Down-Regulation of miR-212-5p Facilitates Homocysteine-Induced Hepatocytes Apoptosis Via Targeting PSMD10

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Research

Keywords: apoptosis, endoplasmic reticulum stress, homocysteine, miR-212-5p, proteasome 26S subunit non-ATPase 10

Posted Date: December 30th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-136080/v1

Abstract

Background: Increasing evidences supported that elevated homocysteine (Hcy) levels contribute to cell apoptosis is implicated in the pathogenesis of liver injury, it correlates with liver disease severity. However, the underlying mechanism of apoptosis in Hcy-mediated liver injury remains obscure.

Results: In this study, we found that homocysteine increases ER stress-mediated apoptosis and aggravates liver injury through up-regulation of PSMD10 expression in *cbs*$^{+/-}$ mice mice fed with high methionine diet and hepatocytes treated with homocysteine in vitro. Knockdown of PSMD10 expression remarkably reduced ER stress or apoptosis-associated protein in hepatocytes exposed to homocysteine. Moreover, bioinformatics analysis revealed that PSMD10 is a potential target gene of miR-212-5p, and luciferase reporter assay also confirmed that miR-212-5p negatively regulated PSMD10 expression by direct binding to its 3'-UTR regions. Subsequently, over-expression of miR-212-5p inhibited ER stress-mediated hepatocytes apoptosis though targeting PSMD10, all of which were abrogated by knockdown of miR-212-5p expression. Further study showed that the interaction between PSMD10 and GRP78 accelerated ER stress-mediated hepatic apoptosis induced by homocysteine.

Conclusion: Taken together, these results demonstrated that down-regulation of miR-212-5p facilitates homocysteine-induced hepatocytes apoptosis via targeting PSMD10, which provides novel insight into the mechanism of homocysteine induced apoptosis in liver injury.

Background

Homocysteine is a non-essential sulfhydryl-containing amino acid that is derived from methionine metabolism. Liver is a major organ of homocysteine metabolism(1). Recently, epidemiological and experimental studies linked homocysteine to a wide range of impaired liver function. Generally, homocysteine secreted into the circulation may induce widespread modifications of plasma proteins that could potentially contribute to the development of liver injury and cardiovascular diseases(2). Meanwhile, hepatocyte apoptosis plays an important role in liver injury, which correlates with liver disease severity and participates in the progress of hepatic fibrosis(3). Therefore, understanding the molecular mechanism of homocysteine-induced hepatocyte apoptosis is crucial for the treatment of liver disease.

Many studies have indicated that homocysteine promotes liver disease through oxidative stress, endoplasmic reticulum (ER) stress and the activation of pro-inflammatory factors(4). ER stress often occurred in the cells demanding a high rate of protein synthesis and secretion as well as harmful stresses, including hypoxia, infection and exposure to a toxic substance. It is generally accepted that the three unfolded protein reaction (UPR) signaling pathways play an independent role in ER stress, of which may present opportunities for targeted therapies(5). Meanwhile, the activation of glucose-regulated protein 78 (GRP78) also plays a critical role in sensing ER stress and triggering the UPR, resulting in cell apoptosis(6). In addition, oncoprotein proteasome 26S subunit non-ATPase 10 (PSMD10) was reported to attenuate ER stress by up-regulating the GRP78 and enhancing the activation of the UPR pathway in

HCC cells(7). Our previous studies demonstrated that dramatic increase in GRP78 expression induced by homocysteine can accelerate ER stress-mediated liver injury(8). However, the concrete mechanism about the regulation of PSMD10 on the GRP78 during homocysteine-induced hepatocytes apoptosis remains unclear.

MicroRNAs (miRNAs) are essential small endogenous non-coding RNAs that negatively regulate gene expression by binding to the 3’-untranslated regions (3’UTR) of target genes. It can either promote or inhibit cell proliferation, differentiation and apoptosis by regulating target gene expression (9). Growing evidences indicated that miRNAs are important in liver development and they may constitute robust biomarkers for liver disease diagnosis and prognosis(10). Among them, miR-212 located at chromosome 17p13.3 is up-regulated in cancers such as oral carcinoma and lung cancer, while it was inhibited in the colorectal cancer, prostate cancer and hepatocellular carcinoma. In addition to cancers, the circulating or liver miR-212 also plays a role in the biology of NAFLD(11). Although miR-212 has showed its important role in the cancer and hepatic disease, whether it can mediate the homocysteine-induced liver injury is currently unknown.

In the present study, we explored the possible role of miR-212-5p in regulating ER stress-mediated hepatocytes apoptosis following treatment with homocysteine. The results showed that miR-212-5p suppresses homocysteine-induced ER stress in liver by targeting PSMD10. This finding demonstrated that PSMD10 is involved in the regulation of homocysteine-induced liver injury, which may provide a novel PSMD10-based therapeutic target for liver injury.

Methods

Animals

All animal experiments were approved and carried out in accordance with the Institutional Animal Care and Use Committee of the University of Ningxia Medical University. Eight to ten weeks old cystathionine beta-synthase (CBS) heterozygous knockout mice (*cbs*$^{+/-}$) were obtained from Jackson Laboratory (Bar Harbor, ME) and maintained in the animal facility center at Ningxia Medical University. The mice were fed with regular diet plus 2.0% methionine chow and water ad libitum. Mice genotypes were determined by PCR.

Cell culture, infection and transfection

Human hepatocytes (HL-7702) were obtained from the Japanese Collection of Research Bioresources and cultured in RPMI-1640 medium (Thermo, USA) containing 10% fetal bovine serum (FBS) (Hyclone, USA) and 1% penicillin (Thermo, Waltham, MA, USA). The cells were infected with adenoviruses encoding PSMD10 (Ad-PSMD10) when they were 80% confluent. The control cells were infected with Ad-GFP. Hepatocytes were transfected with non-silencing small interfering RNA (NC, 5’-UUCUCCGAACGUGUCACGUTT-3’), PSMD10 siRNA (5'-GCCGGGAUGAGAUUGUAAATT-3'), GRP78 siRNA (5’-GGGCAA AGAUGUCAGGAAATT-3’), miR-212-5p mimics (5’-ACCUUGGCUCUAGACUGCUUA CU-3’) and miR-

212-5p inhibitor (5’-AGUAAGCAGUCUAGAGCCAAGGU-3’) using Lipofectamine 2000 according to the manufacturer’s instructions (Invitrogen, Carlsbad, CA, USA).

Liver tissue preparation and morphologic observation

All mice were fasted but supplied with water for 24 h followed by peritoneally injection with 3% pentobarbital sodium in a dose of 2 mL/kg. Abdominal cavity of anesthetic mouse was incised, and then inferior vena cava blood was collected using 10 mL syringe. After standing for 3h at 4 °C, blood samples were centrifuged at 5000 × g for 15 min. Supernatant was stored at −80 °C until future use. Liver was incised and two segments (1.0 cm ×1.0 cm × 0.3 cm) were cut from right lobe of the liver. Two liver segments were fixed in 10 % neutral formalin and embedded in optimum cutting temperature compound (OCT). To observe morphologic changes in liver tissues, liver segments was HE stained.

TUNEL Assay

Liver tissues were stained using a TUNEL Staining Kit (Roche Inc., Basel, Switzerland) and the TUNEL-positive cells were symbolized by fluorescein-dUTP with dNTP according to the manufacturer’s protocol of the in-suit apoptosis Detection Kit (Roche Inc, Basel, Switzerland). Cells with nuclear condensation/fragmentation and apoptotic bodies in the absence of cytoplasmic TUNEL reactivity (green staining of nuclei) were considered as apoptotic cells. Cell nuclei was counterstained with DAPI and visualized by fluorescent microscopy.

Western blot

Western blot analysis was performed as previously described[11]. Liver tissues and HL-7702 cells were lysed in a lysis buffer (KeyGEN, China) containing the protease inhibitor phenylmethanesulfonylfluoride (PMSF, KeyGEN, China) at 4 °C for 30 min followed by centrifugation to remove cell debris. Protein concentration was measured using BCA protein assay kit (Beyotime Institute of Biotechnology). The protein was separated by SDS-PAGE and transferred to PVDF membranes followed by examination with antibodies against PSMD10, cleaved caspase3, caspase12, Bax, Bcl2, PARP, ATF6, p-PERK, PERK, eif2a, p-eif2a, IRE1a, p-IRE1a, CHOP and β-actin (all from Abcam Inc., Cambridge, MA, USA) respectively. Signal intensity was analyzed with Bio-Rad image analysis (Bio-Rad, Hercules, CA, USA).

Real-time PCR (qRT-PCR)

Total RNA was isolated from livers or cultured cells using the miRNA isolation kit (Ambion), and reverse transcribed using TaKaRa Master Mix (Dalian, China). Real-time PCR examination of PSMD10, GAPDH, miR-212-5p and U6 were performed using Maxima SYBR Green/ROX qPCR Master Mix assay (Thermo Scientific). The expression levels of mRNA and miRNA were normalized using GAPDH or U6 as a reference gene. qRT-PCR primer sequences are listed in Table 1.

Immunofluorescence staining

The paraffin embedded mouse liver tissue sections were permeabilized in PBS containing 0.1% Triton X-100 for 5min, and then incubated with blocking solution (5% goat serum in PBS) at room temperature for 30min, followed by incubation with primary antibody (PSMD10, 1:50, KDEL receptor, 1:50) overnight at 4 °C. After washing with PBS for 3 times, tissues were incubated with fluorescein conjugated secondary antibodies (goat anti-mouse or goat anti-rabbit) at RT for 1 h. After washing with PBS for 3 times and they were stained with DAPI for 5 min. Sections were then assessed using fluorescence microscopy (OLMPUS FV1000 confocal laser scanning microscope, Tokyo, Japan).

Live/Dead staining assay

Cell viability was monitored under laser scanning confocal microscope (LSM710) using cell viability Imaging Kit (Roche Diagnostics, 06432379001) according to the manufacturer's instruction.

Cell Apoptosis

Hepatocytes apoptosis was detected using the FITC-Annexin V/PI staining kit. After homocysteine treatment, the cells were washed with ice-cold PBS, and incubated with fluoresce in conjugated Annexin V and PI for 15 min, cell apoptosis was analyzed using a FACS flow cytometer equipped with the FACS talion data management system and Cell Quest software (Becton Dickinson, San Jose, CA, USA).

Dual luciferase assays

Hepatocytes in 6-well plates were co-transfected with 200 ng of either pMIR-PSMD10-wt(5'-GAGAGTGGAAGAAGCAAAACTGCTGGTGTCCCAAGGAGCAAGTATTTACATTGAGAATAA-3') or pMIR-PSMD10 mut (5'-GAGAGTGGAAGAA GCAAAACTGCTGGTGTCGGTTCCAGCAAGTATTTACATTGAGAATAA-3') together with 100 nM of miR-212-5p mimics or non-target miRNA mimics using Lipofectamine 2000. In 48 h after transfection, firefly and renilla luciferase activities were measured using the Dual-Glo luciferase assay kit (Gibco Life Technologies).

Coimmunoprecipitation assays

Cells were lysed in a lysis buffer containing protease inhibitor on ice. After centrifugation, the supernatant was incubated with an anti-PSMD10, anti-GRP78 or normal rabbit IgG respectively at 4°C overnight followed by incubation with Dynabeads Protein G. Immunocomplex was separated by SDS-PAGE and proceeded for western blot analysis.

Statistical analysis

Results are expressed as the mean ± SD from at least three independent experiments. The data were analyzed using one-way ANOVA and additional analysis using the Student Newman-Keuls test for multiple comparisons within treatment groups or t-test for two groups. $P<0.05$ was considered to be statistically significant.

Results

1. **Homocysteine aggravated liver injury by promoting hepatocytes apoptosis**

In order to investigate the role of homocysteine in liver injury, *cbs*$^{+/-}$ mice were fed with 2.0% methionine about 8 weeks to induce hyperhomocysteinemia. As shown in Fig.1A, the levels of homocysteine in the plasma and liver of *cbs*$^{+/-}$ mice were significantly increased compared to that of *cbs*$^{+/+}$ mice, indicating that hyperhomocysteinemia animal model was successfully established. In parallel to that, the levels of liver injury indicators such as aspartate aminotransferase (AST) and alanine transaminase (ALT) in *cbs*$^{+/-}$ mice are much higher than that in *cbs*$^{+/+}$ mice (Fig.1B). The correlation analysis showed a significant positive correlation between the levels of liver homocysteine and AST or ALT (Fig.1C). Meanwhile, the morphological changes of liver were assessed by H&E staining. It was found that hepatic lobules are clear and complete, and hepatic cords arranged radially around the central vein were observed in *cbs*$^{+/+}$ mice. In contrary, a considerable mass of disordered arrangement of hepatocytes, enlargement of space, fragmentation and pyknosis of nuclei, as well as hydropic degeneration of hepatocytes were found in *cbs*$^{+/-}$ mice liver (Fig. 1D). Moreover, homocysteine prominently reduced cell viability in cultured hepatocytes (HL-7720) (Fig.1E). These results suggested that homocysteine promotes hepatocytes injury. To further study the effect and molecular mechanisms of homocysteine-induced hepatocytes injury, TUNEL staining was performed and we observed very few TUNEL-positive hepatocytes in liver tissues from *cbs*$^{+/+}$ mice, whereas hepatocytes apoptosis was markedly increased in *cbs*$^{+/-}$ mice (Fig.1F). In consistent with that, homocysteine enhanced the expression of cleaved caspase3, 12 and Bax, altogether with Bcl2 inhibition in the *cbs*$^{+/-}$ mice (Fig.1G). Similar results were observed in hepatocytes treated with homocysteine in vitro (Fig.1H and I), suggesting that homocysteine promotes hepatocytes apoptosis. Since ER stress serves as a major signaling pathway that activates apoptosis in mammalian cells, TUDCA (a ERs-specific inhibitor) was used to investigate whether ER stress is involved in hepatocytes apoptosis. As shown in Fig.1H and I, TUDCA markedly inhibited the expression of apoptosis-related proteins and apoptosis rate of hepatocytes treated with homocysteine. These results demonstrated that homocysteine aggravates liver injury by promoting hepatocytes apoptosis, ER stress may play a key role in the process of homocysteine-induced hepatocyte apoptosis.

2. **Homocysteine induced ER stress through UPR signaling pathway in hepatocytes**

Since ER stress can induce apoptosis through the UPR pathway, which is initiated by three transmembrane stress sensors of protein kinase RNA-like ER kinase (PERK), inositol-requiring protein 1a (IRE1), and activating transcription factor 6 (ATF6)(12). Next, we determined whether UPR pathway is involved in homocysteine induced ER stress of hepatocytes. As shown in Fig. 2A, ER stress was induced by homocysteine after 8 weeks as evidenced by increased levels of GRP78, PERK, p-PERK, eif2a, p-eif2a, IRE1a, p-IRE1A, ATF6 and CHOP in liver tissue of *cbs*$^{+/-}$ mice compared to *cbs*$^{+/+}$ mice. We also analyzed ER stress signaling by immunostaining of KDEL receptor in the liver sections (Fig. 2B). The KDEL receptor is a retrieval receptor for ER chaperones in the early secretary pathway, and over-expression of a mutant

KDEL receptor disrupted the recycling of misfolded proteins between ER and Golgi, increased the CHOP level and apoptosis, suggesting that homocysteine could induce ER stress signaling (Fig. 2B). To further confirm that homocysteine can cause ER stress, we also examined the expression of ER stress markers in vitro. We found that the expression levels of the ER chaperone GRP78, CHOP, as well as PERK, IRE1a and ATF6 branches of UPR signaling, including p-eIF2a, p-PERK and p-IRE1a were markedly enhanced in the hepatocytes treated with homocysteine, which was reduced by TUDCA (Fig.2C and D). Collectively, these results demonstrated that homocysteine induces ER stress through UPR signaling pathway in hepatocytes.

3. **PSMD10 promoted ER stress and apoptosis of hepatocytes induced by homocysteine**

Previous study has demonstrated that PSMD10 is involved in cell apoptosis through enhancing the UPR signaling pathway(13). To determine whether PSMD10 plays a role in homocysteine-induced hepatocyte apoptosis, we performed co-immunofluorescent staining of CK18 (a positive marker of hepatocytes) and PSMD10 in the liver tissues of mice. Interestingly, more PSMD10 positive cells co-localized with CK-18 in hepatocytes from *cbs*$^{+/-}$ mice (Fig.3A). Similarly, the expression of PSMD10 was remarkably increased in the liver of *cbs*$^{+/-}$ mice and the hepatocytes treated with homocysteine, respectively (Fig.3B and C). To further confirm the role of PSMD10 in regulating of ER stress-mediated apoptosis, PSMD10 was overexpressed or knocked down in hepatocytes (Fig.S1A and B). As depicted in Fig. 3D, knockdown of PSMD10 remarkably decreased the level of ER stress-associated protein, such as GRP78, p-PERK, PERK, p-eIF2α, eIF2α, IRE1a, p-IRE1a, CHOP when treated with homocysteine (Fig. 3D), while it is contrary for over-expression of PSMD10 (Fig.S2A). Similar results were also verified by western blotting examination of apoptosis-associated protein after si-PSMD10 and ad-PSMD10 were transduced into HL-7720 cells (Fig. 3E and Fig.S2B). Consistent with western blot results, the flow cytometry assay showed that the cell apoptosis was obviously reduced when PSMD10 expression was knocked down in homocysteine treated cells, whereas over-expression of PSMD10 obtained the contrary results (Fig.3F). Taken together, these data demonstrated that PSMD10 promotes ER stress and apoptosis of hepatocytes induced by homocysteine.

4. **The interaction between PSMD10 and GRP78 facilitates ER stress-mediated apoptosis**

It was reported that PSMD10 can up-regulate GRP78 expression(14). We performed coimmunoprecipitation (Co-IP) to test that whether PSMD10 and GRP78 interact with each other. The result confirmed that endogenous PSMD10 is able to immunoprecipitate GRP78, implying that PSMD10 physically interacted with GRP78 (Fig.4A). Consistent with the Co-IP results, the co-immunofluorescent staining indicated that homocysteine promoted co-localization between endogenous GRP78 and PSMD10 in the cytoplasm of hepatocytes (Fig.4B). Then, GRP78 expression was knocked down in hepatocytes to investigate its effects on homocysteine-induced hepatocytes apoptosis (Fig.S3A). Western blot showed that knockdown of GRP78 expression results in a significant decrease of the ER stress-associated protein, such as p-PERK, PERK, p-eIF2α, eIF2α, IRE1a, P-IRE1a, ATF6 and CHOP, which is accompanied by a synchronous decreased expression of apoptosis-associated protein, including cleaved

caspase 3, 12 and Bax (Fig.4C and D). Subsequently, flow cytometry analysis also observed obvious reduction of hepatocytes apoptosis after GRP78 knockdown in hepatocytes treated with homocysteine (Fig.4E). These results implied that GRP78 promotes ER stress-mediated apoptosis induced by homocysteine. We further investigated the functional relationship between PSMD10 and GRP78 in vitro by using co-transfection of ad-PSMD10 and si-GRP78 in hepatocytes and found that up-regulation of PSMD10 apparently enhances the expression of apoptosis-related proteins, which was reversed by si-GRP78 transfection in hepatocytes treated with homocysteine (Fig.4F and G). These data indicated a cooperative role of PSMD10 and GRP78 in facilitating ER stress-mediated apoptosis induced by homocysteine.

5. **Homocysteine promotes ER stress-mediated hepatocytes apoptosis though miR-212-5p targeting PSMD10**

Since miRNAs are a class of important posttranscriptional regulators, we sought to investigate whether miRNAs regulate homocysteine-induced hepatocytes apoptosis through PSMD10. Based on the TargetScan databases, a potential binding site of miR-212-5p at the 3'-UTR of PSMD10 was found (Fig. 5A). Luciferase reporter assay further illustrated that miR-212-5p mimic significantly inhibits the luciferase activity in the cells transfected with wild type (wt) 3'-UTR of PSMD10, but not in those transfected with mutant (mut) 3'-UTR of PSMD10 (Fig. 5B). These data suggested that miR-212-5p negatively regulates the expression of PSMD10 by direct binding to their putative sequences. In order to confirm the above results, we analyzed the effect of homocysteine on the miR-212-5p level. As we expected, miR-212-5p level in the liver of *cbs*$^{+/-}$ mice and the hepatocytes was inhibited after treatment with homocysteine (Fig.5C and D). Furthermore, down-regulation of PSMD10 was observed in hepatocytes transfected with miR-212-5p mimics compared to negative control, which is contrary for cells with miR-212-5p inhibitor treatment (Fig.S4A, Fig.5E and F). All of these data again indicated that miR-212-5p down-regulates PSMD10 expression. In adition, we investigated the effect of miR-212-5p on the UPR signaling and ER stress by detecting the ER stress marker of p-IRE1α, p-PERK, p-eIF2α, IRE1α, PERK, eIF2α, ATF6 and GRP78. The results revealed that over-expression and knockdown of miR-212-5p expression in homocysteine treated hepatocytes respectively inhibits or enhances ER stress-associated protein expression (Fig. 5G, and Fig. S5A). In addition, miR-212-5p mimics significantly decreased the expression of cleaved caspase3, 12 and Bax but increased Bcl2 expression (Fig. 5H), which is contrary for cells with miR-212-5p inhibitor (Fig. S5B). In line with the western blot results, flow cytometry analysis showed that miR-212-5p mimics significantly reduced hepatocytes apoptosis induced by homocysteine, whereas miR-212-5p inhibitor obtained the contrary results (Fig. 5I and Fig.S5C). Taken together, these data indicated that down-regulation of miR-212-5p promotes homocysteine-induced ER stress-mediated hepatocytes apoptosis though targeting PSMD10.

Discussion

Homocysteine in the liver is methylated to methionine by methionine synthase (requiring folate) and by betaine-homocysteine methyltransferase or converted to cystathionine by cystathionine-synthase (CBS)

(15). Dysfunction of liver alters methionine metabolism, resulting in elevated homocysteine that is released into the plasma. In return, homocysteine can influence the status of liver as well (16). Our previous study proposed that ER stress might explain the pathogenic effects of homocysteine on liver injury, while its molecular mechanism remains obscure(17). In this study, we investigated the function of PSMD10 in regulating ER stress induced by homocysteine in liver and found that homocysteine remarkably induces liver injury through apoptosis. This led us to investigate alternative mechanisms of the pro-apoptotic effect of homocysteine. Alteration of ER morphology, evidenced by changes in ER dilation, suggested that homocysteine can induce ER stress. This was further confirmed by activation of PERK, p-PERK, IRE1a, p-IRE1A and ATF6, the three major mediators of the ER stress response, as well as eif2a and p-eif2a. Activation of PERK causes phosphorylation of the eukaryotic translation initiation factor 2a and then inhibits protein synthesis. Activated IRE1 catalyzes the removal of a small intron from the XBP-1 mRNA, which triggers the induction of ER chaperones and other genes involved in ER-associated protein degradation(18,19). ATF6 and XBP-1 may combine to the ER stress response element and the UPR element, leading to GRP78 expression. Additionally, induction of CHOP, indicative of prolonged ER stress and pro-apoptotic signaling, further supported a homocysteine-induced ER stress response linked to apoptosis(20). GRP78 is the typical molecule that binds to ATF6, PERK, and IRE1 in normal condition(21). When the cells are under stress, these proteins will be separated from GRP78 and activate downstream signal pathway to initiate UPR(22). We found that homocysteine remarkably up-regulates GRP78 both in vivo and in vitro and GRP78 promoted ER stress-mediated apoptosis induced by homocysteine, which is consisitent with previous reports. Interestingly, the recent discovery that cannabidiol causes activated hepatic satellite cell death through a mechanism of ER stress-induced apoptosis(23), which further confirmed our results that homocysteine promotes ER stress-mediated apoptosis to induce liver injury.

PSMD10 is involved in diverse biological processes, such as cell growth, proliferation, apoptosis and invasion, and it contributes to oval cell-mediated liver regeneration and cell cycle progression(24). In addition to normal biological functions, emerging evidences supported that PSMD10 functions as an oncogene in many cancers including hepatocellular carcinomas, gliomas lung cancer, and colon, et al(25). Moreover, the abnormal expression of PSMD10 is related to the clinicopathological parameters of the disease, elucidating the important function of PSMD10 as a potential biomarker for different disease diagnosis and a therapeutic target of treatment. Consistent over-expression of PSMD10 promotes tumor growth and inhibits apoptosis in hepatocellular carcinomas cells by enhancing the UPR and up-regulating GRP78 expression. As expected, we found that PSMD10 promoted ER stress-mediated hepatocytes apoptosis induced by homocysteine, which is in agreement with previous report that PSMD10 augments CCl4-mediated chronic hepatic injury, inflammation and compensatory proliferation via the Rac1/JNK pathway(26). However, what contradicts with us is that PSMD10 protected hepatocellular carcinoma cells from ER stress-induced apoptosis through the enhancement of UPR signaling. A recent study reported that PSMD10 interacts with the IL-1β/IRAK-1 inflammatory signaling pathway, and thereby induces the binding of the nuclear factor (NF-Y) complex to the PSMD10 promoter, facilitates the recruitment of the E1A-binding protein p300 and CREB-binding protein, and finally increases PSMD10 expression, in

addition, PSMD10 promotes Rb phosphorylation and inactivation through interaction with cyclin-dependent kinase 4 and retinoblastoma protein (Rb), and activates the E2F transcription factor, leading to cell cycle progression(27). Considering the interaction between PSMD10 and proteins, we also investigated if PSMD10 and GRP78 interact with each other. We firstly found that PSMD10 interacts with the GRP78 leading to GRP78 up-regulation and thereby accelerates ER stress-mediated hepatic apoptosis induced by homocysteine.

Additionally, PSMD10 could be regulated by miRNA at the posttranscriptional level. Recent report provided evidence that miR-605 significantly repressed intrahepatic cholangiocarcinoma (ICC) cell proliferation and invasion by down-regulating PSMD10 through binding to the 3'UTR of PSMD10(28). Another study showed that ectopic expression of miR-214 inhibits myeloma cell growth and induces apoptosis by the inhibiting PSMD10 protein(29). Therefore, repressing PSMD10 activity may be a potential anticancer strategy. Previous studies have revealed many functions of miR-212-5p, such as tumor-promoting properties in NSCLC and proliferation-inhibition properties in gastric cancer(30,31). To the best of our knowledge, our study indicated for the first time that miR-212-5p attenuates homocysteine-induced hepatocytes apoptosis by targeting PSMD10, which is consistent with previous reports that a large number of miRNAs negatively regulate gene expression.

Conclusion

In summary, as shown in Fig. 6, we demonstrated that the miR-212-5p is down-regulated and specifically regulates the expression of PSMD10 in homocysteine-induced liver injury. Most notably, the interaction between PSMD10 and GRP78 facilitated ER stress-mediated hepatocytes apoptosis induced by homocysteine. These findings will give us a further insight into the potential of opening up novel therapeutic avenues for liver injury.

Abbreviations

Hcy homocysteine

ER endoplasmic reticulum

UPR unfolded protein reaction

GRP79 glucose-regulated protein 78

PSMD10 proteasome 26S subunit non-ATPase 10

CBS cystathionine beta-synthase

ChIP Chromatin immunoprecipitation

Co-IP co-immunoprecipitation

Declarations

Ethics approval and consent to participate

All animals received humane care in compliance with the Institutional Authority for Laboratory Animal Care of Ningxia Medical University in accordance with the Guide for the Care and Use of Laboratory Animals published by the United States National Institutes of Health.

Consent for publication

The authors declare that they have no competing interests and not applicable.

Availability of data and materials

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Funding

This work was supported by the National Natural Science Foundation of China (8206030439, 81900273,81870225, 81870332, 81760139, 81704065), Key Research and Development Projects in Ningxia Province (2020BEG03005) and Ningxia Provincial Natural Science Foundation of China (2019AAC03075).

Author's contributions

H.Z., K.X., Y.J and S.M conceived the experiment(s); H.Z., K.X., S.M, L.X, N.D, H.Z, L.X and Y.W conducted the experiment(s); H.Z., K.X., S.M, L.X, N.D, and L.X. analyzed the results; H.Z., K.X., S.M and L.X coordinated the statistical analyses, and H.Z., K.X., S.M, L.X, N.D, and L.X. verified the results together with Y.J. wrote the first version of this manuscript. All authors reviewed and approved the manuscript.

Acknowledgements

Not applicable.

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Tables

Table 1. Primer sequences for qRT-PCR analyses

Gene	GenBank	Primer sequence, 5′ to 3′
GAPDH	NM_002814.4	F: GGTGAAGGTCGGTGTGAACG
		R: CTCGCTCCTGGAAGATGGTG
PSMD10	NM_001256799.3	F: GAACTGACCAGGACAGCAGAACTG
		R: AGCAGAAGCCGCAATATGAAGAGG

Figures

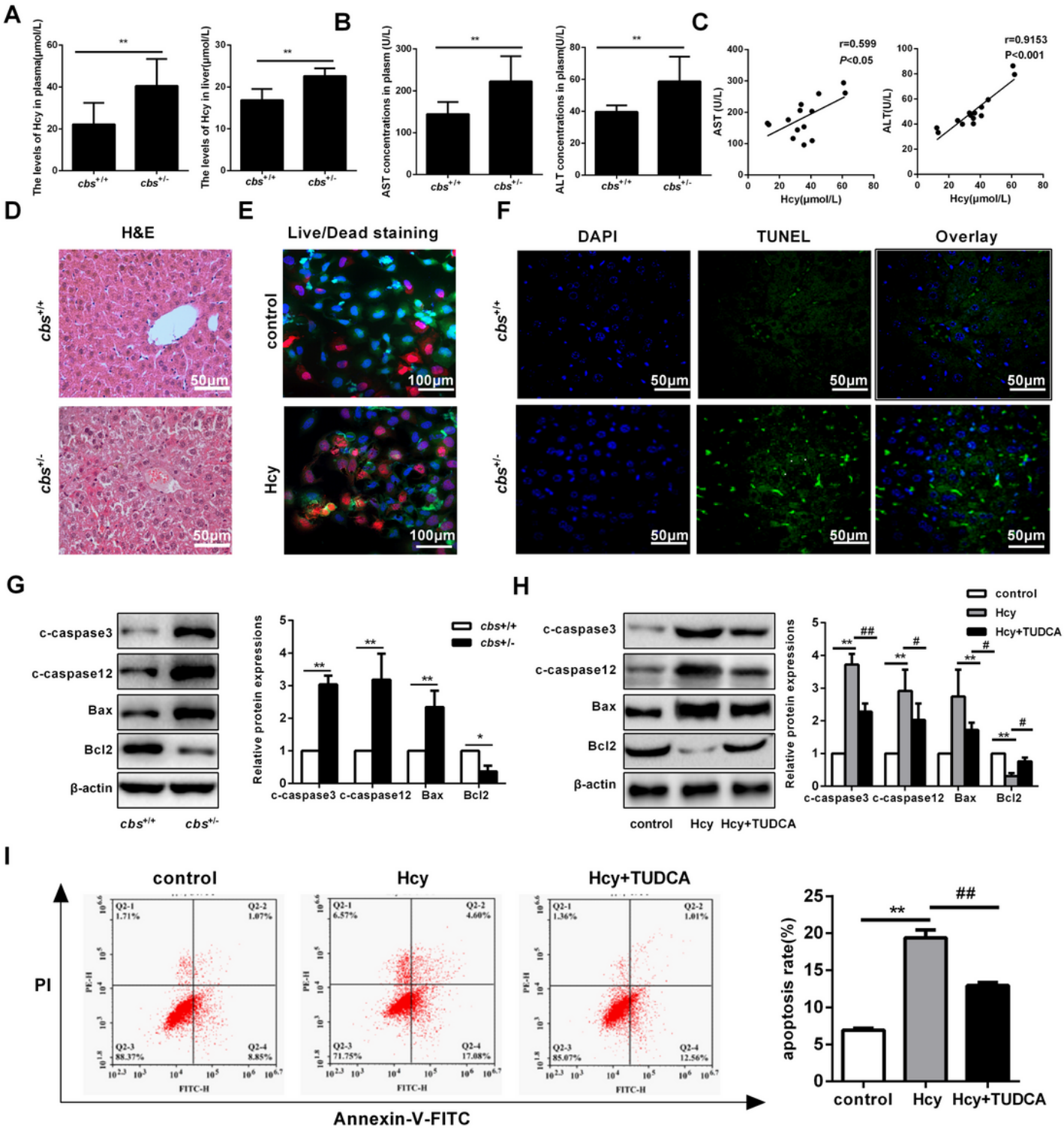

Figure 1

Homocysteine promoted apoptosis of hepatocytes leading to liver injury. (A) The levels of homocysteine in plasma and liver of cbs+/+ and cbs+/- mice were measured by automatic biochemical analysis and Elisa kit, respectively (n=8/group). (B) The levels of serum aspartate aminotransferase (AST) and alanine transaminase (ALT) in cbs+/+ and cbs+/- mice. (C) Correlation analysis of homocysteine with AST (r=0.599, P<0.05) or ALT (r=0.9153, P<0.001), respectively. (D) Representative photomicrographs of

hematoxylin & eosin (HE) staining in liver sections from cbs+/+ and cbs+/- mice (scale bars=50μm, 40×) (E) Representative immunofluorescence images of alive (green) and dead (red) hepatocytes after treatment with 100 μmol/l homocysteine (scale bars=100μm, 20×). Blue represents cell nucleus. (F) Apoptotic hepatocytes in the liver of cbs+/+ and cbs+/- mice were assessed by TUNEL staining (scale bars=50μm, 40×). (G) Representative western blot and quantification of Bax, Bcl2 and cleaved caspase3, 12 in the liver tissue of cbs+/+ and cbs+/-mice (n=8/group). (H) Representative western blot and quantification of Bax, Bcl-2 and cleaved caspase-3, 12 in hepatocytes treated with 100 μmol/L homocysteine or homocysteine + TUDCA for 48h (n=3/group). (I) Apoptosis rate of hepatocytes was measured by flow cytometry after cells were treated with 100 μmol/L homocysteine or homocysteine + TUDCA for 48h. The apoptotic indices are expressed as the number of apoptotic cells/the total number of counted cells ×100%. All data are expressed as mean ± SD. *P<0.05, **P<0.001 versus cbs+/+ or control group(without treated with 100 μmol/L homocysteine). #P<0.05, ##P<0.01 versus homocysteine group.

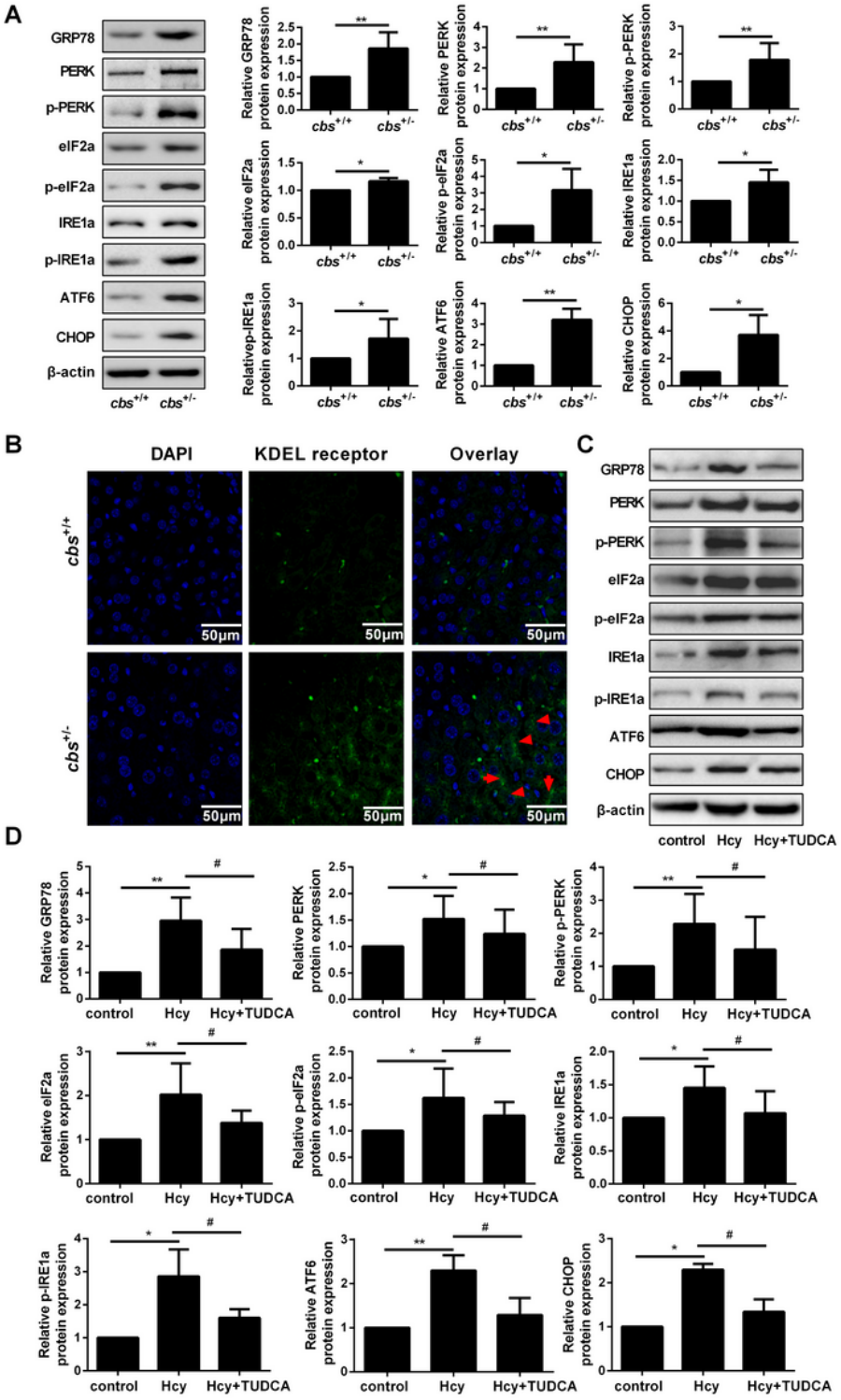

Figure 2

Homocysteine induces ER stress through UPR signaling pathway in hepatocytes. (A) Western blot analysis of ER stress signaling markers, including GRP78, ATF6, p-PERK, PERK, p-eIF2α, eIF2α, IRE1a, P-IRE1a, CHOP and β-actin in the liver of cbs+/+ and cbs+/- mice (n=8/group). (B) Representative images for immunostaining analysis of KDEL receptor-positive cells in liver sections (scale bars=20μm, 40×). (C and D) Western blot analysis of ER stress signaling markers in hepatocytes treated with 100 μmol/L

homocysteine or homocysteine + TUDCA for 48h (n=3/group). All data are expressed as mean±SD. *P<0.05, **P<0.01 versus cbs+/+ or control group. #P<0.05, ##P<0.01 versus homocysteine group.

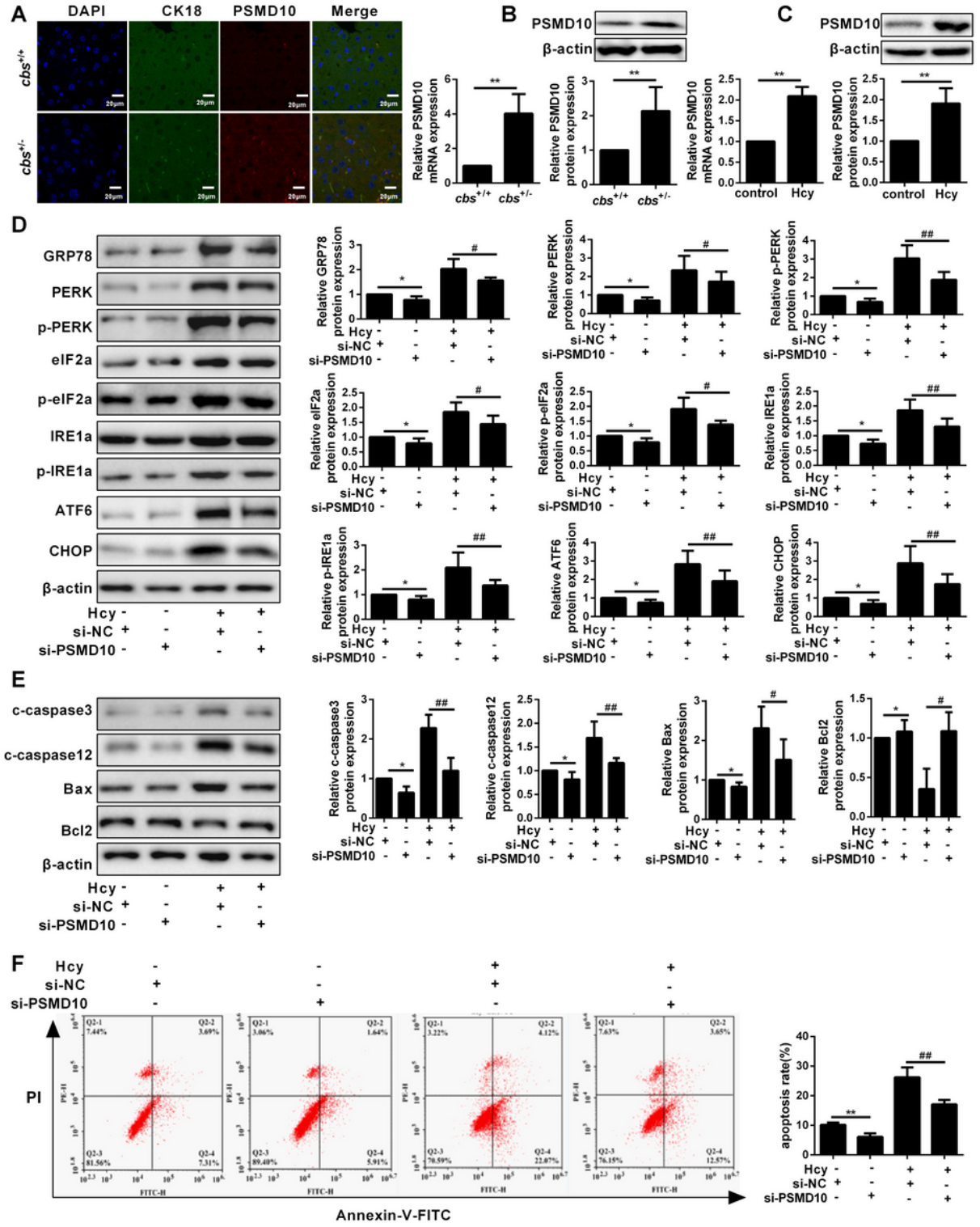

Figure 3

PSMD10 promoted hepatocytes apoptosis induced by homocysteine. (A) Co-immunofluorescent staining of PSMD10 (red) and CK18 (green, a positive marker of hepatocytes), nuclei were stained with DAPI (blue) (scale bars=20μm, 60 ×). (B, C) Western blot and qRT-PCR detected the mRNA and protein

expression of PSMD10 in the liver tissue of cbs+/−mice and the hepatocytes treated with 100 μmol/L homocysteine. (D, E) Western blot analysis of the protein expression of GRP78, p-PERK, PERK, p-eIF2aα, eIF2a, IRE1a, p-IRE1a, CHOP, cleaved caspase3,12, Bax and Bcl2 in hepatocytes after transfected with PSMD10 siRNA (si-PSMD10) in the presence of homocysteine. (F) Apoptosis of hepatocytes was analyzed by flow cytometry after transfected with si-PSMD10 in the presence of homocysteine. All data are expressed as mean ± SD. *P<0.05, **P<0.01 versus cbs+/+, control or si-NC group. #P<0.05, ##P<0.01 versus si-NC +homocysteine group.

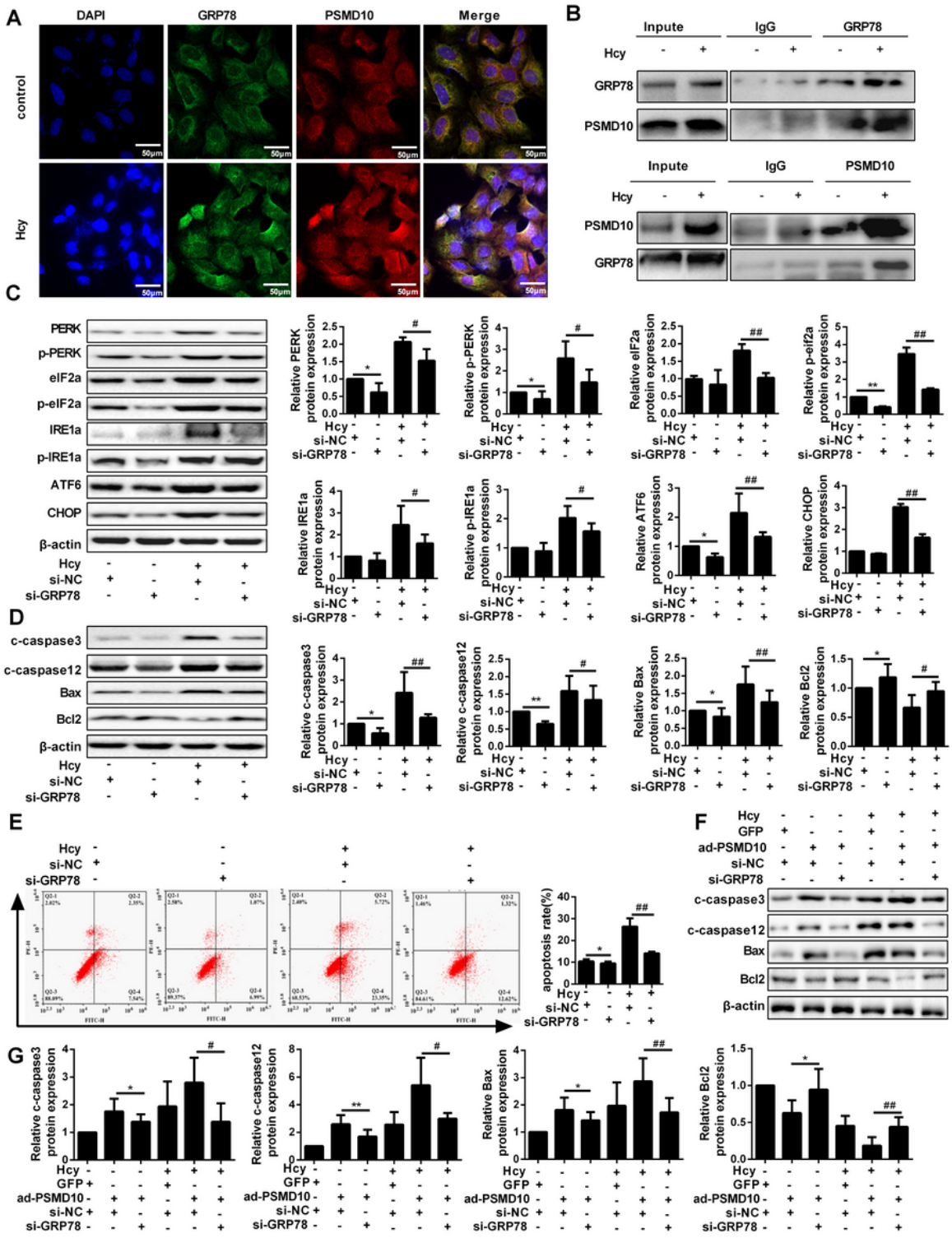

Figure 4

The cooperation between PSMD10 and GRP78 in facilitating ER stress-mediated apoptosis induced by homocysteine. (A) Co-immunofluorescent staining of GRP78 (green) and PSMD10 (red) in hepatocytes treated with 100μmol/L homocysteine was observed by laser confocal microscopy (scale bars=50μm, 40×). Nuclei were stained with DAPI (blue). (B) The interaction between PSMD10 and GRP78 were confirmed by co-immunoprecipitation (Co-IP) in hepatocytes treated with homocysteine. top, Co-IP with anti-GRP78; bottom, Co-IP with anti-PSMD10. (C)Western blot detected the expression of p-PERK, PERK, p-eIF2α, eIF2α, IRE1a, P-IRE1a and CHOP in hepatocytes transfected with si-NC and si-GRP78 in homocysteine treated cells. (D) Expression of apoptosis markers in hepatocytes after transfection with si-NC and si-GRP78. (E) Representative flow cytometry analyses of apoptosis rate after hepatocytes was transfected with si-NC and si-GRP78. (F, G) The protein levels of Bax, Bcl2, cleaved caspase 3,12 were assessed by western blot after hepatocytes were co-transfected with adenoviruses encoding PSMD10 (ad-PSMD10) and si-GRP78. All data are expressed as mean±SD. *P<0.05, **P<0.01 versus si-NC group. #P<0.05, ##P<0.01 versus si-NC +homocysteine group or ad-PSMD10+si-NC+homocysteine group.

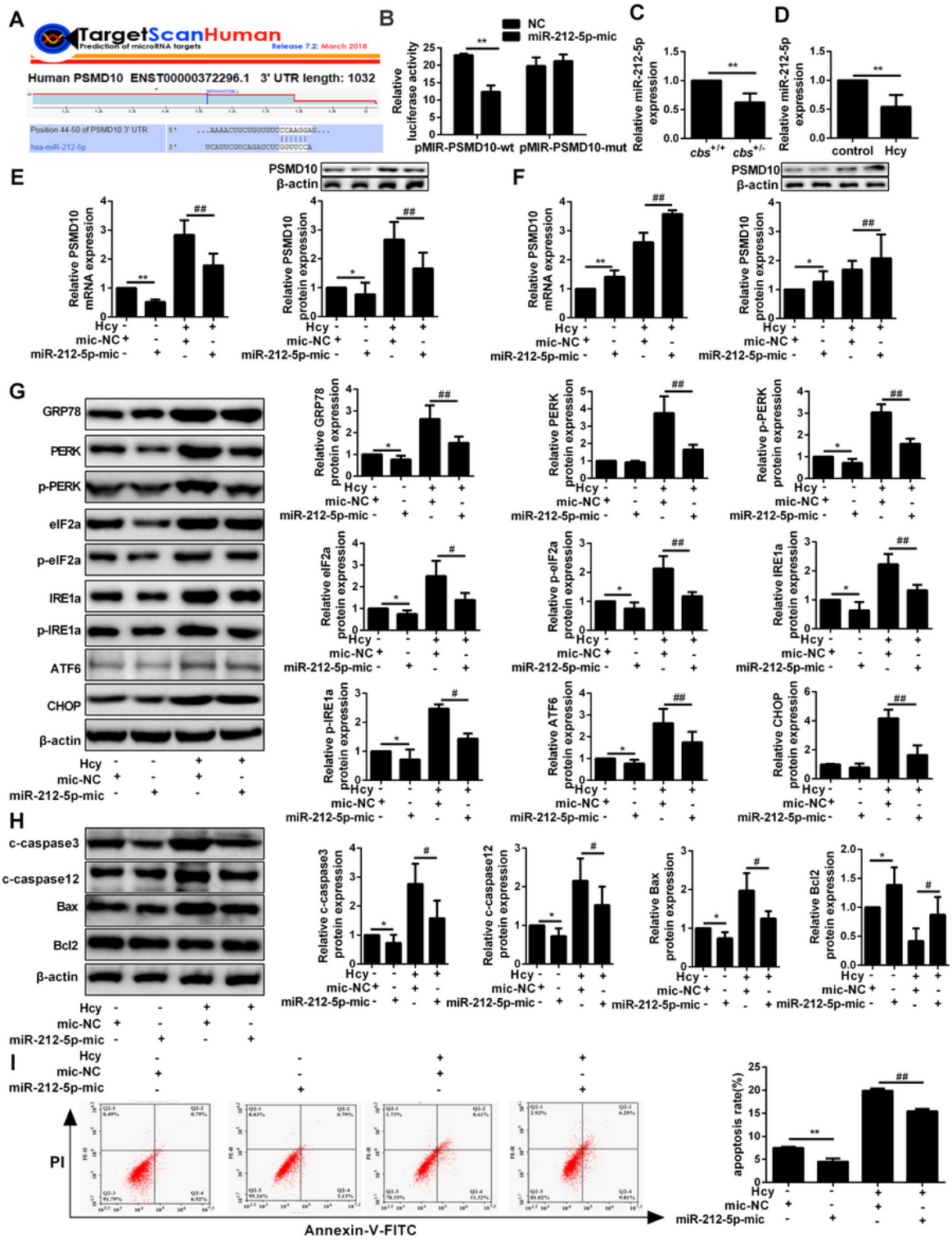

Figure 5

miR-212-5p regulates homocysteine-induced hepatocyte apoptosis via targeting PSMD10. (A) Schematic representation of binding sites of miR-212-5p in the PSMD10 3'UTR by TargetScan database (http://www.targetscan.org/vert_72/). (B)Luciferase activity of pMIR-PSMD10-wt or pMIR-PSMD10-mut reporters in the presence of miR-212-5p mimics and a negative control (N-control) (n=3/group, *P<0.05, **P<0.01). (C, D) The levels of miR-212-5p in cbs+/- mice livers and the hepatocytes treating with 100

μmol/L homocysteine were detected by qRT-PCR and normalized by U6. (E, F) qRT-PCR and western blot analysis of PSMD10 expression in hepatocytes transfected with miR-212-5p mimics, miR-212-5p inhibitor and miR-neg control. (G, H) Western blot analysis of GRP78, p-PERK, PERK, p-eIF2α, eIF2α, CHOP, ATF6, cleaved caspase3,12, Bcl2 and Bax in hepatocytes transfected with the miR-212-5p mimic under 100μmol/L homocysteine or without homocysteine treatment. (I) Representative cell apoptosis assay by flow cytometry analyses after hepatocytes transfected with the miR-212-5p mimic under 100μmol/L homocysteine or without homocysteine treatment. All data are expressed as mean ± SD. *P<0.05, **P<0.01 versus cbs+/+, control, mic-NC or inh-NC group #P<0.05, ##P<0.01 versus mic-NC +homocysteine or inh-NC +homocysteine group.

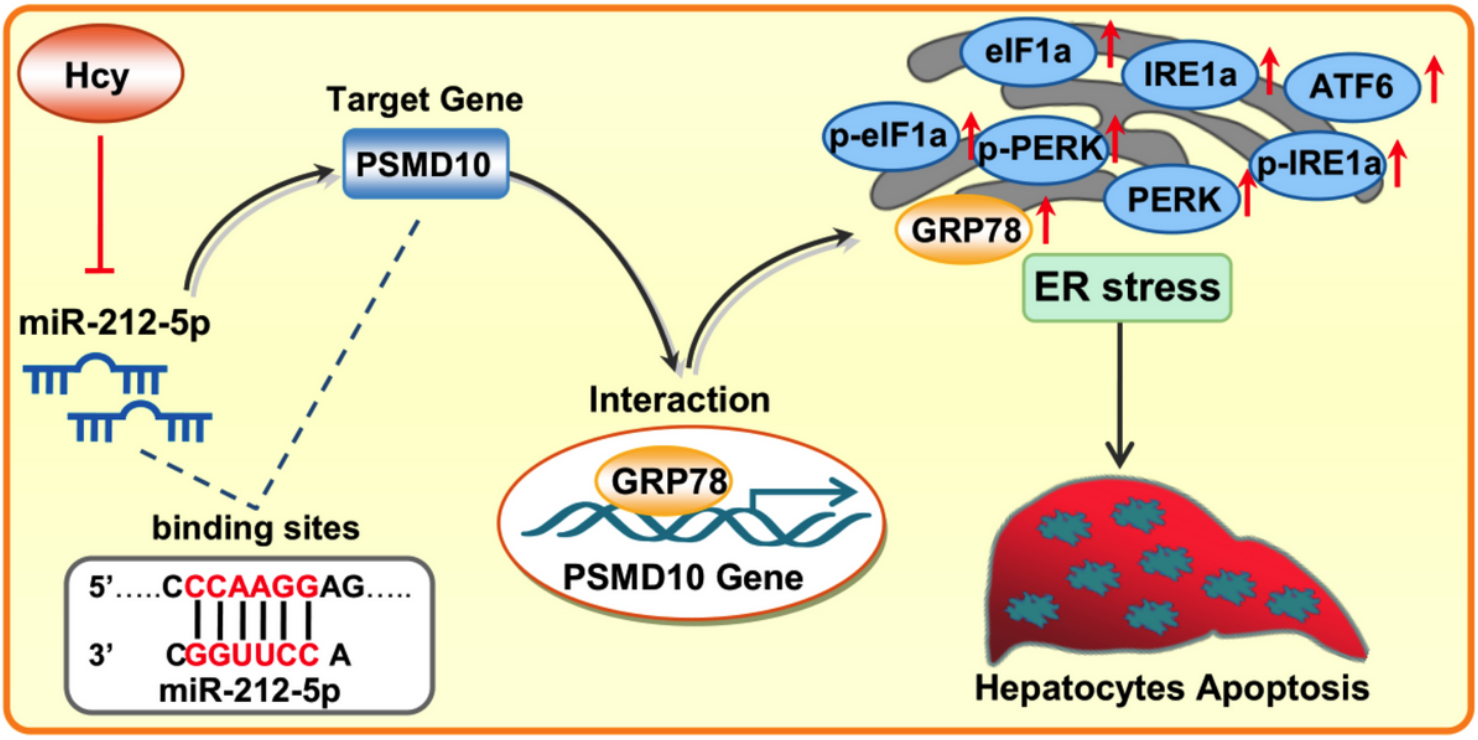

Figure 6

A proposed model of miRNA expression regulation in HHcy-induced liver injury. miR-212-5p down-regulated and specifically regulates the expression of PSMD10 in homocysteine-induced liver injury, which attributed to the interaction between PSMD10 and GRP78 facilitated ER stress-mediated hepatocytes apoptosis.

Supplementary Files

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