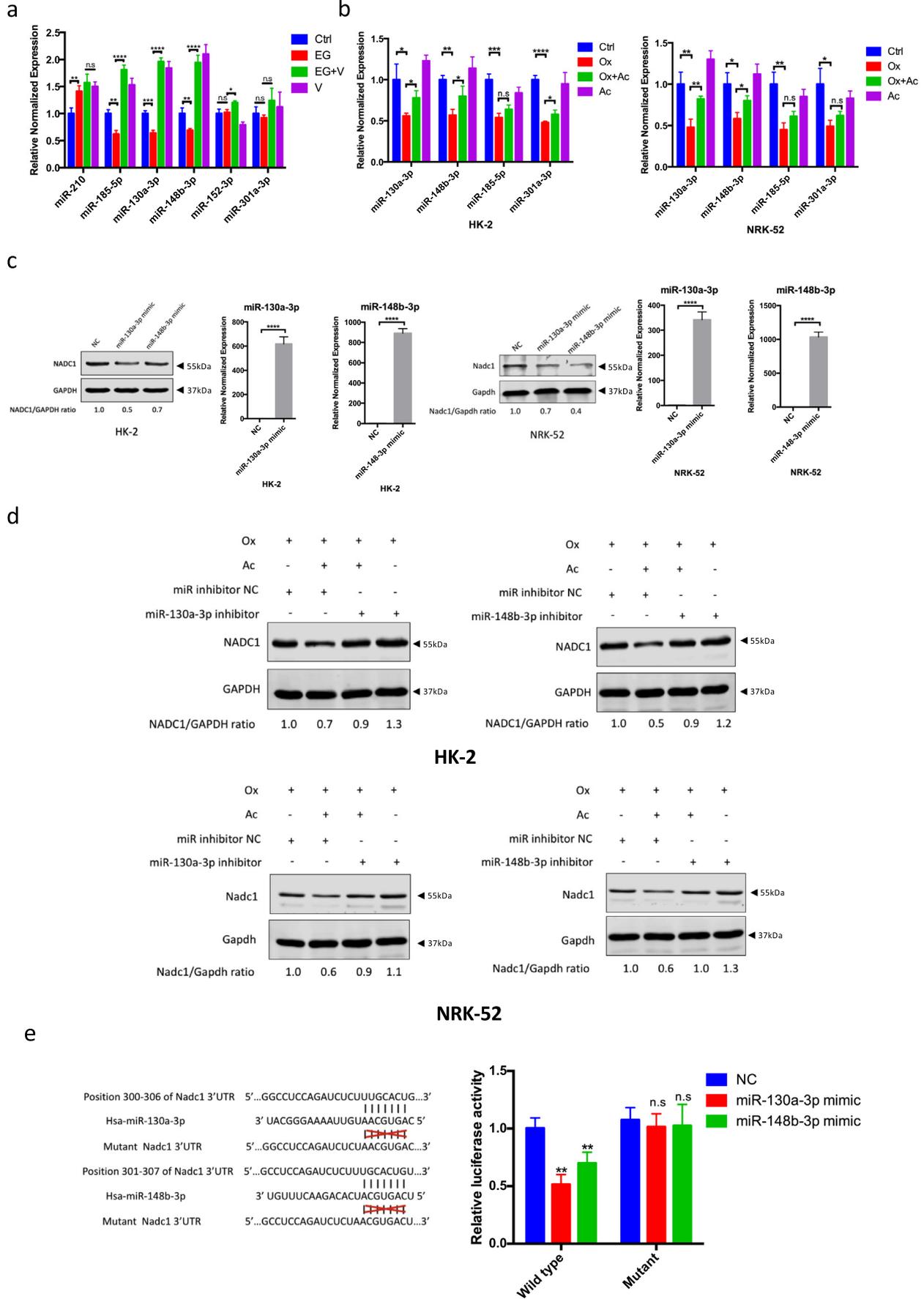
### 2.17. miRNA in situ hybridization assay (MISH)

The in situ hybridization for miRNAs was performed on fixed paraffin-embedded sections as previously described [17,18]. Oligonucleotide probes complementary to hsa-miR-130a-3p, hsa-miR-148b-3p, and hsa-miR-374b-5p were purchased from the Exonbio Lab (Guangzhou, China). The probe sequences were as follows: 5'- ATGC CCTTTAACATTGCACTG-3' (miR-130a-3p), 5'- ACAAGTTCGTGATG CACTGA-3' (miR-148b-3p), 5'- CACTTAGCAGGTTGTATTATAT-3' (miR-374b-5p). These oligonucleotides contain 2'-fluoro-modified RNA residues (2'-F RNA) at the 3, 6, 15 and 20 base. Both 5' and 3' ends were labeled by digoxin (DIG). 10 µm thick sections from kidney tissues were deparaffinized, dehydrated and subsequently immersed in 0.2 M HCl for 15 min. Slides were then immersed in PBS solution. Proteinase K (working solution: 200 µg/ml in PBS) digestion was used to treat tissues at 37 °C for 5 min. After digestion, slides were immersed in RNase-free water for 5 min and air dried. The slides were then prehybridized in hybridization (Exonbio Lab, Guangzhou, China) buffer at 37 °C for 2 h, followed by the hybridization with probe at 37 °C for 48 h. After hybridization, slides were washed 2xSSC with 0.5% Tween-20 twice for 5 min at room temperature. DNA was counterstained with DAPI (1 mg/ml). Images of miRNA signals in slides were captured

**Fig. 1.** Effect of vinegar on urinary composition and renal CaOx crystals formation in the EG-treated rats. (a) Detection of 24-h oxalate excretion in urine samples of each group of rats. (b) Detection of 24-h citrate excretion in urine samples of each group of rats. (c) Detection of 24-h calcium excretion in urine samples of each group rats. (d) Pizzolato staining (left) illustrating CaOx crystal deposition in the kidney and its quantification (right panel). Crystals components in rats kidneys were isolated as CaOx crystals with Fourier-transform infrared spectroscopy analysis (right panel). (e) Representative macrophotographs of Masson-stained kidneys in rats. (f) Kidney weight, serum BUN and creatinine (Cr) concentration in rats. (g) q-PCR analysis of inflammation-related genes expression in kidney from rats. Ctrl, control. EG, ethylene glycol. V, vinegar. Data are given as mean ± SD, from 6 to 8 rats in each group. n.s, not significant, \**P* < .05, \*\**P* < .01, \*\*\**P* < .001, \*\*\*\**P* < .0001 (One-way ANOVA).

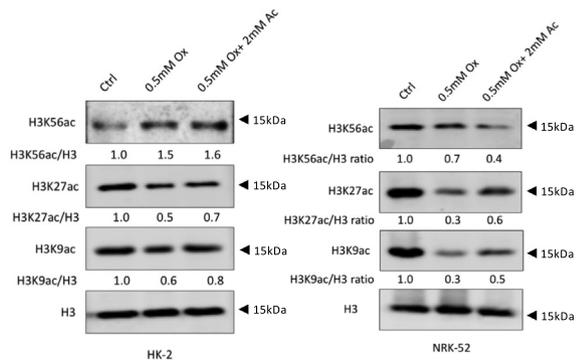


**Fig. 2.** Vinegar increased urinary citrate excretion via down-regulating the Nadc1 expression. (a) q-PCR analysis of genes expression of citrate transporters in kidney from rats. (b) IHC staining of Nadc1 in kidney tissues from each group of rats (quantitation on the right). (c) Immunofluorescence costaining for Nadc1 and proximal tubular marker Aqp-1 in kidney tissues for each group of rats. (d) Western blot analysis of Nadc1 protein expression after 2 mM sodium acetate and/or 0.5 mM oxalate treatment in HK-2 or NRK-52 cells. (e) q-PCR analysis of Nadc1 mRNA expression after 2 mM sodium acetate and/or 0.5 mM oxalate treatment for 24 h in HK-2 or NRK-52 cells. (f) Western blot analysis of validation of knockdown of Nadc1 using siRNAs. Ctrl, control. EG, ethylene glycol. V, vinegar. Ox, oxalate. Ac, sodium acetate. IRS, immune-reactive score. Data are given as mean  $\pm$  SD, from 6 to 8 rats in each group. n.s., not significant,  $^{**}P < .01$ ,  $^{****}P < .0001$  (One-way ANOVA).

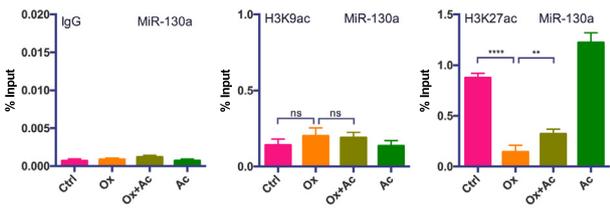


**Fig. 3.** Acetate suppressed Nadc1 protein expression via up-regulating *miR-130a-3p* and *miR-148b-3p* expression. (a) 6 potential miRNAs candidates were screened by q-PCR assay in kidney from rats. (b) q-PCR analysis of 4 miRNA expressions after 2 mM sodium acetate and/or 0.5 mM oxalate treatment for 24 h in HK-2 or NRK-52 cells. (c) HK-2 or NRK-52 cells were transfected with the *miR-130a-3p* or *miR-148b-3p* mimic or a negative control (NC). Nadc1 expression was analyzed 48 h later by Western blot. Gapdh serves as a loading control. (d) HK-2 or NRK-52 cells were transfected with the *miR-130a-3p* or *miR-148b-3p* inhibitor or a negative control (NC). 24 h later cells were treated with 0.5 mM oxalate or/and 2.0 mM sodium acetate. Nadc1 expression was analyzed 24 h later by Western blot. (e) Co-transfection of Nadc1 3'UTR constructs containing wild type or mutant seed regions with *miR-130a-3p* and *miR-148b-3p* into HEK-293 cells and luciferase assay was applied to detect the luciferase activity. Ctrl, control. EG, ethylene glycol. V, vinegar. Ox, oxalate. Ac, sodium acetate. Data are given as mean  $\pm$  SD, from 6 to 8 rats in each group. n.s, not significant, \* $P < .05$ , \*\* $P < .01$ , \*\*\* $P < .001$ , \*\*\*\* $P < .0001$  (Student's *t*-test).

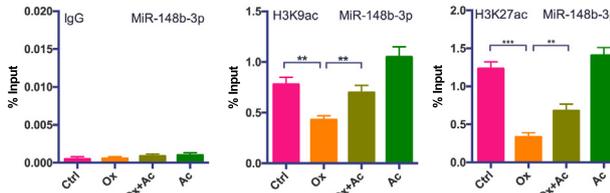
**a**



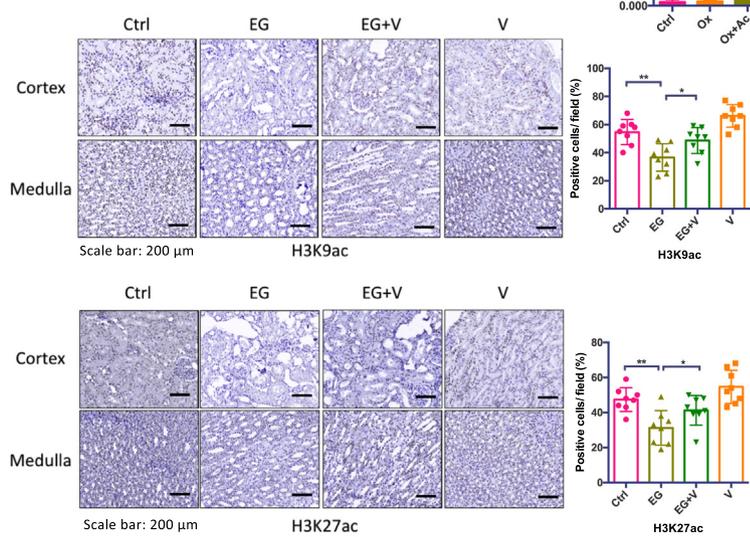
**b**



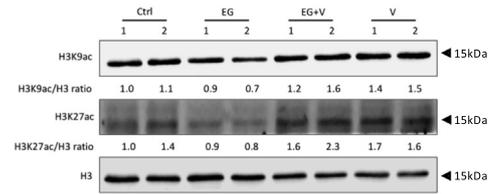
**c**



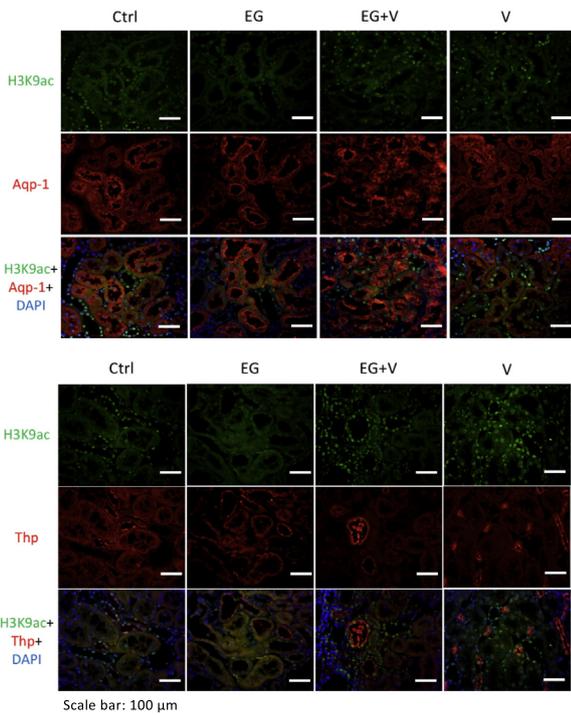
**d**



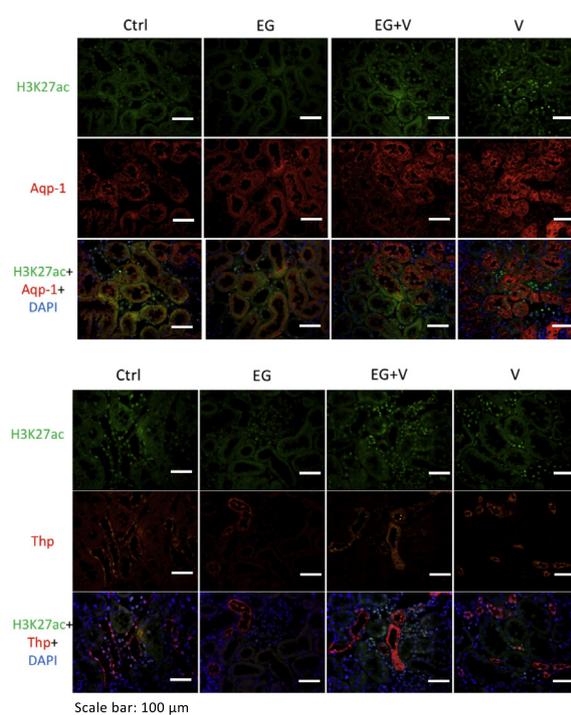
**e**



**f**



**g**



by fluorescent microscope. For quantitative assessments, the images were analyzed by using Image J software.

### 2.18. MISH and IF co-staining

To assess whether miRNAs were expressed in renal tubular cells, renal sections were processed for double IF staining to visualize the simultaneous locations of miRNAs (green) and a primary antibody for AQP-1 (a known proximal tubule marker) or THP (a TAL marker). First, we performed MISH to detect miRNAs, and then, we used IF to label renal tubules. For quantitative assessments, the images were analyzed by using Image J software.

### 2.19. Prospective randomized study design

A prospective randomized clinical trial to test the impact of vinegar on 24-h urine composition and preventing recurrent calcium oxalate nephrolithiasis was performed at the First Affiliated Hospital of Guangzhou Medical University since March 2017. The study protocol has been approved by the local Institutional Ethics Committee and registered on [www.clinicaltrials.gov](http://www.clinicaltrials.gov). The inclusion and exclusion criteria were in the Supplementary Table 4. Subjects were randomly assigned in a 1:1 ratio to receive mature vinegar or control pure water. The sequence of randomization was computer generated and were performed by the hospital's pharmacy service, whoever administered 5 ml mature vinegar (Ninghuafu®) three times a day and control as liquid of the same type in identical bottles.

From the beginning of intervention, participants were scheduled to receive a brief (< 10 min) individual telephone contact once per month and weekly text messages. The telephone contacts were conducted by intervention staff and followed a standard script. Text message was provided once per week and was used to remind participants in intervention group of drinking mature vinegar. 24-h urine and serum specimens were obtained at baseline (with values documented as the average of the two sets of measurements performed before randomization) and 3–12 months after randomization. Stone recurrences were diagnosed on the basis of renal ultrasound performed at 3 months, 6 months, and 1 year after randomization. If renal stones were detected, CT were performed. The imaging studies were performed by a central radiologic service, and the radiologist have no knowledge of the trial or the group assignment.

### 2.20. Statistical analysis

Statistical analysis was performed using Statistical Package for the SPSS 13.00 software. Kaplan–Meier analyses was used to determine the cumulative incidence of recurrent stones. Continuous data were analyzed using the one-way ANOVA and Student's *t*-test. Categorical data, shown as the number and/or percentage, were analyzed using the chi-square or Fisher's exact tests. A two-sided  $P < .05$  was considered to indicate statistical significance.

## 3. Results

### 3.1. Analysis of urine chemistry in adults with daily consumption of vinegar

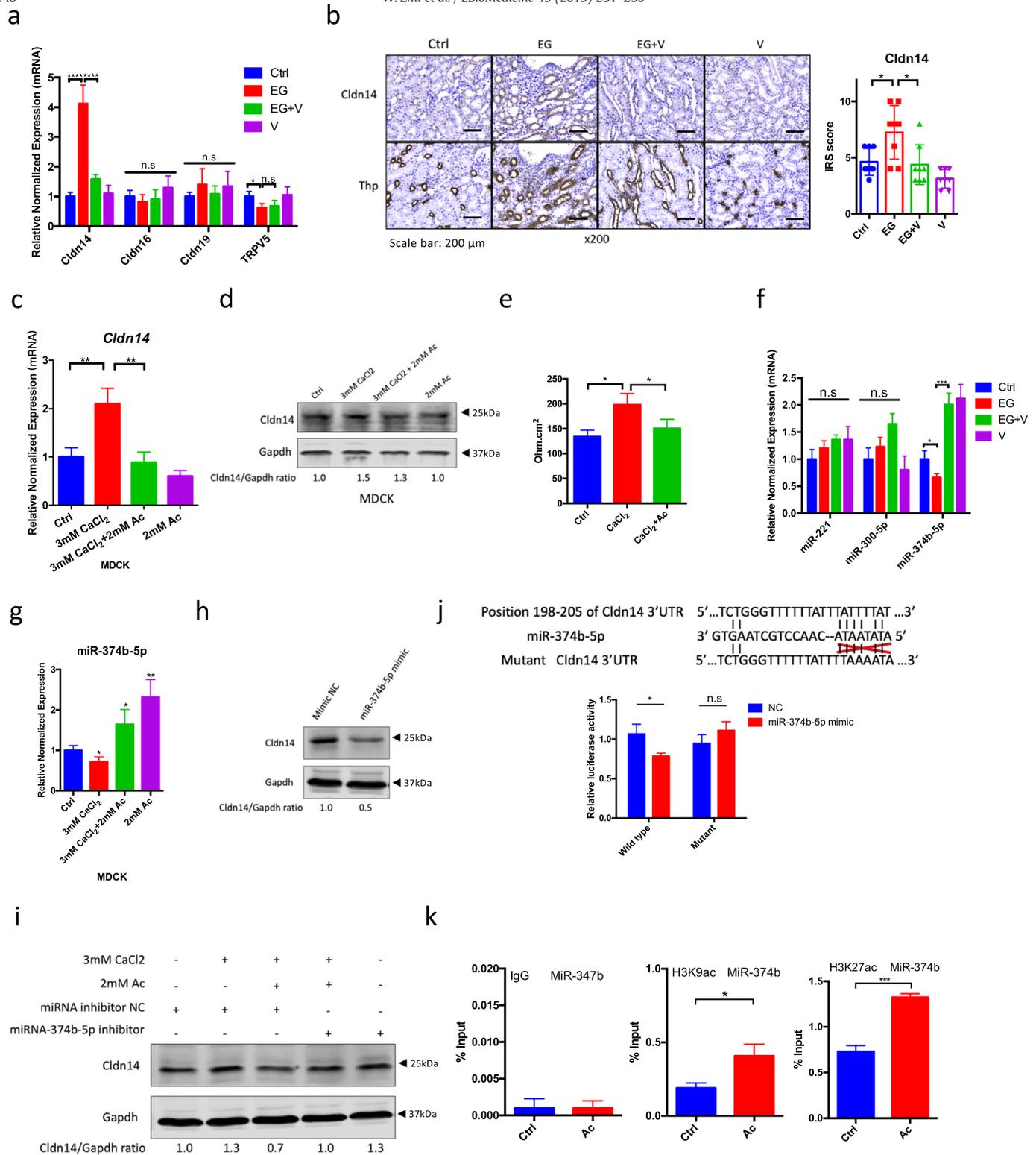
We performed a clustered, stratified, and multi-stage screening for urolithiasis in six different cities of China between 2013 and 2014 [1]. In order to examine the impact of vinegar on the kidney stone formation, we collected urine samples of individuals without nephrolithiasis, gout, hyperparathyroidism, or gastroenteric diseases. After obtaining informed consent, each participant completed an initial questionnaire – a self-reporting system that collected information regarding his or her social and demographic status (e.g. gender and age), personal and family health history, lifestyle, and the use of vitamins and medications [19–22]. The 24-h urine profiles of 61 adults who consume vinegar daily (>15 ml/day) were compared with those of 61 adults who do not using a propensity score-matching analysis. Cases were matched sequentially using the following criteria: gender, age, BMI, history of hypertension and diabetes. The results showed that individuals with daily consumption of vinegar compared to those without have a higher urinary citrate excretion with a higher urine pH, a lower urinary calcium, uric acid, and creatinine excretion, a lower AP(CaOx) index and AP(CaP) index [23–25], well-known indexes representing the risk of CaOx crystallization in urine (Table 1). Taken together, these analyses indicate that dietary vinegar is correlated with urine chemistry, particularly citrate and calcium excretion that might suppress kidney stone formation in adults.

### 3.2. Oral administration of vinegar reduced CaOx crystals formation via increased citrate and reduced calcium in urinary excretion in a rat model

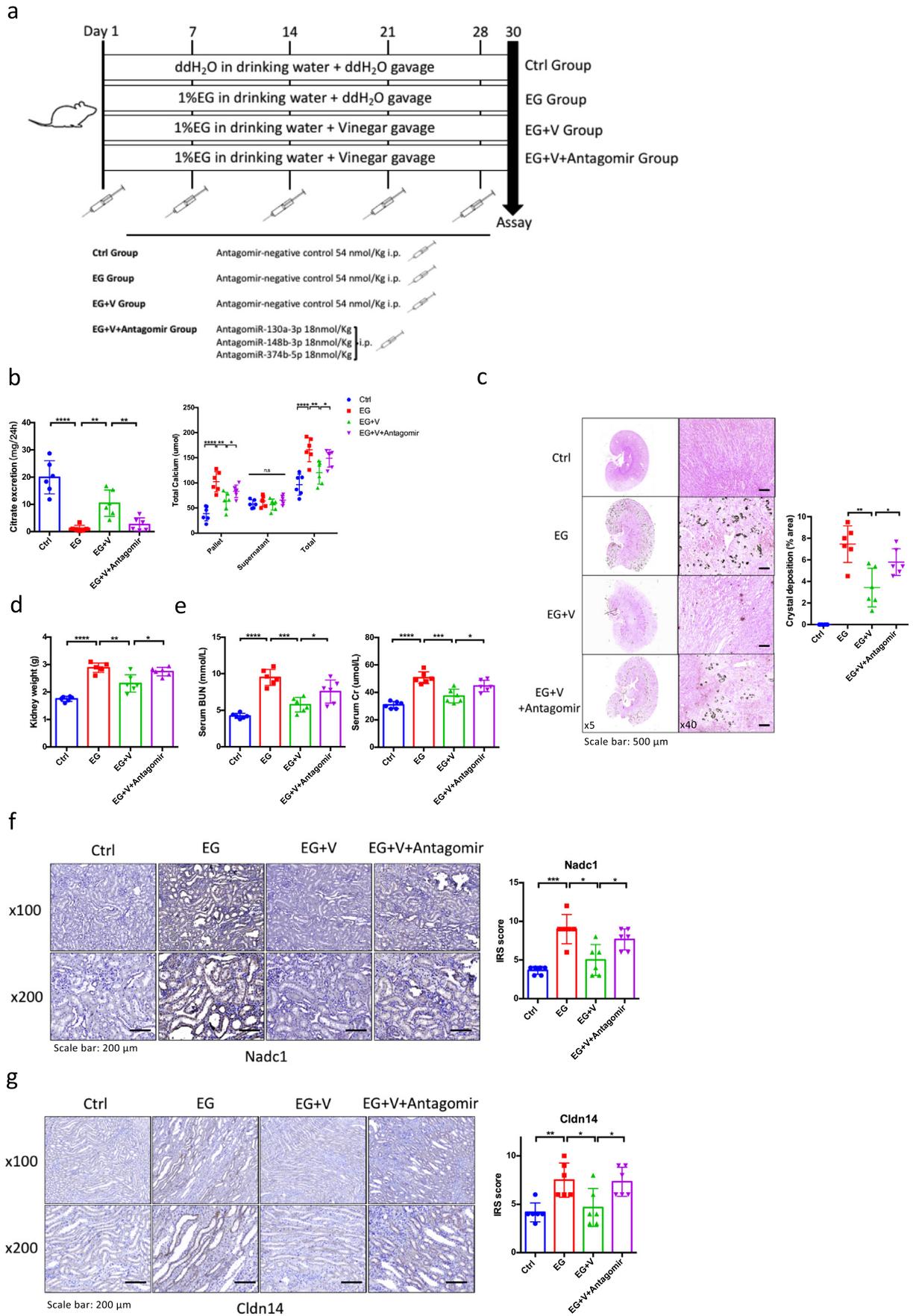
In order to examine the mechanisms responsible for the protection by vinegar against nephrolithiasis in humans, we tested the impact of vinegar on ethylene glycol (EG)-induced CaOx crystal formation in a rat model. This model displays hyperoxaluria, hypercalciuria, and hypocitraturia, conditions promoting CaOx kidney stone formation in humans [26,27]. 8-week-old Sprague-Dawley rats were treated with vinegar (2 ml/kg) or distilled H<sub>2</sub>O every day by gavage. In the meantime, all rats received 1% EG in drinking water to induce urinary abnormalities and CaOx crystals formation. After 4-weeks administration of vinegar, we analyzed 24-h urine compositions and examined CaOx crystal formation in the kidney tissue. We found that administration of vinegar did not influence the urinary oxalate excretion (Fig. 1a). However, administration of vinegar significantly increased urine citrate excretion (Fig. 1b), a well-known inhibitor of CaOx crystallization [28,29], and decreased urine calcium excretion which is a component and a strong promoter of CaOx crystal formation (Fig. 1c) [30,31]. Using Pizzolato staining, we found that there were fewer CaOx crystals in the vinegar plus EG rats than in the EG-treated rats (Fig. 1d).

We further examined the effect of vinegar on histological changes and renal functions in the different groups of rats. By Masson's trichrome staining (MTS) of the kidney, the decrease in the cortical tubular area in the EG group was slightly but significantly recovered in the EG plus vinegar group (Fig. 1e). In contrast, the fibrotic area and interstitial collagen deposition in the damaged kidneys were decreased in the

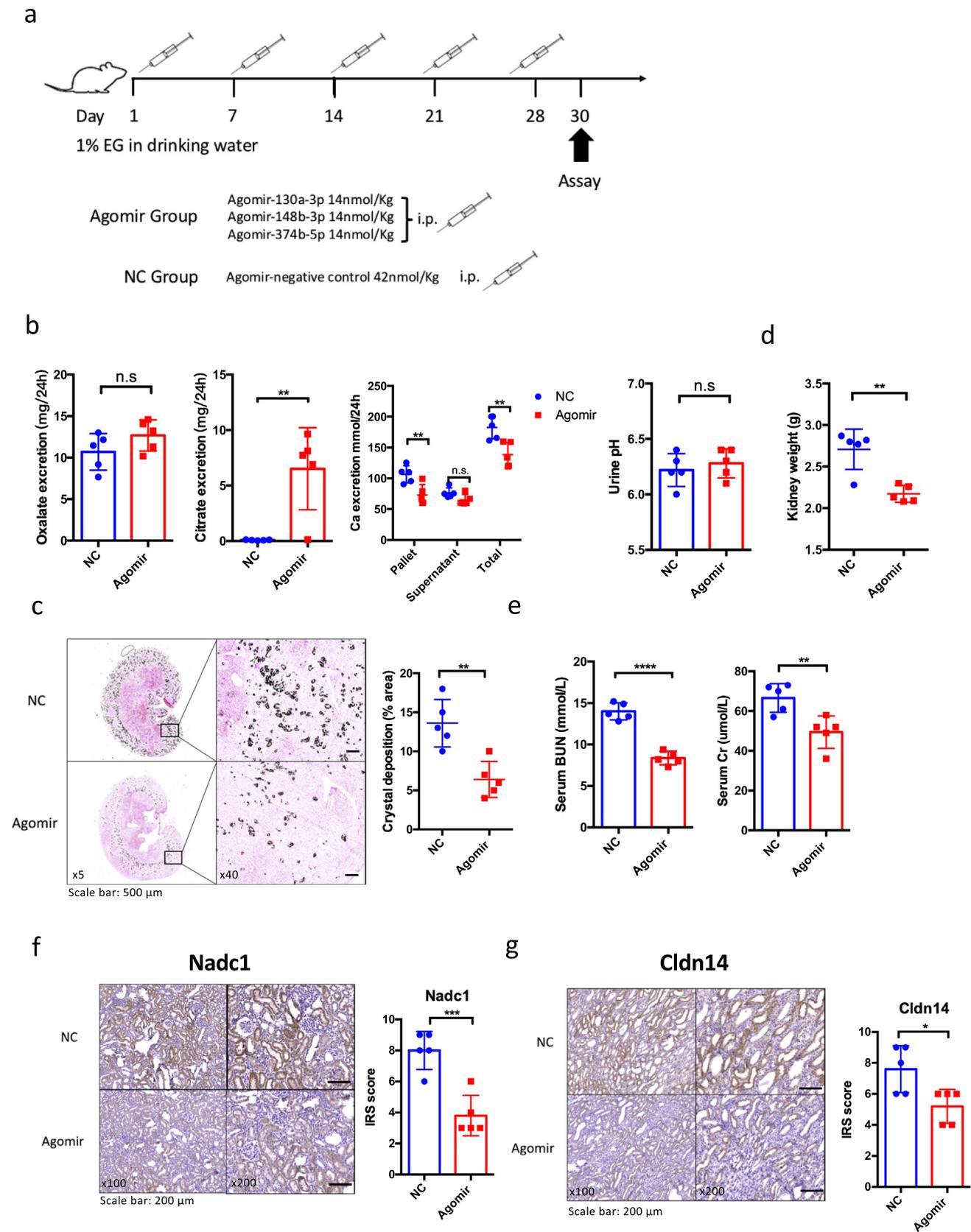
**Fig. 4.** Acetate activated *miR-130a-3p* and *miR-148b-3p* through epigenetic regulation. (a) Acetate rescues hyper-oxalate-reduced H3K9 and H3K27 acetylation levels. HK-2 or NRK-52 cells were treated with or without 2 mM sodium acetate under hyper-oxalate condition (0.5 mM) for 24 h. The histone acetylation levels were determined by Western blot. Total H3 served as a loading control. (b) ChIP-qPCR assays showing histone acetylation enrich at *miR-130a* promoter region in HK-2 cells treated with or without 2 mM sodium acetate under normal or hyper-oxalate condition (0.5 mM) for 24 h. Rabbit IgG was included as a negative control. (c) ChIP-qPCR assays showing histone acetylation enrichment at *miR-148b* promoter region in HK-2 cells treated as in (b). Each histogram was presented as mean  $\pm$  SD of triplicate experiments (ns, no significant, \*\* $P < .01$ , \*\*\*\* $P < .001$ ; by Student's *t*-test). (d) IHC staining of H3K9ac and H3K27ac in kidney tissues from each group rats (quantitation on the right) (amplification x200). (e) H3K9 and H3K27 acetylation levels (normalized against Histone H3) in kidney tissues from each group rats were analyzed by western blot. Two pairs of representative samples were shown. (f) Immunofluorescence co-staining for H3K9ac and tubular markers (Aqp-1 or Thp) in kidney tissues from each group rats (amplification x400). (g) Immunofluorescence costaining for H3K27ac and tubular markers (Aqp-1 or Thp) in kidney tissues from each group rats. Ctrl, control. EG, ethylene glycol. V, vinegar. Ox, oxalate. Ac, sodium acetate. n.s, not significant, \* $P < .05$ , \*\* $P < .01$ , \*\*\* $P < .001$ , \*\*\*\* $P < .0001$  (One-way ANOVA).



**Fig. 5.** Vinegar decreased urinary calcium excretion via epigenetic regulation of miR-374b-5p/Cldn14 signaling. (a) q-PCR analysis of genes expression of calcium transporters in kidney from rats. (b) IHC staining of serial sections for Cldn14 and Thp in kidney tissues from each group rats (quantitation on the right). (c) q-PCR analysis of Cldn14 mRNA expression after 2 mM sodium acetate and/or 3 mM calcium chloride treatment for 24 h in MDCK cells. (d) Western blot analysis of Cldn14 protein expression after 2 mM sodium acetate and/or 3 mM calcium chloride treatment for 24 h in MDCK cells. (e) Cldn14 function was determined by measurement of trans-epithelial electrical resistance (TER) in MDCK cells treated with 1.5 mM calcium chloride and/or 2 mM sodium acetate for 24 h. (f) 3 potential miRNAs candidates were screened by q-PCR assay in kidney tissue from rats. (g) q-PCR analysis of miR-374b-5p expressions after 3 mM calcium chloride and/or 2 mM sodium acetate treatment for 24 h in MDCK cells. (h) MDCK cells were transfected with the miR-374b-5p mimic or a negative control (NC). Cldn14 expression was analyzed 48 h later by Western blot. Gapdh serves as a loading control. (i) MDCK cells were transfected with the miR-374b-5p inhibitor or a negative control (NC). 24 h later cells were treated with 3 mM calcium chloride or/and 2.0 mM sodium acetate. Cldn14 expression was analyzed 24 h later by Western blot. (j) Co-transfection of Cldn14 3'UTR constructs containing wild type or mutant seed regions with miR-374b-5p into HEK-293 cells and luciferase assay was applied to detect the luciferase activity. (k) ChIP-qPCR assays showing histone acetylation enrich at miR-374b promoter region in MDCK cells treated with or without 2 mM sodium acetate for 24 h. Rabbit IgG was included as a negative control. Ctrl, control. EG, ethylene glycol. V, vinegar. Ac, sodium acetate. CaCl2, calcium chloride. Data are given as mean  $\pm$  SD, from 6 to 8 rats in each group. n.s, not significant, \* $P < .05$ , \*\* $P < .01$ , \*\*\* $P < .001$ , \*\*\*\* $P < .0001$  (One-way ANOVA for a, b, c, e, f and g; Student's *t*-test for j and k).



**Fig. 6.** Antagomir treatments attenuated vinegar effects of regulating urinary citrate and calcium and suppressing CaOx crystal formation. (a) A diagram describing the injection schedule for antagomir; EG, ethylene glycol; i.p., intraperitoneal injection. (b) Detection of 24-h citrate (left) and calcium (right) excretion in urine samples of each group rats. (c) Pizzolato staining illustrating CaOx crystal deposition in the kidney and its quantification. (d) Kidney weight in rats. (e) Serum BUN and Cr concentration in rats. (f) IHC staining of Nadc1 in kidney tissues from each group rats (quantitation on the right). (g) IHC staining of Cldn14 in kidney tissues from each group rats (quantitation on the right). Ctrl, control. EG, ethylene glycol. V, vinegar. Data are given as mean  $\pm$  SD, from 6 rats in each group. n.s, no significant, \* $P < .05$ , \*\* $P < .01$ , \*\*\* $P < .001$ , \*\*\*\* $P < .0001$  (One-way ANOVA).



**Fig. 7.** Agomir treatments mimicked vinegar effects in regulating urinary citrate and calcium and suppressing CaOx crystal formation. (a) A diagram describing the injection schedule for antagonomir; EG, ethylene glycol; i.p., intraperitoneal injection. (b) Detection of 24-h oxalate (left), citrate (second from left), calcium (second from right) excretion and pH value (right) in urine samples of each group rats. (c) Pizzolato staining illustrating CaOx crystal deposition in the kidney and its quantification. (d) Kidney weight in rats. (e) Serum BUN and Cr concentration in rats. (f) IHC staining of Nadc1 in kidney tissues from each group rats (quantitation on the right). (g) IHC staining of Cldn14 in kidney tissues from each group rats (quantitation on the right). NC, negative control. Data are given as mean  $\pm$  SD, from 5 rats in each group. n.s., not significant, \* $P < .05$ , \*\* $P < .01$ , \*\*\* $P < .001$ , \*\*\*\* $P < .0001$  (Student's t-test).

**Table 2**

The characteristics of adults who do or do not consume vinegar daily.

| Mean ± SE                           | Control (n = 17) | Vinegar (n = 18) | P value |
|-------------------------------------|------------------|------------------|---------|
| Age (Year)                          | 53.56 ± 1.81     | 48.35 ± 3.62     | 0.200   |
| BMI                                 | 23.89 ± 0.78     | 22.70 ± 1.07     | 0.372   |
| Vinegar consumption duration (year) | 0                | 41.05 ± 3.84     | <0.001  |
| Vinegar consumption per day (ml)    | 0                | 19.65 ± 0.71     | <0.001  |
| Serum creatinine level (μmol/L)     | 84.08 ± 5.10     | 85.12 ± 11.57    | 0.934   |
| Serum calcium level (μmol/L)        | 2.23 ± 0.07      | 2.36 ± 0.05      | 0.155   |

EG plus vinegar group compared with the EG group (Fig. 1e). Levels of BUN and plasma creatinine, as well as the kidney weight, at 4 weeks were used as markers of kidney function and kidney injury. Treatment with EG alone significantly increased all three measures of injury, whereas intervention with vinegar blocked increases in all three measures (level of plasma creatinine in the EG plus vinegar group was not different from those in the control group) (Fig. 1f).

We also evaluated renal inflammation and metabolic functions by quantitative real-time PCR (q-PCR) of genes associated with these processes. The results indicated a significant reduction in the expression of inflammation-related genes (*Opn*, *CD68*, *CD44*, *Il-6*, *Il-1b*, *Tgf-β*) in the EG plus vinegar rats as compared to EG-treated rats (Fig. 1g). Consistent with previous microarray analysis of gene expression in EG-treated rats [32], most of metabolic genes were changed significantly in the EG group compared with the control group, while simultaneous vinegar intake significantly reversed these changes (Supplementary Fig. 1).

As the principal bioactive component of vinegar is acetic acid, we directly tested the effect of 5% acetic acid (2 ml/kg/day) instead of vinegar in the rat model. After 4 weeks EG and acetic acid treatment, similar results were observed that higher urinary citrate, lower urinary calcium, fewer CaOx crystals and better renal functions were found in the EG plus acetic acid rats than in the EG-treated rats (Supplementary Fig. 2a-c).

Together, these data suggest that, consistent with urine chemistry analysis in humans, vinegar/acetic acid could protect against CaOx crystals formation in the rat model via increased urinary citrate excretion and reduced urinary calcium excretion.

### 3.3. Mechanism dissection how vinegar/acetate can increase urinary citrate excretion: via down-regulating the *Nadc1* expression

To dissect the molecular mechanisms how vinegar/acetate can increase urinary citrate excretion, we first examined commonly known renal citrate transporters including *Nadc1*, Organic Acid Transporter (*Oat*) and *Nadc3* [33]. Using q-PCR, we found that the expression of renal citrate transporters in kidney showed no significant difference between EG-treated group and EG plus vinegar group (Fig. 2a). However, using immunohistochemistry (IHC) and immunofluorescence staining (IF) we found that EG treatment in rats significantly increased the expression of *Nadc1* in renal proximal tubules expressing Aquaporin-1 (*Aqp-1*), which promotes tubular reabsorption of the citrate in kidney [34,35], while administration of vinegar suppressed the EG-induced *Nadc1* expression (Fig. 2b and Fig. 2c). Similar results were also observed in EG treatment rat model when we replaced the vinegar with 5% acetic acid (Supplementary Fig. 2d). We also found that EG or vinegar treatment minimally impacted expression of *Oat* and *Nadc3* (Supplementary Fig. 3).

We then confirmed these results in cell lines studies in vitro. Using q-PCR and western blot assays, we found that in human proximal tubule epithelial HK-2 cells and rat proximal tubule epithelial NRK-52 cells, 0.5 mM oxalate significantly increased the expression of NADC1 at protein level (Fig. 2d), but not at the mRNA level (Fig. 2e). Adding 2 mM acetate in both HK-2 and NRK-52 cells suppressed the oxalate-induced expression of NADC1 at protein level (Fig. 2d), but not at the mRNA

level (Fig. 2e). The validity of NADC1 staining was confirmed by siRNA-mediated reduction of NADC1 expression level (Fig. 2f).

Together, results from Fig. 2a-f suggest that vinegar/acetate could increase urinary citrate excretion via suppressing the NADC1 expression in the renal proximal tubular cells.

### 3.4. Mechanism dissection how acetate suppressed *Nadc1* expression: via up-regulating *miR-130a-3p* and *miR-148b-3p* expression

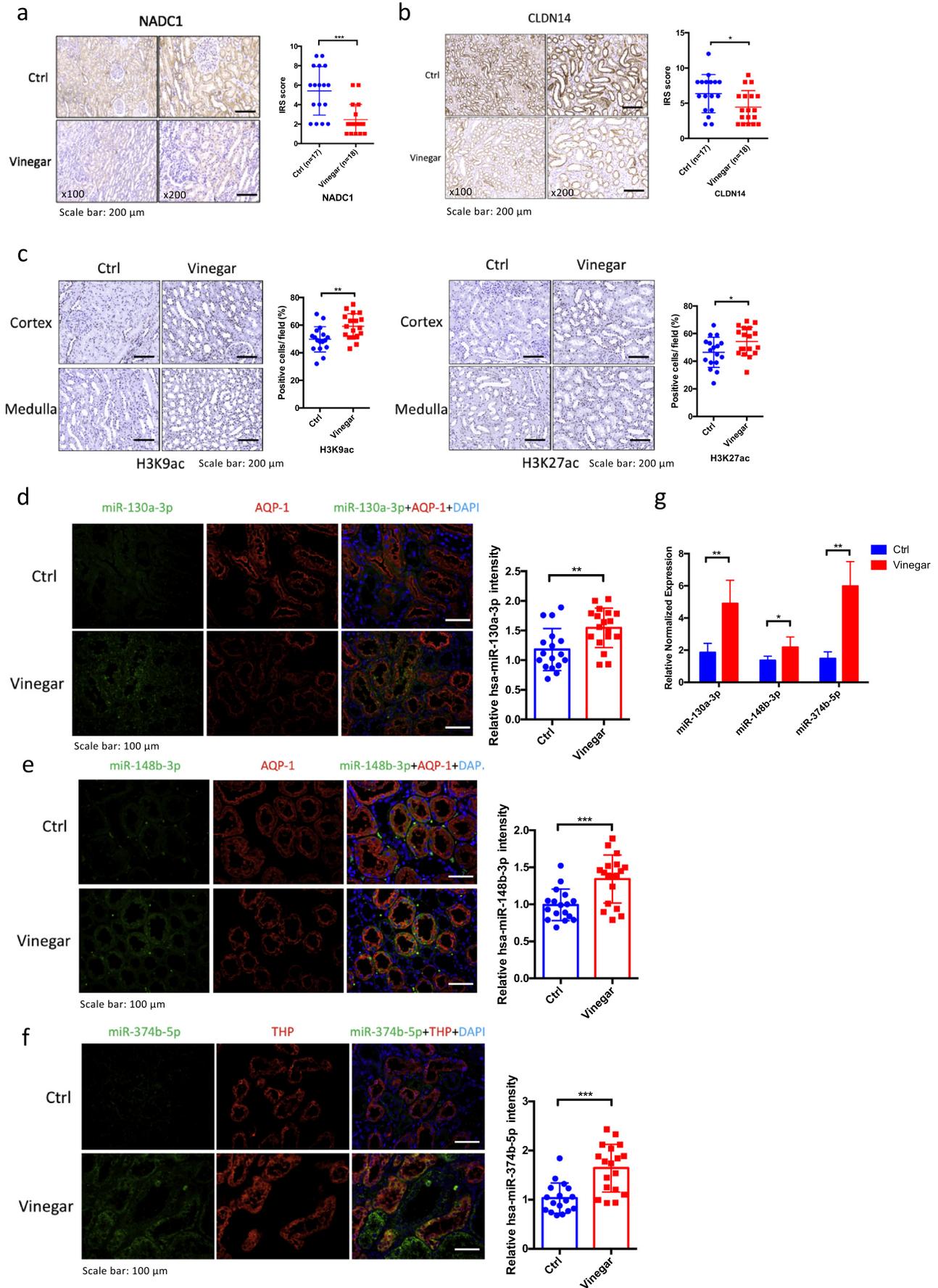
The finding that vinegar/acetate down regulated *Nadc1* expression at the protein level but not at the mRNA level both in the animal model and cultured cells suggested that *Nadc1* expression is regulated at the post-transcriptional level, involving mechanisms such as differential miRNA expression [36]. To directly test this hypothesis, we examined the expression of miRNAs that potentially regulate *Nadc1* based on the search of online databases (DIANA miRGen, MicroCosm Targets, RNA22) and published literature [10,37], and results suggest that *miR-130a-3p* and *miR-148b-3p* were the most likely candidates that were up-regulated by vinegar or acetate in both in vivo (Fig. 3a) and in vitro (Fig. 3b) models. More significantly, direct transfection of the *miR-130a-3p* or *miR-148b-3p* mimetics into HK-2 and NRK-52 cells resulted in suppression of NADC1 expression using western blot assays (Fig. 3c). Conversely, inhibition of *miR-130a-3p* or *miR-148b-3p* by transient transfection with antisense inhibitors led to the reversal of repression of NADC1 expression caused by acetate in HK-2 and NRK-52 (Fig. 3d), strongly suggesting that *miR-130a-3p* and *miR-148b-3p* can mediate the effect of acetate in suppressing the NADC1 expression.

Next, to directly test that acetate-induced *miR-130a-3p* and *miR-148b-3p* could suppress the *Nadc1* expression, we searched and identified the potential binding sites of *miR-130a-3p* and *miR-148b-3p* in the 3'UTR of *Nadc1* mRNA, and generated a luciferase reporter construct bearing the 3'UTR of *Nadc1* gene using a dual-luciferase reporter backbone pmiR-RB-REPORT™ as well as a mutated version at the predicted target sites. The luciferase assay results revealed that *miR-130a-3p* and *miR-148b-3p* could suppress luciferase expression of the wild-type *Nadc1* 3'UTR construct, but not the mutant *Nadc1* 3'UTR construct, thus *miR-130a-3p* and *miR-148b-3p* could directly target *Nadc1* 3'UTR to suppress its expression (Fig. 3e).

Together, results from Fig. 3a-e suggested that acetate can suppress *Nadc1* expression through increasing *miR-130a-3p* and *miR-148b-3p* expression in renal proximal tubular cells.

### 3.5. Acetate promoted *miR-130a-3p* and *miR-148b-3p* expression via restoring histone acetylation

To dissect the molecular mechanisms how acetate promoted expression of *miR-130a-3p* and *miR-148b-3p*, we first focused on histone acetylation since previous studies showed that acetate could function as an epigenetic metabolite to regulate gene expression [38]. We treated HK-2 and NRK-52 cells with acetate under hyper-oxalate condition and found that acetate counteracted the decline of histone acetylation from hyper-oxalate treatment. Of particular interest, acetate induced a significant increase of H3K9 and H3K27 acetylation levels, but not H3K56 acetylation levels (Fig. 4a), indicating that acetate rescued oxalate-reduced histone acetylation with certain specificity. To link the genome-wide histone acetylation change with locus-specific transcription of *miR-130a* and *miR-148b*, we carried out chromatin immunoprecipitation (ChIP)-qPCR assays for the histones located at the presumptive promoters of *miR-130a* and *miR-148b* and found that the acetylation level (H3K27ac) at *miR-130a* promoter (Fig. 4b) and the acetylation level (H3K9ac and H3K27ac) at *miR-148b* promoter (Fig. 4c) were decreased after oxalate treatment, which were derepressed by acetate treatment in HK-2 cells.



Furthermore, using IHC (Fig. 4d), western blot (Fig. 4e) and IF (Fig. 4f–g) assays we found that EG treatment in rats significantly weaken renal H3K9ac and H3K27ac signal, and administration of vinegar reversed this decrease. Similar results were also observed in EG treatment rat model when we replaced the vinegar with 5% acetic acid (Supplementary Fig. 2f–g).

Collectively, these data support the notion that acetate promotes expression of *miR-130a-3p* and *miR-148b-3p* through epigenetic regulations.

### 3.6. Mechanism dissection how vinegar/acetate can decrease urinary calcium excretion: via down-regulating the *Cldn14* expression

To dissect the molecular mechanisms how vinegar/acetate can decrease urinary calcium excretion, we followed similar strategy outlined in the analysis of *Nadcl* expression. Among commonly known renal calcium transporters including *Cldn14*, *Cldn16*, *Cldn19*, and *TRPV5* we found that the renal expression of *Cldn14*, which inhibits calcium absorption in the thick ascending limb (TAL) in response to increased interstitial calcium [39,40], increased at mRNA level in EG group as compared to control group, while vinegar suppressed the EG-induced *Cldn14* expression (Fig. 5a). We therefore used IHC of kidney serial sections to investigate *Cldn14* expression in TAL cells marked by Uromodulin (commonly known as THP) expression. The results showed that *Cldn14* expression was downregulated in TAL cells after vinegar treatment (Fig. 5b). Similar results were also observed in EG treatment rat model when we replaced the vinegar with 5% acetic acid (Supplementary Fig. 2e).

We then confirmed these results in cell lines in vitro. Early studies clearly documented that the *CLDN14* expression was dose-dependent on and linear within the physiological range of extracellular  $\text{Ca}^{2+}$  (0–3 mM), and maximal induction of *CLDN14* protein level was observed with 3 mM  $\text{Ca}^{2+}$  [41]. Therefore, we applied the 3 mM  $\text{Ca}^{2+}$  to induce the *Cldn14* expression in cells in vitro. Using q-PCR and western blot assays, we found that adding 3 mM  $\text{Ca}^{2+}$  in dog distal tubule epithelial MDCK cells significantly increased the expression of *Cldn14* at mRNA and protein level (Fig. 5c–d). Adding 2 mM acetate in MDCK cells suppressed the  $\text{Ca}^{2+}$ -induced expression of *Cldn14*. Importantly, we also tested whether acetate influenced the function of *Cldn14*. We measured the transepithelial resistance (TER) and found that acetate decreased the TER in MDCK cells in culture, consistent with its effect on the expression of *Cldn14* at the protein level (Fig. 5e).

Together, results from Fig. 5a–e suggested that vinegar/acetate decreased urinary calcium excretion via suppressing the *Cldn14* expression.

### 3.7. Mechanism dissection how acetate suppressed *Cldn14* expression: via up-regulating *miR-374b-5p* expression

Next, to dissect the detailed mechanism(s) how acetate can suppress the *Cldn14* expression, we focused on miRNA regulation as previous studies reported that *Cldn14* expression could be regulated by miRNAs [40]. We tested the expression of miRNAs that can target *Cldn14* expression based on the consensus of bioinformatic predictions (DIANA miRGen, MicroCosm Targets, RNA22) as well as published literature [10,37]. Consistent with previous finding on miRNA regulation of

*Cldn14* [40], we found that *miR-374b-5p* was significantly suppressed in kidney from EG-treated rats, while vinegar treatment significantly increased its expression (Fig. 5f). Extending this in vivo finding, we found that *miR-374b-5p* was down-regulated in MDCK cells by  $\text{Ca}^{2+}$  treatments, while was up-regulated by acetate treatment (Fig. 5g).

We then transfected the *miR-374b-5p* mimetics into MDCK cells and found that overexpression of *miR-374b-5p* suppressed the *Cldn14* expression in MDCK cells (Fig. 5h). Importantly, using the interruption assay with antisense inhibitors for *miR-374b-5p*, we found the expressions of *Cldn14* suppressed by acetate in MDCK cells were significantly reversed (Fig. 5i).

Next, to dissect the mechanism how acetate-induced *miR-374b-5p* can suppress the *Cldn14* expression, we searched for the potential binding sites of *miR-374b-5p* located on the 3'UTR of *Cldn14* mRNA, and identified likely miRNA target that matched the seed sequence of *miR-374b-5p* in the 3'UTR of *Cldn14* gene. We then inserted the whole *Cldn14* 3'UTR into a dual-luciferase reporter backbone pmiR-RB-REPORT™ as well as a mutated version at the predicted target site. The luciferase assay results revealed that *miR-374b-5p* could suppress luciferase expression of the wild-type *Cldn14* 3'UTR construct, but not the mutant *Cldn14* 3'UTR construct, suggesting that *miR-374b-5p* could directly target *Cldn14* 3'UTR to decrease its expression (Fig. 5j).

Lastly, we carried out ChIP-qPCR assays to define the underlying mechanism of acetate-induced *miR-374b-5p* expression. The results showed that the acetylation levels (H3K9ac and H3K27ac) at *miR-374b* promoter were up-regulated by acetate supplementation (Fig. 5k).

Collectively, these data support the conclusion that acetate can function through epigenetic regulation of *miR-374b-5p* expression to suppress the *Cldn14* expression.

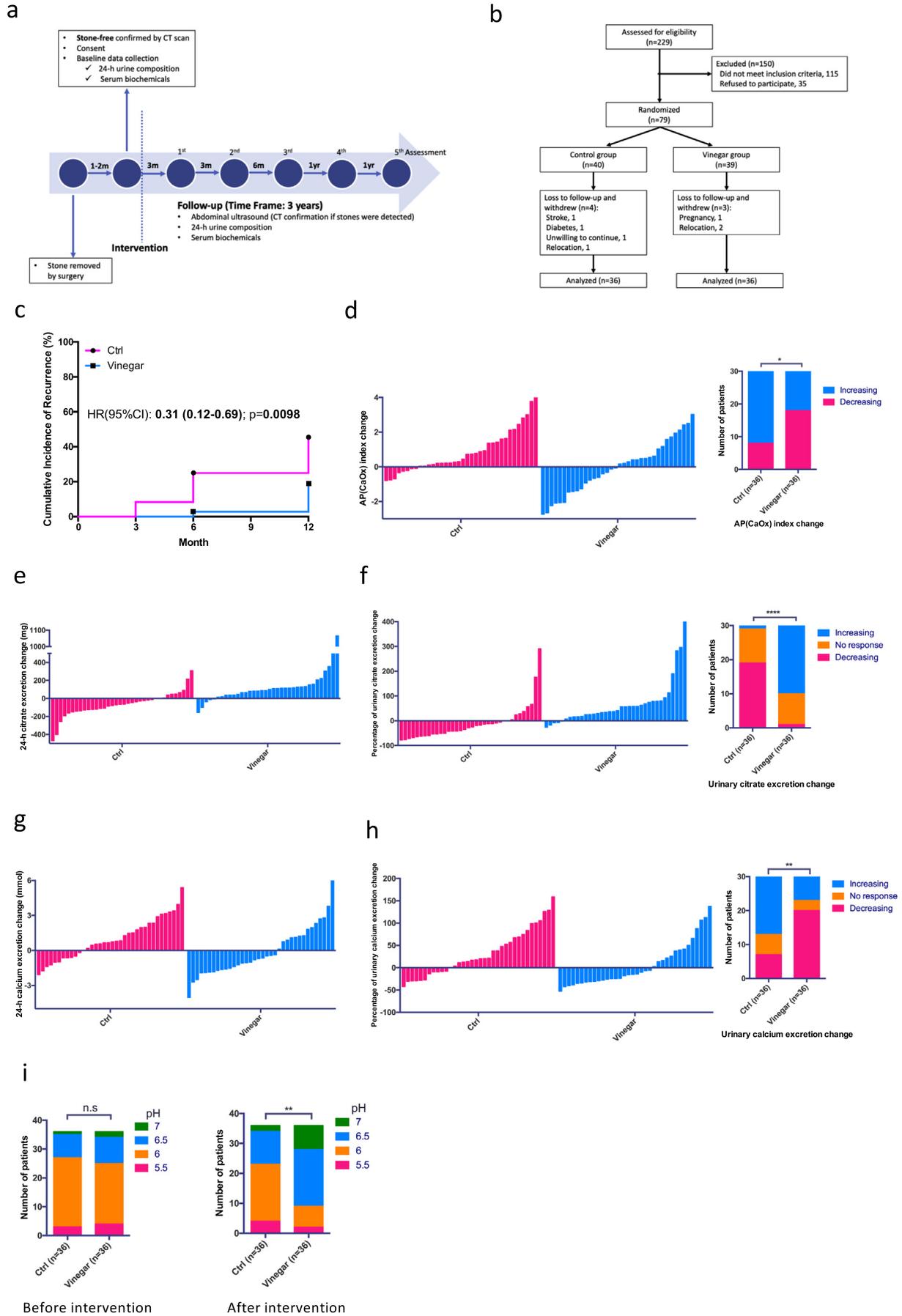
### 3.8. In vivo miRNAs are critical for the vinegar effects in regulating urinary citrate and calcium and suppressing CaOx crystal formation

To directly test whether the acetate-induced miRNAs are mediating the effect of vinegar in regulating urinary citrate and calcium in the animal model, we performed an experiment in which chemically modified antisense oligonucleotides [42,43] specific to *miR-130a-3p* (Antagomir-130a-3p), *miR-148b-3p* (Antagomir-148b-3p), *miR-374b-5p* (Antagomir-374b-5p) were injected intraperitoneally (18 nmol/kg/week) into rats that had received 2 ml/kg/day vinegar by gavage and 1% EG in drinking water. Antagomir-scramble sequence (NC group) rats were used as control (see detail in Fig. 6a). We analyzed 24-h urinary composition in various groups of rats. The results revealed that treatment of antagomirs could partly reverse the vinegar effects in increasing urine citrate excretion and decreasing urine calcium excretion (Fig. 6b). As expected, antagomirs administration could also partly reverse the vinegar effects in suppressing CaOx crystal formation (Fig. 6c) and improvement of renal functions (Fig. 6d–e). Using the IHC, the *Nadcl* and *Cldn14* expressions were higher in kidney of rats with antagomirs treatment compared with NC groups (Fig. 6f–g).

These in vivo results from Fig. 6a–g confirmed that vinegar regulated urinary citrate and calcium, and decreased the CaOx crystals formation at least via up-regulating the expression of *miR-130a-3p*, *miR-148b-3p* and *miR-374b-5p*.

Similarly, we also tested whether these miRNAs were sufficient to mimic vinegar effects in regulating urinary citrate and calcium and

**Fig. 8.** Vinegar consumption positively correlated with histone acetylation and miRNA expressions, and negatively correlated with *NADCl* and *CLDN14* expressions in human kidney tissue. (a) IHC staining of *NADCl* in kidney tissues from 18 individuals who consumed vinegar daily and 17 individuals who did not (quantitation on the right). (b) IHC staining of *CLDN14* in kidney tissues from 18 individuals who consumed vinegar daily and 17 individuals who did not (quantitation on the right). (c) Representative IHC staining results for H3K9/K27 acetylation in kidney tissues from individuals who consumed vinegar daily and individuals who did not (quantitation on the right). (d) Representative MISH combined IF staining results for hsa-miR-130a-3p in proximal tubule (AQP-1 as marker) in kidney tissues from 18 individuals who consumed vinegar daily and 17 individuals who did not (quantitation on the right) (amplification x400). (e) Representative MISH-IF staining for hsa-miR-148b-3p in proximal tubules (AQP-1 positive) in kidney tissues from 18 individuals who consumed vinegar daily and 17 individuals who did not (quantitation on the right) (amplification x400). (f) Representative MISH-IF staining for hsa-miR-374b-5p in TAL (THP positive) in kidney tissues from 18 individuals who consumed vinegar daily and 17 individuals who did not (quantitation on the right) (amplification x400). (g) q-PCR results for miRNA expression (Hsa-miR-130a-3p, -148b-3p, -374b-5p) in kidney tissues from individuals who consumed vinegar daily and individuals who did not. n.s, no significant, \*P < .05, \*\*P < .01, \*\*\*P < .001, \*\*\*\*P < .0001 (Student's t-test).



suppressing CaOx crystal formation in the animal model. To do so, agomir-negative control (NC group) or agomirs containing *miR-130a-3p*, *miR-148b-3p* and *miR-374b-5p* (agomir group) was injected intraperitoneally (14 nmol/kg/week) in rats that received 1% EG in drinking water (see detail in Fig. 7a) for 30 days. We also analyzed 24-h urinary composition in the two groups of rats, and the results revealed that treatment of agomirs did not influence the urinary oxalate excretion and urine pH, while significantly increased urine citrate excretion and decreased urine calcium excretion (Fig. 7b). As shown in Fig. 7c, the renal CaOx crystals formation was significantly reduced in the agomirs-treated rats, compared with the rats with agomir-NC. Moreover, the kidney weight and the level of serum creatinine and BUN also showed that agomirs treatment ameliorated CaOx crystals-induced renal injury (Fig. 7d–e). Using IHC we found that the Nadc1 and Cldn14 expressions were also lower in kidney of rats with agomirs treatment (Fig. 7f–g).

Together, results from Fig. 6–7 showed that *miRNAs-130a-3p*, *-148b-3p* and *-374b-5p* are both necessary and sufficient to mediate the vinegar's effects in regulating urine citrate and calcium, and suppressing CaOx crystal formation in the animal model.

### 3.9. Correlations among vinegar consumption, NADC1, CLDN14, miRNAs expressions and histone acetylation in clinical samples

To test whether the molecular mechanisms characterized in the animal model and tissue culture cells are underlying the vinegar effects in humans, we detected NADC1, CLDN14, and H3 acetylation levels in kidneys from 18 individuals who had daily consumption of vinegar from Shanxi province and 17 individuals who did not elsewhere (Table 2). Human kidney tissue samples were derived from the areas of normal kidney tissues of radical nephrectomy for renal cell carcinoma. The results from IHC staining revealed weaker NADC1 (Fig. 8a) and CLDN14 (Fig. 8b) signals in individuals who consumed vinegar daily than individuals who did not. We also found that H3K9ac and H3K27ac were significantly stronger in individuals who consumed vinegar daily than individuals who did not (Fig. 8c). More importantly, using miRNA in situ hybridization (MISH) and q-PCR techniques, we found that the expression of *miR-130a-3p*, *miR-148b-3p* and *miR-374b-5p* was higher in kidney tissue from individuals with daily dietary consumption of vinegar than that from individuals without (Fig. 8d–g).

Together, results from Fig. 8a–g confirmed that vinegar consumption is associated with a lower expression of NADC1 and CLDN14 in human kidney tissues. These results strongly support a positive correlation between vinegar consumption and *miR-130a-3p*, *miR-148b-3p*, *miR-374b-5p* expression via histone acetylation in human kidney tissues.

### 3.10. Pilot clinical study to evaluate vinegar effect in CaOx stone formers

To directly test the efficacy of vinegar consumption in suppressing kidney stone formation in humans through the mechanisms outlined in the above analysis, we performed a prospective randomized controlled study to test the impact of vinegar on stone recurrence and 24-h urine composition in CaOx kidney stone formers. The study protocol was approved by the Institutional Ethics Committee and was registered on [www.clinicaltrials.gov](http://www.clinicaltrials.gov) (NCT03092908) (Fig. 9a).

From March 2017 to October 2018, a total of 79 patients who had a history of CaOx nephrolithiasis were enrolled and randomized to receive mature vinegar or control (pure water). All patients were stone-

**Table 3**

Baseline characteristics of the pilot study-participants before intervention in both groups.

| Mean ± SD or n(%)              | Control (n = 36) | Vinegar (n = 36) | P value |
|--------------------------------|------------------|------------------|---------|
| Age (year)                     | 45.81 ± 9.89     | 47.36 ± 10.99    | 0.53    |
| Gender (Male)                  | 21 (58.33)       | 24 (66.66)       | 0.63    |
| Family history of urolithiasis | 17 (47.22)       | 16 (44.44)       | 1.00    |
| BMI                            | 23.81 ± 3.18     | 24.73 ± 2.69     | 0.19    |
| Serum biochemical              |                  |                  |         |
| Calcium (mM)                   | 2.34 ± 0.11      | 2.30 ± 0.12      | 0.11    |
| Potassium (mM)                 | 3.95 ± 0.33      | 4.03 ± 0.30      | 0.32    |
| Sodium (mM)                    | 138.7 ± 2.22     | 139.6 ± 2.40     | 0.13    |
| Uric acid (μM)                 | 380.7 ± 112.1    | 402.9 ± 87.61    | 0.35    |
| Creatinine (μM)                | 77.13 ± 20.48    | 81.99 ± 22.29    | 0.18    |
| 24-h urinary excretion         |                  |                  |         |
| Oxalate (mg)                   | 31.86 ± 18.29    | 37.31 ± 22.78    | 0.28    |
| Citrate (mg)                   | 262.2 ± 155.4    | 277.5 ± 190.6    | 0.71    |
| Urine volume (ml)              | 2000 ± 885.6     | 2205 ± 736.8     | 0.29    |
| Chloride (mmol)                | 132.8 ± 59.18    | 135.6 ± 50.11    | 0.83    |
| Potassium (mmol)               | 33.60 ± 10.62    | 37.14 ± 15.72    | 0.27    |
| Sodium (mmol)                  | 142.7 ± 70.04    | 141.1 ± 51.93    | 0.91    |
| Calcium (mmol)                 | 4.13 ± 1.71      | 4.76 ± 1.83      | 0.14    |
| Phosphate (mmol)               | 18.17 ± 17.99    | 17.71 ± 11.08    | 0.90    |
| Magnesium (mmol)               | 3.05 ± 1.00      | 3.11 ± 1.45      | 0.84    |
| Creatinine (mmol)              | 10.86 ± 3.11     | 11.47 ± 3.10     | 0.41    |
| Uric acid (mmol)               | 3.58 ± 1.20      | 3.56 ± 0.99      | 0.92    |

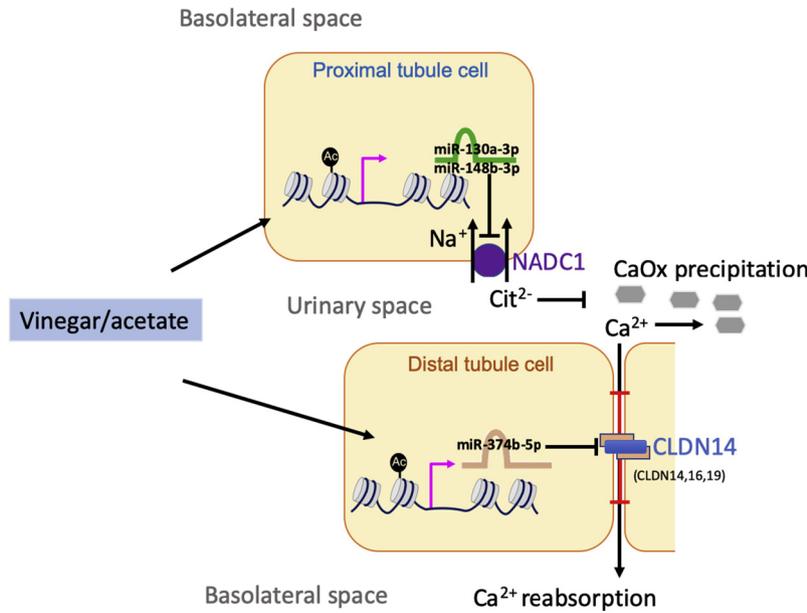
free as confirmed by a CT scan before intervention. The sequence of randomization was computer generated and were performed by the hospital's pharmacy service. Participants administered 5 ml mature vinegar (Ninghuafu®) three times a day (containing 12.5 mEq. acetate per day) or control as liquid of the same type in identical bottles. Seven patients withdrew from the study with no follow-up. Until March 2019, 50 and 22 patients were followed-up for more than one year and 6 months, respectively (Fig. 9b).

The baseline characteristics of the pilot study participants before intervention in both groups are in Table 3. The results showed that the cumulative incidence of recurrent stones by imaging analysis was lower in patients of vinegar group as compared to control group (Fig. 9c). The relative risk of a recurrence among the patients in the vinegar group, as compared with the patients in control groups, was 0.31 (95% confidence interval, 0.12 to 0.69;  $P = .0098$ ). After 6–12 months vinegar consumption, AP(CaOx) index was decreased in 18 (50%) patients of vinegar group while in 8 (22%) patients in control group (Fig. 9d). Meanwhile, urinary citrate excretion was improved in 26 (72%) patients of vinegar group while in 7 (19%) patients in control group (Fig. 9e–f). Also, urinary calcium was reduced in 20 (56%) patients of vinegar group while in 7 (19%) patients in control group (Fig. 9g–h). In addition, vinegar group had higher urine pH than controls (Fig. 9i). These results are consistent with our mechanistic studies both in vitro and in vivo.

## 4. Discussion

Vinegar has been used as a flavoring agent world-wide and is very popular in Asian countries. Shanxi province in northern China is well known for its vinegar production and consumption. Compared to other regions in China, approximately 30–50 times more vinegar is consumed in Shanxi. Our epidemiological survey of nephrolithiasis prevalence raised the possibility that daily intake of vinegar contributes to a

**Fig. 9.** Vinegar effect in CaOx kidney stone patients. (a) Study design overview. (b) Chart showing patient selection and assessment at 6 or 12-month follow-up. (c) Kaplan–Meier estimates of the cumulative incidence of recurrent stones in both groups. HI, hazard ratio. CI, confidence interval. (d) Waterfall plots of change of AP(CaOx) indexes after 6–12 months intervention in both groups. (e) Waterfall plots of actual value change of urinary citrate excretion after 6–12 months intervention in both groups. (f) Waterfall plots of urinary citrate excretion change compared to baseline after 6–12 months intervention in both groups. We defined lesser or equal to 20% change in citrate level from baseline after intervention as no response. (g) Waterfall plots of actual value change of urinary calcium excretion after 6–12 months intervention in both groups. (h) Waterfall plots of urinary calcium excretion compared to baseline after 6–12 months intervention in both groups. We defined lesser or equal to 10% change in calcium level from baseline after intervention as no response. (i) Urine pH change after 6–12 months intervention in both groups. \* $P < .05$ , \*\* $P < .01$ , \*\*\*\* $P < .0001$  (Chi-square test).



**Fig. 10.** A scheme of vinegar effect. The scheme summarizes the pathway described: vinegar or acetate enhances the H3K9 or/and H3K27 acetylation levels at miR-130a, miR-148b, miR-374b promoter regions, which downregulate Nadc1 and Cldn14 expression, and increase urinary citrate excretion while decrease calcium excretion to suppress CaOx crystal formations.

lesser risk of nephrolithiasis likely due to regulation of urinary excretion of citrate and calcium as reported here.

The principal bioactive component of vinegar is acetic acid, which is a short-chain fatty acid (SCFA). SCFAs *in vivo* are principally derived from fermentable nondigestible substrates including acetic, butyric, and propionic acids [44,45]. Acetate has been reported to be readily absorbed in the intestines, transported into the blood stream, and easily incorporated in tissues [46,47]. Several studies reported that treatment with SCFAs, especially acetate, reduced kidney damage in different kidney injury animal models [48]. In this study, we found for the first time that vinegar inhibits CaOx kidney stone formation likely through regulating urinary excretion of citrate and calcium in humans as well as in the animal model. This anti-CaOx stone formation effect of vinegar at least derives from acetic acid in vinegar as acetic acid alone can mimic the effect of vinegar in the rat model in suppressing CaOx crystal formation. In addition, inhibition of miRNAs that were found to be mediating the effect of acetate in tissue cultured cells can block the effect of vinegar *in vivo* while miRNAs themselves can fully mimic the protection of vinegar in the rat model. Thus although vinegar contains many components that might influence its efficacy of suppressing kidney stone, a principal molecule must be the acetic acid itself.

Acetate does so likely through epigenetic regulation of histone acetylation in addition to its potential binding to G-protein membrane receptors (GPR41 and GPR43) [49]. Consistent with a previous finding that acetate can function as an epigenetic metabolite to promote cancer cell survival under hypoxic stress [38], we found that acetate could influence urinary citrate and calcium excretion to inhibit renal CaOx crystals formation. It does so likely through regulating histone acetylation at H3K9 and H3K27 with a consequent activation of transcription of *miR-130a-3p*, *miR-148b-3p* and *miR-374b-5p*, which in turn can suppress the expression of NADC1 and CLDN14, two key regulators of renal citrate and calcium excretion (Fig. 10).

Citrate is a well-known inhibitor of stone formation [50]. It directly inhibits spontaneous nucleation of calcium oxalate by combining with calcium ion to form a nondissociable but soluble complex, leading to less free calcium to bind with oxalate [51]. It also hinders CaOx and calcium phosphate crystal growth, agglomeration, and aggregation [52]. A deficiency of citrate in the renal tubules can lead to nephrolithiasis, as hypocitraturia is involved in 20–60% of calcium nephrolithiasis caused by renal tubular acidosis, carbonic anhydrase inhibitors as well as

genetic deficiencies [53]. NADC1 is a sodium-dependent dicarboxylate cotransporter expressed mainly in the epithelial cells of the kidney proximal tubule and is thought to be involved in the reabsorption of citrate. He et al. [35] reported that increased expression of NADC1 has been correlated to a decline in urinary citrate excretion and the occurrence of nephrolithiasis. Okamoto et al. [54] found that NADC1 gene polymorphism is associated with hypocitraturia in recurrent renal stone formers. Ho et al. [34] generated *Nadc1* knockout mice and found these knockout mice excreted significantly high amount of citrate in their urine without other phenotypic changes. Consistent with these findings, we found that vinegar/acetate could function through epigenetically altering the *miR-130a-3p/miR-148b-3p* expression to suppress NADC1 expression and hypocitraturia, which may provide us a novel therapy via targeting NADC1 with vinegar or acetate to suppress the CaOx nephrolithiasis.

Hypercalciuria is the greatest risk factor for the development of calcium-containing kidney stone and a major cause for disturbed transport of  $\text{Ca}^{2+}$  in kidney. A large genome-wide association study found single nucleotide polymorphisms (SNPs) in human *CLDN14* are strongly associated with kidney stones and lower bone mineral density, suggesting a role for CLDN14 in the pathogenesis of idiopathic hypercalciuria [55]. Sato et al. [39] generated *Cldn14* knockout mice and found that systemic deletion of *Cldn14* completely rescued the hypercalciuria phenotype in *Ksp-cre/Pth1r<sup>fl/fl</sup>* mice, suggesting targeting CLDN14 could provide a potential treatment to hypercalciuria. Gong et al. [40]. reported that *miR-9* and *miR-374* could silence the *Cldn14* gene and abrogated hypercalciuria. Extending these studies, we found that vinegar/acetate could reduce the urinary calcium excretion via stimulating the *miR-374b-5p* gene transcription to suppress the CLDN14 expression, therefore reduced renal CaOx crystal formation.

In conclusion, our findings from clinical, animal models and *in vitro* studies show that vinegar prevents CaOx nephrolithiasis through influencing urinary citrate and calcium excretion via epigenetic regulations. Vinegar consumption is a promising strategy to prevent CaOx nephrolithiasis occurrence and recurrence.

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### Author contributions

Guohua Zeng had full access to all the data in the study and takes responsibility for the data and the accuracy of the data analysis.

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Administrative, technical, or material support: Li Wang, Yan Sun.

Supervision: None.

Other: None.

### Declarations of interests

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

### Ethics approval and consent to participate

The tissue samples used for the study were approved by the ethics committee of the People's Hospital of Changzhi and the First Affiliated Hospital of Guangzhou Medical University. The pilot clinical study protocol has been approved by the ethics committee of the First Affiliated Hospital of Guangzhou Medical University. Informed consent was obtained from all patients. All of the rat experiments were performed under protocols approved by the Institutional Animal Care and Use Committee of the Guangzhou Medical University.

### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ebiom.2019.06.004>.

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