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Functional analysis reveals calcium-sensing receptor gene regulating cell–cell junction in renal tubular epithelial cells

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Abstract

Purpose Calcium-sensing receptor (CASR) influences the expression pattern of multiple genes in renal tubular epithelial cells. The objective of this inquiry was to explore the molecular mechanisms of CASR in renal tubular epithelial cells and nephrolithiasis.

Methods HK-2 cells were transfected with lentiviruses carrying either CASR (named CASR) or an empty vector negative control (named NC), as well as shRNA intended to target CASR (named shCASR) or its corresponding negative control (named shNC). CCK-8 assay was used to detect the effect of CASR on the proliferation of HK-2 cells. RNA-Sequencing was applied to explore potential pathways regulated by CASR in HK-2 cells.

Results PCR and western blot results showed that CASR expression was significantly increased in CASR cells and was decreased in shCASR cells when compared to their corresponding negative control, respectively. CCK-8 assay revealed that CASR inhibited the proliferation of HK-2 cells. RNA-Sequencing results suggested that the shCASR HK-2 cells exhibited a significant up-regulation of 345 genes and a down-regulation of 366 genes. These differentially expressed genes (DEGs) were related to cell apoptosis and cell development. In CASR HK-2 cells, 1103 DEGs primarily functioned in mitochondrial energy metabolism, and amino acid metabolism. With the Venn diagram, 4 DEGs (Clorf116, ENPP3, IL20RB, and CLDN2) were selected as the hub genes regulated by CASR. Enrichment analysis revealed that these hub genes were involved in cell–cell junction, and epithelial cell development.

Conclusions In summary, our investigation has the potential to offer novel perspectives on CASR regulating cell–cell junction in HK-2 cells.

Keywords Calcium-sensing receptor · Human renal tubular epithelial cells · Lentiviral transfection · RNA-Seq · Nephrolithiasis

Introduction

Renal tubular epithelial cells can affect the composition of the urine and, in turn, the formation of kidney stones by reabsorbing or secreting certain substances [1]. Meanwhile, nephrolithiasis can also damage renal tubular epithelial cells when crystals deposit in the renal tubules [2]. Calcium-sensing receptor (CASR), a dimeric G protein-coupled receptor, plays an important role in regulating parathyroid hormone (PTH) release and Ca^{2+} homeostasis [3]. Empirical evidence highlights that CASR finds high expression in parathyroid glands, kidneys, intestines, and bones [4]. Maintaining and regulating the body's calcium homeostasis is the most important physiological function of CASR. When stimulated by high serum Ca^{2+} concentrations, CASR inhibits the production of cAMP

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and the action of PTH, further reducing Ca^{2+} reabsorption, promoting the precipitation of calcium phosphate, and increasing citrate excretion [5]. In addition, CASR increases water and proton excretion in the collecting duct [6]. In contrast, when extracellular calcium levels are low, CASR reduces Ca^{2+} excretion by the kidneys, promoting its reabsorption back into the bloodstream.

Previous studies have demonstrated that the critical mechanism of forming calcium-containing stones is CASR activation in the cell surface, leading to a reduction in the abundance of aquaporin-2 in the membrane, which limits water conservation and provides protection against the formation of stones [3]. However, several studies have assessed the role of CASR in nephrolithiasis with mixed conclusions. Li and others found that CASR could promote calcium oxalate crystal adhesion and renal injury in rats through reactive oxygen species (ROS) generation [7]. In contrast, Ibeh et al. found that CASR and TRPC3 existed in the proximal tubule and can mediate transcellular Ca^{2+} transport, mitigate formation of the calcium crystals and subsequent formation of stones [8]. Subsequent research revealed that genetic variants of CASR may be associated with the onset of nephrolithiasis in patient-based studies and some data indicate that minor alleles at the associated polymorphism leads to a loss of CASR expression in the kidney [5, 6, 9–11]. Although these studies have indicated that CASR is a candidate gene to explain the individual predisposition to nephrolithiasis, the special molecular mechanisms of CASR in renal tubular epithelial cells and nephrolithiasis remain unknown.

The present study aimed to investigate the mechanisms of CASR in renal tubular epithelial cells and in the development of nephrolithiasis through RNA-Sequencing. To achieve this, lentiviral transduction was used to either up-regulate or down-regulate CASR expression levels in HK-2 cells, and a bioinformatics analysis of gene expression profiling was undertaken after CASR lentiviral transduction. Our results have shed light on a new avenue of exploration and comprehension in the study of nephrolithiasis.

Materials and methods

Cell culture

Human renal proximal tubular epithelial cell line, HK-2 and HKC-8 cells were purchased from the Chinese Academy of Sciences (Shanghai, China). HK-2 and HKC-8 cells were cultured in DMEM/F12 medium (Servicebio, Wuhan, China) supplemented with 10% fetal bovine serum (FBS, VivaCell, Shanghai, China) and 1% penicillin/streptomycin at 37 °C in a humidified 5% CO_2 atmosphere.

Lentiviral transduction

The lentivirus construction of CASR knockdown or overexpress was obtained from HANBIO (Shanghai, China). HK-2 and HKC-8 cells were plated in 6 wells dishes at 50% confluence and infected with CASR overexpression lentivirus (termed as CASR), a negative control (termed as NC), CASR knockdown lentivirus (termed as shCASR), or scramble control (termed as shNC), respectively.

Real-time quantitative PCR (RT-qPCR), western blot, and cell proliferation assay

The efficiencies of each transfection reaction were evaluated using RT-qPCR and western blot assays. Total RNA was extracted using the RNA simple Total Kit following the protocol (Vazyme, Nanjing, China) and then reverse-transcribed into cDNA. The RT-qPCR was performed using the SYBR Green PCR system (Vazyme, Nanjing, China) with the following primer: CASR forward, 5′-CCAACTTGACGCTGG GATACA-3′; CASR reverse, 5′-CAGCAATCGTAGAGG GAATGTG-3′.

Proteins derived from whole cell lysate were resolved in 8% SDS–PAGE gel and then transferred onto a polyvinylidene difluoride (PVDF) membrane. Non-specific bindings were blocked with 5% skim milk in TBST at RT for 2 h. The membrane was incubated with rabbit polyclonal anti-CASR antibody (1:1000, ab137408, Abcam, USA) at 4 °C overnight. After being incubated with a secondary antibody and washed with TBST, the images were detected with a gel imaging system.

HK-2 and HKC-8 cells with different transfection of were established in 96-well plates, respectively. Cell proliferation was monitored using the Cell Counting Kit-8 (CCK-8) at 37 °C for 1 h with 450 nm absorbance on days 1, 2, 3 and 4.

RNA-Sequencing (RNA-Seq)

TRIzol reagent was utilized to extract total HK-2 cells RNA. The removal of ribosomal RNA (rRNA) was achieved through the use of the RiboGone™ rRNA Removal Kit. The purity and quantity of RNA were assessed through the employment of Nanodrop equipment and SDS gel electrophoresis. To prepare libraries, NEBNext® Ultra™ DNA Library, Prep Kit for Illumina® from NEB in the USA was utilized. The sequencing libraries were generated through random fragmentation of the cDNA sample, which was then followed by ligation of 5′ and 3′ adapters. To produce clusters, the library was loaded onto a flow cell, where the fragments were captured on a surface-bound oligos lawn that

corresponded to the library adapters. Once cluster generation was complete, the libraries were prepared for sequencing via Illumina HiSeq 4000. The RNA-Seq analysis was performed by Bioprofifile (Shanghai, China).

Data preprocessing and screening of differentially expressed genes (DEGs)

The raw data underwent normalization through the quantile algorithm function of the Agilent Gene Spring Software 11.0. The DEGs were selected based on the cutoff criteria of $P < 0.05$ and | fold-change |> 1.5.

Protein–protein interaction (PPI) network and enrichment analysis

The Gene Multiple Association Network Integration Algorithm (GeneMANIA, http://www.genemania.org/) was used to reveal physical and genetic protein–protein interactions. Moreover, the significant Gene Ontology (GO) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis were conducted for the genes in the PPI network using clusterProfiler," and "enrichplot" R packages with a corrected P value < 0.05. The gseKEGG function in the "clusterProfiler" R package was used to perform Gene Set Enrichment Analysis (GSEA) of the KEGG pathway in terms of normalized enrichment scores (NES) with false discovery rate (FDR) < 0.25.

GSE117518 analysis

The data set GSE117518, titled "Analysis of Long Noncoding RNA and mRNA Expression Profiles in Randall's Plaques of CaOx Stone Patients," was acquired from the Gene Expression Omnibus (GEO) database. This data set utilized microarray analysis to evaluate mRNA expression levels within three pairs of Randall's plaque (RP) tissues from patients with CaOx renal stones and normal renal papillary tissues. RP tissues from patients with CaOx renal stones were collected from individuals diagnosed with idiopathic CaOx renal stones who underwent percutaneous nephrolithotomy. Normal renal papillary tissues were sourced from patients with renal tumors who underwent nephrectomy without any tumor invasion. The analysis revealed 448 significantly differentially expressed mRNAs in RP tissues compared to normal papillary tissues ($P < 0.05$ using the Wilcox test), among which included CASR and its associated hub genes.

Statistical analysis

The statistical analysis was conducted using R software (version 4.1.1) and GraphPad Prism (version 8.0). The Student's *t* test was employed to compare two independent test series, while the ANOVA test was utilized to compare more than two test series.

Results

Expression levels of CASR

CASR expression in HK-2 after different transfection conditions was measured by RT-qPCR (Fig. 1A) and western blot (Fig. 1B). RT-qPCR and western blot analyses demonstrated a marked reduction in the expression level of CASR in shCASR cells. Conversely, the expression level of CASR was higher in CASR cells compared to NC cells. CCK-8 assay revealed that blocking CASR significantly promoted the proliferation of HK-2 cells, while elevated expression of CASR inhibited proliferation of HK-2 cells (Fig. 1C).

Identification of DEGs and functional enrichment analysis

In shCASR cells, the hierarchical clustering analysis of DEGs is shown in Fig. 2A. Compared with the shNC cells, 345 genes were significantly higher and 366 genes were lower in the shCASR cells (Fig. 2B). GO analysis demonstrated that the top 20 significant functions of DEGs in shCASR cells were identified, including cellular developmental process, regulation of cell population proliferation regulation of T-cell apoptotic process, negative regulation of leukocyte apoptotic process, negative regulation of lymphocyte apoptotic process, response to oxygen-containing compound, cellular response to chemical stimulus, response to lipid, and so on (Fig. 2C), a large proportion of which relate to cell apoptosis and cell development.

In CASR cells, the heatmap showed the expression of significant DEGs (Fig. 3A). Compared with the NC cells, 485 genes were down-regulated in the CASR HK-2 cells and 618 genes up-regulated (Fig. 3B). In Fig. 3C, GO analysis revealed that the top 20 significantly enriched GO terms mainly focused on glycometabolism, mitochondrial energy metabolism, and amino acid metabolism.

Pathway enrichment analysis

In shCASR cells, the significantly enriched pathways of the DEGs analyzed by KEGG were shown in Fig. 4A. The DEGs were primarily enriched in the classical intracellular signaling transduction pathways (TNF signaling pathway, FoxO signaling pathway, TGF-beta-signaling pathway, JAK–STAT signaling pathway, and NOD-like receptor signaling pathway), and metabolism disorders (primary bile acid

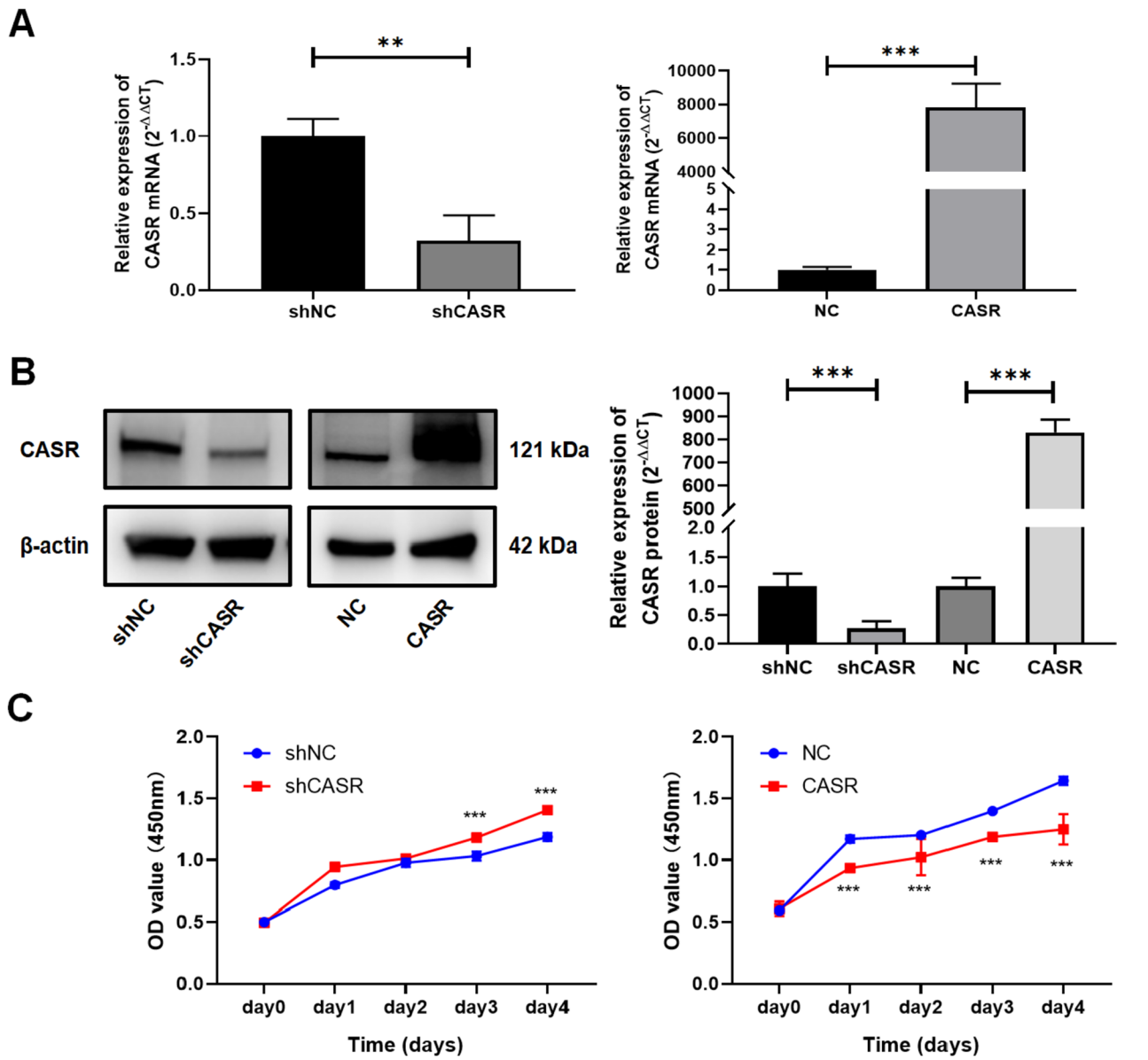

Fig.1 CASR expression in shNC, shCASR, NC, and CASR HK-2 cells after lentiviral transfection. **A, B** RT-qPCR (**A**) and western blot (**B**) assays validated CASR expression in HK-2 cells with different lentiviral transfection. **C** CCK-8 assay reveals blocking CASR significantly promoted the proliferation of HK-2 cells, while elevated expression of CASR inhibited proliferation of HK-2 cells

biosynthesis, pyrimidine metabolism, and steroid hormone biosynthesis).

In CASR cells, KEGG pathways of DEGs were displayed in Fig. 4B. The enriched pathways and diseases were diverse, involving ferroptosis, focal adhesion, the classical intracellular signaling transduction pathways (MAPK signaling pathway, Rap1-signaling pathway, and PI3K–Akt signaling pathway), and metabolism diseases (glycine, serine and threonine metabolism, diabetic cardiomyopathy, purine metabolism, non-alcoholic fatty liver disease, and parathyroid hormone synthesis, secretion and action). For example, 7 DEGs (SAT1, ACSL4, MAP1LC3B2, ACSL1, ATG7, TP53, and NCOA4) participated in ferroptosis. Ferroptosis is a novel type of regulatory cell death based on iron-dependent lipid peroxidation [12]. Studies have confirmed that ferroptosis is positively correlated with the severity of renal tubular epithelial cell injury caused by calcium oxalate crystals [13, 14].

GSEA analysis of CASR

GSEA results further showed that shCASR was positively correlated with AMPK SIGNALING PATHWAY, and ARGININE AND PROLINE METABOLISM, while was

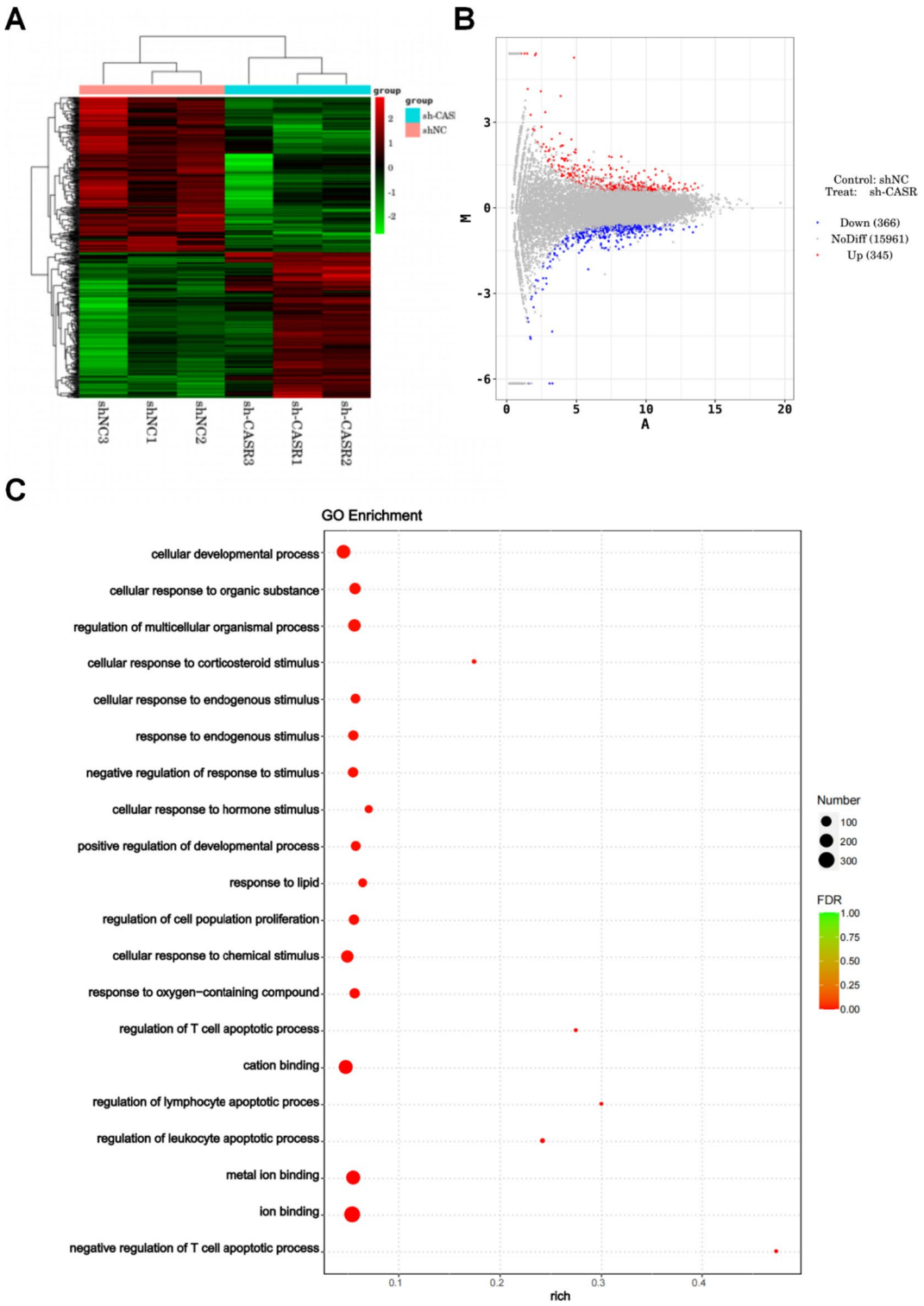

Fig. 2 Significant differentially expressed genes (DEGs) and enriched functions for DEGs in shCASR cells. **A, B** Heat map (**A**) and volcano map (**B**) analysis of DEGs in shCASR cells. **C** Enriched functions for DEGs in shCASR cells

negatively correlated with MATURITY ONSET DIABETES OF THE YOUNG (Fig. 5A). As shown in Fig. 5B, CASR was positively correlated with COLLECTING DUCT ACID SECRETION, and TH17 CELL DIFFERENTIATION, while was negatively correlated with DNA REPLICATION.

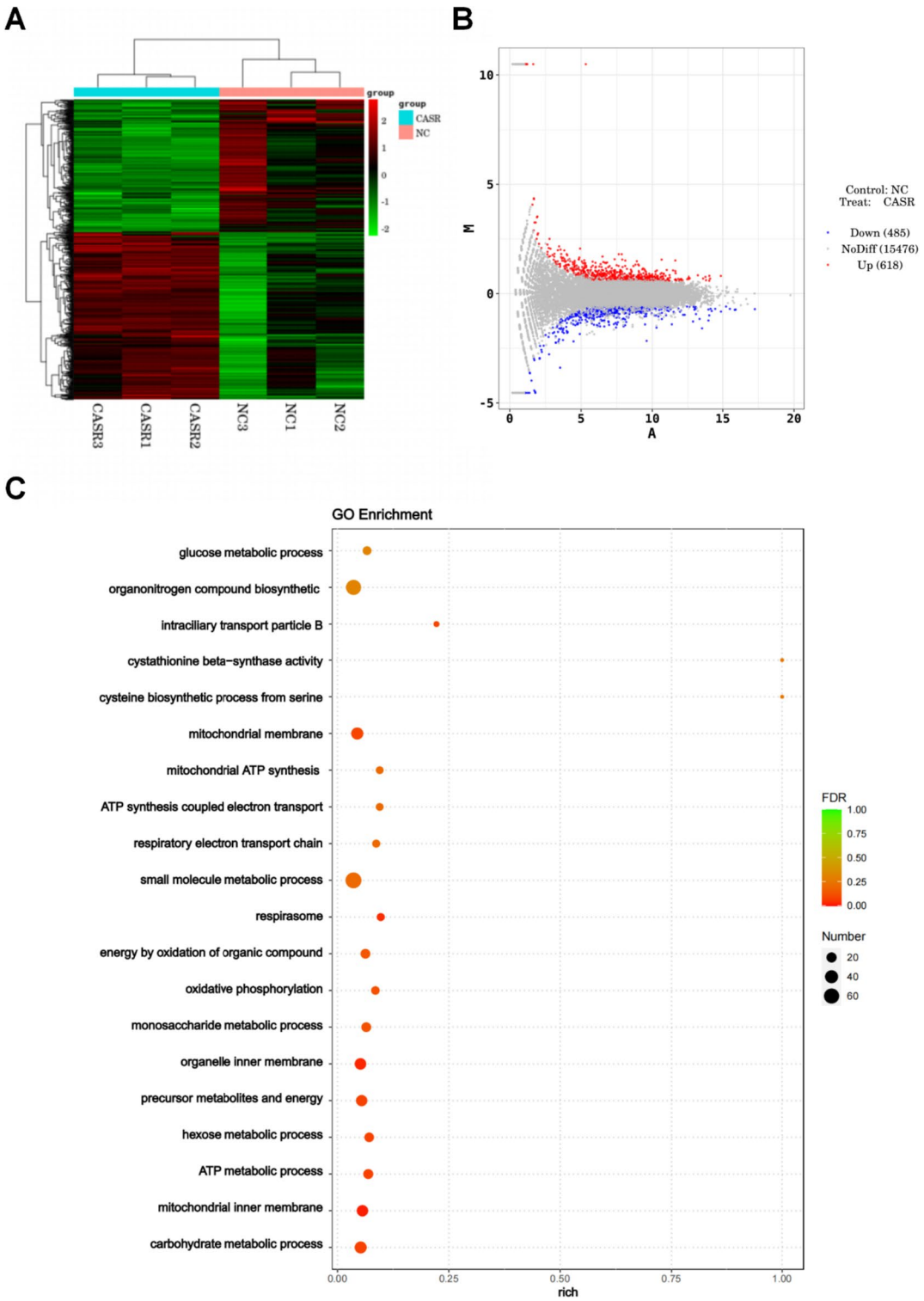

Fig. 3 Significant differentially expressed genes (DEGs) and enriched functions for DEGs in CASR cells. **A, B** Heat map (**A**) and volcano map (**B**) analysis of DEGs in CASR cells. **C** Enriched functions for DEGs in CASR cells

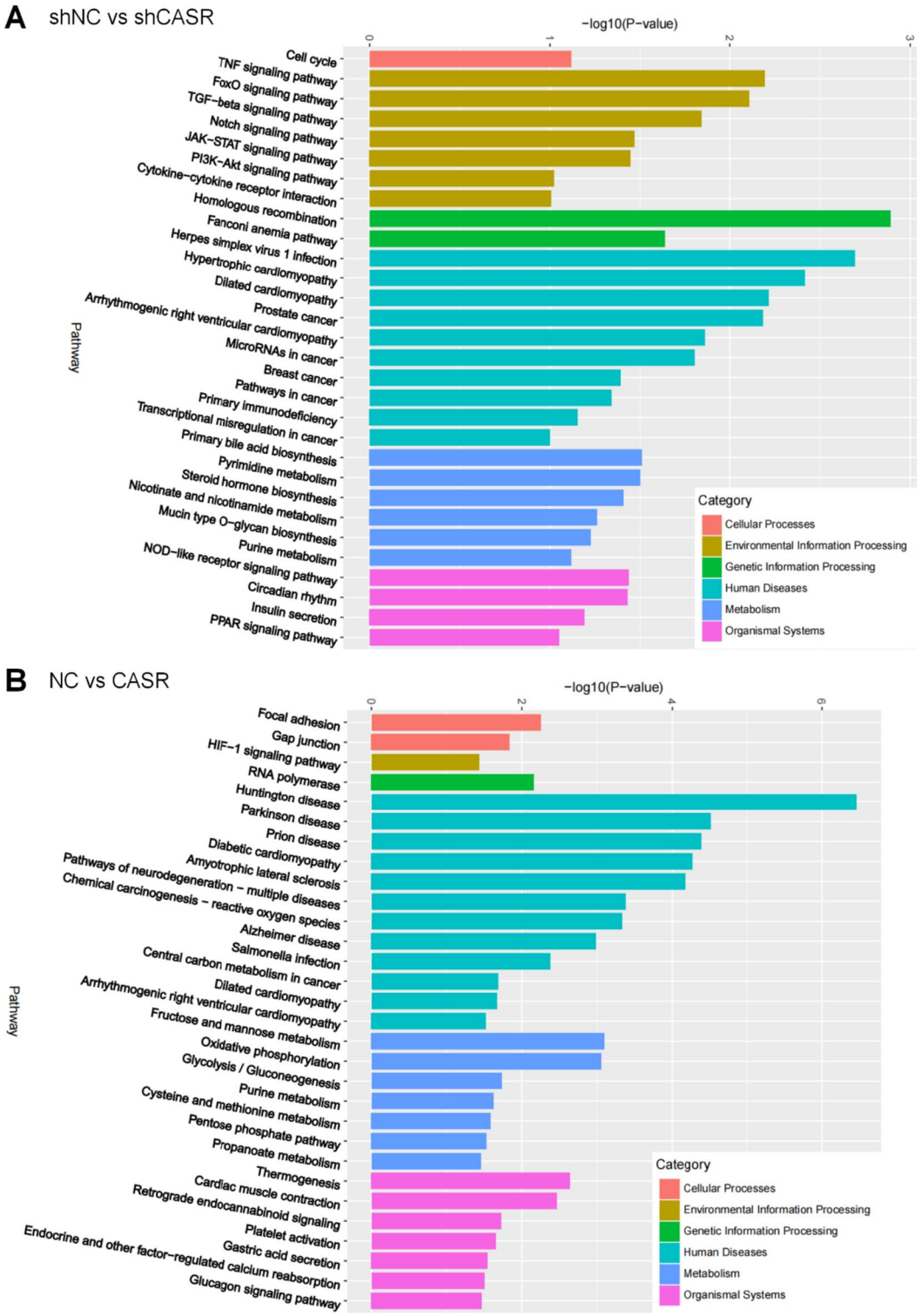

Fig. 4 KEGG pathway analysis of the DEGs. **A, B** KEGG pathway analysis of the DEGs in shCASR (**A**) and in CASR (**B**) HK-2 cells

Identification of hub genes and PPI interaction network construction

Compared with the control cells, 3 genes (ENPP3, IL20RB, and CLDN2) were significantly down-regulated in the CASR HK-2 cells and higher in the shCASR HK-2 cells, respectively (Fig. 6A). Meanwhile, Clorf116 was significantly down-regulated in the shCASR HK-2 cells while

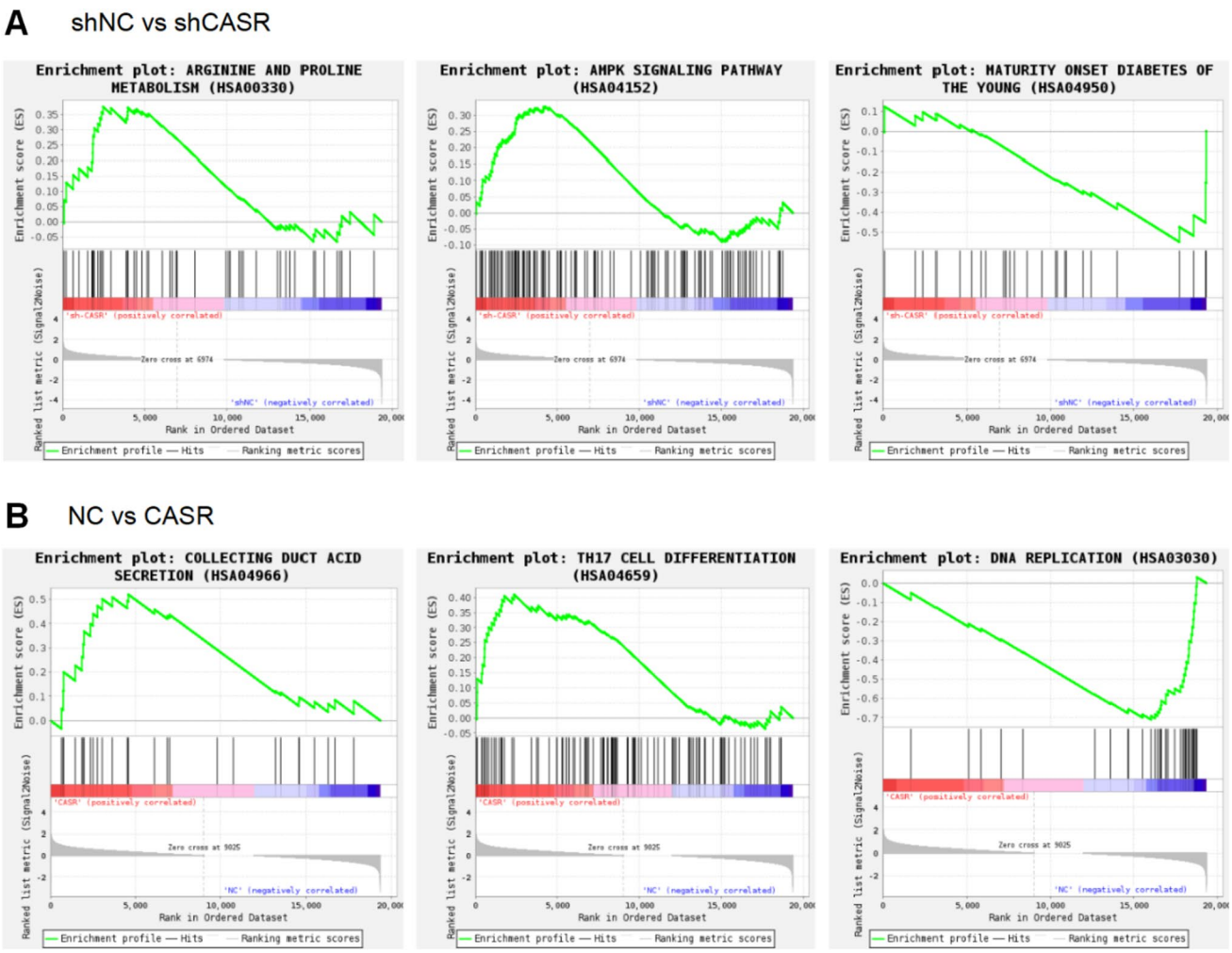

Fig. 5 GSEA enrichment analysis. **A**, **B** Results of the GSEA reporting the significant Kyoto Encyclopedia of Genes and Genomes pathways correlated with shCASR (**A**) and CASR (**B**). (threshold values, NES > 1, NES < − 1 and FDR < 0.25)

up-regulated in the CASR HK-2 cells (Fig. 6B). Hence, a total of 4 genes (C1orf116, ENPP3, IL20RB, and CLDN2) regulated by CASR were identified as the hub genes. Next, we validated the hub genes in HK-2 cells by RT-qPCR, which is consistent with the results of RNA-Seq (Fig. 6C). The constructed PPI network for these hub genes was shown in Fig. 6D. Function data showed that the hub genes may play a role in tight junction, cell–cell junction, cell–cell junction organization, epithelial cell development, and regulation of metal ion transport. Similarly, GO and KEGG analysis revealed the function and pathways of these hub genes (Fig. 6E). In terms of biological process (BP), the hub genes were involved in calcium-independent cell–cell adhesion via plasma membrane cell-adhesion molecules. Regarding cellular components (CC), and molecular functions (MF), tight junction and cytokine receptor activity were enrichment. KEGG results showed that the hub genes mainly regulated nucleotide metabolism, and pyrimidine metabolism.

Validation of hub genes in HKC-8 cells

The in vitro assays were repeated in HKC-8 cell line. CASR expression in HKC-8 after different transfection conditions were measured by RT-qPCR (Fig. 7A) and western blot (Fig. 7B). RT-qPCR and western blot analyses demonstrated a marked reduction in the expression level of CASR in shCASR cells. Conversely, the expression level of CASR was higher in CASR cells compared to NC cells. CCK-8 assay revealed that blocking CASR significantly promoted the proliferation of HKC-8 cells, while elevated expression of CASR inhibited proliferation of HKC-8 cells (Fig. 7C). Next, we validated the hub genes in HKC-8 cells by RT-qPCR, which is consistent with the results in HK-2 cells (Fig. 7D). Finally, the expression of the core genes was validated in human data sets of nephrolithiasis, GSE117518 data set (Fig. 7E). The GSE117518 contains three samples from normal renal

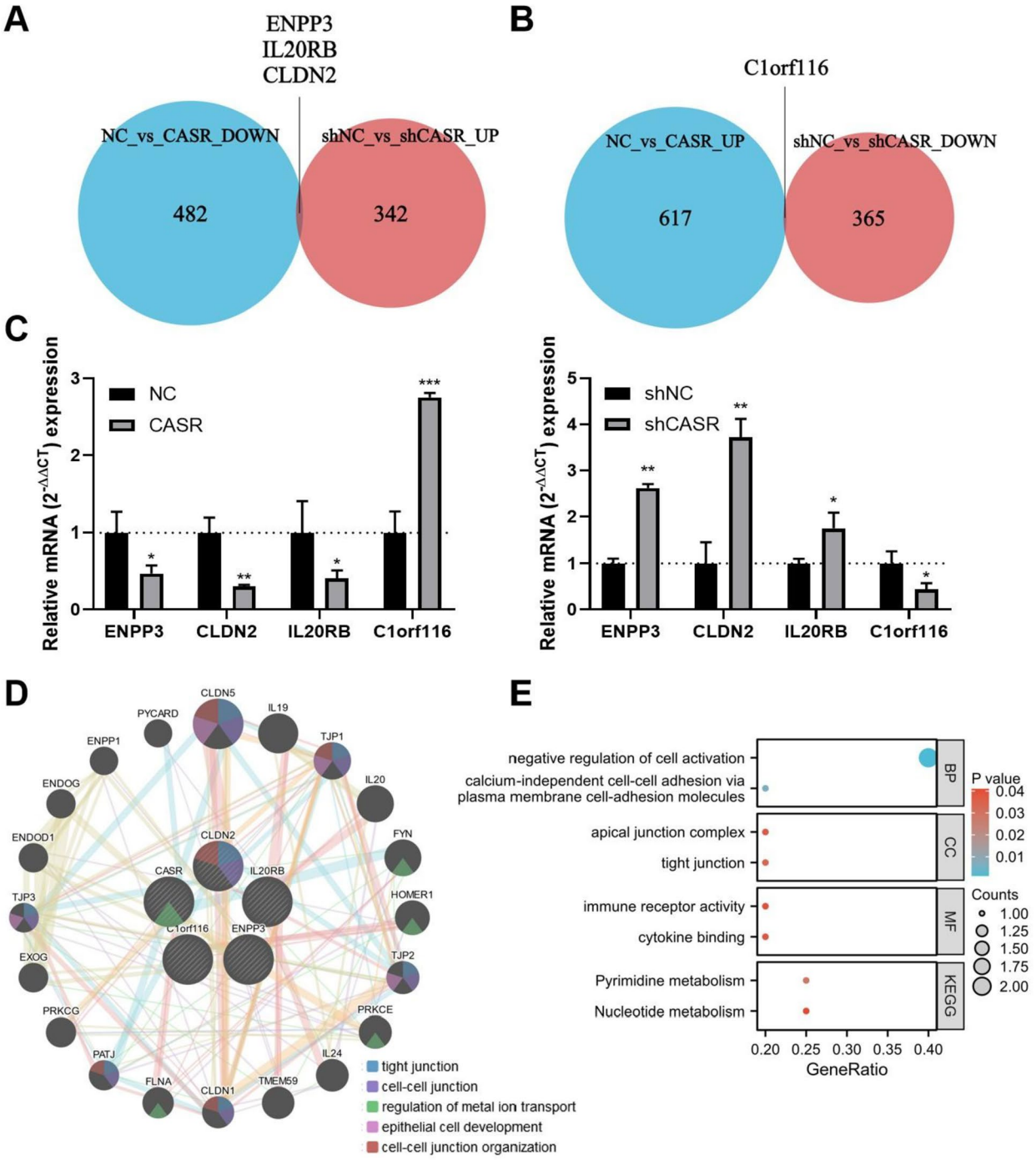

Fig. 6 Screened hub genes and the constructed protein–protein interaction (PPI) network. **A** Hub genes lower in the CASR group and higher in the shCASR group. **B** Hub genes expression in the shCASR group and in the CASR group. **C** Validation of the hub genes in HK-2 cells by RT-qPCR. **D** PPI network for the hub genes based on GeneMANIA. **E** GO and KEGG function analysis of the hub genes

papillary tissues (named Normal), and three samples from Randall's plaques tissues of calcium oxalate (CaOx) renal stones patients (named Nephrolithiasis). CASR expression is increased in human papilla from CaOx renal stones patients. The expression trends of Clorf116 and CLDN2 are consistent with the results of RT-PCR. The inconsistent expression trends of ENPP3 and IL20RB may be due to the small sample size and the limited diversity in stone types (CaOx).

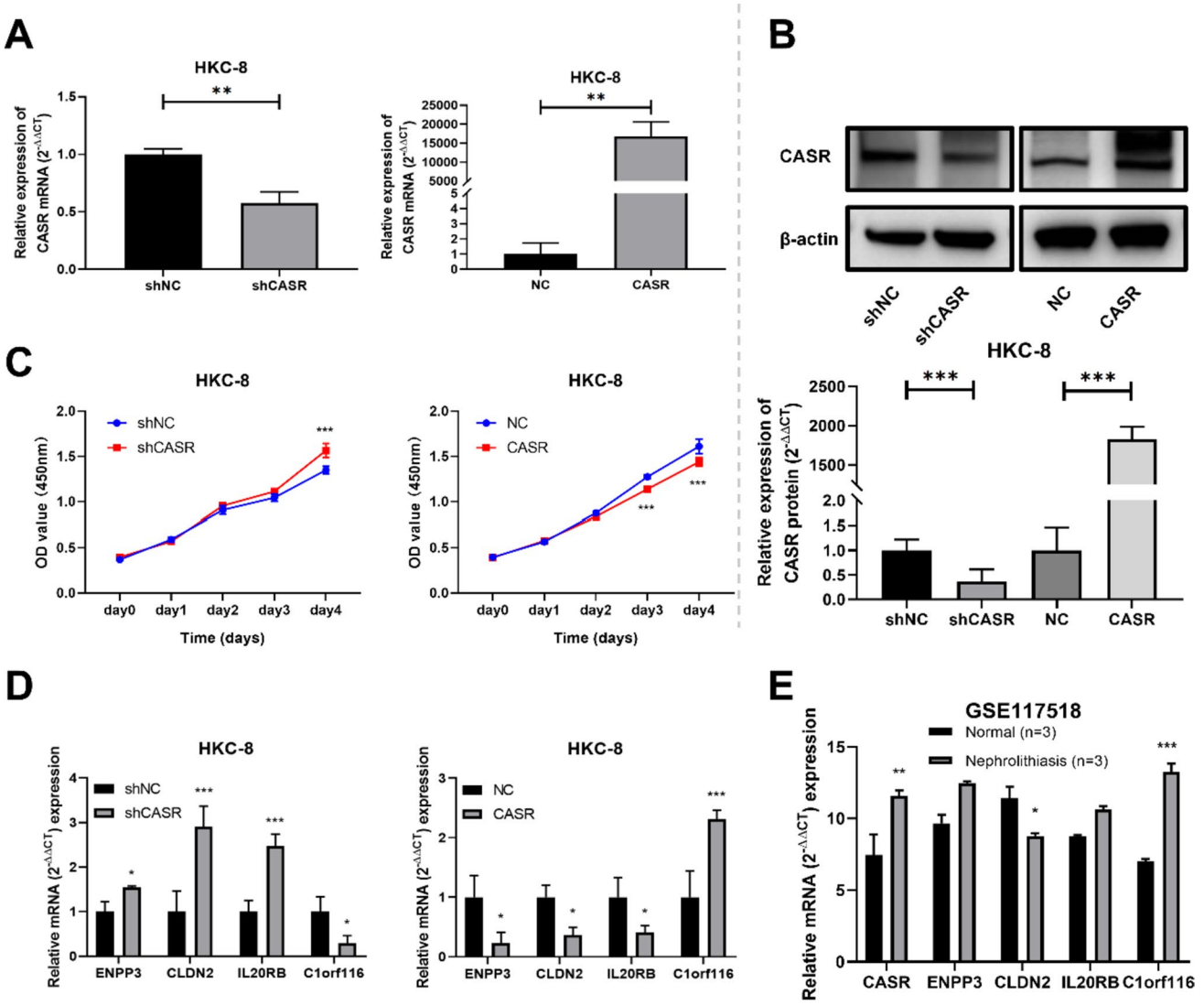

Fig.7 CASR expression in HKC-8 cells after different lentiviral transfection. **A**, **B** RT-qPCR (**A**) and western blot (**B**) assays validated CASR expression in HKC-8 cells with different lentiviral transfection. **C** CCK-8 assay reveals blocking CASR significantly promoted the proliferation of HKC-8 cells, while elevated expression of CASR inhibited proliferation of HKC-8 cells. **D** Hub genes expression in the shCASR group and in the CASR group. **E** Hub genes and CASR expression in GSE117518 data set

Discussion

Previous studies have reported that mutation of CASR could cause familial hypocalciuric hypercalcaemia and primary hyperparathyroidism, and in turn result in basal ganglia calcifications, seizures, cramps, tetany nephrolithiasis [15, 16]. In our previous study, we found a notable association between polymorphisms of CASR-related genes (WDR72, DGKH, and CLDN14) and calcium nephrolithiasis in Chinese Han population [17]. Some data indicate that minor alleles at the associated polymorphism leads to a loss of CASR expression in the kidney. In this work, we further explored cellular mechanisms of HK-2 cells and nephrolithiasis underlying CASR based on DEGs via RNA-Seq in HK-2 cells. Pathway analysis was employed to pinpoint the salient pathways of the DEGs of CASR. Although several studies have found CASR could cause stimulation of intracellular signaling pathways, including intracellular Ca^{2+} release, and MAPK stimulation [18], we discovered novel pathways regulated by CASR, including tight junction and cell–cell junction.

GO analysis demonstrated that the DEGs in shCASR cells, mainly function in cell apoptosis and cell development. When renal tubular cells undergo apoptosis, they release small vesicles called apoptotic bodies that contain calcium and oxalate ions. These ions can then bind together to form crystals, which can grow into kidney stones [19]. For instance, Peng et al. report that renal tubular epithelial cell

apoptosis induced by testosterone was an important pathophysiological process contributing to the development of nephrolithiasis [20]. In CASR cells, the DEGs mainly performed mitochondrial energy metabolism, and amino acid metabolism. Recent studies have suggested that defects in mitochondrial function and oxidative stress in renal tubular epithelial cells can lead to the formation of calcium oxalate crystals [21].

KEGG analysis results indicated that the DEGs in shCASR cells focused on the multiple signal transduction pathways, including TNF signaling pathway, FoxO signaling pathway, TGF-beta signaling pathway, JAK–STAT signaling pathway, and NOD-like receptor signaling pathway. The FoxO signaling pathway and TGF-beta signaling pathway are complex regulatory networks of cellular processes, including cell differentiation, proliferation, apoptosis, and stress response [22]. In CASR cells, the DEGs function in MAPK signaling pathway, Rap1 signaling pathway, and PI3K–Akt signaling pathway. It has previously been suggested that MAPK signaling and PI3K–Akt signaling involve in lipotoxicity, while lipotoxicity and oxidative stress in renal tubular epithelial cells are the driving force for the loss of nephrons, the predominant pathophysiological changes of nephrolithiasis [23]. Overall, the signaling and regulatory pathways underlying the DEGs of CASR suggest important functional roles in renal tubular epithelial cells and nephrolithiasis.

Combining the DEGs in shCASR and CASR cells, we identified 4 hub genes that CASR regulated, including C1orf116, ENPP3, IL20RB, and CLDN2. C1orf116 (chromosome 1 open reading frame 116, also named as specifically androgen-regulated gene, SARG) was reported to regulate the epithelial to mesenchymal transition (EMT) pathway [24]. Moreover, evidence has shown that EMT of renal tubular epithelial cells occurs in the early stage of CaOx crystals formation and deposition [25]. ENPP3, also known as ectonucleotide pyrophosphatase/phosphodiesterase 3, codes for the protein called CD203c [26]. CD203c/ENPP3 has been implicated in the regulation of extracellular pyrophosphate levels, which is important for the mineralization of bone and the formation of kidney stones [27]. IL20RB, also known as interleukin-20 receptor subunit beta, functions in immune responses, inflammation, and tissue repair. Meanwhile, CASR plays a role in inflammation and in the cardiovascular system like atherosclerosis [28], indicating a potential correlation between CASR and IL20RB. Nonetheless, similar to C1orf116, the direct link between ENPP3 or IL20RB and nephrolithiasis is still unknown, and more research is needed to determine the precise mechanisms involved. CLDN2 (Claudin-2) is a protein that is part of the tight junctions for maintaining the barrier function of epithelial cells and regulating the movement of ions and molecules across cell layers [29]. Previous studies have suggested that decreased expression of CLDN2 in the kidney can lead to a reduction in reabsorption of sodium and calcium via regulating the permeability of the epithelial barrier in the kidney, further promoting the development of nephrolithiasis [30]. New findings demonstrate that common genetic variants in CLDN2 gene were associated with decreased tissue expression of CLDN2 and increased risk of nephrolithiasis, and a rare missense variant in CLDN2 (p.Gly161Arg) has marked hypercalciuria and nephrolithiasis [31]. Therefore, CLDN2 regulated by CASR is a key regulator of calcium excretion and a potential target for therapies to prevent nephrolithiasis. Nevertheless, the specific mechanism of CASR regulating CLDN2 requires further investigation.

However, the hub gene expression in renal proximal tubule epithelia cells were not totally consistent with the results in samples of renal papilla (GSE117518). On the one hand, the number of tests for GSE117518 analysis was too small to have sure results; on the other hand, the in vitro analysis of one cell type lids to different results respect to the analysis of bulk RNA seq of renal tissue. Nephrolithiasis can be intricately related to both the papilla and proximal tubules within the kidney, but the complexity of the two systems is different. For instance, Wiener et al. revealed that the earliest identifiable biominerals in the human renal papilla were plate-like calcium phosphate deposits located within proximal intratubular areas. They proposed a plausible step-by-step process in renal biomineralization; initial proximal intratubular calcium phosphate deposits could progress to interstitial regions rich in calcium phosphate, eventually maturing into a base upon which a CaOx stone forms within the kidney's collecting system [32]. Similarly, Sherer and colleagues demonstrated a continuum in the mineralization process leading to calcium-based nephrolithiasis, encompassing four distinct anatomical and structural biomineralization stages: (1) mineralization within the renal pyramid's proximal intratubular areas; (2) interstitial regions near the papilla's tip; (3) the emergence of plaque formations (stems); and (4) the formation of heterogeneous stones within the kidney [33].

Functional and pathway enrichment analysis revealed that these hub genes regulated by CASR were involved in tight junction, cell–cell junction, cell–cell junction organization, and epithelial cell development. Prior research has demonstrated that tight junctions in renal tubular epithelial cells play a critical role in maintaining the proper balance of ions and molecules in the urine, helping prevent the formation of kidney stones [34]. In the initiation of stone formation, calcium oxalate could induce a loss of intercellular tight junctions and renal tubular epithelial cells' attachment to the basement membrane [35]. Meanwhile, the subsequent impairment of tight junction barrier and fence functions due to exposure to calcium oxalate crystals can cause renal tubulointerstitial injury [36]. As

mentioned above, CLDN2 primarily functions in maintaining the integrity of epithelial barriers. Therefore, CASR may regulate CLDN2 through tight junction or cell–cell junction of in nephrolithiasis pathogenesis.

Our present study is the first exploratory investigation of CASR aspect using the RNA-Seq approach. The present study has yielded a fresh perspective on the mechanistic role of CASR in HK-2 cells and in the pathogenesis of nephrolithiasis. Notably, our findings suggest that CASR exerts its effects on HK-2 cells and nephrolithiasis through diverse signaling pathways, and that the interaction between CASR and CLDN2 may be pivotal in the formation of kidney stones. The limitation of this study is that we just detected mRNA changes but did not validate at protein levels. Secondly, we did not select areas of the rat kidney or human kidney for sequencing analyses and we are not so sure whether the mRNAs observed in HK-2 cells are consistent with the natural pathogenesis of nephrolithiasis. The results obtained from analyzing a specific cell type in vitro may differ from those obtained by analyzing bulk RNA sequencing of renal tissue. Thirdly, the study of CASR in HK-2 cells is not sufficient since CASR is expressed across the entire length of the nephron. Meanwhile, overexpression or downregulation of CASR gene is not sufficient to investigated the CASR polymorphisms associated with nephrolithiasis.

Conclusion

Our report of RNA-Seq events in HK-2 cells fills a gap in the field of CASR in basic research on nephrolithiasis. The analytical results provide new insights into the mechanism of CASR in renal tubular epithelial cells and nephrolithiasis, and provide a basis for subsequent research on the specific regulatory mechanisms of CASR in nephrolithiasis.

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Author contributions ZZJ, WLJ, and WZ conceived and designed the experiments; ZZJ, YYY, and GP performed the experiments; WLJ and DQ analyzed the data; ZZJ wrote the manuscript; ZTT, WZ and WLJ reviewed the manuscript; ZZJ and ZTT revised the manuscript.

Data availability All data experimental data are available upon request.

Declarations

Conflict of interest The authors declare no conflict of interest.

Human and/or animals rights This article does not contain any studies with animals.

References

1. Lieske JC, Toback FG (1996) Interaction of urinary crystals with renal epithelial cells in the pathogenesis of nephrolithiasis. Semin Nephrol 16:458–473
2. Ding T, Zhao T, Li Y, Liu Z, Ding J, Ji B, Wang Y, Guo Z (2021) Vitexin exerts protective effects against calcium oxalate crystal-induced kidney pyroptosis in vivo and in vitro. Phytomedicine 86:153562
3. Riccardi D, Valenti G (2016) Localization and function of the renal calcium-sensing receptor. Nat Rev Nephrol 12:414–425
4. Riccardi D, Brown EM (2010) Physiology and pathophysiology of the calcium-sensing receptor in the kidney. Am J Physiol Renal Physiol 298:F485-499
5. Vezzoli G, Terranegra A, Soldati L (2012) Calcium-sensing receptor gene polymorphisms in patients with calcium nephrolithiasis. Curr Opin Nephrol Hypertens 21:355–361
6. Vezzoli G, Macrina L, Magni G, Arcidiacono T (2019) Calcium-sensing receptor: evidence and hypothesis for its role in nephrolithiasis. Urolithiasis 47:23–33
7. Li X, Chen S, Feng D, Fu Y, Wu H, Lu J, Bao J (2021) Calcium-sensing receptor promotes calcium oxalate crystal adhesion and renal injury in Wistar rats by promoting ROS production and subsequent regulation of PS ectropion, OPN, KIM-1, and ERK expression. Ren Fail 43:465–476
8. Ibeh CL, Yiu AJ, Kanaras YL, Paal E, Birnbaumer L, Jose PA, Bandyopadhyay BC (2019) Evidence for a regulated Ca(2+) entry in proximal tubular cells and its implication in calcium stone formation. J Cell Sci 132:jcs225268
9. Renkema KY, Bindels RJ, Hoenderop JG (2011) Role of the calcium-sensing receptor in reducing the risk for calcium stones. Clin J Am Soc Nephrol 6:2076–2082
10. Renkema KY, Velic A, Dijkman HB, Verkaart S, van der Kemp AW, Nowik M, Timmermans K, Doucet A, Wagner CA, Bindels RJ, Hoenderop JG (2009) The calcium-sensing receptor promotes urinary acidification to prevent nephrolithiasis. J Am Soc Nephrol 20:1705–1713
11. Vezzoli G, Terranegra A, Rainone F, Arcidiacono T, Cozzolino M, Aloia A, Dogliotti E, Cusi D, Soldati L (2011) Calcium-sensing receptor and calcium kidney stones. J Transl Med 9:201
12. Zhao J, Wu Y, Zhou K, Huang M, Sun Y, Kang J, Su Q, Zhao Y, Liu Q, Li C (2023) Ferroptosis in calcium oxalate kidney stone formation and the possible regulatory mechanism of ANKRD1. Biochim Biophys Acta Mol Cell Res 1870:119452
13. Dong C, Song C, He Z, Song Q, Song T, Liu J, Xiong Y, Su X, Zhou J, Yang S, Liao W (2023) Protective efficacy of Schizandrin B on ameliorating nephrolithiasis via regulating GSK3β/Nrf2 signaling-mediated ferroptosis in vivo and in vitro. Int Immunopharmacol 117:110042
14. He Z, Liao W, Song Q, Li B, Liu J, Xiong Y, Song C, Yang S (2021) Role of ferroptosis induced by a high concentration of calcium oxalate in the formation and development of urolithiasis. Int J Mol Med 47:289–301
15. Hill F, Sayer JA (2019) Precision medicine in renal stone-formers. Urolithiasis 47:99–105
16. Magno AL, Leatherbarrow KM, Brown SJ, Wilson SG, Walsh JP, Ward BK (2020) Functional analysis of calcium-sensing receptor variants identified in families provisionally diagnosed with familial hypocalciuric hypercalcaemia. Calcif Tissue Int 107:230–239
17. Wang L, Zhou Z, Yang Y, Gao P, Lin X, Wu Z (2022) A genetic polymorphism in the WDR72 gene is associated with calcium nephrolithiasis in the Chinese han population. Front Genet 13:897051
18. Howles SA, Wiberg A, Goldsworthy M, Bayliss AL, Gluck AK, Ng M, Grout E, Tanikawa C, Kamatani Y, Terao C et al (2019)

Genetic variants of calcium and vitamin D metabolism in kidney stone disease. Nat Commun 10:5175
19. Peng Y, Yang C, Shi X, Li L, Dong H, Liu C, Fang Z, Wang Z, Ming S, Liu M et al (2019) Sirt3 suppresses calcium oxalate-induced renal tubular epithelial cell injury via modification of FoxO3a-mediated autophagy. Cell Death Dis 10:34
20. Peng Y, Fang Z, Liu M, Wang Z, Li L, Ming S, Lu C, Dong H, Zhang W, Wang Q et al (2019) Testosterone induces renal tubular epithelial cell death through the HIF-1α/BNIP3 pathway. J Transl Med 17:62
21. Gu Y, Shen Y, Chen W, He H, Ma Y, Mei X, Ju D, Liu H (2022) Protective effects of interleukin-22 on oxalate-induced crystalline renal injury via alleviating mitochondrial damage and inflammatory response. Appl Microbiol Biotechnol 106:2637–2649
22. Qiao X, Rao P, Zhang Y, Liu L, Pang M, Wang H, Hu M, Tian X, Zhang J, Zhao Y et al (2018) Redirecting TGF-β signaling through the β-catenin/foxo complex prevents kidney fibrosis. J Am Soc Nephrol 29:557–570
23. Yuan Q, Tang B, Zhang C (2022) Signaling pathways of chronic kidney diseases, implications for therapeutics. Signal Transduct Target Ther 7:182
24. Parsana P, Amend SR, Hernandez J, Pienta KJ, Battle A (2017) Identifying global expression patterns and key regulators in epithelial to mesenchymal transition through multi-study integration. BMC Cancer 17:447
25. Li S, Lan Y, Wu W, Duan X, Kong Z, Wu W, Zeng G (2019) Peroxisome proliferator-activated receptor γ modulates renal crystal retention associated with high oxalate concentration by regulating tubular epithelial cellular transdifferentiation. J Cell Physiol 234:2837–2850
26. Tsai SH, Kinoshita M, Kusu T, Kayama H, Okumura R, Ikeda K, Shimada Y, Takeda A, Yoshikawa S, Obata-Ninomiya K et al (2015) The ectoenzyme E-NPP3 negatively regulates ATP-dependent chronic allergic responses by basophils and mast cells. Immunity 42:279–293
27. Letavernier E, Kauffenstein G, Huguet L, Navasiolava N, Boud-erlique E, Tang E, Delaitre L, Bazin D, de Frutos M, Gay C et al (2018) ABCC6 deficiency promotes development of randall plaque. J Am Soc Nephrol 29:2337–2347
28. Sundararaman SS, van der Vorst EPC (2021) Calcium-sensing receptor (CaSR), its impact on inflammation and the consequences on cardiovascular health. Int J Mol Sci 22:2478
29. Raju P, Shashikanth N, Tsai PY, Pongkorpsakol P, Chanez-Paredes S, Steinhagen PR, Kuo WT, Singh G, Tsukita S, Turner JR (2020) Inactivation of paracellular cation-selective claudin-2 channels attenuates immune-mediated experimental colitis in mice. J Clin Invest 130:5197–5208
30. Gamero-Estevez E, Andonian S, Jean-Claude B, Gupta I, Ryan AK (2019) Temporal effects of quercetin on tight junction barrier properties and claudin expression and localization in MDCK II cells. Int J Mol Sci 20:4889
31. Curry JN, Saurette M, Askari M, Pei L, Filla MB, Beggs MR, Rowe PS, Fields T, Sommer AJ, Tanikawa C et al (2020) Claudin-2 deficiency associates with hypercalciuria in mice and human kidney stone disease. J Clin Invest 130:1948–1960
32. Wiener SV, Chen L, Shimotake AR, Kang M, Stoller ML, Ho SP (2018) Novel insights into renal mineralization and stone formation through advanced imaging modalities. Connect Tissue Res 59:102–110
33. Sherer BA, Chen L, Kang M, Shimotake AR, Wiener SV, Chi T, Stoller ML, Ho SP (2018) A continuum of mineralization from human renal pyramid to stones on stems. Acta Biomater 71:72–85
34. Mandel N, Riese R (1991) Crystal-cell interactions: crystal binding to rat renal papillary tip collecting duct cells in culture. Am J Kidney Dis 17:402–406
35. Peerapen P, Thongboonkerd V (2011) Effects of calcium oxalate monohydrate crystals on expression and function of tight junction of renal tubular epithelial cells. Lab Invest 91:97–105
36. Hadpech S, Peerapen P, Thongboonkerd V (2022) Alpha-tubulin relocalization is involved in calcium oxalate-induced tight junction disruption in renal epithelial cells. Chem Biol Interact 368:110236