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Original Article

Osteopontin Promotes Macrophage M1 Polarization by Activation of the JAK1/STAT1/HMGB1 Signaling Pathway in Nonalcoholic Fatty Liver Disease

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Abstract

Background and Aims: Osteopontin (OPN) is reported to be associated with the pathogenesis of nonalcoholic fatty liver disease (NAFLD). However, the function of OPN in NAFLD is still inconclusive. Therefore, our aim in this study was to evaluate the role of OPN in NAFLD and clarify the involved mechanisms. ***Methods:*** We analyzed the expression change of OPN in NAFLD by bioinformatic analysis, qRT-PCR, western blotting and immunofluorescence staining. To clarify the role of OPN in NAFLD, the effect of OPN from HepG2 cells on macrophage polarization and the involved mechanisms were examined by FACS and western blotting. ***Results:*** *OPN* was significantly upregulated in NAFLD patients compared with normal volunteers by microarray data, and the high expression of OPN was related with disease stage and progression. OPN level was also significantly increased in liver tissue samples of NAFLD from human and mouse, and in HepG2 cells treated with oleic acid (OA). Furthermore, the supernatants of OPN-treated HepG2 cells promoted the macrophage M1 polarization. Mechanistically, OPN activated the janus kinase 1(JAK1)/signal transducers and activators of transcription 1 (STAT1) signaling pathway in HepG2 cells, and consequently HepG2 cells secreted more high-mobility group box 1 (HMGB1), thereby promoting macrophage M1 polarization. ***Conclusions:*** OPN promoted macrophage M1 polarization by increasing JAK1/STAT1-induced HMGB1 secretion in hepatocytes.

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Keywords: OPN; NAFLD; Macrophage M1 polarization; HMGB1; JAK1/STAT1 signaling.

Abbreviations: Alb, Albumin; BMDMs, bone marrow-derived macrophages; DEGs, differentially expressed genes; FACS, fluorescence-activated cell sorting assay; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GEO, Gene Expression Omnibus; HFD, high-fat diet; HMGB1, high-mobility group box 1; HE, Hematoxylin and eosin; IL, interleukin; IFNγ, interferon γ; JAK1, janus kinase 1; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; NAS, NAFLD activity score; ND, normal diet; OA, oleic acid; OPN, osteopontin; qRT-PCR, quantitative real-time polymerase chain reaction; ROS, reactive oxygen species; STAT1, signal transducers and activators of transcription 1; TC, total cholesterol; TG, triglyceride; TLR, toll like receptor.

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Introduction

NAFLD is now considered as the most frequent reason of liver disease worldwide.[1,2] It is characterized as pathological accumulation of triglycerides (TGs) in liver cells without heavy drinking. Moreover, on account of other related metabolic particularities including obesity and type II diabetes, NAFLD is also thought to be a hepatic manifestation of metabolic syndrome.[3] The uncontrolled NAFLD may deteriorate to nonalcoholic steatohepatitis (NASH), liver fibrosis and cirrhosis, and eventually become NAFLD-related hepatocellular carcinoma.[3–6] Extensive studies have identified several pathological mechanisms relevant to NAFLD. NAFLD begins with the accumulation of TGs in hepatocytes, while obvious hepatic TG accumulation is the risk factor for disease progression.[7] Obesity, type 2 diabetes, insulin resistance and so on have been shown to increase the burden of the liver and induce the development of hepatic fibrosis in NAFLD.[8,9] Moreover, proinflammatory cytokines are shown to contribute to the pathologies of NAFLD to some extent.[10] Studies about macrophage amassment have demonstrated that hepatic macrophages take part in the initiation and development of diverse liver diseases including NAFLD.[11]

Hepatic macrophages, which include recruited macrophages and Kupffer cells (KCs) are the largest group of hepatic immune cells. Based on the programs of activation, hepatic macrophages can be roughly divided into two main types: classically activated pro-inflammatory (known as M1) and alternatively activated anti-inflammatory (known as M2) phenotypes.[11] M1 macrophages prone to drive he-

patic inflammation and damage in diverse liver diseases via activating some signaling pathways, such as myeloid differentiation factor 88 (MyD88), STAT1, to release tumor necrosis factor-alpha (TNF-α), interleukin (IL)-6, IL-1β, and reactive oxygen species (ROS).[12,13] M2 macrophages contribute to produce large numbers of anti-inflammatory cytokines including IL-10 and arginase 1 (Arg1) to prevent the further progress of the inflammatory response and promote tissue repair.[14] Upon hepatocyte injury, it releases various danger-associated molecular pattern (DAMP) molecules, like high-mobility group box 1 (HMGB1), mitochondrial DNA and free RNA, to activate hepatic macrophages, which in turn secrete pro-inflammatory cytokines, such as TNF-α and IL-1β which lead to further hepatocyte injury.[13]

Most HMGB1 is localized in the nucleus and functions to bind DNA in its homeostatic state as a DAMP molecule. However, it can be secreted from cells responding to cellular stress or lytic cell death.[15,16] HMGB1 is defined as a crucial pro-inflammatory cytokine, but current reports of HMGB1-mediated macrophage polarization are inconsistent. Some studies reported that HMGB1 induced macrophages into not only M1-like phenotype[17,18] but also M2-like phenotype.[19] Additionally, HMGB1-C1q complexes was reported to induce macrophages towards an M2-like phenotype.[20] Therefore, in different conditions, macrophages can polarize towards M1 or M2 phenotype by HMGB1.[21] Moreover, it is reported that HMGB1 nuclear translocation and cytoplasmic accumulation for subsequent release is mediated by JAK/STAT1 pathway.[22]

OPN, also named as secreted phosphoprotein 1 (abbreviated as SPP1), is a multifunctional glycosylated protein which is widely expressed in various cells including activated macrophages, endothelial cells, osteoclasts and hepatocytes.[23] OPN plays a crucial role in inflammation, fibrosis, angiogenesis, bone resorption, and wound healing.[24] Importantly, OPN is very sensitive to oxidative stress and markedly triggered the liver injury in NAFLD.[25] Neutralizing OPN with antibodies demonstrated protective against D-galactosamine-induced inflammatory liver damage,[23] and OPN also can function as a negative regulator of autophagy to accelerate lipid accumulation during the progression of NAFLD.[26] However, other studies showed that OPN knockout mice with streptozotocin-induced diabetes had enhanced liver TG,[9,27] and liver OPN is necessary to prevent the development of age-related NAFLD.[28] So the role of OPN in NAFLD are still controversial. Therefore, to clarify the signal pathway regulated by OPN helps to reveal the role of OPN in NAFLD, especially that the effect of OPN-regulated hepatocyte signaling pathway on macrophage polarization has not been well investigated.

In this study, we explored the signaling pathway in liver cells by which OPN regulates the macrophage polarization in NAFLD. We used bioinformatics methods, liver samples from human NAFLD patients, animal models of NAFLD, and cell lines to identify that OPN expression is increased in NAFLD and that the high expression of OPN is also related with disease progression and stage. Furthermore, we observed that the supernatants of OPN-treated HepG2 cells promoted the macrophage M1 polarization, which was attributed to OPN-activated JAK1/STAT1/HMGB1 signaling pathway in hepatocytes. Our study expanded current understanding of OPN role in NAFLD, which may provide a novel theoretical basis for the treatment of NAFLD.

Methods

Reagents

Rabbit anti-STAT1 (Cat#: ab109320), phospho-STAT1 (pi-STAT1; Cat#: ab109461), HMGB1 (Cat#: ab18256), anti-JAK1 (Cat#: ab133666), phospho-JAK1 (pi-JAK1; Cat#: ab138005) antibodies were purchased from Abcam (Cambridge, UK). Mouse anti-GAPDH (Cat#: sc-47724) antibody was purchased from Santa Cruz Biotechnologies (Dallas, TX, USA). Rabbit anti-OPN (Cat#: 22952-1-AP) antibody and mouse anti-Albumin (Alb; Cat#: 66051-1) was purchased from Proteintech (Rosemont, IL, USA). Rabbit PE-conjugated anti-mouse CD86 (Cat#: 159202) and MHCII (Cat#: 107615) antibodies were purchased from Biolegend (San Diego, CA, USA). IFNγ (Cat#: 315-05) was purchased from PeproTech (Rocky Hill, NJ, USA). Recombinant human OPN (Cat#: 1433-OP-050) was purchased from R&D Systems (Minneappolis, MN, USA).

Identification of differentially expressed genes (DEGs) and meta-analysis

Normal liver tissues and NAFLD or NASH tissues from GSE33814 dataset were used to get the DEGs by using limma, which is linear modeling for microarray data in Bioconductor (http://www.bioconductor.org/). Genes, if they met the cutoff criteria of Z score >2.5 and *t* test >4 were identified as DEGs. In order to increase the reliability of expression data, another 7 publicly available gene expression profiles (GSE61260, GSE48452, GSE63067, GSE37031, GSE17470, GSE24807, GSE33814 and GSE71989) were used in our study. R package was used to conduct the background correction and data normalization for all datasets profiles,[29] and all samples in every dataset realized admissible homogeneity. Based on control and case, samples were divided into two groups, and the mean plus SD (standard deviation) were measured respectively followed by performing meta-analysis to generate a forest map of the genes.[30]

Expression level of OPN in the progression of NAFLD

Clinical information of GSE61260 were obtained from GEO2R which includes relevant pathological stage of the case group patients. The box plot[31] was generated by integrating the data, which described OPN expression level in the progression of NAFLD (healthy obese and NAFLD/NASH). Similarly, GSE114564 was analyzed to determine the expression level of OPN between normal and liver cirrhosis. Furthermore, GSE130970, GSE135251 and GSE162694 were included to determine the relationship between OPN levels and fibrosis stages, NAFLD activity score (NAS), sex, age, and the functions of hepatic macrophages in NAFLD.

Human samples

All investigations involving human samples were approved by the Ethics Committee of Second Affiliated Hospital of Nanchang University (Nanchang, China) and complied strictly with the Declaration of Helsinki Principle 2008. The informed written consent forms were signed by patients in order to collect the samples. Liver pathological specimens were obtained from two patients with NAFLD undergoing liver biopsy in the Second Affiliated Hospital of Nanchang University. Paracancerous liver tissues were obtained from two patients with hepatocellular carcinoma and used as normal controls (NCs); these patients did not have a history of alcohol abuse and other liver diseases.

Animal and in vivo studies

The protocols for procedures in mice were approved by the

Table 1. qRT-PCR primer sequences

Gene	Forward (5′-3′)	Reverse (5′-3′)
Homo OPN	CTCCATTGACTCGAACGACTC	CAGGTCTGCGAAACTTCTTAGAT
Mus OPN	AGCAAGAAACTCTTCCAAGCAA	GTGAGATTCGTCAGATTCATCCG
Homo GAPDH	GGTGGTCTCCTCTGACTTCAACA	GTTGCTGTAGCCAAATTCGTTGT
Mus GAPDH	ACCCAGAAGACTGTGGATGG	ACACATTGGGGGTAGGAACA

Animal Ethics Committee of Nanchang University and conformed to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH). The wild-type (WT) C57BL/6 mice (8 weeks old, about 22 g body weight) were raised in the transgenic animal Facility of Nanchang University (Nanchang, China) under standard temperature (23±3°C) and lighting conditions (12 h dark/12 h light cycles).

To establish the NAFLD models, the mice were randomly divided into two groups of six each. They were separately fed with normal diet (ND) or a high-fat diet (HFD) with 60 kcal% fat (D12492; Research Diets, New Brunswick, USA) for 16 weeks. After treatment, the animals were weighed and euthanatized in a CO_2 chamber, followed by collection of liver and blood. Serum TGs and total cholesterol (TC) were assayed with kits purchased from PPLYGEN (Beijing, China).

Hepatic lipid content

Livers were weighed and photographed, and a piece of liver (~50 mg) was collected to measure the TG content with an assay kit from PPLYGEN following the manufacturer's protocol. Briefly, the liver was homogenized in lysate (~1.1 mL), and 1 mL homogenate was left standing for 10 m before heating the supernatant to 70°C for 10 m followed by centrifugation at 2,000 rpm for 6 m. Absorbance was measured at 550 nm with a microplate reader. A 100 μL volume of homogenate was saved for determination of protein content, which was used to normalize TG levels.

Hematoxylin and eosin (HE) and oil red O staining

A piece of the liver was removed to prepare frozen or paraffin sections by conventional procedures. Frozen sections were stained with fresh Oil Red O solution for 30 m and then rinsed with 60% isopropanol followed by stained the nuclei with hematoxylin solution for 30 s. Finally, the sections were rinsed with distilled water and mounted in glycerin jelly. The paraffin sections were stained with HE. Images were obtained with an Olympus microscope.

Cell culture and treatment

HepG2, the human hepatocellular carcinoma cells, and LO2, the normal human liver cells were purchased from the American Type Culture Commission and cultured in complete Dulbecco's Minimal Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS) (BI) and 50 μg/mL penicillin/streptomycin at 37°C in a humidified atmosphere containing 5% CO_2. HepG2 or LO2 cells in 6-well plates at ~80% confluence were switched to serum-free medium, and then treated with or without 10 ng/mL recombinant human OPN for 0.5, 1, 2, or 6 h followed by extraction of proteins to determine the phosphorylation level of STAT and JAK1. To determine the effect of OA on OPN expression, HepG2 cells in 6-well plates at ~80% confluence were switched to serum-free medium and then treated with various doses of OA for 24 h or with 0.2 mM OA for various times followed by extraction of protein and RNA.

RNA extraction and quantitative real-time polymerase chain reaction (qRT-PCR)

After treatment, liver tissue or cultured cells were homogenized or lysed in Trizol reagent (Takara, Shiga, Japan) to isolate total RNA.[32] cDNA was synthesized with 1 μg of total RNA with a reverse transcriptase kit (Vazyme, Nanjing, Jiangsu, China). qRT-PCR was performed with SYBR green PCR master mix (Vazyme) with the primers listed in Table 1. OPN mRNA expression was normalized to corresponding GAPDH levels.

Western blotting

Total proteins were extracted from liver tissues or HepG2/LO2 cells by lysis buffer as described previously.[33] After measurement of total protein content, the levels of OPN, p-STAT1, STAT1, p-JAK1, JAK1, HMGB1 and GAPDH were determined by western blotting.[34]

Immunofluorescence staining

OPN expression in the liver was assayed by immunofluorescent staining with 5 μm frozen sections. The sections were permeabilized with 0.1% (v/v) Triton X-100 for 15 m, washed three times with phosphate buffered saline (PBS), blocked with 2% bovine serum albumin (BSA) for 1 h at room temperature, and then incubated with OPN antibody (1:100) or/and Alb antibody (1:100) overnight at 4°C. After washing three times with PBS, the sections were incubated with the secondary antibody conjugated to fluoresceine isothiocyanate (FITC) for 1 h at room temperature. The nuclei were then stained with diamidino-phenylindole (DAPI) solution, mounted with antifluorescence quenching agent and photographed with a fluorescence microscope (Olympus, Tokyo, Japan).

ROS and HepG2 supernatant preparation

HepG2 cells were incubated with 100 μM H_2O_2 or plus OPN (10 ng/mL) for 24 h. The medium was collected and concentrated for the HMGB1 assay. Cells were also collected to extract the proteins to determine the expression of HMGB1. To collect the HepG2 supernatants (referred to here as CM), HepG2 cells were treated as above and then were switched to fresh serum-free medium for another 24 h followed by collecting CM1 (ROS-treated supernatant) or CM2 (OPN and ROS-treated supernatant) to treat macrophages.

Induction of M1-polarized macrophages

Bone marrow cells were collected from mouse femurs and tibias followed by removal of erythrocytes, and culture in DMEM medium containing 10% FBS and 30% L929-conditioned medium for 7 days to generate bone marrow-derived macrophages (BMDMs), or M0-macrophages, of which half of the medium is needed to switch on the fourth day of culture. And then, BMDMs were switched to either medium with 10 ng/mL IFN-γ or plus CM1 or plus CM2 for 24 h followed by collecting the cells for the FACS assay to determine the content of M1-polarized macrophages.

FACS assays

Following the manufacturer's instructions, CD86 and MHC class II staining kit were used to detect M1 polarization of BMDMs by FACS. In brief, treated BMDMs were collected and washed twice with FACS buffer (1× PBS, 2% FBS and 2 mM EDTA). Cells (1×10^6) were initially blocked with 500 μL of PBS containing 5% BSA for 1 h at RT, and then 200 μL of PE-conjugated anti-CD86 or -MHCII antibody was added for 1 h at RT. After incubation, cells were washed three times with FACS buffer before performing the assay.

Statistical analysis

Data are reported as means±standard error of the means and the statistical analysis was performed with GraphPad Prism7 (La Jolla, CA, USA). One-way analysis of variance was used to compare the differences among more than two groups. Unpaired student's *t*-test was used to compare the differences between two groups. *P*-values <0.05 were considered statistically significant.

Results

OPN was upregulated in NAFLD and was associated with diseases progression

To explore the role of OPN in liver diseases, the GSE33814 dataset was chosen to analyze the expression changes of OPN in NAFLD or NASH. Thirteen normal controls and thirty-one patients with steatosis and steatohepatitis were included in the dataset. After filtering and normalization, 91 DEGs were identified in patients and control group, including 44 that were upregulated and 47 that were downregulated (Fig. 1A). Among the upregulated DEGs, *OPN* had a significant *p*-value and Z-score (p=0.00113, Z score=4.21). Furthermore, we integrated seven datasets to perform a meta-analysis and concluded that *OPN* was consistently and significantly upregulated in all seven datasets (Fig. 1B).

We further analyzed the expression level of OPN in NAFLD disease progression. The results in Figure 1C shows that *OPN* expression level increased with NAFLD progression from healthy obese to NAFLD and NASH. The *OPN* level was also increased in patients with liver cirrhosis compared with normal controls (Fig. 1D), and was associated with fibrosis stages and the NAS of patients (Fig. 1E, F), but was independent of sex and age (Supplementary Fig. 1). Collectively, the results suggested that OPN was upregulated in NAFLD and associated with disease progression.

OPN level was elevated in human NAFLD patients

We collected liver specimens from human NAFLD and NC groups to validate the changes of OPN level in NAFLD. The results indicated that OPN protein level was elevated in patients with NAFLD (Fig. 2A, B). Similarly, the OPN mRNA level was also elevated in NAFLD compared with NCs (Fig. 2C). The increase of OPN protein level was further confirmed by immunofluorescent staining (Fig. 2D). Collectively, the results demonstrated that OPN level was upregulated in NAFLD.

OPN level was elevated in mouse NAFLD models

We established mouse NAFLD models to further validate the increased expression of OPN in the liver. Compared with ND, body weight was increased (Fig. 3A) and liver color was decreased (Fig. 3B) by HFD. HFD also increased hepatic lipid accumulation (Fig. 3C), liver damage (Fig. 3D), and TG content (Fig. 3E). Similarly, serum TG and TC content were also increased by HFD (Fig. 3F, G). Furthermore, OPN expression was significantly increased by HFD (Fig. 3H–J). Finally, the increase of OPN protein level in liver of HFD mice was further confirmed by immunofluorescent staining, but the results also showed that HFD increased Alb expression, a hepatocyte marker, whereas had no effect on the colocalization of OPN and Alb (Fig. 3K). Taken together, the results suggested that OPN expression was increased in mouse NAFLD models.

OPN level was increased by OA in HepG2 cell lines

We determined the expression of OPN in HepG2 cells treated with OA. OA induced OPN protein expression in a dose-dependent manner (Fig. 4A, B). OA also significantly induced OPN protein expression in a time-dependent manner (Fig. 4C, D). Similarly, OA also induced OPN mRNA expression in dose and time-dependent manners (Fig. 4E, F). The results suggested that OPN level in HepG2 cells was increased by OA.

OPN promoted macrophage M1 polarization by activating JAK1/STAT1 pathway of HepG2 to release HMGB1

It is reported that hepatic macrophages take part in the initiation and development of a variety of liver diseases including NAFLD. In NAFLD, injury hepatocytes release a variety of DAMP molecules to activate hepatic macrophages that in turn secrete proinflammatory cytokines thereby leading to further hepatocyte injury.[13] Therefore, to explore the role of OPN, we firstly analyzed the association of OPN and the functions of hepatic macrophages by a bioinformatic method. As shown in Supplementary Figure 2, high levels of OPN promoted macrophage M1 polarization. Next, we further determined the effect of OPN-treated HepG2 cells medium on macrophage M1 polarization by FACS assay. As shown in Fig. 5A, B, macrophages treated with CM2 expressed more M1-related surface markers (CD86 or MHCII) compared with CM1 treatment.

To determine the molecular mechanisms of OPN-induced macrophage M1 polarization, the expression and secretion of HMGB1 were examined in HepG2 cells treated with OPN because HMGB1 has been reported to polarize macrophages towards the M1 phenotype.[17,18] As shown in Figure 5C–E, OPN increased the expression and secretion of HMGB1 induced by ROS in HepG2 cells. Furthermore, the macrophage

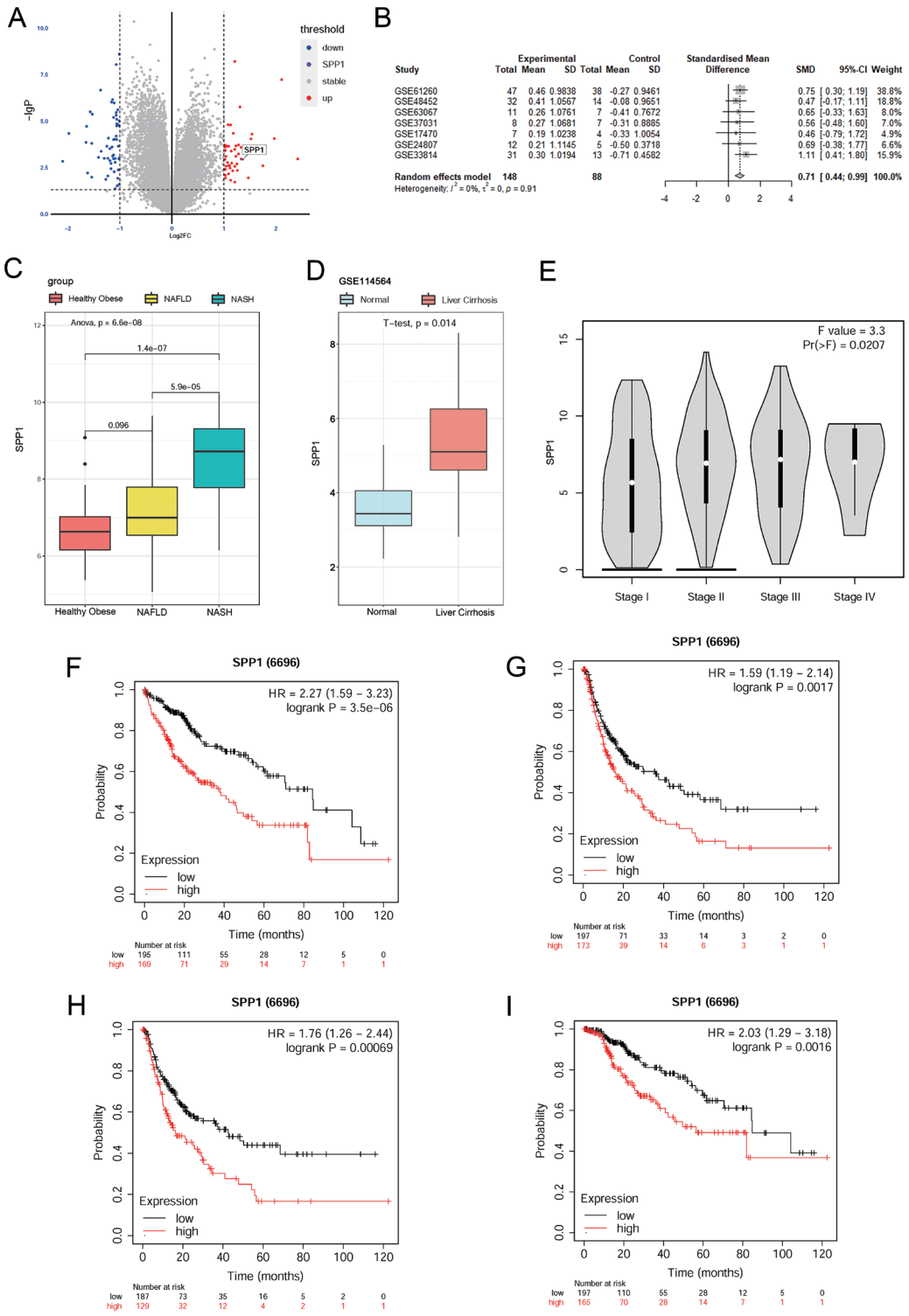

Fig. 1. OPN was upregulated in NAFLD and associated with disease progression. (A) Volcano plot of gene expression profile between normal people and NAFLD and NASH patients. Red dots represent upregulated genes; blue dots represent the downregulated genes in NAFLD. p=0.00113. (B) The consistency of changes in OPN expression was performed by meta-analysis. An SMD of <0 demonstrated that the gene was a protective factor. An SMD >0 demonstrated that the gene was a risk factor in the progression of NAFLD. The Forest map and corresponding confidence intervals are indicated. (C) GSE61260 was downloaded and OPN expression was analyzed by GEO2R in healthy obese, NAFLD, and NASH patients. (D) GSE114564 was downloaded and OPN expression was analyzed by GEO2R in health people and liver cirrhosis patients. (E) The association of OPN level and fibrosis stage was analyzed by using GSE130970, GSE135251 and GSE162694 datasets. (F) The association of OPN expression and NAS was analyzed by using GSE135251 and GSE162694 datasets. GEO, Gene Expression Omnibus (www.ncbi.nlm.nih.gov/geo/); NAFLD, non-alcoholic fatty liver disease; NAS, NAFLD activity score; NASH, nonalcoholic steatohepatitis; OPN, osteopontin.

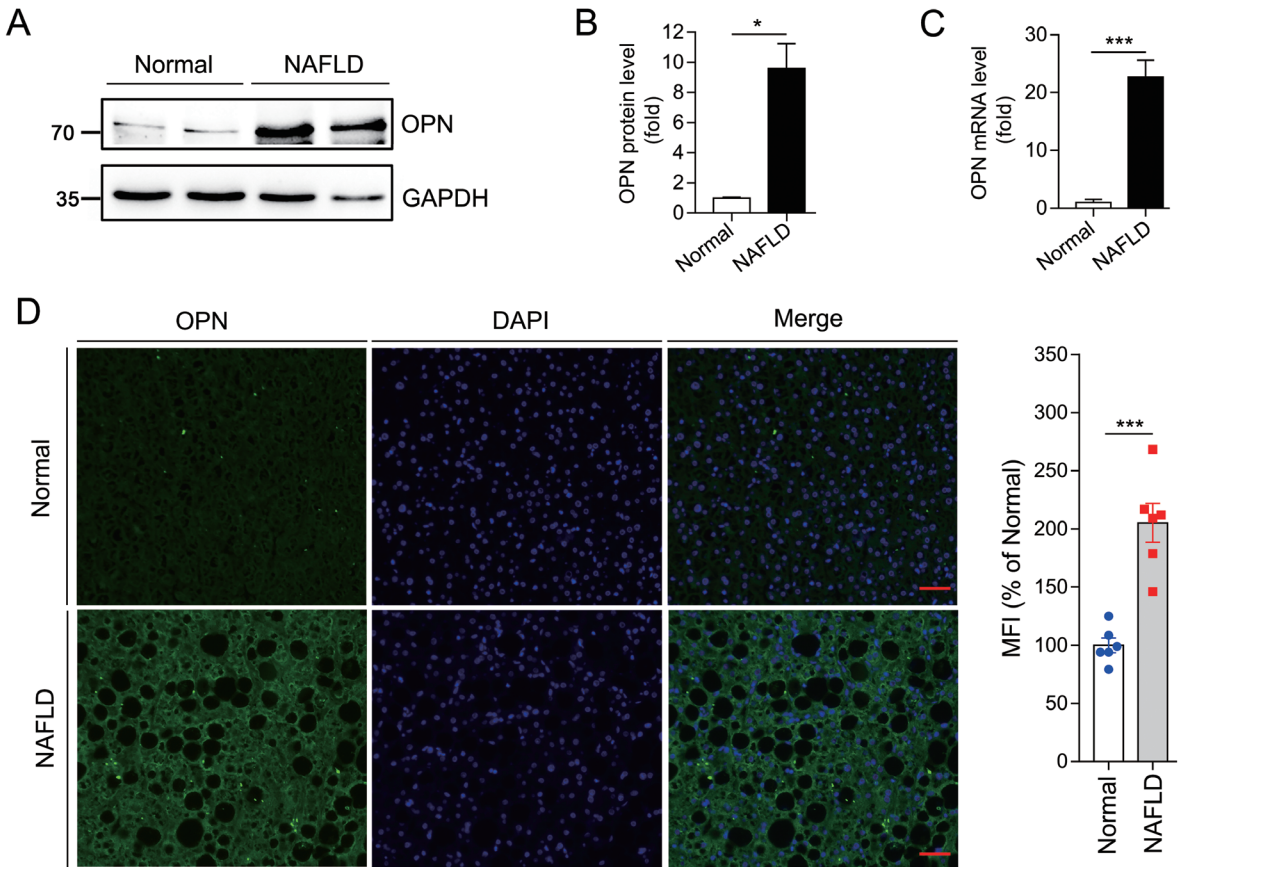

Fig. 2. OPN level was increased in liver specimens from human patients with NAFLD. (A) OPN protein level in liver specimens from NAFLD and NC groups was analyzed by western blotting. (B) Quantitative analysis of OPN protein levels. *p<0.05 (n=2). (C) OPN mRNA levels in liver specimens from NAFLD and NC groups were analyzed by qRT-PCR. ***p<0.001 (n=2). (D) OPN expression was assessed by immunofluorescent staining with OPN antibody in liver specimens from NAFLD and NC groups. Scale bar, 50 μm. ***p<0.001 (n=6). NAFLD, non-alcoholic fatty liver disease; NC, normal control; OPN, osteopontin; qRT-PCR, quantitative real-time polymerase chain reaction.

M1 polarization was decreased when anti-HMGB1 polyclonal antibody was used to block HMGB1 (Fig. 5F), suggesting that OPN promoted macrophage M1 polarization via HMGB1.

It has been reported that HMGB1 nuclear translocation and cytoplasmic accumulation for subsequent release is mediated by the JAK/STAT1 pathway.[22] Therefore, we further examined the activation of JAK1/STAT1 pathway in order to clarify the molecular mechanisms of OPN increasing the expression and secretion of HMGB1. As shown in Supplementary Figure 3, OPN increased the phosphorylation of JAK1 and STAT1 but had little effect on JAK1 and STAT1 expression levels in HepG2 cells. Moreover, OPN also increased the phosphorylation of JAK1 and STAT1 in a time dependent manner in LO2 cells (Fig. 6A–E). Taken together, the results indicate that OPN activated the JAK1/STAT1 signaling pathway to release HMGB1, thereby promoting macrophage M1 polarization.

Discussion

NAFLD is the most common chronic liver disease and is considered to be an independent risk factor for cardiovascular disease. However, there are still no FDA-approved drugs for the treatment of NAFLD. Therefore, it is of great significance to clarify the pathogenesis of NAFLD and identify targeted genes for treating of NAFLD.

The pathogenesis of NAFLD is a complex and involving the interaction of diverse factors. The primary step in the development of NAFLD is the activation of hepatic macrophages participating in inflammation and tissue repair. Various cytokines, hormones and chemokines mediate the activation of macrophages, one of which is OPN. OPN takes part in the regulation of immunity and inflammation,[35] angiogenesis and tumor progression,[36] and fibrosis.[37] However, its role in NAFLD and the underlying mechanisms is still not well described.

In this study, we first identified 91 DEGs by analysis of a GSE33814 dataset from the Gene Expression Omnibus (GEO) and found that OPN expression was increased in NAFLD. It was further confirmed that *OPN* was significantly and steadily upregulated by a meta-analysis of GSE17470, GSE24807, GSE33814, GSE37031, GSE48452, GSE61260 and GSE63067. We further observed that OPN expression increased with the pathological progression of NAFLD from healthy obese to NAFLD and NASH, and was associated with fibrosis stages and NAS. Next, we validated changes in OPN expression in liver tissues from human NAFLD patients, animal model of NAFLD, and cell model with lipid accumulation. These results suggest that OPN was upregulated in the models, which is consistent with the conclusions of related reports.[9] Therefore, we think that OPN level may reflect the pathological stage of NAFLD and can be used as biomarker or target to diagnose or treat NAFLD by detecting the OPN

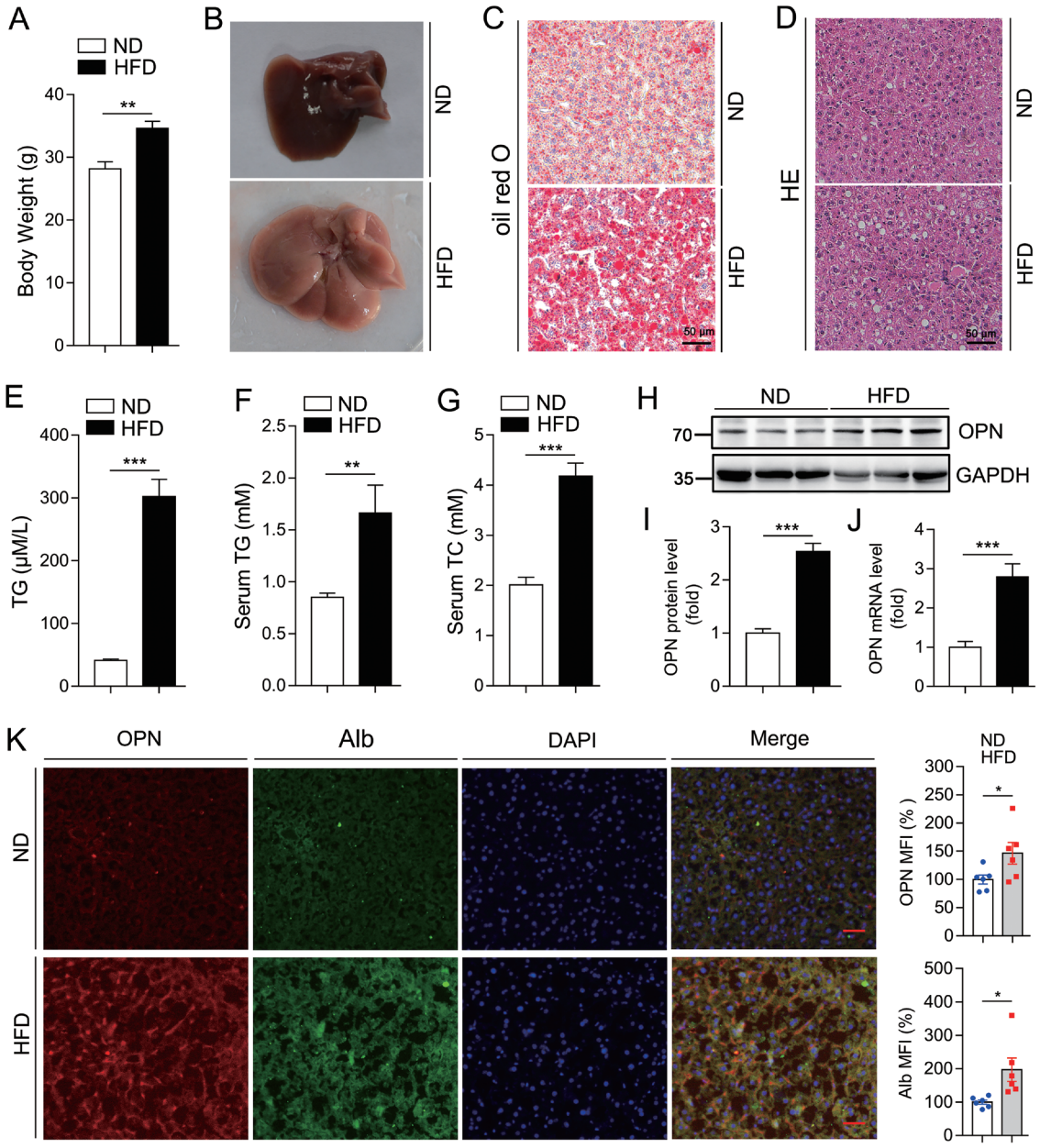

Fig. 3. OPN level was increased in livers from mice with NAFLD. (A) Body weight, (B) liver image, (C) oil red O staining, (D) HE staining, (E) liver TG content, (F) serum TG and (G) serum TC were assayed in HFD mice compared with ND mice. (H) Expression of OPN protein in liver tissue from HFD and ND mice was assayed by western blotting. (I) Quantitative analysis of OPN protein levels. (J) OPN mRNA level in liver tissues from HFD and ND mice were determined by qRT-PCR. (K) OPN level and the colocalization with Alb were assayed by immunofluorescence staining with OPN and Alb antibody in liver tissues in the ND and HFD groups. Scale bar, 50 μm. *p<0.05, **p<0.01, ***p<0.001 (n=6). Alb, albumin; HFD, high-fat diet; NAFLD, non-alcoholic fatty liver disease; ND, normal diet; OPN, osteopontin; TC, total cholesterol, TG, triglyceride.

level in the serum or using the OPN neutralizing antibody.

Because hepatocytes and hepatic macrophages interact in liver injury, we investigated the effect of OPN from hepatocyte on macrophage M1 polarization. Bioinformatic analysis showed that high levels of OPN significantly promoted macrophage M1 polarization in NAFLD, but inhibited macrophage M2 polarization, although it was not statistically significant. Moreover, the results shown in Figure 5 indicate that the CM2 (OPN and ROS-treated supernatant) promoted macrophage M1 polarization that is dependent on HMGB1, and that OPN increased the expression and secretion of HMGB1 in HepG2 cells. How HMGB1 promoted macrophage M1 polarization is not known. A previous study reported that HMGB1 aggravated the inflammation response in lung injury and induced macrophages M1 polarization by toll like receptor (TLR) 2, TLR4, and RAGE/NF-κB signaling pathways.[38] Therefore, we will determine the activation of TLR2, TLR4, and RAGE/NF-κB signaling pathways in OPN-CM treated macrophages in our future work.

It has been reported that JAK/STAT1 pathway mediated HMGB1 nuclear translocation and cytoplasmic accumulation for subsequent release.[22] The JAK/STAT1 pathway partici-

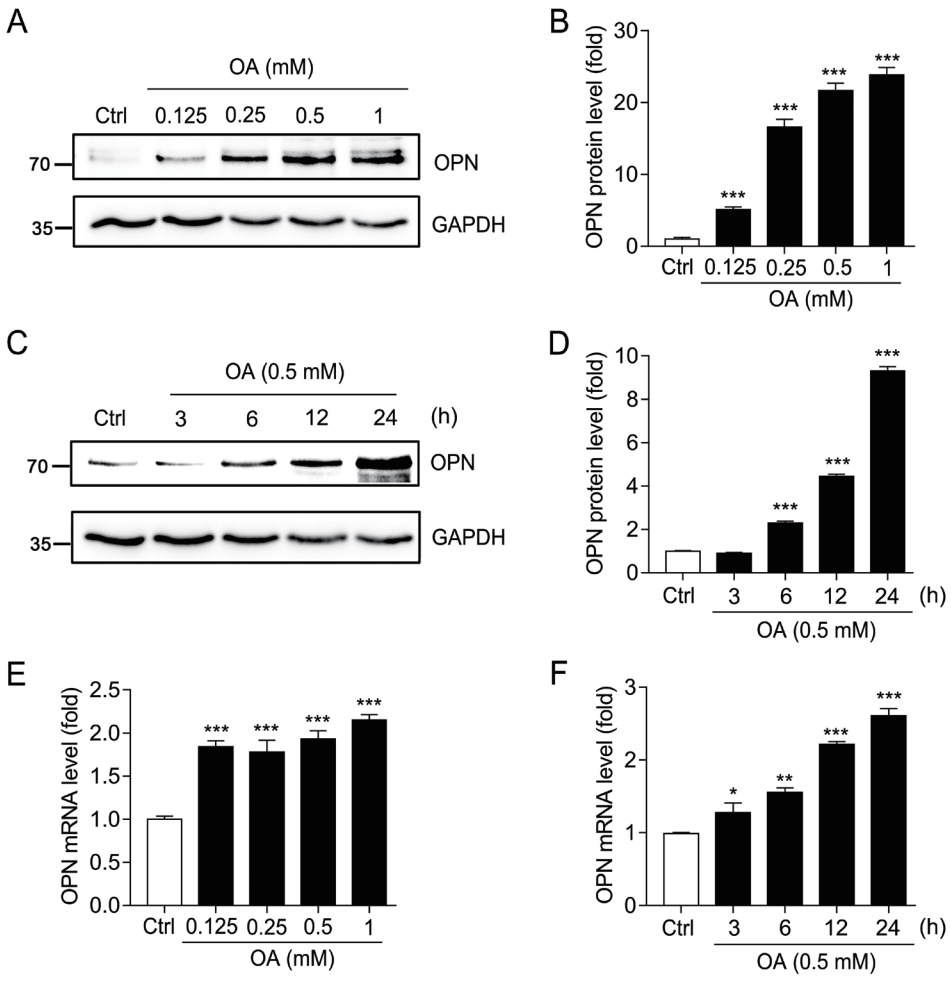

Fig. 4. OPN level in HepG2 cells was increased by OA. (A–D) HepG2 cells were treated with OA at the indicated concentrations for 24 h (A, B) or with 0.5 mM OA for the indicated times (C, D) following by isolation of total proteins. OPN protein level was assayed by western blotting. (E, F) HepG2 cells were treated with OA at the indicated concentrations for 24 h (E) or with 0.5 mM OA for the indicated times (F) following extraction of total RNA, the level of OPN mRNA level was determined by qRT-PCR. **p*<0.05; ***p* < 0.01; ****p*<0.001 compared with Ctrl. OA, oleic acid; OPN, osteopontin.

pates in multiple biological functions, such as glucose metastasis, diabetes, and inflammation.[39] Moreover, although a study based on mouse microarray has found that OPN was associated with the JAK/STAT1 pathway, that has not been further confirmed.[40] Therefore, it is reasonable to assume that OPN may activate JAK/STAT pathway in NAFLD to promote inflammation and disease progression. In order to verify that assumption, we examined the levels of phosphorylated of JAK1 and STAT1, and found that OPN significantly activated JAK1 and STAT1.

In summary, we observed that OPN promoted macrophage M1 polarization by increasing JAK1/STAT1-induced HMGB1 secretion in hepatocytes (Fig. 7). Our findings of the OPN/JAK1/STAT1/HMGB1 axis have expanded current understanding of OPN role in NAFLD, which may provide a novel insight to explore pathogenesis and treatment of NAFLD.

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Conflict of interest

The authors have no conflict of interests related to this publication.

Author contributions

Study concept and design (MJ, LD), acquisition of data (ZX, FX, XD, YN, DW, CP, XZ), analysis and interpretation of

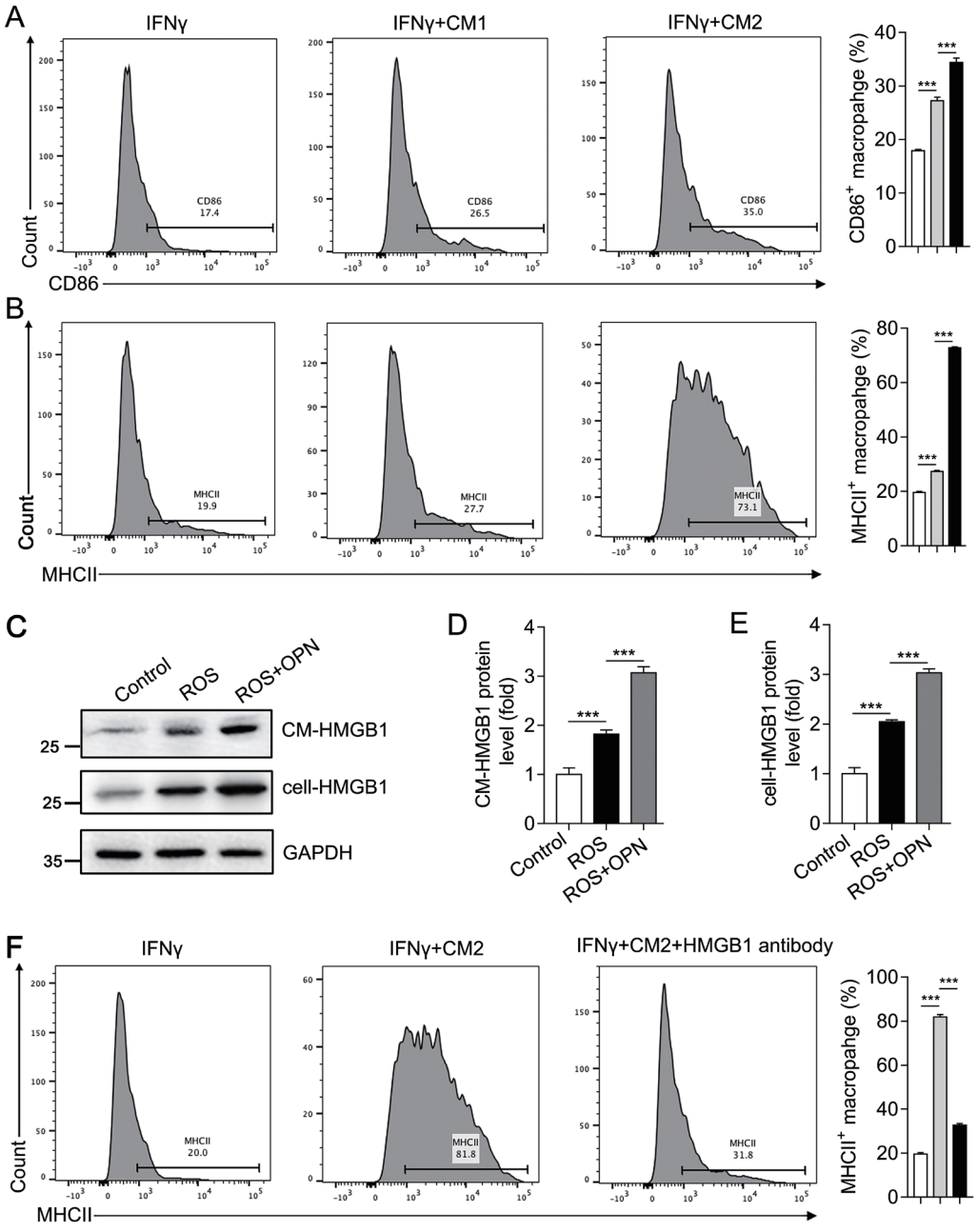

Fig. 5. OPN promoted macrophage M1 polarization via HMGB1. (A, B) The effect of IFNγ plus CM1 or CM2 on macrophage M1 polarization was assayed by FACS. (C) HepG2 cells were treated with ROS or ROS plus OPN for 24 h followed by collecting the medium and extraction of protein to examine the HMGB1 expression in culture medium and cells by western blotting. (D) Quantitative analysis of CM-HMGB1 protein levels. (E) Quantitative analysis of cell-HMGB1 protein levels. (F) The effect of HMGB1 antibody on macrophage M1 polarization was analyzed by FACS. ***p<0.001. FACS, fluorescence-activated cell sorting assay; HMGB1, high-mobility group box 1; OPN, osteopontin; ROS, reactive oxygen species.

data (WL, NS, CC, ZZ, MJ), drafting of the manuscript (FX), critical revision of the manuscript for important intellectual content (MJ, LD), administrative, technical, or material support, study supervision (MJ, LD).

Ethical statement

The protocols for procedures in mice were approved by the Animal Ethics Committee of Nanchang University and conformed to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH).

Data sharing statement

The datasets generated for this study can be found in the Gene Expression Omnibus (GEO): GSE17470, GSE24807, GSE33814, GSE37031, GSE48452, GSE61260, GSE63067, GSE114564, GSE130970, GSE135251, and GSE162694.

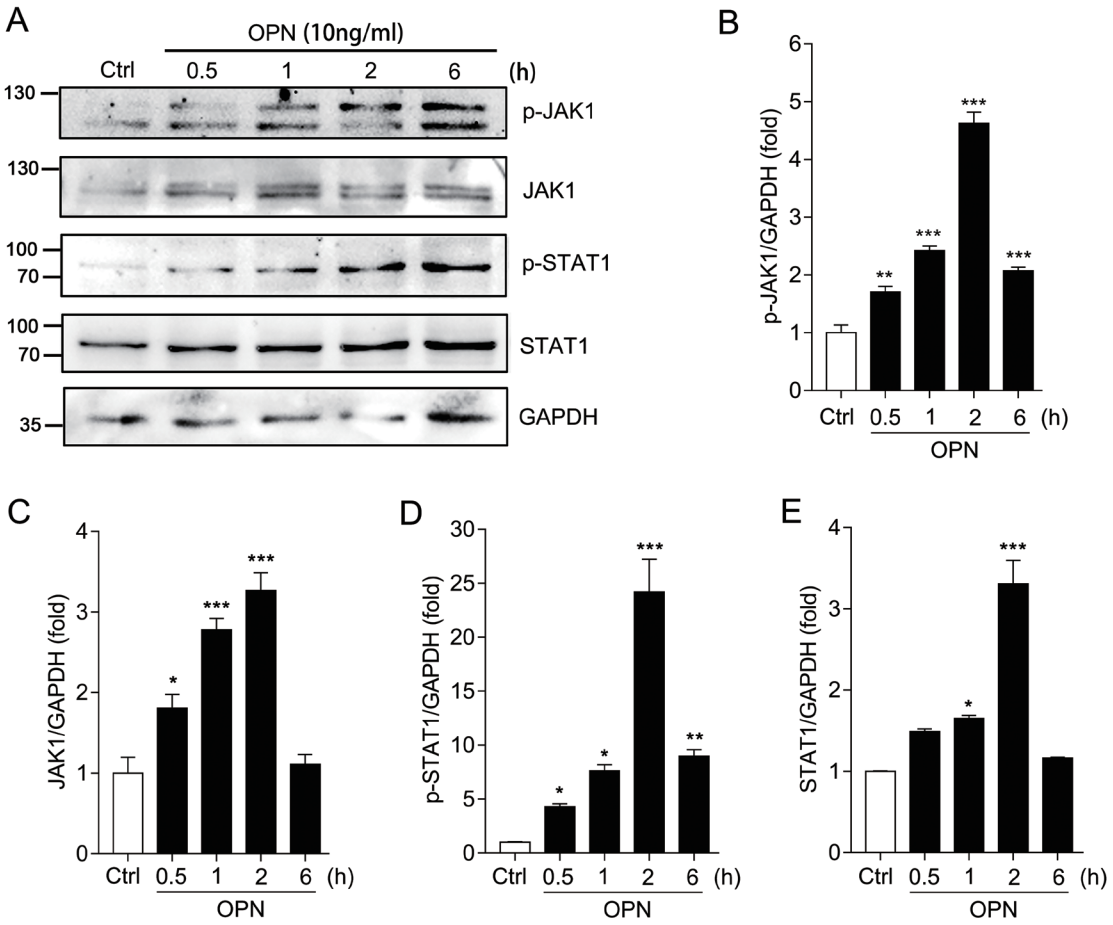

Fig. 6. OPN activated the JAK1/STAT1 signaling pathway in LO2 cells. (A) LO2 cells were treated with 10 ng/mL OPN for 0.5, 1, 2, and 6 h, and then total protein was extracted for determination of phosphorylation and expression of JAK1 and STAT1 by western blotting. (B) Quantitative analysis of JAK1 phosphorylation level normalized to GAPDH levels. (C) Quantitative analysis of JAK1 protein levels normalized to GAPDH levels. (D) Quantitative analysis of STAT1 phosphorylation level normalized to GAPDH levels. (E) Quantitative analysis of STAT1 protein levels normalized to GAPDH levels. *p<0.05; **p<0.01; ***p<0.001. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; JAK1, janus kinase 1; OPN, osteopontin; STAT1, signal transducers and activators of transcription 1.

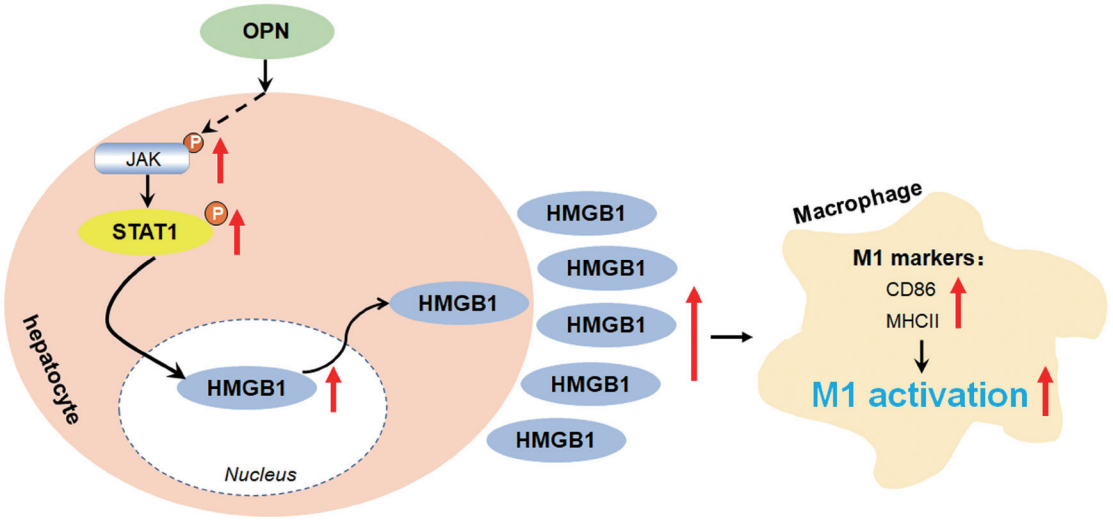

Fig. 7. A depiction of the signaling pathway by which OPN promotes macrophage M1 polarization. OPN activates the JAK1/STAT1 signaling pathway to produce and secrete more HMGB1 in hepatocytes, and the secreted HMGB1 further promoted macrophage M1 polarization. HMGB1, high-mobility group box 1; JAK1, janus kinase 1; OPN, osteopontin; STAT1, signal transducers and activators of transcription 1.

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