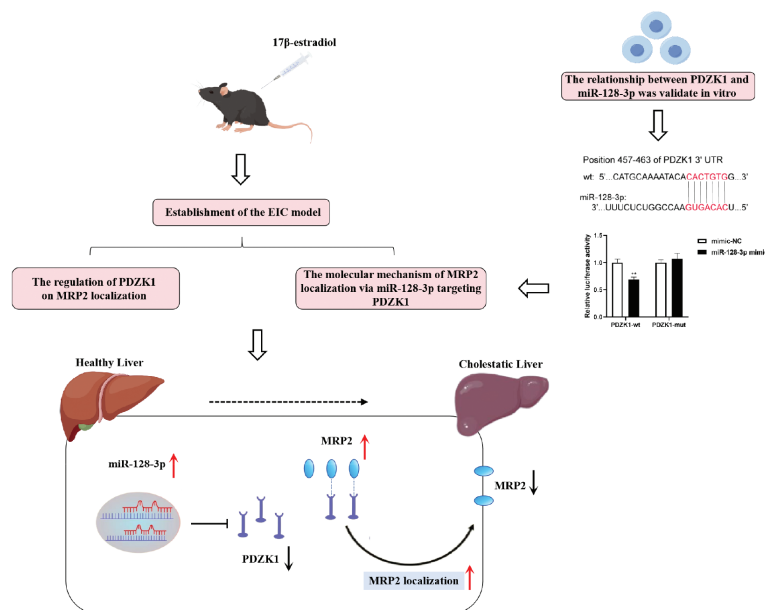


## Research Article

# MiR-128-3p mediates MRP2 internalization in estrogen-induced cholestasis through targeting PDZK1

## Graphical abstract



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## In brief

miR-128-3p inhibited the expression of PDZK1 by targeting PDZK1, leading to the internalization of MRP2 and exacerbating cholestatic liver disease, which might be a crucial mechanism involved in the progression of EIC. These findings illuminated the miR-128-3p function in hepatic PDZK1/ MRP2 regulation and the exact mechanism via which this regulation occurs, highlighting the existence of different targets and potential strategies in the treatment of EIC.

## Highlights

- PDZK1 downregulation promoted MRP2 internalization in the EIC model: Our study revealed that PDZK1 expression was downregulated, increasing internalization of multidrug resistance-associated protein 2 (MRP2) in the liver of estrogen-induced cholestasis (EIC).
- miR-128-3p targeted PDZK1, regulating the localization of MRP2 in EIC: We identified miR-128-3p as a key microRNA that targets the 3'-untranslated region (3'-UTR) of PDZK1, inhibiting the expression of PDZK1, finally promoting MRP2 internalization and exacerbating cholestatic liver injury.
- Therapeutic potential of restoring PDZK1 expression: Suppressing miR-128-3p or increasing PDZK1 expression could enhance the membrane localization of MRP2, thus alleviating EIC severity.

# MiR-128-3p mediates MRP2 internalization in estrogen-induced cholestasis through targeting PDZK1

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## ABSTRACT

Estrogens have been reported to cause dysfunction in biliary transport systems, thereby inducing cholestasis. Multidrug resistance-associated protein 2 (MRP2) is a transporter responsible for independent bile flow. Emerging evidence indicates that PDZ domain containing 1 (PDZK1) regulates localization of MRP2; however, PDZK1's role and regulatory machinery in MRP2-mediated estrogen-induced cholestasis (EIC) remain unclear. Herein, in a mouse model of EIC, we observed downregulated PDZK1 expression in the liver and enhanced intracellular domain MRP2 internalization. Notably, expression of miR-128-3p, a potential biomarker of estrogen-related cholestasis discovered by our group, was significantly elevated. We demonstrated that miR-128-3p targeted the 3'-untranslated region of PDZK1 in EIC and consequently promoted MRP2 internalization. Accordingly, miR-128-3p suppression upregulated PDZK1, thereby suppressing MRP2 internalization and significantly attenuating cholestatic liver disease. Furthermore, we observed MRP2 internalization and PDZK1 downregulation, as well as excessive miR-128-3p, in clinical samples from patients with cholestatic liver injury. Overall, our findings illustrate that miR-128-3p inhibits PDZK1 expression, thereby inhibiting the membrane localization of MRP2 in EIC. Enhancing or restoring PDZK1 expression might therefore have therapeutic potential for cholestatic liver injury.

**Keywords:** miR-128-3p, estrogen-induced cholestasis, MRP2, PDZK1, localization

## 1. INTRODUCTION

Cholestatic liver diseases include a variety of disorders that affect bile formation and/or flow, thus generally resulting in progressive hepatobiliary injury, and ultimately fibrosis and cirrhosis [1-3]. Estrogen-induced cholestasis (EIC) occurs in pregnant people, who are particularly vulnerable to the condition during the second or third trimester; this condition is also called intrahepatic cholestasis of pregnancy (ICP) [4]. EIC varies in incidence from 0.2% to 5.6%, and is strongly associated with both race and geographic region [4, 5]. The only FDA-approved medication for EIC treatment is ursodeoxycholic acid [6]; however, 40% of patients have an unsatisfactory treatment response [7, 8]. Therefore,

further clarifying the pathogenesis of EIC is critical to enable the identification of new therapeutic targets.

Aberrant expression and function of bile transporters lead to dysfunction in the hepatobiliary transport system and are currently considered important contributors to estrogen-induced liver damage [9-12]. The dynamic plasma membrane insertion and internalization of multidrug resistance-associated protein 2 (MRP2) are involved in bile formation [13, 14]. Razori and Yano et al. have found that internalization of MRP2 on the plasma membrane correlates with cholestasis severity [15, 16]. In addition, MRP2 internalization is involved in estradiol-17 $\beta$ -d-glucuronide-induced cholestasis [17]. However, the regulation of MRP2 membrane localization, as well as estrogen-mediated regulation of MRP2

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localization and function, remain to be clarified. Thus, further exploration of the mechanism regulating MRP2 membrane localization in the liver of EIC remains necessary.

The accurate expression and localization of transporters on the plasma membrane require interactions among various proteins, particularly scaffold proteins. PDZ domain containing 1 (PDZK1) is a protein scaffold with distinct domains for protein-PDZ interaction. PDZK1 modifies the intracellular signals transduction, membrane localization, and transport function of many membrane transporters, particularly ion channels and drug transport proteins [18, 19]. MRP2 and several organic anion transport proteins are interaction partners of PDZK1; this interaction increases membrane localization and the activity of these transporters [12, 20-23]. However, whether MRP2 membrane localization regulated by PDZK1 plays a role in EIC remains unknown.

MicroRNAs (miRNAs), comprising 18–25 nucleotides, bind mRNA 3'-untranslated regions (3'-UTRs) and post-transcriptionally suppress the expression of target genes [24]. These RNAs participate in several cellular processes, including proliferation, apoptosis, and polarization [24]. Accumulating evidence indicates a strong link between the aberrant expression of specific miRNAs and the incidence of liver disease [25-27]. The miRNA miR-199a-5p is upregulated in obstructive cholestasis and inhibits BSEP expression [28]. Aberrantly upregulated miR-let7a-5p has been found to downregulate MRP2 expression in obstructive cholestasis [29]. In our previous study, the miRNAs miR-128-3p, miR-7706, and miR-877-3p were found to be markedly upregulated in patients with ICP and therefore might be potential biomarkers for ICP diagnosis [30]. However, whether these miRNAs regulate PDZK1 expression and MRP2 localization in EIC remains unclear.

This study examined a mouse model with 17 $\beta$ -estradiol (17 $\beta$ -EE)-induced cholestasis and demonstrated that miR-128-3p expression was associated with PDZK1 downregulation, and subsequently resulted in abnormal localization of MRP2 and cholestatic liver disease. Our findings therefore suggest a potential alternative direction and target for EIC diagnosis and treatment.

## 2. MATERIALS AND METHODS

### 2.1 Experimental animals

Male C57BL/6 mice (6–8 weeks of age, weighing 19–20 g each) were obtained from the Center of Experimental Animals of Tongji Hospital, Tongji Medical College, Huazhong Science and Technology University. All animal experiments were approved by the institution's Ethics Committee on Animal Experimentation (TJH-202106008). Recommended procedures for the handling and care of experimental animals, as outlined in National Institutes of Health guidelines, were strictly followed. The mice were reared under a 12-hour light/dark cycle and fed a standard laboratory pellet diet.

They were provided with an unrestricted supply of food and water. Before the commencement of the tests, all mice acclimated for 1 week.

For 5 days, a 17 $\beta$ -EE propylene glycol solution was administered via subcutaneous injection to establish the EIC model [8]. Mice were injected with miR-128-3p antagomir (100  $\mu$ M) or miR-antagomir negative control (NC) (100  $\mu$ M) via the tail vein. In addition, *in vivo* viral transduction was performed by tail vein injection of purified AAV9 containing a liver-specific PDZK1 promoter at  $4.81 \times 10^{11}$  viral particles per mouse. After 6 days of overnight fasting, all mice were euthanized. Serum and hepatic tissue were collected and stored at  $-80^{\circ}\text{C}$ .

### 2.2 Serum TBIL, ALP, TBA, AST, and ALT

We used commercial kits from JianCheng in Nanjing, China, to determine the enzymatic activity of total bilirubin (TBIL), total bile acid (TBA), alkaline phosphatase (ALP), aspartate aminotransferase (AST), and alanine aminotransferase (ALT), according to the manufacturer's directions.

### 2.3 Cell culture and transfection

Dulbecco's modified Eagle's medium (Gibco, China) containing 10% fetal bovine serum, 0.25 ng/mL amphotericin B, and 100  $\mu$ g/mL streptomycin was used to culture L02 and Aml12 cells, which were incubated at  $37^{\circ}\text{C}$  in a humid environment with 5%  $\text{CO}_2$ . The medium was changed at intervals of 48 hours. Transfection was performed 24 hours after cells had been seeded onto six-well plates. Subsequently, quantitative real-time PCR (qRT-PCR) and western blotting analysis were conducted on the collected cells.

### 2.4 Assessment of MRP2 activity

Primary mouse hepatocytes were harvested by liver perfusion, as previously reported [31]. Medium containing 100  $\mu$ M dinitrophenyl S-glutathione (DNP-SG) was used to culture cells for half an hour. Subsequently, the cells were incubated for 15 min with 17 $\beta$ -EE after the transfection of AAV9-PDZK1 and AAV9-Vector with Lipofectamine 2000. In addition, primary hepatocytes were transfected with miR-128-3p mimic, mimic NC, miR-128-3p inhibitor, or inhibitor NC (QINGKE, China) with Lipofectamine 2000 (Invitrogen), according to the manufacturer's directions.

The transport activity of MRP2 in primary hepatocytes was evaluated by measurement of DNP-SG excretion, and MRP2 content was evaluated with high-performance liquid chromatography in all samples, with authenticated standards [32].

### 2.5 Hematoxylin and eosin staining

Sections 5  $\mu$ m thick were cut from the liver after fixation in 4% paraformaldehyde for 24 hours and embedding in paraffin. Hematoxylin and eosin (H&E) solution (Beyotime, China) was used to stain the sections according

to the manufacturer's instructions. An optical microscope (Zeiss, Germany) was used to capture the images.

2.6 qRT-PCR

PDZK1, transporter genes, miRNAs (Table 1), and their relative expression in mouse liver cells and tissues were quantified with qRT-PCR. In brief, we followed the manufacturer's guidelines for extracting total RNA from cells or tissues with TRIzol reagent (Invitrogen Life Technologies, Carlsbad, USA). Subsequently, miRNAs and mRNAs were detected with a Mir-X miRNA qRT-PCR TB Green kit (Takara Biotechnology Co., Ltd., Japan) and PrimeScript RT Master Mix (Takara Biotechnology Co., Ltd., Japan). SYBR Premix Ex Taq (Takara Biotechnology Co., Ltd., Japan), and a StepOnePlus Real-Time PCR system (Applied Biosystems, USA) were used for qRT-PCR evaluation of gene expression. The  $2^{-\Delta\Delta C_t}$  approach was used to quantify expression levels relative to  $\beta$ -actin and U6 mRNA and miRNA normalization standards, respectively. To ensure statistical validity, each sample was examined three times. The gene primer sequences are illustrated in Table 2.

2.7 Preparation of liver membranes

Differential centrifugation was used to prepare membrane fractions enriched in plasma membranes [17, 33]. Protein concentrations were measured with the method of Gilboa et al. [34], with bovine serum albumin (BSA) as a standard.

Table 1 | Sequences of miRNAs.

| Genes       | Sequences                      |
|-------------|--------------------------------|
| miR-7706    | 5'-UGAAGCGCCUGUGCUCUGCCGAGA-3' |
| miR-877-3p  | 5'-UGUCCUCUUCUCCCUCCUCCCA-3'   |
| miR-128-3p  | 5'-UCACAGUGAACCGGUCUCUUU-3'    |
| miR-1306-5p | 5'-CCACCUCCCCUGCAAACGUCCA-3'   |
| miR-30c-5p  | 5'-UGUAAACAUCUACACUCUCAGC-3'   |

Table 2 | Primer sequences.

| Genes          | Forward primer               | Reverse primer              |
|----------------|------------------------------|-----------------------------|
| PDZK1          | 5'-CCAGGCCTCACATTGGAATCTA-3' | 5'-TTCTGCCCTCTTGTGTTGGA-3'  |
| MRP2           | 5'-AACATCTGCTTCCCTTGAGGC-3'  | 5'-TCCAGGGCACTTCTGTACG-3'   |
| BSEP           | 5'-CTTCTCCTGGCTCCCTCAAA-3'   | 5'-CAGGTAGTGTCAGTGGCCTTT-3' |
| MDR            | 5'-CAGTGGCTCTGAAGCCGTA-3'    | 5'-TCCTTCGAAAGTCAGCCAA-3'   |
| NTCP           | 5'-CCCAACGTACCCAGAAAGTT-3'   | 5'-ATACGTTGTGCGCCTCCATC-3'  |
| OATP1A1        | 5'-AGCATTGGCCTGTCCTATCT-3'   | 5'-TCTGGCCATGTGCAATCTTT-3'  |
| $\beta$ -actin | 5'-GAGCGCAAGTACTCTGTGTG-3'   | 5'-AAACGCAGCTCAGTAACAGTC-3' |
| U6             | 5'-CTCGCTTCGGCAGCAC-3'       | 5'-AACGCTTCACGAATTTGCGT-3'  |

2.8 Western blot analysis

A phosphatase inhibitor A/B, protease inhibitor cocktail, and 1 mM phenylmethanesulfonyl fluoride were added to the RIPA lysis solution used to isolate total protein. A BCA protein assay reagent kit (Beyotime Biotechnology, China) was used to measure protein concentrations. Thereafter, total proteins were isolated through 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis, then transferred to a PVDF membrane (Merck Millipore Ltd., Ireland), which was blocked for 1 hour at 37°C in Tris-buffered saline with 5% nonfat dry milk. The following antibodies were added to the membrane and incubated overnight at 4°C: mouse anti-PDZK1 (Santa Cruz Biotechnology, Inc.) (1:1000), rabbit anti-MRP2 (Cell Signaling Technology, CST, USA) (1:1000), rabbit anti-BSEP (Cell Signaling Technology, CST, USA) (1:1000), rabbit anti-NTCP (Absin) (1:1000), rabbit anti-MDR (Beijing Boaoson Biotechnology Co., Ltd.) (1:1000), rabbit anti-OATP1A1 (Novus Biologicals, USA) (1:1000), and rabbit anti- $\beta$ -actin (Abcam, Cambridge, UK) (1:10000). Subsequently, the membrane was treated with corresponding horseradish peroxidase-conjugated secondary antibodies and incubated for 1 hour at 20°C. A G:BOX Chemi XRQ Imaging System (Syngene, UK) was used to visualize the protein bands, and ImageJ was used for quantification.

2.9 Dual-luciferase reporter gene assays

Fragments of the wild-type or mutant 3'-UTR of PDZK1 containing miR-128-3p binding domains were cloned into the pmirGLO Dual-Luciferase miRNA Target Expression Vector. According to the manufacturer's guidelines, miR-128-3p mimic or mimic control (mimic-NC) was introduced into Aml12 cells via Lipofectamine 2000 transfection. A Dual-Luciferase Reporter Assay System Protocol was used to detect luciferase activity.

2.10 Liver samples from patients with PBC

Sections of normal human liver tissue adjoining the cancerous tissue were used as controls. These samples were obtained from Tongji Hospital at Huazhong University

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of Science and Technology. Surgical resection was used to acquire liver slices (4–5  $\mu\text{m}$  thick) from individuals diagnosed with PBC. The study was approved by the Human Ethics Committee of Tongji Hospital, Huazhong University of Science and Technology (TJ-IRB20230901).

### 2.11 In situ hybridization of miR-128-3p

FAM-labeled U6 or miR-128-3p probe (Baiqiandu Biotechnology Co., Ltd.) was used to hybridize liver sections overnight at 55°C in a hybridization solution. After slides were rinsed with 1× SSC buffer, sheep anti-FAM-POD (Abcam, Cambridge, MA) diluted 1:250 in blocking solution was applied and incubated at ambient temperature for 1 hour. Thereafter, the Alexa Fluor-conjugated secondary antibody Alexa Fluor 549 goat anti-rabbit immunoglobulin G (IgG) (Cell Signaling Technology) was added and incubated 1 hour at ambient temperature. The nuclei in the slides were stained by incubation with DAPI (1 mg/mL) for 8 minutes. A confocal microscope was used to acquire the images.

### 2.12 Immunofluorescence

Fluorescence staining of PDZK1 and transporters was performed to determine the changes in their expression and localization in EIC. Liver samples were paraffin-embedded after fixation for 24 hours with 4% paraformaldehyde, then sliced at a thickness of 5  $\mu\text{m}$ . Before incubation of samples with antibodies to PDZK1 and transporters overnight, the slides were blocked with phosphate-buffered saline containing 1% bovine serum albumin and 0.3% Triton X-100, and pretreated with 0.3% BSA to prevent non-specific binding. Next, the slides were treated with goat anti-mouse IgG Alexa Fluor 488 or goat anti-rabbit IgG Alexa Fluor 568 secondary antibodies in the dark for 1 hour. A confocal laser scanning microscope was used to acquire the images.

### 2.13 Statistical analysis

GraphPad Prism 8 was used for data analysis. A *t*-test was used to compare two groups, whereas a one-way analysis of variance was used to examine differences among multiple groups. Data are reported as mean  $\pm$  SD. *p* < 0.05 was chosen as the threshold for statistical significance.

## 3. RESULTS

### 3.1 Establishment of the EIC model

To evaluate the successful establishment of the EIC model, we assessed body weight, liver index, liver histology and plasma biochemical markers [3, 6]. The body weight in EIC mice was initially elevated, but on the third day was lower than that observed in the control mice (Figure 1A), whereas the liver index was markedly elevated (Figure 1B). Serum AST, ALT, ALP, TBA, and TBIL levels in the model group were considerably higher than those in the control group (Figure 1C). Histological evaluation of the liver revealed a normal structure in the

control mice, but inflammatory cell infiltration and a disordered hepatic structure in the EIC mice (Figure 1D). According to the above findings, the EIC model appeared to have been successfully established.

### 3.2 Expression of liver transporters in EIC and co-localization with PDZK1

To explore the roles of liver transporters and PDZK1 in EIC, we detected the expression of transporters and their co-localization with PDZK1. On day 5 of the modeling study, the hepatic mRNA expression of NTCP, OATP1A1, and MRP2 was lower, whereas no significant difference in BSEP and Mdr mRNA levels was observed, in EIC compared with control mice (Figure 2A). Moreover, NTCP, OATP1A1, BSEP, MDR, and MRP2 protein expression decreased in 17 $\beta$ -EE-treated mice on day 5 (Figure 2B). Co-localization of PDZK1 and the major hepatic transporters NTCP, OATP1A1, BSEP, MDR, and MRP2 was assessed. The results revealed co-localization of PDZK1 with OATP1A1 or MRP2, but less co-localization was observed in EIC mice than control mice (Figure 2C). Overall, these findings demonstrated diminished NTCP, OATP1A1, BSEP, MDR, and MRP2 protein expression, and co-localization of PDZK1 with OATP1A1 and MRP2, in EIC.

### 3.3 Diminished PDZK1 expression and MRP2 membrane localization in EIC

A previous study has indicated that PDZK1 modulates intracellular trafficking of OATP1A1 through the recruitment of motor proteins to vesicles containing intracellular OATP1A1 [20]. Therefore, we focused on the association between MRP2 localization and PDZK1. According to our findings, 17 $\beta$ -EE substantially decreased PDZK1 expression (Figure 3A, B). To explore the changes in MRP2 localization in EIC, we detected the expression of MRP2 in hepatocyte cytoplasm and membranes. In EIC mice, compared with control mice, MRP2 expression was markedly lower in membranes, but higher in the cytoplasm (Figure 3C, D). Changes in MRP2 localization revealed that MRP2 was confined primarily to the membranes in control mice (Figure 3E). After 17 $\beta$ -EE administration, the cholestatic agent altered MRP2 membrane localization, as indicated by an increase in MRP2 containing vesicular-like structures in the hepatocyte cytoplasm (Figure 3E). Together, these findings indicated diminished PDZK1 expression and MRP2 internalization in EIC.

### 3.4 PDZK1 overexpression ameliorates liver injury by increasing MRP2 membrane localization and transport activity in EIC mice

To confirm the function of PDZK1 in EIC, we generated the plasmids AAV9-PDZK1 and AAV9-Null (control) by cloning PDZK1 and flanking sequences into an AAV9 vector under control of a mouse albumin promoter. The diminished expression of PDZK1 in EIC was reversed

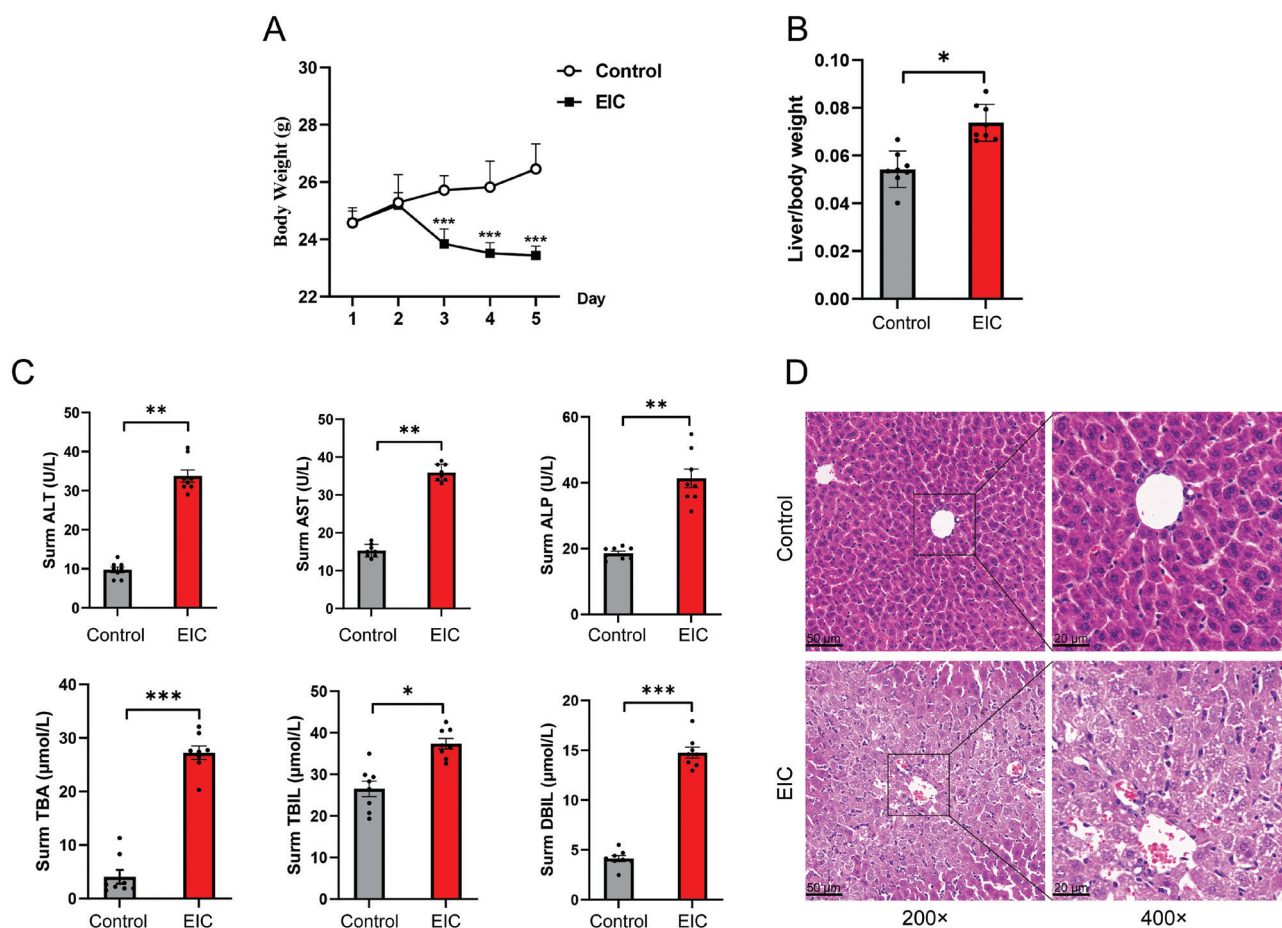


**Figure 1 | Cholestatic liver injury is induced in mice by 17β-EE.**

(A) Patterns of changes in body weight within 5 days after administration of 17β-EE. (B) Liver index. (C) DBIL, AST, ALP, ALT, TBA, and TBIL levels in the serum in each group. (D) H&E images of hepatic sections (200x and 400x magnification). Results are displayed as mean ± SD (n = 8). \* $p < 0.05$ , \*\* $p < 0.01$  and \*\*\* $p < 0.001$ , vs the control group.

by AAV9-PDZK1 (Figure 4A–C). PDZK1 overexpression resulted in significantly lower levels of AST, ALT, ALP, TBA, TBIL, and DBIL levels than observed in the 17β-EE + AAV9-Null group (Figure 4D), and ameliorated the inflammatory cell infiltration and disordered hepatic structure in EIC mice (Figure 4E). These results suggested that PDZK1 protects EIC mice against cholestatic liver injury.

Furthermore, we observed diminished expression of MRP2 on membranes in the 17β-EE + Vector group; however this affect was reversed in the 17β-EE + AAV9-PDZK1 group *in vitro* and *in vivo* (Figure 5A, B), and the internalization of MRP2 was ameliorated (Figure 5C) in the 17β-EE + AAV9-PDZK1 group. In addition, PDZK1 overexpression attenuated the decreased excretion of DNP-5G by hepatocytes treated with 17β-EE (Figure 5D). Overall, these findings demonstrated that the hepatocyte-specific expression of PDZK1 ameliorated EIC through a mechanism involving increased MRP2 expression, membrane localization, and transport activity.

### 3.5 miR-128-3p suppresses PDZK1 expression by binding the 3'-UTR of PDZK1

We previously analyzed miRNA microarray profiles in blood samples from patients with ICP and healthy pregnant people, and identified five candidate upregulated miRNAs [30]. Herein, we measured the expression of these miRNAs *in vitro* and *in vivo*, to determine the functions of the dysregulated miRNAs in EIC. The EIC model showed much higher levels of miR-128-3p expression *in vitro* and *in vivo* than observed in controls (Figure 6A, B). We performed bioinformatic prediction with TargetScan8.0 ([https://www.targetscan.org/vert\\_80/](https://www.targetscan.org/vert_80/)) and identified potential miR-128-3p binding sites in the PDZK1's 3'-UTR (Figure 6C). We examined the possibility that miR-128-3p's binding site in the 3'-UTR of PDZK1 might inhibit the protein. The miR-128-3p mimic effectively suppressed luciferase activity. Interestingly, this suppression was eliminated after mutation of the binding site in the 3'-UTR of PDZK1 (Figure 6C). The relationship between PDZK1 and miR-128-3p was also

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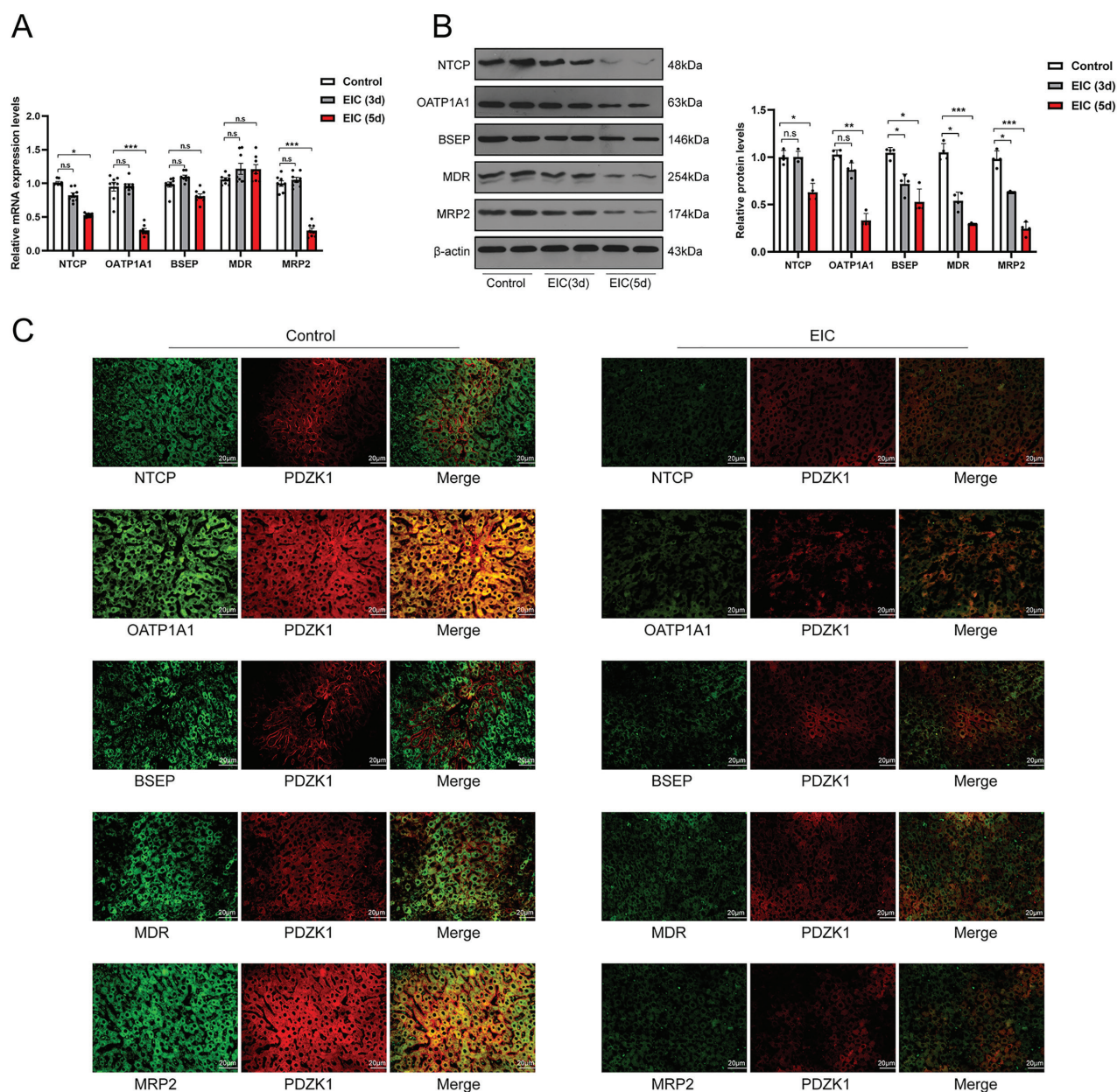


**Figure 2 | Liver transporter expression in EIC and co-localization with PDZK1 in EIC mice.**

(A) Liver transporter-related gene expression in control and EIC mice, detected by qRT-PCR ( $n = 8$ ). (B) NTCP, OATP1A1, BSEP, MDR, and MRP2 expression, detected by western blotting ( $n = 4$ ). (C) Immunofluorescence staining of transporters (green) and PDZK1 (red). Results are shown as mean  $\pm$  SD. \* $p < 0.05$ , \*\* $p < 0.01$  and \*\*\* $p < 0.001$ , vs the control group.

detected in Aml12 and L02 cells transfected with the miR-128-3p mimic/inhibitor or NC. The results were consistent with those of the luciferase reporter gene assays. Indeed, *in vitro*, the miR-128-3p mimic downregulated PDZK1 expression to a greater extent than the NC; however, the miR-128-3p inhibitor group exhibited opposite effects (Figure 6D, E). According to these findings, miR-128-3p was found to negatively regulate and directly target PDZK1.

### 3.6 miR-128-3p inhibition ameliorates EIC liver injury

We used miR-128-3p antagomir to suppress hepatic miR-128-3p expression to further investigate the function of this miRNA in EIC. Significant downregulation of miR-128-3p expression was observed after antagomir treatment (Figure 7A), thus resulting in a lower liver index in the EIC group than the 17 $\beta$ -EE + miR-antagomir NC group (Figure 7B). Furthermore, miR-128-3p

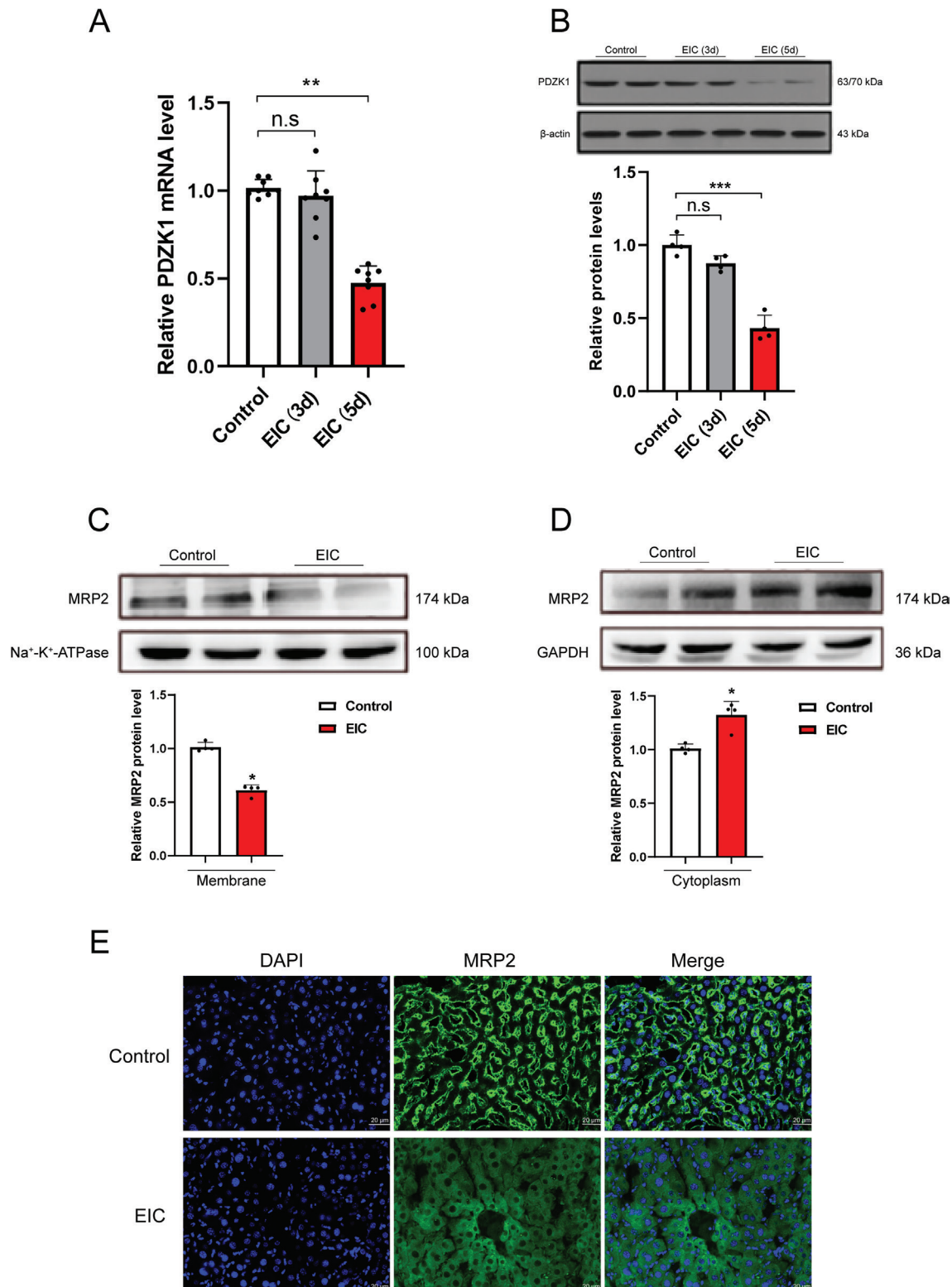


**Figure 3 | PDZK1 expression is downregulated, and the membrane localization of MRP2 is diminished, in the liver in EIC.**

(A) PDZK1 gene expression, detected by RT-qPCR (n = 8). (B) PDZK1 protein expression and quantification, detected by western blotting (n = 4). (C, D) MRP2 expression on hepatocyte membranes (C) and cytoplasm (D), estimated through western blotting (n = 4). (E) Localization of MRP2 on hepatocyte membranes, detected by immunostaining. Results are shown as mean  $\pm$  SD. \* $p$  < 0.05, \*\* $p$  < 0.01 and \*\*\* $p$  < 0.001, vs the control group.

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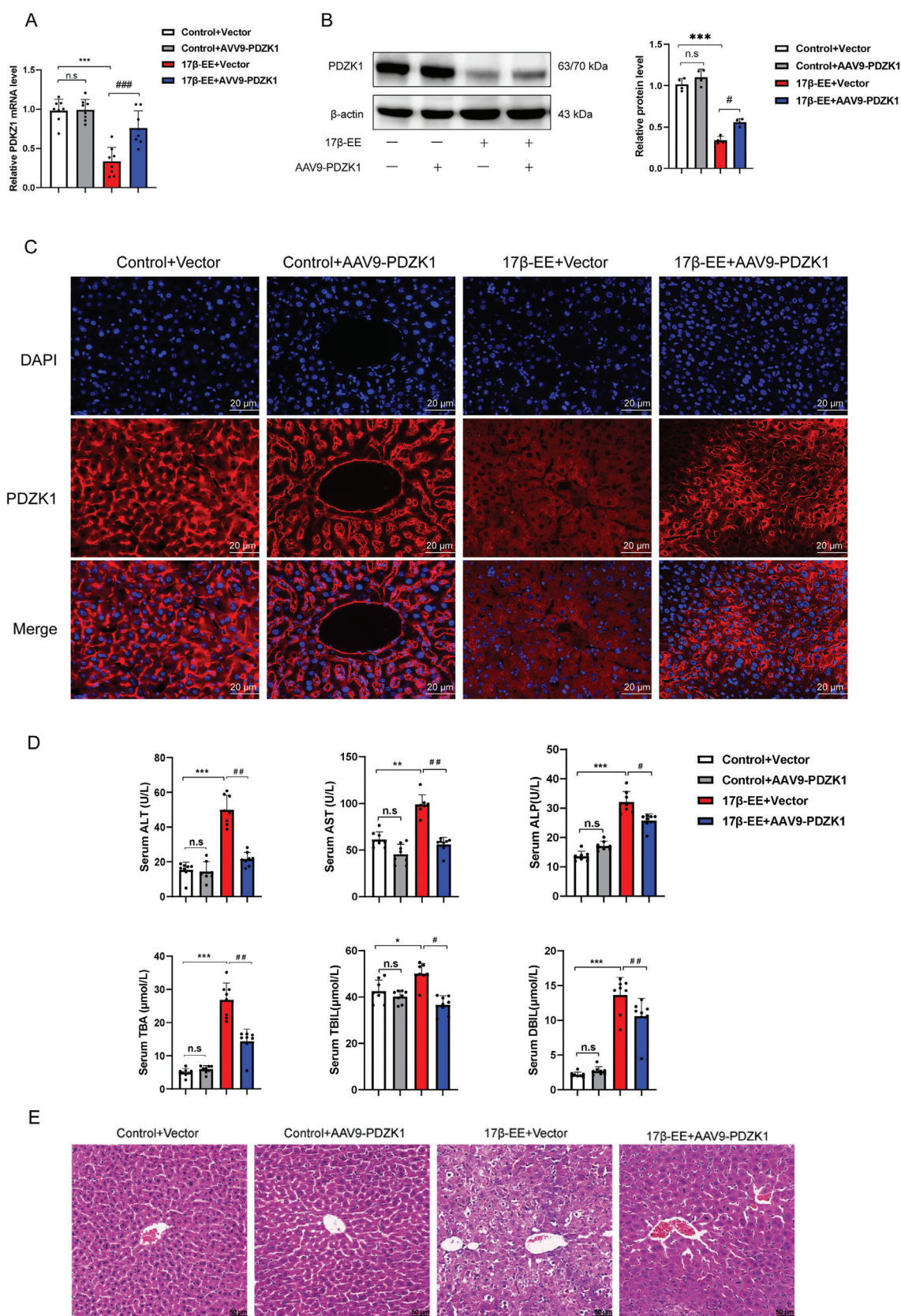


Figure 4 | PDZK1 overexpression alleviates cholestatic liver injury in EIC.

(A) Relative PDZK1 gene expression, detected by qRT-PCR in mice transfected with AAV9-PDZK1 (n = 8). (B) PDZK1 protein expression, detected by western blotting in mice transfected with AAV9-PDZK1 (n = 4). (C) Immunofluorescence staining of PDZK1. (D) DBIL, AST, ALP, ALT, TBA, and TBIL levels in the serum in each group (n = 8). (E) Representative liver H&E staining from mice. Results are shown as mean  $\pm$  SD. \* $p < 0.05$ , \*\* $p < 0.01$  and \*\*\* $p < 0.001$ , vs the control + vector group; # $p < 0.05$ , ## $p < 0.01$  and ### $p < 0.001$ , vs 17 $\beta$ -EE + Vector group.

downregulation significantly suppressed the 17 $\beta$ -EE-induced increases in serum DBIL, TBA, ALP, AST, TBIL, and ALT levels (Figure 7C), and alleviated EIC liver injury (Figure 7D). Therefore, miR-128-3p suppression decreased the hepatotoxicity mediated by 17 $\beta$ -EE.

### 3.7 miR-128-3p perturbs MRP2 membrane localization and transport activity by targeting PDZK1 in EIC

To further investigate the molecular mechanism through which miR-128-3p modulates biological functions in EIC, we assessed the levels of PDZK1 expression and membrane localization of MRP2 in control mice and EIC mice that received miR-128-3p antagomir injections. PDZK1 expression was restored in the liver when miR-128-3p was suppressed (Figure 8A, B). In addition, MRP2 expression on membranes was greater than that observed in the 17 $\beta$ -EE + miR-antagomir NC group (Figure 8C), and the internalization was abrogated in the 17 $\beta$ -EE + miR-128-3p antagomir group (Figure 8D). The secretory rate of DNP-SG was also assessed to determine whether MRP2 transport activity was compromised during its internalization from the membrane. The concentration of MRP2 substrate markedly decreased after 15 minutes of miR-128-3p mimic treatment but was restored by miR-128-3p inhibitor treatment (Figure 8E). Overall, miR-128-3p was found to have an indispensable role in facilitating MRP2 internalization by blocking PDZK1, and miR-128-3p suppression abolished the decrease in MRP2 transport activity in EIC.

### 3.8 miR-128-3p is upregulated, and PDZK1 and MRP2 membrane levels are diminished, in the livers of patients with PBC

Our *in vitro* and *in vivo* studies indicated that miR-128-3p considerably influenced the internalization of MRP2 in EIC through its regulation of PDZK1. To determine the applicability of our findings in cholestatic mice and humans, we studied the livers of patients with PBC and control livers (normal liver tissue adjacent to liver cancer tissues), given that specimens from patients with EIC are typically inaccessible. The hepatic miR-128-3p levels were higher (Supplementary Figure 1A), whereas the hepatic protein level of PDZK1 was markedly lower, in PBC livers than control livers (Supplementary Figure 1B). In addition, the MRP2 internalization from the plasma membrane into the intracellular domains increased in PBC liver (Supplementary Figure 1B). These results further supported that miR-128-3p mediates MRP2 internalization in cholestatic liver injury by targeting PDZK1.

## 4. DISCUSSION

In this study, upregulation of miR-128-3p expression in EIC mice increased MRP2 internalization by inhibiting the PDZK1, thereby exacerbating cholestatic liver injury. Our findings revealed a regulatory role of miR-128-3p in MRP2 localization and function through PDZK1 (Figure 9), thus suggesting a promising therapeutic strategy for EIC treatment.

Bile transporters such as BSEP and MRP2 are dynamically internalized and inserted into the membrane, where they regulate bile flow from the intracellular pool of vesicles [17, 35]. Excessive internalization of bile transporters leads to cholestasis. MRP2 contributes to the formation of bile flow in a manner not dependent on bile salts, through ATP-dependent transport of glutathione and glutathione conjugates [18, 36]. Estradiol-17 $\beta$ -d-glucuronide induces changes in the activity and localization of MRP2, manifesting as endocytic internalization of MRP2 and sustained cholestasis [17, 37]. However, the regulatory mechanism underlying MRP2 internalization by estrogens remains unclear.

Important functions of PDZK1 include targeting proteins to cell membranes, controlling the activity of transmembrane proteins, and assembling proteins into signaling complexes for effective transmission [12, 18]. The interaction of PDZK1 with organic anion transporting polypeptides 2B1 (OATP2B1) influences their localization and function, thereby contributing to distinct pharmacokinetic mechanisms mediated by OATP2B1 [21, 38]. In addition, PDZK1 stabilizes and sorts OATP1A2, OCTN2, and OATP1A [21, 39, 40]. Recently, studies have demonstrated that MRP2 physically interacts with PDZK1 [12, 41]. This interaction is achieved primarily through the C-terminal sequence of MRP2, which promotes the localization and transport function of MRP2 on the membrane [42]. The PDZ domains of PDZK1 fold, thereby forming peptide-binding clefts and mediating interactions with the C-terminal NSTKF of MRP2 [23, 43]. Moreover, pull-down studies have identified MRP2's interaction with PDZK1, via the PDZ domain motif in the C-terminus of MRP2 [19]. The phosphorylation of the C-terminus of MRP2 increases binding to PDZK1 and enhances the membrane localization of MRP2 [12]. Normal MRP2 membrane localization is considerably compromised by removal of the PDZ domain motif [23]. We have also observed diminished PDZK1 and MRP2 expression in our previous study on EIC [18]. However, the mechanism through which PDZK1 regulates MRP2 localization has not been studied in EIC. Therefore, additional *in vitro* and *in vivo* investigations

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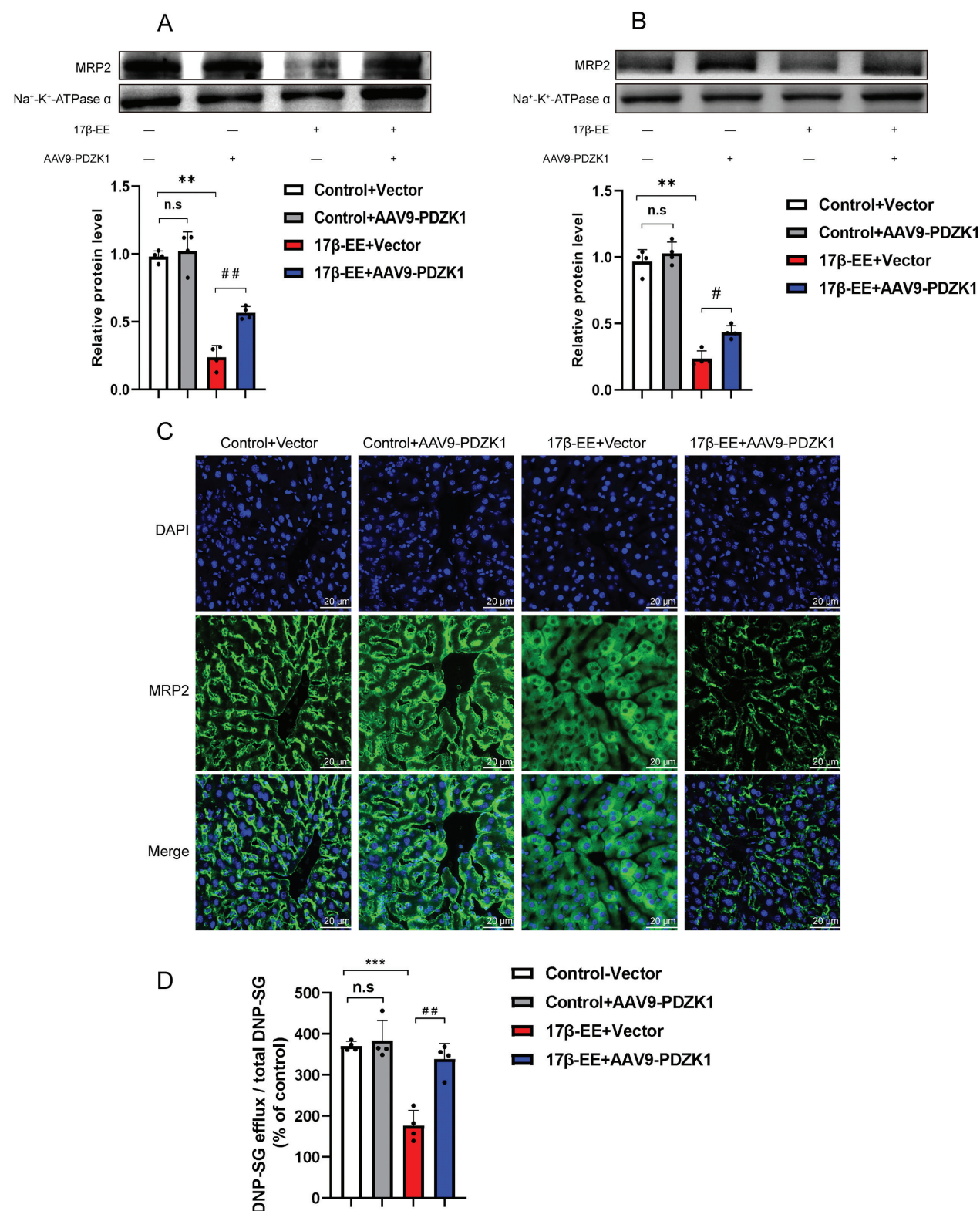


**Figure 5 | PDZK1 overexpression alleviates internalization of MRP2 in EIC.**

(A, B) MRP2 expression on membranes *in vitro* (A) and *in vivo* (B), detected by western blotting (n = 4). (C) Membrane localization of MRP2, detected by immunostaining. (D) Excretion of the MRP2 substrate DNP-SG by mouse primary hepatocytes (n = 4). Results are shown as mean ± SD. \**p* < 0.05, \*\**p* < 0.01 and \*\*\**p* < 0.001, vs the control + vector group; #*p* < 0.05, ##*p* < 0.01 and ###*p* < 0.001, vs 17β-EE + Vector group.

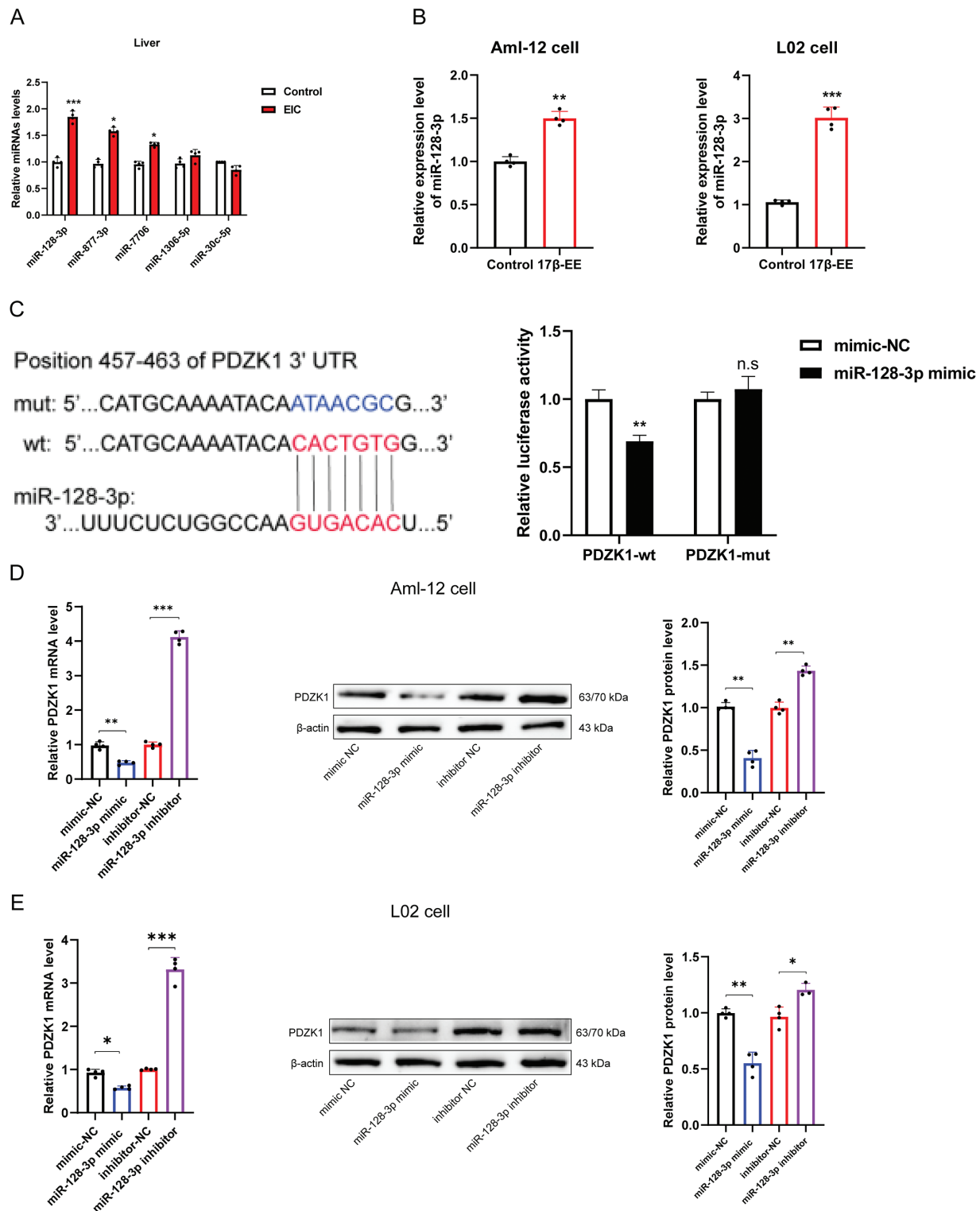


**Figure 6 | PDZK1 is negatively regulated by miR-128-3p.**

(A) Levels of five candidate upregulated miRNAs in the liver in EIC mice (miR-128-3p, miR-877-3p, miR-7706, miR-1306-5p, and miR-30c-5p). (B) miR-128-3p expression in Aml12 and L02 cells. (C) Sequences of potential miR-128-3p binding sites in PDZK1's 3'-UTR and dual luciferase activity assay to confirm direct association between miR-128-3p and PDZK1's 3'-UTR. (D) PDZK1 expression in Aml12 cells, determined with qRT-PCR and western blotting. (E) PDZK1 expression in L02 cells, determined with qRT-PCR and western blotting. Results are shown as mean  $\pm$  SD ( $n = 4$ ). \* $p < 0.05$ , \*\* $p < 0.01$  and \*\*\* $p < 0.001$ , vs the control group or mimic/inhibitor NC group.

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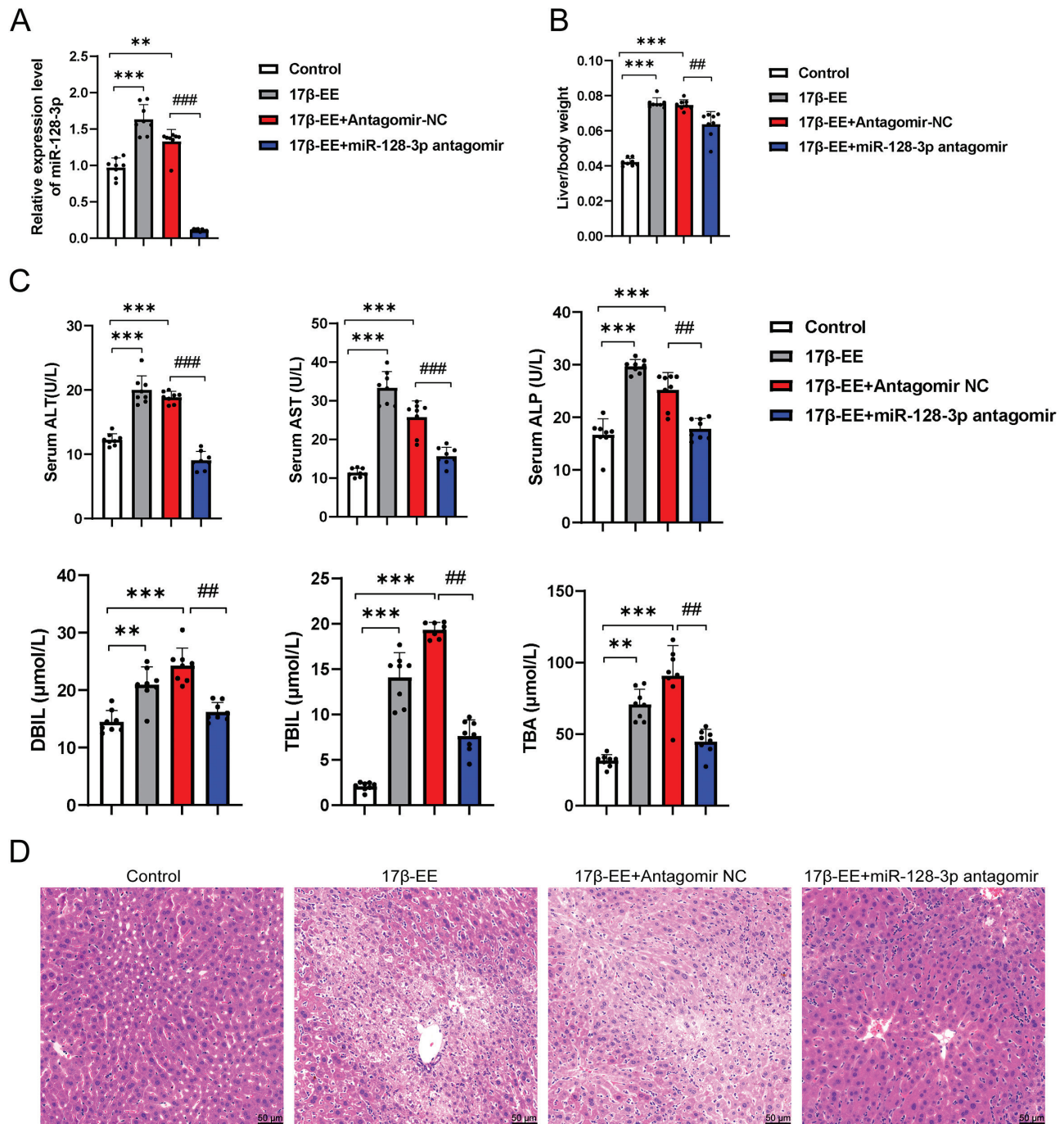


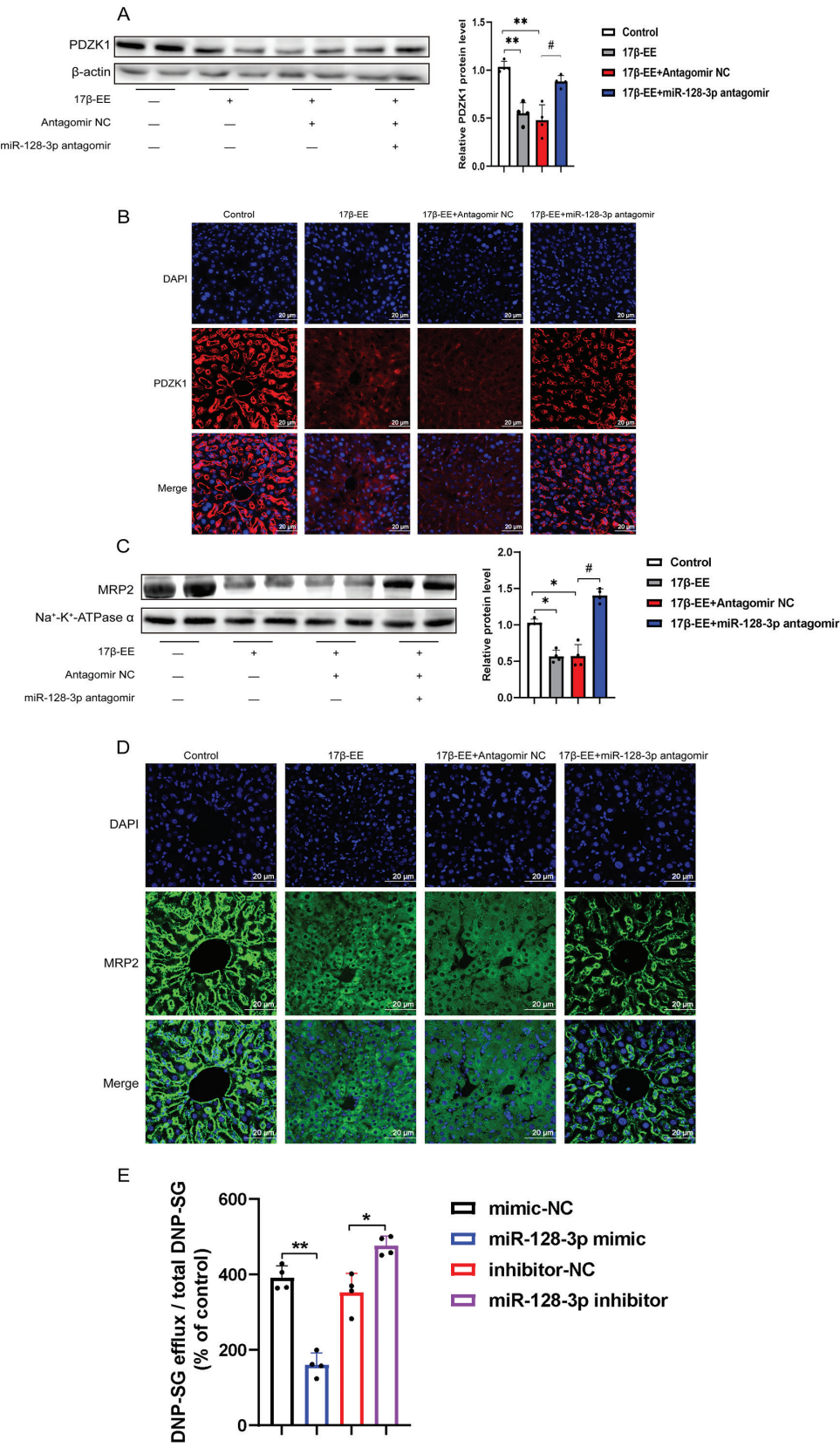
**Figure 7 | Inhibition of miR-128-3p ameliorates EIC liver injury.**

(A) miR-128-3p expression in the liver in EIC mice. (B) Liver index. (C) DBIL, AST, ALP, ALT, TBA, and TBIL levels in the serum in each group. (D) H&E image of liver section (200 $\times$  magnification). Results are displayed as mean  $\pm$  SD ( $n = 8$ ). \* $p < 0.05$ , \*\* $p < 0.01$  and \*\*\* $p < 0.001$ , vs the control group; # $p < 0.05$ , ## $p < 0.01$  and ### $p < 0.001$ , vs the 17 $\beta$ -EE + Antagomir NC group.

of MRP2 membrane localization and its liver regulatory mechanism remain necessary.

Our study indicated PDZK1 colocalization with MRP2 in the liver, whereas this colocalization was diminished in EIC mice. In addition, 17 $\beta$ -EE induced internalization of

MRP2 from the membrane into the cytoplasm. However, PDZK1 overexpression promoted MRP2 expression on the membrane, reversed its internalization, and ameliorated EIC. Our results confirmed the inhibition of PDZK1-mediated MRP2 localization as an important



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**Figure 8 | miR-128-3p mediates MRP2 internalization in EIC by regulating PDZK1.**

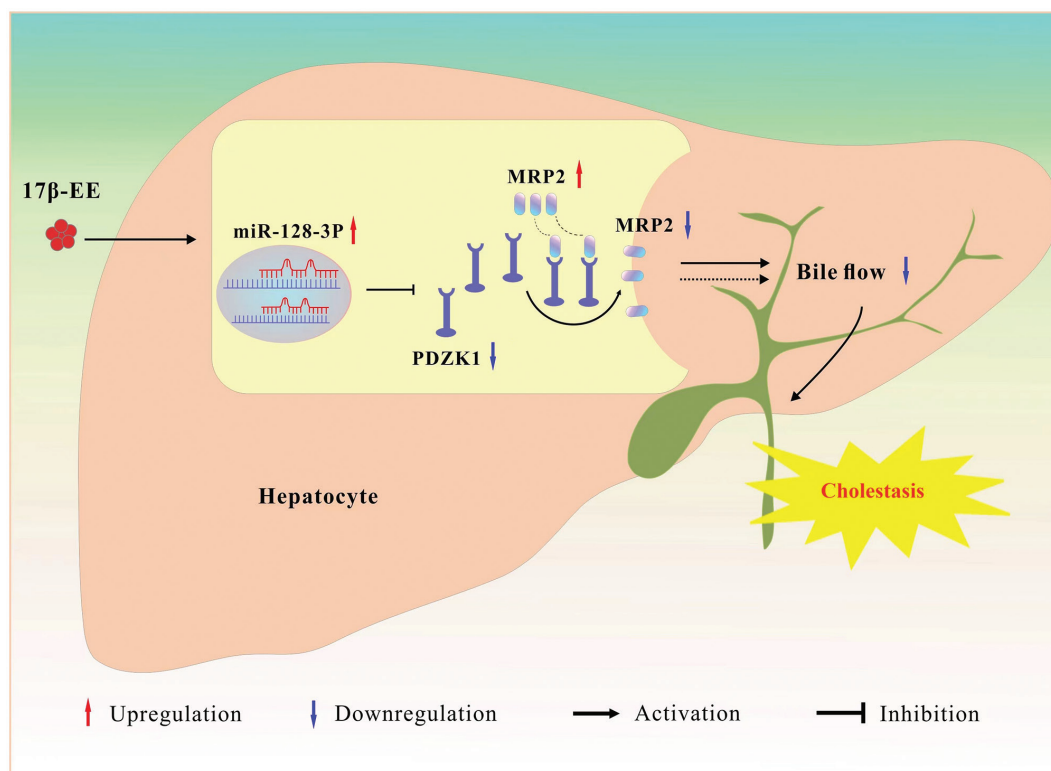
(A) PDZK1 protein expression, detected by western blotting. (B) PDZK1 protein expression, assessed with immunofluorescence. (C) MRP2 on the membrane, assessed with western blotting. (D) MRP2 on the membrane, assessed with immunostaining. (E) Excretion of DNP-SG, an MRP2 substrate, by mouse primary hepatocytes. Results are shown as mean  $\pm$  SD (n=4). \* $p < 0.05$  and \*\* $p < 0.01$ , vs the control group, mimic/inhibitor NC; # $p < 0.05$  and ## $p < 0.01$  vs 17 $\beta$ -EE + antagomir NC group.

pathological mechanism in EIC. However, the regulatory mechanism underlying estrogen-mediated impairment of PDZK1/MRP2 remains unknown.

MiRNAs influence various processes in the body, such as proliferation, apoptosis, differentiation, inflammation, and metabolism, by binding complementary sequences in the 3'-UTRs of specific mRNAs and leading to mRNA degradation or translational repression [44]. The expression of miRNAs is altered in acute and chronic liver disease. One possible mechanism through which miRNA dysregulation might contribute to the pathophysiology of liver disease is interfering with gene regulatory networks [45]. Interest in the possible function of microRNAs (miRNAs) in the bile transport process of EIC has recently surged [28, 29]. Our previous study has indicated significantly upregulated expression of five miRNAs in patients with ICP [30]. The roles of these upregulated miRNAs in EIC were evaluated in this study through detection of their expression in EIC and prediction of their target genes. We discovered that miR-128-3p was upregulated in EIC mice and patients with ICP [30], and subsequently bound the 3'-UTR of PDZK1,

thereby regulating PDZK1 expression and function. Moreover, miR-128-3p has been studied in several disorders, including lung cancer [46], liver cancer [47], and rheumatoid arthritis [48]. Zhao et al. have shown that miRNA-128-3p, targeting sirtuin 1, can promote oxidative stress and aggravate doxorubicin-mediated liver damage [25]. miR-128-3p had not previously been associated with regulation of MRP2 and bile formation, and its effect in EIC had not been well studied. We hypothesize that miR-128-3p decreases MRP2 expression and membrane localization through regulation of PDZK1, thus limiting bile efflux and aggravating EIC.

This study provides the first definitive evidence that miR-128-3p contributes to the progression of EIC *in vitro* and *in vivo* models. Our *in vitro* experiments demonstrated that miR-128-3p mimics decreased PDZK1-3'-UTR-luciferase construct activity, as well as PDZK1 protein and mRNA expression. Moreover, miR-128-3p inhibitor treatment led to substantial upregulation of PDZK1 and MRP2, and reversed endocytosis, thus ultimately alleviating EIC. In addition, we collected the livers of patients with PBC and control livers (normal liver

**Figure 9 | miR-128-3p mediates MRP2 internalization in EIC by targeting PDZK1.**

tissue adjacent to liver cancer tissues) for examination, because bio-specimens from EIC patients are usually inaccessible. The livers of patients with PBC also showed upregulation of miR-128-3p, marked downregulation of PDZK1, and internalization of MRP2 into intracellular domains, thus suggesting that miR128-3p/PDZK1/MRP2 might be a shared mechanism underlying multiple forms of cholestatic liver injury. However, additional research is required to validate this hypothesis.

In conclusion, miR-128-3p inhibited MRP2 membrane localization and transport activity by directly downregulating PDZK1; this mechanism might be crucial in the progression of EIC. These findings illuminated miR-128-3p's function in hepatic PDZK1/MRP2 regulation and the exact mechanism underlying this regulation, thus highlighting the existence of novel targets and potential strategies for EIC treatment. Therefore, suppressing miR-128-3p or increasing PDZK1 expression might enhance the membrane localization of MRP2 and consequently alleviate EIC severity.

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#### CONFLICTS OF INTEREST

The authors disclose no possible conflicts of interest regarding the research, authorship, and/or publication of this paper.

#### SUPPLEMENTARY DATA

Supplementary data for this article can be found online at: [https://amm-journal.org/wp-content/uploads/2025/02/amm20240053\\_Suppl.zip](https://amm-journal.org/wp-content/uploads/2025/02/amm20240053_Suppl.zip).

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