Hepatic HRC induces hepatocyte pyroptosis and HSCs activation via NLRP3/caspase-1 pathway

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Abstract

The histidine-rich calcium-binding protein (HRC) is a regulator of Ca^{2+} homeostasis and it plays a significant role in liver fibrosis. Pyroptosis, a specific inflammatory cell death, can lead to HSCs activation and liver fibrosis. However, the role of HRC in pyroptosis has not been explored. In this study, we demonstrated that HRC, mainly located in the hepatocyte, was overexpressed in fibrotic liver tissues. We further found that enforced expression of HRC in hepatocytes induced pyroptosis and HMGB1 release, and subsequently led to HSCs activation by NLRP3/caspase-1 pathway. In addition, the proliferation and migration of HSCs were also enhanced by HRC overexpression in hepatocytes. Furthermore, NLRP3 inhibitor MCC950 and caspase-1 inhibitor VX-765 alleviated hepatic HRC-mediated hepatocytes pyroptosis and HSCs activation. This study demonstrated that hepatic HRC promoted HSCs activation by inducing hepatocyte pyroptosis, which suggests HRC may be a promising therapeutic target to prevent liver fibrosis.

1 Introduction

Liver fibrosis is characterized as a wound-healing response to continuous liver injury, which progressively develops into cirrhosis or hepatocellular carcinoma[1]. Activated hepatic stellate cells (HSCs) are a pivotal participant in promoting the synthesis and deposition of extracellular matrix (ECM) protein during liver fibrosis[2]. HSCs activation depends on intricate crosstalk with parenchymal or mesenchymal cells and their released damage-associated molecular patterns (DAMPs)[3]. Numerous studies that focus on the development and progression of liver fibrogenesis have been identified in the past. At present, there is no effective intervention and treatment for progressive fibrosis.

Emerging evidence has shed light on the vital pro-fibrotic roles of hepatocyte death[4]. Pyroptosis, a novel programmed and inflammatory cell death, is characterized as cell swelling, pore formation of the plasma membrane, and subsequently the release of pro-inflammatory contents[5]. Pyroptosis is initially defined as “caspase-1 dependent necrosis”. However, recent studies show that gasdermin D (GSDMD) is the sole executor of pyroptosis, and the expression of N-terminal fragment of GSDMD (GSDMD-N) is sufficient to induce pyroptosis without caspase activation[6]. Pyroptosis occurs in many cell types, such as the monocytic lineage, hepatocytes, HSCs, eosinophils and iNKT cells[7–11]. Recently, there is convincing evidence that hepatocyte pyroptosis plays a pivotal role in the progression of liver diseases, including liver fibrosis.

The histidine-rich calcium-binding protein (HRC) is a regulator of Ca^{2+} homeostasis[12]. Calcium mobilization is reported to be a critical upstream event in the activation of the NLRP3 inflammasome[13]. NLRP3 activation governs the cleavage and activation of caspase-1, which is responsible for hepatocyte pyroptosis and cytokines secretion, including IL-1 β , IL-18 and high mobility group box 1 (HMGB1)[8, 14]. Furthermore, HSCs can be activated either by IL-1 β or IL-18 to induce ECM deposition[15]. Recent studies have shown that HMGB1, a prototypical DAMP molecule, can also induce HSCs activation and liver fibrosis[16, 17]. Our previous study showed that HRC promoted the progression of liver fibrosis[18].

However, the specific contribution of HRC in hepatocytes to liver fibrosis and the molecular mechanisms involved in this process remain incompletely understood.

In this study, we demonstrated that hepatic HRC expression was upregulated during liver fibrosis. Moreover, hepatic HRC induced hepatocyte pyroptosis and HMGB1 release, which in turn, promoted the activation, proliferation and migration of HSCs through the NLRP3/caspase-1 pathway.

2 Materials And Methods

Cells and reagents

The L02 (human hepatic cell line) and LX-2 (human hepatic stellate cell line) were acquired from the Institute of Liver and Gastrointestinal Diseases, HUST, and cultured in DMEM with 10% FBS.

Human sample

The human sample (n = 38) were divided into four groups according to the severity of liver fibrosis, including S0 (n = 9), S1 (n = 10), S2 (n = 10), S3 (n = 9), which were diagnosed by the pathologists at Tongji Hospital of Tongji Medical College, Huazhong University of Science and Technology. The study acquired approval from the Ethics Committee of Tongji Hospital, and all procedures involving human participates were performed after obtaining their informed consent.

CCl4-induced fibrosis model

Mice fibrotic model: mice (n = 6 per group) were randomly divided into 3 groups: vehicle group, CCl₄-injected 4w group, and CCl₄-injected 8w group. To establish the hepatic fibrosis model, mice were intraperitoneally injected with 25% CCl₄ solved in olive oil or saline (twice a week). Several weeks' injections were made to establish fibrosis with different degrees of severity. All experimental procedures were conducted under the approval of The Institution Ethics Committee of Tongji Medical College, Huazhong University of Science and Technology.

Immunohistochemistry

The sample was used 4% paraformaldehyde for fixation, and paraffin was applied for embedding. The Hematoxylin and Eosin (H&E), Masson, and Sirius Red stain were performed to determine the degree of liver fibrosis. HRC (HPA004833, ATLAS ANTIBODIES), α -SMA (Ab124961, Abcam) were used for immunohistochemistry.

Enzyme-linked Immunosorbent Assay (ELISA)

Cellular supernatant IL-1 β , IL-18, IL-6, TNF- α , and HMGB1 levels were detected by enzyme-linked immunosorbent assay (ELISA) kit. Serum alanine transaminase (ALT), aspartate transaminase (AST) levels of mice were detected by kits (Nanjing Jiancheng Bioengineering Institute, China) according to the manufactures' instructions.

RNA extraction and qRT-PCR

Total RNA was extracted using TRIzol reagent (Invitrogen, CA, USA), and cDNA was synthesized using the PrimeScript RT reagent kit (Abclonal, China). The real-time polymerase chain reaction was performed using SYBR Premix ExTaq (Aclonal, China) on an ABI QuantaStudio Real-Time System (Applied Biosystem, Carlsbad, CA, USA). The sequences of the primers used for PCR are listed in Table 1.

Table 1
primer sequences used for this study

Targeted genes	Forward primer sequences (5'-3')	Reverse primer sequences (3'-5')
hHRC	TAGGCTTCAGAAACCGGAACA	AGAAATGATGCCCATTCTCTGTG
hNLRP1	CCACAACCCTCTGTCTACATTAC	GCCCCATCTAACCCATGCTTC
hNLRP3	GATCTTCGCTGCGATCAACAG	CGTGCATTATCTGAACCCAC
hNLRP4	TGCATCATTGAAGGGGAATCTG	GATTGTGCCAGGTATATCCAGG
hGSDMD	GTGTGTCAACCTGTCTATCAAGG	CATGGCATCGTAGAAGTGGAAG
hCaspase-1	CCGAAGGTGATCATCATCCA	ATAGCATCATCCTCAAACCTTTCTG
hIL-1 β	AGCTACGAATCTCCGACCAC	CGTTATCCCATGTGTCTGAAGAA
hHMGB1	TATGGCAAAAGCGGACAAGG	CTTCGCAACATCACCAATGGA
h α -SMA	AAAAGACAGCTACGTGGGTGA	GCCATGTTCTATCGGGTACTTC
hCOL1A1	GAGGGCCAAGACGAAGACATC	CAGATCACGTCATCGCACAAC
hCCNB1	AATAAGGCGAAGATCAACATGGC	TTTGTTACCAATGTCCCCAAGAG
hCCND1	GCTGCGAAGTGGAACCATC	CCTCCTTCTGCACACATTTGAA
hMMP2	CCAGATGTGGCCAACTACAA	GGTCAGGTGTGTAACCAATGA
hMMP9	CAGTACCGAGAGAAAGCCTATT	CAGGATGTCATAGGTCACGTAG
hGAPDH	TCATCTCCGACTTTGTGGTCTT	GAAGATGGTGATGGGATTTC
mHRC	CAACCGATGTACCGAATGTGA	GGTAGCAGAATTGACAGTGCTG
m α -SMA	GCGTGGCTATTCTTCGTGACTAC	CGTCAGGCAGTTCGTAGCTCTTC
mGAPDH	AGGTCGGTGTGAACGGATTTG	TGTAGACCATGTAGTTGAGGTCA

Western blotting

The cells or liver tissues were extracted using RIPA lysis buffer containing phosphatase inhibitor and PMSF. The proteins were transferred to PVDF membranes. The membranes were blocked with 5% Skim milk at room temperature for 1h and subsequently were incubated with primary antibodies against HRC,

NLRP3, caspase-1, GSDMD, IL-1 β , HMGB1, and GAPDH overnight at 4°C. Then followed by anti-mouse or -rabbit IgG, and the signals were detected with an ECL kit.

Conditioned medium

L02 cells were infected with lenti-HRC or lenti-Vector for 24h, and then the cells were cultured with 1ng/ml puromycin. Infected cells were seeded onto 6-well plates and cultured for 24h in DMEM containing 10% FBS. Supernatant from infected cells was centrifuged at 1500 $\times$ g for 15min, and the resulting supernatant was transferred to a new tube and stored at -80°C.

Cell counting kit-8 (CCK-8) assay

CCK-8 assay was performed to detect cell proliferation capability. The LX-2 cell line was digested and re-seeded in 96-well plates (2000 cells per well) after incubated with a conditioned medium 24h. the culture medium of each well was replaced with a 100ul mixed medium containing 10% CCK-8. The absorbance value was assessed at 450nm wavelength.

Transwell assay

Transwell assay was performed to value HSC migration. Briefly, 3 $\times$ 10⁴ cells were resuspended in 0.2ml DMEM (serum-free) and then plated in the upper chamber. 0.6ml DMEM containing 10% FBS was added to the lower chamber. After 24h, cells that migrated through the membrane to the lower surface were fixed with 4% paraformaldehyde, stained with crystal violet. Then, photos were taken by the microscope, and numbers were counted.

Lactate dehydrogenase (LDH) assay

The LDH level of culture supernatant was assessed by the LDH cytotoxicity assay kit (Beyotime, Shanghai, China) according to the manufacturer's instructions.

Statistical analysis

Data are presented as means $\pm$ SD. Student's t-test was performed to assess the significance of differences between the two groups. P-value $\leq$ 0.05 was considered statistically significant.

3 Results

Hepatic HRC expression is gradually increased in liver fibrosis

The advanced clinical stage of liver fibrosis is accompanied by IHC analysis for high expression of α -SMA. The results also showed that HRC expression, which is mainly located in hepatocytes, gradually increased along with the severity of liver fibrosis (Fig. 1A). Furthermore, we established the CCl₄-induced fibrosis model, confirmed by serum ALT/AST, hematoxylin/ eosin (H&E) staining, Masson's trichrome staining and Sirius red staining (Fig. 1B, 1C). Similarly, IHC staining revealed HRC positive staining is also

mostly localized in hepatocytes, and we observed higher expression of α -SMA and HRC in fibrotic mice (Fig. 1D). In addition, the mRNA and protein levels of α -SMA and HRC in fibrotic mice were both significantly elevated compared to normal (Fig. 1E). Collectively, our data revealed that the expression of HRC in liver tissue was positively correlated with the degree of liver fibrosis.

Hepatic HRC triggers hepatocyte pyroptosis and hmgb1 secretion

To explore the role of HRC in pyroptosis *in vitro*, we established an HRC over-expression cell model via transfection lenti-HRC or lenti-Vector to L02, which was confirmed by qRT-PCR and western blot (Fig. 2A). We firstly measured the LDH level of supernatant by LDH assay. The result showed that the LDH level of L02-HRC was significantly elevated than control (Fig. 2B). Then, we detected the frequencies of caspase-1/PI double-positive by flow cytometry and observed a significant increase in the number of pyroptotic hepatocytes compared to the control (Fig. 2C). We verified the significant upregulation of HRC in L02-HRC cells compared to L02-Vector cells (Fig. 2D). In addition, the results also showed that HRC overexpression in hepatocytes markedly increased the protein level of GSDMD-N, which is the sole executor of pyroptosis (Fig. 2E). Recent data have indicated that pyroptosis induces the release of pro-inflammatory contents [19, 20]. We further detected the level of IL-1 β , IL-6, IL-18, TNF- α and HMGB1 in the supernatant of generated L02 cells. Our result showed that the HMGB1 level was significantly higher in L02-HRC compared with the control, but there were no significant changes of other cytokines (Fig. 2F). As expected, the protein level of HMGB1 was significantly upregulated in L02-HRC compared with the control cells (Fig. 2G). Taken together, these findings indicated that hepatic HRC could induce hepatocyte pyroptosis and HMGB1 release.

Hepatocyte pyroptosis promotes HSCs activation, proliferation and migration

Activation of HSCs is the most critical participant in response to accumulated levels of inflammatory signals derived from the damaged hepatocyte in the development of liver fibrosis [2, 21]. To determine the relation between HRC-induced hepatocyte pyroptosis and HSCs activation, we collected conditioned medium (CM) from L02-HRC (L02-HRC-CM) and control cells (L02-Vector-CM) to treat LX-2 cells. As seen, treatment with the CM from L02-HRC cells significantly elevated α -SMA and COL1A1 expression in LX-2 cells compared with the treatment of L02-Vector-CM (Fig. 3A). Next, flow cytometry analysis revealed that L02-HRC-CM did not affect the apoptosis of LX-2 cells (Fig. 3B). Then cell proliferation of LX-2 cells was assessed by CCK-8 and Edu assay. The results showed that L02-HRC-CM promoted proliferation of LX-2 cells compared to control (Fig. 3C, 3D), accompanied by an increase in the mRNA and protein levels of cyclin B1 (CCNB1) and cyclin D1 (CCND1) (Fig. 3E), which are markers of cell proliferation. In addition, Transwell assay showed that L02-HRC-CM also enhanced the migration of LX-2 cells (Fig. 3F), accompanied by increased expression of metalloproteinase 2 (MMP2) and metalloproteinase 9 (MMP9) (Fig. 3G).

Hepatic HRC induces hepatocyte pyroptosis via NLRP3/caspase-1 pathway

Next, we made a further investigation to explore the underlying mechanism of HRC-mediated hepatocyte pyroptosis. Inflammasome activation is a crucial member in triggering pyroptosis, and NLRP3/caspase-1 pathway plays an important role in hepatocytes pyroptosis[22, 23]. Consistent with our expectation, the expression of NLRP3 and caspase-1, but not NLRP1 and NLRC4, were significantly increased in L02-HRC cells (Fig. 4A). Moreover, caspase-1 activity was significantly increased in L02-HRC cells compared with control (Fig. 4B). To clarify the effect of the NLRP3/caspase-1 pathway on HRC-mediated pyroptosis. L02-HRC and control cells were pre-treated with NLRP3 inhibitor MCC950 or caspase-1 inhibitor VX-765 for 24h. Then the results revealed that inhibition of NLRP3/caspase-1 significantly decreased the level of supernatant LDH and proportion of caspase-1/PI double-positive staining of L02-HRC compared with control cells (Fig. 4C, 4D). Furthermore, increased expression of NLRP3, caspase-1, GSDMD, and HMGB1 induced by HRC was suppressed in L02-HRC cells pretreated with MCC950 or VX-765 (Fig. 4E). HMGB1 release from L02-HRC cells was abolished by MCC950 or VX-765 pretreatment compared to control (Fig. 4F).

Inhibition of NLRP3/caspase-1 abolished the pro-fibrotic properties of HSCs mediated by hepatic HRC

In addition, we explored whether the pro-fibrotic properties of HSCs induced by hepatic HRC could be abolished by inhibition of NLRP3/caspase-1. The results showed the expression of α -SMA and COL1A1 were not markedly changed in LX-2 cells incubated with CM from L02-HRC (L02-HRC-CM) plus MCC950 (Fig. 5A). CCK-8, Edu and Transwell assay showed that the enhanced proliferation and migration ability of LX-2 cells in L02-HRC-CM were repressed by MCC950 pretreatment (Fig. 5B, 5C, 5D). As expected, inhibition of caspase-1 (VX-765) also abolished the effect of CM from L02-HRC on the activation, proliferation and migration of LX-2 cells compared with the control group (Fig. 5E, 5F, 5G, 5H). These data suggest that hepatic HRC-induced HSCs activation, proliferation and migration were abolished by inhibiting the NLRP3/caspase-1 pathway.

4 Discussion

Increasing evidence has demonstrated that hepatocyte death is an initial driver of hepatic fibrogenesis[24, 25]. Pyroptosis, a novel programmed cell death, plays a pivotal role in the progression of liver fibrosis[26]. Our previous study showed that HRC was involved in the activation of HSCs during liver fibrosis, whether HRC participated in pyroptosis remains unknown. This study demonstrated that the expression of hepatic HRC was gradually upregulated in fibrotic liver tissues. Furthermore, we uncovered an important role of HRC in hepatocyte pyroptosis, which in turn induced HMGB1 secretion, thereby leading to HSCs activation, proliferation and migration *in vitro*. Mechanistic studies revealed that NLRP3/caspase-1 pathway was involved in hepatic HRC-mediated hepatocytes pyroptosis and pro-fibrotic properties of HSCs. Collectively, we clarify the role of HRC in the cross-talk between hepatocytes and HSCs in liver fibrosis.

Pyroptosis is an inflammatory process of caspase-1 or caspase 4/5/11-dependent programmed cell death, which has been reported to be closely associated with many diseases, including liver fibrosis[27].

GSDMD, identified as the main effector in pyroptosis, is a direct substrate of caspase-1/4/5/11 which can be specifically cleaved by inflammatory caspase. The N-terminal fragment of GSDMD releases and forms the pores in the plasma membrane, generating cell swelling and plasma membrane lysis, resulting in the secretion of pro-inflammatory contents, including IL-1 β , IL-18 and HMGB1[6, 28]. Our experiments showed that HRC induced hepatocyte pyroptosis because overexpression of HRC in hepatocytes increased the expression of GSDMD-N and LDH. A recent study shows that hepatocyte pyroptosis and the release of inflammasome particles can induce HSCs activation and liver fibrosis[29]. Moreover, HMGB1, a non-histone nuclear protein, which can be actively secreted by hepatocytes in response to pyroptosis, is also involved in the pathogenesis of liver fibrosis[30–32]. In line with these researches, our study demonstrated that the release of HMGB1, rather than IL-1 β or IL-18, was increased by hepatic HRC-mediated hepatocyte pyroptosis, and thereby enhancing HSCs activation, proliferation and migration.

Inflammasomes are multimolecular complexes that can trigger pyroptosis by stimulating inflammatory responses. Canonical inflammasomes, including Nod-like receptors (NLRP1, NLRP3, NLRC4) and AIM2, activate caspase-1 after linking microbial and endogenous “danger” signals[33]. NLRP3 inflammasome is the best-characterized inflammasome, which comprises the NLR protein NLRP3, the adapter ASC and the pro-caspase-1. Once activated, NLRP3 acts as a platform to trigger the activation of caspase-1, which is involved in the maturation of the pro-IL-18 and pro-IL-1 β [34]. Moreover, the activation of caspase-1 can also form plasma-membrane pores of 1.1–2.4 nm, which are associated with pyroptosis and the secretion of the pro-inflammatory mediators HMGB1[35]. Furthermore, recent research suggests that NLRP1 and NLRC4 inflammasomes also participated in pyroptosis[36, 37]. Here we showed that overexpression of HRC in hepatocytes led to activation of NLRP3 and caspase-1, and pre-treated with NLRP3 inflammasome inhibitor MCC950 or caspase-1 inhibitor VX-765 abrogated hepatic HRC-mediated hepatocyte pyroptosis, HMGB1 secretion and HSCs activation. These results indicate that NLRP3/caspase-1 pathway is responsible for hepatic HRC-induced liver fibrosis.

In conclusion, we demonstrated that hepatic HRC promotes paracrine activation of HSCs by inducing hepatocyte pyroptosis via NLRP3/caspase-1/HMGB1 pathway in liver fibrosis *in vitro* (Fig. 6). Inhibition of HRC may be a promising relevance to against or slow down the progression of fibrogenesis. Unfortunately, the present study lacks *in vivo* experiments, which should be carried out in further research.

Abbreviations

HRC: histidine-rich calcium-binding protein; α -SMA: α -smooth actin; CCl₄: carbon tetrachloride; ALT: aminotransferase; AST: aspartic aminotransferase; LDH: lactate dehydrogenase; H&E: hematoxylin-eosin; IHC: immunohistochemistry; GSDMD: gasdermin D; GSDMD-N: N-terminal fragment of GSDMD; NLRP3: nod-like receptor protein 3; IL-1 β : interleukin-1 β ; IL-18: interleukin-18; HMGB1: high mobility group box 1.

Declarations

Ethics Statement

The animal study was reviewed and approved by The Institution Ethics Committee of Tongji Medical College, Huazhong University of Science and Technology. This study was performed according to the principles of the Declaration of Helsinki and was approved by the Ethics Committee of Tongji Hospital, Huazhong University of Science and Technology following the ethical and institutional guidelines.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author Contributions

JM. Liu and DA. Tian designed the study. JW. Wu, SH. Xia and WD. Zhou performed the experiments. P. Han, K. Zhao and MY. Zhang analyzed the data. JW. Wu, JM. Liu and KXP prepared the manuscript. JM. Liu and JZ. Liao provide financial support. All authors contributed to the article and approved the submitted version.

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Data Availability Statement

The data used to support the findings of this study are available from the corresponding author upon request.

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Figures

Figure 1

Hepatic HRC is gradually increased in the fibrotic liver

(A) Representative immunohistochemical (IHC) analysis shows that the expression of α -SMA and HRC in human normal and fibrotic liver tissue. S0 refers to no inflammation and fibrosis of the liver, S1 refers to fibrosis around the hepatic sinus or scar of the lobule, S2 refers to the formation of fibrosis septum, S3 refers to numerous septa forming separation and destroying liver lobules. Scale bars: 100 μ m (100 $\times$), 50 μ m (200 $\times$). **(B)** The fibrosis model was assessed by the detection of serum alanine transaminase (ALT) and aspartate transaminase (AST). **(C)** Established CCl₄-induced fibrosis model was assessed by representative H&E staining, Masson's trichrome staining and Sirius staining in liver tissues. **(D)** Representative IHC stain for α -SMA and HRC in the CCl₄-induced fibrosis model. Scale bars: 100 μ m (100 $\times$), 50 μ m (200 $\times$). 4w represents mice treated with CCl₄ for 4 weeks. 8w represents mice treated with CCl₄ for 8 weeks. **(E)** The expression of α -SMA and HRC was assessed by qRT-PCR and western blot in liver tissues from the CCl₄-induced fibrosis model. Significance * P < 0.05, ** P < 0.01, *** P < 0.001.

Figure 2

HRC triggers hepatocyte pyroptosis and releases HMGB1

(A) Generated L02-HRC was confirmed by qRT-PCR and western blot. **(B)** The LDH level of supernatant was assessed by LDH assay. **(C)** The proportion of caspase-1/PI double-positive cells was assessed by

flow cytometry. **(D)** The expression of HRC, GSDMD, IL-1 β was determined in L02 cells transfected with lenti-HRC or lenti-Vector by qRT-PCR. **(E)** The expression of HRC, GSDMD, IL-1 β was determined in L02 cells transfected with lenti-HRC or lenti-Vector by western blot. **(F)** Cytokines and HMGB1 level of cell supernatant were assessed by ELISA. **(G)** The expression of HMGB1 was assessed by qRT-PCR and western blot in L02-Vector and L02-HRC cells. Significance * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$.

Figure 3

The supernatant of L02-HRC promotes activation, proliferation and migration of LX-2 cells

(A) The expression of markers of HSC activation was assessed by qRT-PCR and western blot in LX-2 incubated with conditioned medium from L02-Vector/L02-HRC. **(B)** The apoptosis of cocultured LX-2 cells was assessed by flow cytometry. **(C)** CCK-8 assay showed the proliferation of cocultured LX-2 cells. **(D)** Edu assay showed the proliferation of cocultured LX-2 cells. **(E)** The expression of markers of proliferation was assessed by qRT-PCR and western blot in LX-2 cells incubated with conditioned medium from L02-Vector/L02-HRC. **(F)** The migration of cocultured LX-2 cells was assessed by Transwell assay. **(G)** The expression of markers of migration was assessed by qRT-PCR and western blot in cocultured LX-2 cells. Significance * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$.

Figure 4

NLRP3/caspase-1 pathway is responsible for hepatocyte pyroptosis induced by hepatic HRC

(A) The expression of NLRP1, NLRP3, NLRC4 and caspase-1 were assessed by qRT-PCR and western blot. **(B)** The activity of caspase-1 was assessed by caspase activity detection kit. **(C)** After MCC950 or VX-765 pretreatment for 24h, the LDH level of supernatant was assessed by LDH assay. **(D)** The proportion of caspase-1/PI double-positive cells was assessed by flow cytometry. **(E)** The expression of the pyroptotic-associated protein was assessed by western blot. **(F)** The HMGB1 level of supernatant was assessed by ELISA. Significance * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$.

Figure 5

Inhibition of NLRP3/caspase-1 abolished hepatic HRC-induced pro-fibrotic properties of HSCs

(A) The expression of α -SMA and COL1A1 were assessed by western blot in LX-2 cells incubated with L02-HRC+MCC950-CM. **(B)** The proliferation of LX-2 cells incubated with L02-HRC+MCC950-CM was assessed by CCK-8. **(C)** The proliferation of LX-2 cells incubated with L02-HRC+MCC950-CM was

assessed by Edu assay. **(D)** The migration of LX-2 cells incubated with L02-HRC+MCC950-CM was assessed by Transwell assay. **(E)** The expression of α -SMA and COL1A1 were assessed by western blot in LX-2 cells incubated with L02-HRC+VX-765-CM. **(F)** The proliferation of LX-2 cells incubated with L02-HRC+VX-765-CM was assessed by CCK-8. **(G)** The proliferation of LX-2 cells incubated with L02-HRC+VX-765-CM was assessed by Edu assay. **(H)** The migration of LX-2 cells incubated with L02-HRC+VX-765-CM was assessed by Transwell assay. Significance * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$.

Figure 6

The schematic illustration demonstrates that the role of HRC in hepatocyte pyroptosis

Hepatic HRC triggers hepatocyte pyroptosis and releases HMGB1, which promotes the pro-fibrotic properties of HSC by the NLRP3/caspase-1 pathway.