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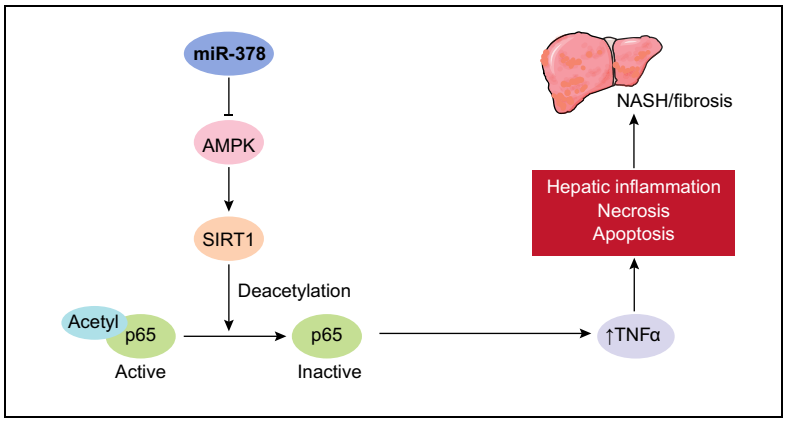
MicroRNA-378 promotes hepatic inflammation and fibrosis via modulation of the NF-κB-TNFα pathway

Graphical abstract



Highlights

- Hepatic expression of miR-378 is significantly upregulated in fatty livers of mice and patients with NASH.
- miR-378 is a potent inhibitor of AMPK signaling.
- miR-378 facilitates an inflammatory pathway of NFκB-TNFα by targeting *Prkag2*.
- miR-378 robustly promotes hepatic inflammation and fibrosis in dietary obese mice.
- TNFα signaling is required for miR-378 to induce NASH progression.

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Lay summary

The recent epidemic of obesity has been associated with a sharp rise in the incidence of non-alcoholic fatty liver disease (NAFLD). However, the underlying mechanism(s) remains poorly described and effective therapeutic approaches against NAFLD are lacking. The results establish that microRNA-378 facilitates the development of hepatic inflammation and fibrosis and suggests the therapeutic potential of microRNA-378 inhibitor for the treatment of NAFLD.

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MicroRNA-378 promotes hepatic inflammation and fibrosis via modulation of the NF-κB-TNFα pathway

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Background & Aims: The progression of hepatosteatosis to non-alcoholic steatohepatitis (NASH) is a critical step in the pathogenesis of hepatocellular cancer. However, the underlying mechanism(s) for this progression is essentially unknown. This study was designed to determine the role of miR-378 in regulating NASH progression.

Methods: We used immunohistochemistry, luciferase assays and immunoblotting to study the role of miR-378 in modulating an inflammatory pathway. Wild-type mice kept on a high-fat diet (HFD) were injected with miR-378 inhibitors or a mini-circle expression system containing miR-378, to study loss and gain-of functions of miR-378.

Results: MiR-378 expression is increased in livers of dietary obese mice and patients with NASH. Further studies revealed that miR-378 directly targeted *Prkag2* that encodes AMP-activated protein kinase γ 2 (AMPKγ2). AMPK signaling negatively regulates the NF-κB-TNFα inflammatory axis by increasing deacetylase activity of sirtuin 1. By targeting *Prkag2*, miR-378 reduced sirtuin 1 activity and facilitated an inflammatory pathway involving NF-κB-TNFα. In contrast, miR-378 knockdown induced expression of *Prkag2*, increased sirtuin 1 activity and blocked the NF-κB-TNFα axis. Additionally, knockdown of increased *Prkag2* offset the inhibitory effects of miR-378 inhibitor on the NF-κB-TNFα axis, suggesting that AMPK signaling mediates the role of miR-378 in facilitating this inflammatory pathway. Liver-specific expression of miR-378 triggered the development of NASH and fibrosis by activating TNFα signaling. Ablation of TNFα in miR-378-treated mice impaired the ability of miR-378 to facilitate hepatic inflammation and fibrosis, suggesting that TNFα signaling is required for miR-378 to promote NASH.

Conclusion: MiR-378 plays a key role in the development of hepatic inflammation and fibrosis by positively regulating the NF-κB-TNFα axis. MiR-378 is a potential therapeutic target for the treatment of NASH.

Lay summary: The recent epidemic of obesity has been associated with a sharp rise in the incidence of non-alcoholic fatty liver disease (NAFLD). However, the underlying mechanism(s) remains poorly described and effective therapeutic approaches against NAFLD are lacking. The results establish that microRNA-378 facilitates the development of hepatic inflammation and fibrosis and suggests the therapeutic potential of microRNA-378 inhibitor for the treatment of NAFLD.

Introduction

The incidence of non-alcoholic fatty liver disease (NAFLD) is estimated to be 20–45% in the general population of Western countries.[1,2] Although NAFLD carries a relatively benign prognosis, a significant proportion of patients can progress to non-alcoholic steatohepatitis (NASH) and later cirrhosis with the risk of developing hepatocellular carcinoma (HCC).[3,4] It is estimated that NASH-related HCC accounts for ≥13% of cases in the US.[5–7] Although the progression of hepatosteatosis to NASH has been studied extensively, the precise mechanism(s) is still under intense investigation.

Chronic hepatic inflammation is considered a major culprit for the development of NASH. Both hepatocytes and Kupffer cells have been reported to contribute to hepatic inflammation.[8] In hepatosteatosis, free fatty acid delivery to the liver is markedly increased, and now known to be harmful to hepatocytes.[9] This so-called lipotoxicity leads to oxidative and endoplasmic reticulum stress.[9] Thus, prolonged cellular stresses can cause severe damage, leading to hepatocyte death.[9] Lipotoxicity from free fatty acids, hepatocyte injury and apoptosis can activate Kupffer cells and promote inflammatory responses.[10] Despite the important role of Kupffer cells in promoting NASH, injured hepatocytes also generate a number of pro-inflammatory cytokines and chemokines including tumor necrosis factor alpha (TNFα), which contributes to hepatic inflammation.[10] Mechanistically, a complex regulatory network of transcription factors, including nuclear factor kappa B (NF-κB) and peroxisome proliferator-activated receptors, orchestrate these events in hepatic cells.[11,12] NF-κB signaling is also activated in

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damaged hepatocytes, which can activate expression of genes encoding TNFα and interleukin 6 (IL6),[13] 2 important pro-inflammatory cytokines. A positive feedback loop between NF-κB and TNFα appears to aggravate the levels of inflammation and injury to hepatocytes.[13] The underlying mechanisms involved in the pathogenesis of NASH are complex and involve heretofore unrecognized regulatory networks.

The discovery of a class of naturally-occurring small non-coding RNAs, termed microRNAs (miRNAs),[14] has stimulated a new field of research on the mechanism of NASH progression. Alterations in miRNA expression have been reported in patients with hepatosteatosis and NASH.[15,16] Some miRNAs such as miR-155 and miR-122, appear to be involved in NASH, as studied in mouse models treated with methionine-choline-deficient diet (MCD).[17,18] Unfortunately, the metabolic profile of the MCD model is the opposite to that of human NASH.[19,20] In contrast, the histopathology and pathophysiology of an high-fat diet (HFD)-induced NASH mouse model most resembles the pathophysiology of human NASH.[19,20] In this study, we used HFD-treated mice to evaluate the role of miRNAs in the pathogenesis of NASH.

Previously, we observed that hepatosteatosis robustly induces transcription of miR-378.[21] miR-378 is embedded in the first intron of *Ppargc1β*,[11] which encodes peroxisome proliferator-activated receptor γ coactivator 1 (PGC1β), a key regulator of thermogenesis and glucose and fatty acid metabolism.[22] Accumulating evidence showed that miR-378 functions as a promoter of hepatosteatosis and insulin resistance.[21–24] However, its role in the progression of NASH is poorly described. Our preliminary data, as detailed in this study, showed that hepatocyte-specific expression of miR-378 significantly enhanced activity of NF-κB and activated expression of *Tnfα*. Our findings, combined with those of others, led to our hypothesis that miR-378 could facilitate hepatic inflammation and fibrosis by promoting the synthesis of hepatocyte-derived pro-inflammatory mediators. Both gain- and loss-of function studies of miR-378 were performed to determine its role in facilitating pro-inflammatory pathways and triggering hepatic inflammation and fibrosis in HFD-treated mice.

Materials and methods

Human liver biopsies

Human liver biopsies were obtained during liver transplantation for histological analysis by fully trained pathologists and surgeons (University of Kansas Medical (KUMC) and the Union Hospital, Tongji Medical College, China). n = 38 specimens with NASH or hepatosteatosis and n = 24 control subjects (normal liver) without NASH were used for our study. Tissues were obtained and handled in accordance with the guidelines set by the medical ethical committees of KUMC and Tongji Medical College. This project utilized only pathological specimens that were de-identified and publicly available and hence the research meets the definition of "exempt" under Exemption 4 (page 213 (III-30)) on SF424. The etiology of NAFLD and normal liver samples is available (Table S1).

Establishment of dietary obese mice

Eight-week-old wild-type male C57Bl/6 mice (Jackson Laboratory) were maintained on either a normal chow diet (Open Source D12450B: 10% kcal fat, 70% kcal carbohydrate, and 20% kcal protein) or an HFD (Open Source D12492: 60% kcal fat, 20% kcal protein, and 20% kcal sucrose) for 8 weeks. After such time, livers were collected for miRNA and gene expression analysis.

Preparation of mini-circle expression vectors for miR-378 and shRNA of TNFα

Mini-circle vectors were purchased from System Biosciences (Cat. MN511A-1). Specifically, we inserted the mouse miR-378 precursor into the mini-circle parental plasmid. A transthyretin gene (*TTR*) promoter was used to ensure liver-specific expression of miR-378.[25] This construct was referred to as MC-*TTR*-miR-378. To rule out non-specific effects of the plasmid, we generated a miR-378 mis-matched-expression vector by mutating the seed region of miR-378, termed MC-*TTR*-miR-378-MM. For expression vectors of shRNAs, we inserted the verified shRNA of *Tnfα* or *Prkag2* (AMP-activated protein kinase non-catalytic subunit gamma 2 [AMPKγ2]) into the mini-circle vectors and the *TTR* promoter was used to ensure hepatic expression. This vector was referred to as to MC-*TTR*-*Tnfα*-shRNA or MC-*TTR*-*Prkag2*-shRNA. To prepare the mini-circle, parental mini-circle vector was transformed into a special host *E. coli* bacterial strain ZYCY10P3S2T (System Biosciences, Cat: MN900A-1).

Intravenous injection of mice

Eight-week-old wild-type C57Bl/6 mice were maintained on the HFD for 8 weeks. After that, mice kept on HFD were injected with MC-*TTR*-miR-378 (n = 8) or MC-*TTR*-miR-378-MM (n = 8). A group of mice before MC-*TTR*-miR-378 treatment (n = 8) served as another control. Mice received a dose of 1.5 μg/g MC-*TTR*-miR-378 or MC-*TTR*-miR-378-MM complexed with *in vivo*-jetPEI (Polyplus Transfection, Strasbourg, France) weekly for 9 weeks via tail vein injection as optimized before.[21]

To examine whether TNFα is required for miR-378 to trigger NASH, 8-week-old wild-type C57Bl/6 mice were maintained on the HFD for 8 weeks. At 16 weeks of age, mice were injected with MC-*TTR*-miR-378-MM (control, n = 8), MC-*TTR*-miR-378 (n = 8), MC-*TTR*-Tnfα-shRNA (n = 8), or a combination of MC-*TTR*-miR-378 and MC-*TTR*-Tnfα-shRNA (n = 8) as described above. A group of mice before MC-*TTR*-378 treatment served as another control. All mice were injected weekly for 10 weeks. Mice were then anesthetized, and blood was collected by way of cardiac puncture. Livers were harvested for gene expression and histological analysis.

Statement on institutional approval for mice experimentation

Eight-week-old wild-type male C57BL/6J mice (Jackson Laboratory) were used for experiments. All mice were housed in a barrier facility on 12 h:12 h light cycle with free access to water and normal chow diet (Open Source D12450B: 10% kcal fat, 70% kcal carbohydrate, and 20% kcal protein) or an HFD (Open Source D12492: 60% kcal fat, 20% kcal protein, and 20% kcal sucrose). Animal care, plasmid injection, and surgical procedures were conducted in compliance with an approved IACUC protocol by University of Minnesota, and those set forth in the "Guide for the Care and Use of Laboratory Animals" as published by the National Research Council.

Statistical analysis

Statistical analysis was performed using GraphPad Prism Software®. Statistical significance between 2 groups was

assessed by a 2-tailed Student *t* test or Mann-Whitney test. ANOVA was used to compare statistical difference among multiple groups. All the experiments were repeated at least 3 times on separate occasions. $p < 0.05$ was considered to be statistically significant.

Full details of these and other methods can be found in the Supplementary Materials and Methods.

Results

MiR-378 is robustly induced in livers of dietary obese mice, patients with NASH and HepG2 cells with accumulated lipid

miRNA profiles of NASH tissues in various animal models and human NASH have been reported and extensively studied in the past.[15,26] However, most of the animal studies used the MCD mouse model, which does not recapitulate the human counterpart. Since characteristics of HFD-induced NASH in mice most resemble those of humans,[19] we used that model system to investigate the roles of miRNAs in NASH. Previously, we have established that miR-378 is highly-expressed in hepatocytes.[21] To study the role of miR-378 in steatohepatitis, we treated wild-type C57Bl/6 mice with an HFD for 8 weeks. HFD treatment significantly increased hepatosteatosis (Fig. S1A-B, Table S2). Quantitative reverse transcription PCR revealed that miR-378 was robustly increased in the livers of HFD-treated mice (Fig. 1A). Further analysis revealed that hepatocytes represented the main source of miR-378 expression, while no significant change in miR-378 was observed in Kupffer cells after HFD treatment (Fig. 1B-C). Oleic acids are the most abundant unsaturated fatty acids in liver triglycerides in human individuals.[27] Human HepG2 cells were used for our *in vitro* models because of their increased sensitivity to fat accumulation. Oleic acid treatment increased intracellular lipids (Fig. S1C), which were associated with increased miR-378 in HepG2 cells (Fig. 1D). miR-378 expression is also increased in the livers of patients with NASH (Fig. 1E). Together, our studies indicated that hepatic lipid accumulation activates expression of miR-378 in hepatocytes of both humans and mice.

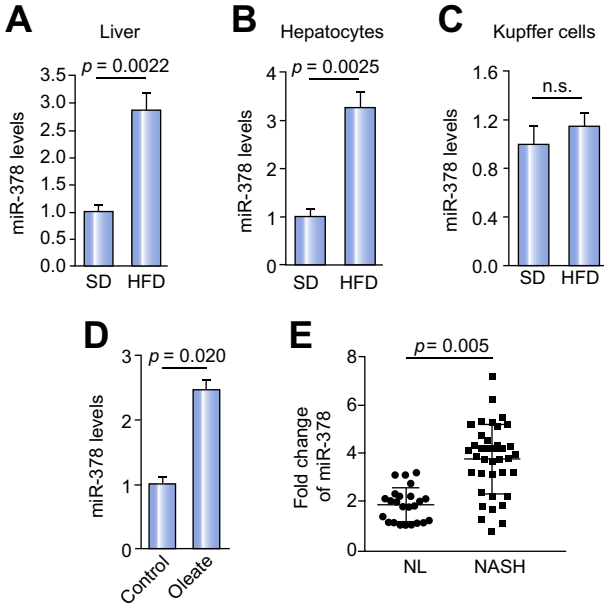

Fig. 1. miR-378 expression is increased in fatty livers of mice, human hepatoma cells with accumulated lipid and patients with NASH. (A) Levels of miR-378 in livers of HFD-fed mice (n = 6) compared to SD-fed mice (n = 6). (B) miR-378 had a 3-fold increase in hepatocytes of HFD-treated mice compared to SD-treated mice. (C) No significant change in levels of miR-378 in Kupffer cells isolated from mice treated with HFD. (D) Oleate treatment increased expression of miR-378 in HepG2 cells. HepG2 cells treated with DMEM medium without oleate served as the control. (E) A 2-fold increase in miR-378 expression in human NASH samples (n = 38) compared to human normal livers (n = 24). Data represent mean ± SEM (Student's t test). HFD, high-fat diet; NASH, non-alcoholic steatohepatitis; NS, no significance; SD, standard diet.

Prkag2 is a direct target of miR-378

Potential effects of miR-378 on NASH progression prompted us to explore the downstream effectors of miR-378. Using TargetScan algorithm,[28] we identified *Prkag2* as a potential target of miR-378 (Fig. 2A). *Prkag2* encodes AMPKγ2; and AMPK signaling can inhibit inflammatory responses by impairing NF-κB signaling.[29,30] These findings suggested that miR-378 might play a critical role in facilitating hepatic inflammation by blocking the AMPK axis. Consistent with increased miR-378, expression of *Prkag2* was reduced in fatty livers of HFD-treated mice (Fig. 2B).

To investigate whether miR-378 suppresses *Prkag2* directly through its putative binding site within the 3′UTR of *Prkag2*, we employed a construct in which the 3′UTR with wild-type or mutated miR-378 binding site were inserted downstream of the luciferase reporter. Co-transfection of the luciferase reporters and MC-*TTR*-miR-378 into Hepa1-6 cells led to increased miR-378, and luciferase expression was repressed by increased miR-378 (Fig. S2A and Fig. 2C), whereas expression of the luciferase with the mutated binding site for miR-378 within 3′UTR of *Prkag2* was not altered significantly (Fig. S2B and Fig. 2D). Overexpression of miR-378 reduced mRNA and protein levels of *Prkag2* in Hepa1-6 cells (Fig. S2C and Fig. 2E-F); while miR-378 knockdown led to an opposite effect (Fig. S2D and Fig. 2G-H). Delivery of MC-*TTR*-miR-378 into livers of mice also repressed protein levels of *Prkag2* (Fig. S2E and Fig. 2I). Compared to normal human livers, mRNA levels of *PRKAG2* are significantly reduced in livers of patients with NAFLD/NASH (Fig. 2J). Our results indicate that *Prkag2* is a direct target of miR-378 *in vivo* and *in vitro*.

MiR-378 activates TNFα signaling by targeting Prkag2

AMPK signaling is an activator of sirtuin 1 (SIRT1) deacetylase that deacetylates the p65 subunit of NF-κB.[31,32] Deacetylation of p65 impairs the ability of the NF-κB complexity to transactivate *Tnfα*.[31,32] These findings led us to posit that miR-378 could potentially facilitate TNFα signaling via the AMPK-SIRT1-NF-κB axis. Gain- and loss-of function analyses of miR-378 were performed to explore the potential effect of miR-378 on TNFα signaling. Four groups of Hepa1-6 cells were treated with MC-*TTR*-miR-378-MM (control), MC-*TTR*-miR-378, MC-*TTR*-Prkag2 or a combination of MC-*TTR*-miR-378 and MC-*TTR*-Prkag2. Overexpression of miR-378 led to a considerable decrease in protein levels of AMPKγ2 and activity of SIRT1 (Fig. 3A-B, Fig. S3A). The reduction in SIRT1 activity promoted acetylation of p65 and activated TNFα signaling, which was reflected by increased acetylated p65 and TNFα (Fig. 3A, Fig. S3A).

AMPK exists as an obligate heterotrimer, containing a catalytic subunit (α) and 2 regulatory subunits (β and γ).[33] MiR-378 did not change levels of total p65 and AMPKα (Fig. 3A, Fig. S3B), suggesting that miR-378 facilitated the AMPK axis via AMPKγ2 rather than AMPKα. To confirm this, we re-introduced an open reading frame (ORF) of *Prkag2* into

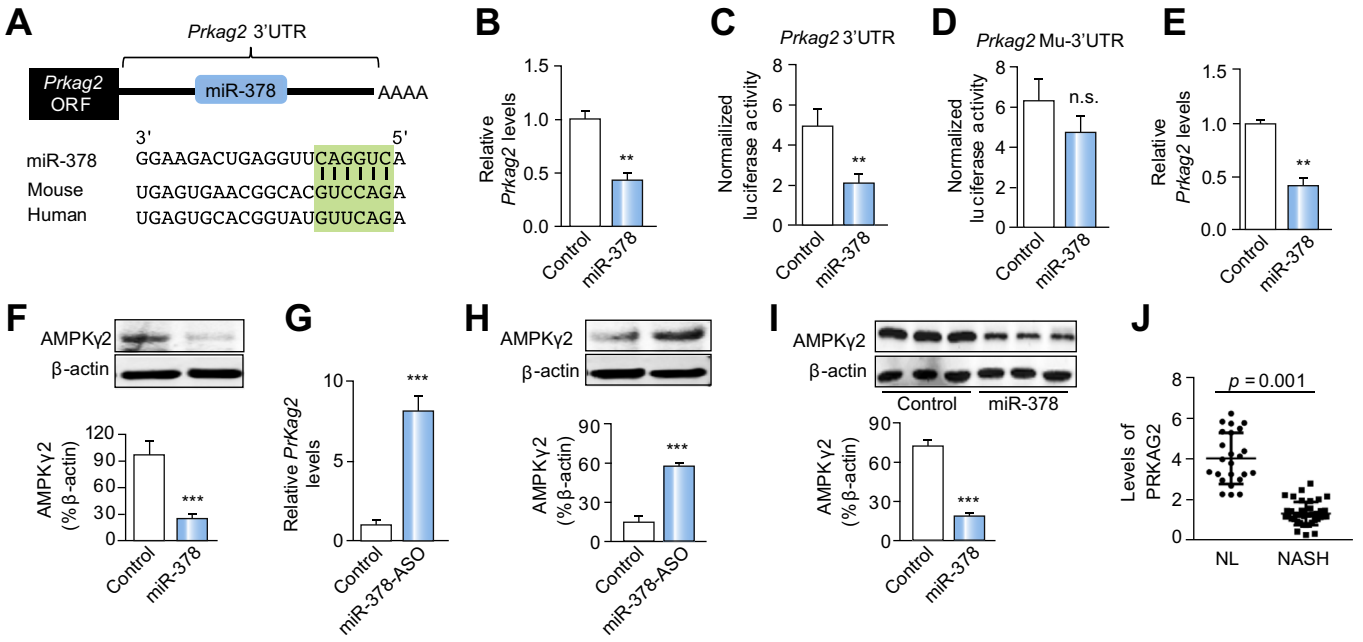

Fig. 2. *Prkag2* is a direct target of miR-378. (A) Graphic representation of the conserved miR-378 binding motifs within the 3′UTR of *Prkag2*. Complementary sequences to the seed regions of miR-378 within the 3′UTRs are conserved between human and mouse (highlighted in green). (B) Reduced mRNA levels of *Prkag2* in livers of HFD-treated mice (n = 6) compared to SD-treated mice. (C-D) Luciferase activity of the reporter constructs containing either WT or mutated 3′UTR of murine *Prkag2* after treatment with MC-*TTR*-miR-378. Hepa1-6 cells transfected with the reporter construct and MC-*TTR*-miR-378 served as the control. (E-F) RT-qPCR and immunoblot analysis revealing reduced mRNA and protein levels of *Prkag2* after MC-*TTR*-miR-378 transfection into Hepa1-6 cells. Hepa1-6 cells transfected with MC-*TTR*-miR-378-MM served as the control. (G-H) Increased mRNA and protein levels of *Prkag2* after miR-378-ASO transfection into Hepa1-6 cells, as revealed by RT-qPCR and Western blot analysis. Hepa1-6 cells received scramble served as the control. (I) Reduced protein and mRNA levels of *Prkag2* after MC-*TTR*-miR-378 injection into dietary obese mice (n = 8). The control mice received MC-*TTR*-miR-378-MM (n = 8). (J) mRNA levels of *PRKAG2* in livers of 24 normal individuals and 38 patients with NAFLD/NASH. Data represent mean ± SEM. $^{**}p$ <0.01 and $^{***}p$ <0.001 (Student's t test). Densitometry quantification of Western blot bands is graphed as Mean ± SEM. HFD, high-fat diet; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; RT-qPCR, quantitative reverse transcription PCR; SD, standard diet; UTR, untranslated region; WT, wild-type.

Hepa1-6 cells transfected with or without miR-378. Expression of *Prkag2* was not suppressed by miR-378 since the ORF did not contain 3′UTR (Fig. 3A, Fig. S3A). A single treatment of *Prkag2* impaired the NF-κB-TNFα axis, which was reflected by increased SIRT1 activity and reduced acetylated p65 and TNFα. Compared with miR-378-treated Hepa1-6 cells, treatment of a combination of miR-378 and *Prkag2* offset the action of miR-378 in facilitating the NF-κB-TNFα axis, which was reflected by increased SIRT1 activity and decreased levels of acetylated p65 and TNFα (Fig. 3A-B, Fig. S3A). These results suggested that miR-378, via targeting *Prkag2*, activated the NF-κB-TNFα pathway. Consistent with activated NF-κB-TNFα axis, miR-378 also activated expression of NF-κB induced genes and re-introduction of *Prkag2* into Hepa1-6 cells treated with miR-378 repressed expression of these genes. Among these genes, 2 were central to hepatic fibrogenesis, including a TGF superfamily member (*Tgfb1*) and a tissue inhibitor of metalloproteinase (*Timp1*) (Fig. 3C).

Next, we sought to examine whether miR-378 deficiency impairs NF-κB-TNFα signaling. Hepa1-6 cells were transfected with scramble control, miR-378-ASO (anti-sense olio), *Prkag2* shRNA or a combination of *Prkag2* shRNA and miR-378-ASO. miR-378 knockdown led to increased levels of AMPKγ2 and SIRT1 activity and reduced acetylated p65 and TNFα (Fig. 3D-E, Fig. S3C). However, no significant change in total p65 and AMPKα was observed after miR-378 knockdown (Fig. 3D, Fig. S3D). To determine whether AMPK signaling mediates the effect of miR-378 on NF-κB-TNFα, we compared Hepa1-6 cells treated with scramble control with those treated with *Prkag2* shRNA or a combination of miR-378-ASO and *Prkag2* shRNA. Compared to the control Hepa1-6 cells, knockdown of *Prkag2* facilitated the NF-κB-TNFα axis (Fig. 3D-E, Fig. S3C). Compared to Hepa1-6 cells treated with miR-378-ASO, additional treatment of *Prkag2* shRNA inhibited expression of *Prkag2* that was induced by miR-378-ASO and offset the inhibitory effect of miR-378-ASO on the NF-κB-TNFα axis. These findings indicated that *Prkag2* mediates the inhibitory effect of miR-378-ASO on the NF-κB-TNFα axis. By blocking NF-κB-TNFα, miR-378 knockdown impaired expression of *Tgfb1* and *Timp1* (Fig. 3F). Compared to Hepa1-6 cells treated with miR-378-ASO, a combined treatment of miR-378-ASO and *Prkag2* shRNA recovered expression of these 2 genes (Fig. 3F), providing additional evidence that AMPK signaling is a key component in the inflammatory and fibrotic pathways activated by miR-378.

Liver-specific expression of miR-378 promotes hepatic inflammation and fibrosis

Activation of the NF-κB-TNFα pathway by miR-378 led us to hypothesize that miR-378 might promote the development of NASH and fibrosis. To test this, we generated a miR-378 *in vivo* expression vector using a mini-circle episomal DNA vector devoid of bacterial plasmid DNA (Fig. 4A).[34] C57Bl6 mice, which had been on the HFD for 8 weeks, were injected with either MC-*TTR*-miR-378-MM (control) or MC-*TTR*-miR-378 for another 9 weeks. A group of mice before MC-*TTR*-miR-378 served as another control. Injection of MC-*TTR*-miR-378 into dietary obese mice led to increased miR-378 in hepatocytes, but no significant change in Kupffer cells and other organs

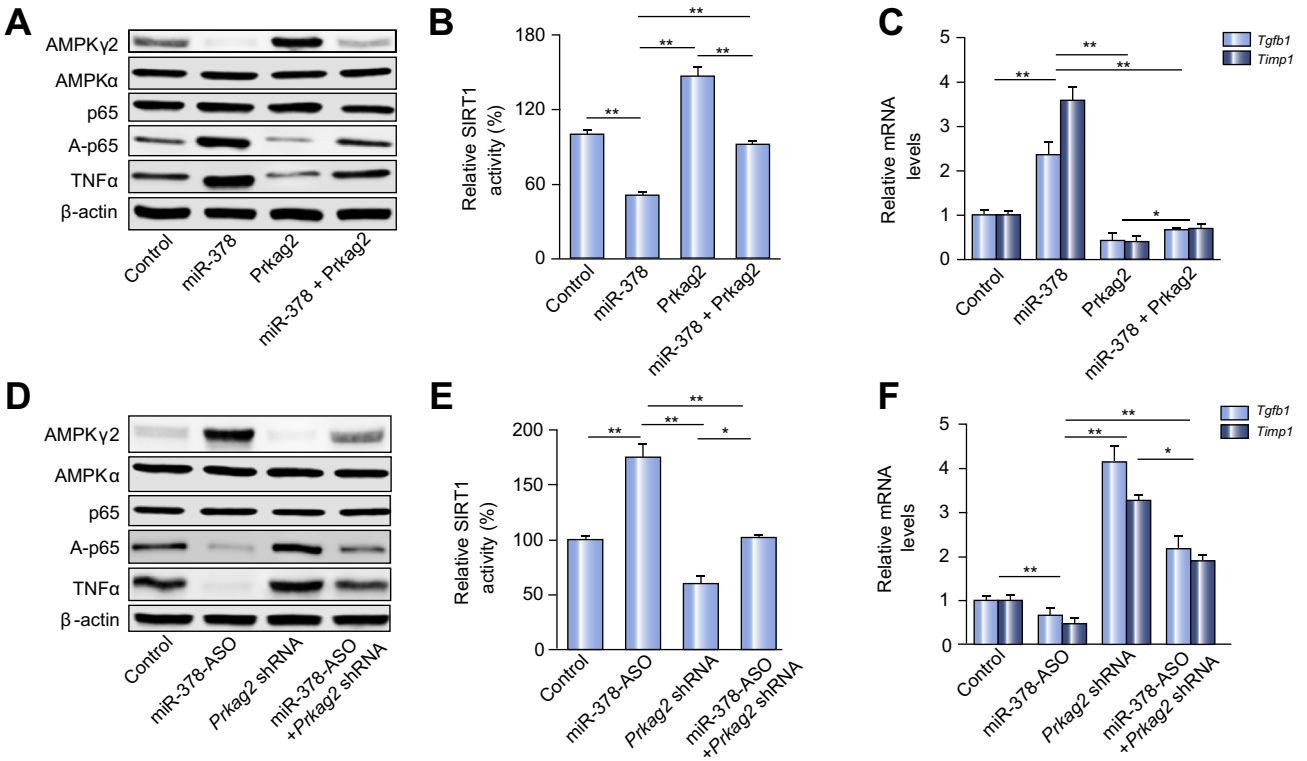

Fig. 3. miR-378 facilitates NF-κB-TNFα signaling via targeting *Prkag2*. (A) Protein levels of AMPKγ2, AMPKα, total p65, acetylated p65 (A-p65), TNFα, and β-Actin (internal control) in Hepa1-6 cells transfected with the MC-*TTR*-miR-378-MM (control), MC-*TTR*-miR-378, MC-*TTR*-*Prkag2* or a combination of MC-*TTR*-miR-378 and MC-*TTR*-*Prkag2*. (B) SIRT1 activities in the above 4 groups of Hepa1-6 cells. (C) mRNA levels of *Tgfb1* and *Timp1* in the above 4 groups of cells. (D) Protein levels of AMPKγ2, AMPKα, total p65, acetylated p65 (A-p65), TNFα, and β-Actin in Hepa1-6 cells treated with miR-378-ASO, MC-*TTR*-*Prkag2*-shRNA or a combination of miR-378-ASO and MC-*TTR*-*Prkag2*-shRNA. Hepa1-6 cells transfected with scramble and MC-*TTR*-miR-378-MM served as the control. (E) SIRT1 activities in the above 4 groups of Hepa1-6 cells. (F) mRNA levels of *Tgfb1* and *Timp1* in the above 4 groups of cells. Data represent mean ± SEM. $^{*}p$ <0.05 and $^{**}p$ <0.01 (ANOVA Test).

(Fig. 4B, Fig. S4A). Compared to miR-378-MM treated control mice, miR-378 treatment had no significant effect on body weight but slightly enhanced fat deposition (Fig. S4B-C, Table S3); and alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were markedly increased in miR-378 treated mice (Fig. 4C), encouraging us to further determine whether miR-378 can promote steatohepatitis. By 9 weeks post injection, miR-378-MM-treated mice exhibited slight hepatic inflammation, while miR-378 treatment robustly promoted the development of steatohepatitis as indicated by H&E staining and inflammation score (Fig. 4D-E). miR-378 treatment also significantly aggravated hepatocyte injury, which was reflected by a significant increase in scores for necrosis and hepatocyte ballooning in miR-378-treated mice (Fig. 4D, F-G). Along with the aggravated hepatic inflammation and injury in miR-378-treated mice, miR-378-treated mice also exhibited extensive fibrosis with collagen deposition as demonstrated by Sirius Red staining and fibrosis score (Fig. 4H-I). NAFLD activity score (NAS) in miR-378-treated mice was 6.3 (Fig. 4J), well within the range of 5–8 which is diagnostic of NASH. In summary, miR-378 dramatically induced hepatic inflammation, hepatocyte injury and fibrosis in HFD-treated mice.

Liver-specific expression of miR-378 led to activation of NF-κB-TNFα axis in dietary obese mice

We then determined whether the NF-κB-TNFα pathway is activated in dietary obese mice treated with miR-378. Compared to the mice before miR-378 treatment, another 9 weeks of HFD treatment slightly reduced AMPKγ2 and increased acetylated p65 and TNFα (Fig. 5A, Fig. S5A). miR-378 treatment accelerated this process and led to a dramatic activation of the NF-κB-TNFα axis, which was reflected by reduced AMPKγ2, decreased SIRT1 activity, and increased acetylated p65 (active form) and TNFα in livers (Fig. 5A-B, Fig. S5A). No significant change was observed in levels of total p65 and AMPKα among 3 groups of mice (Fig. 5A, Fig. S5B). Consistent with activation of the NF-κB-TNFα axis, liver-specific expression of miR-378 significantly induced expression of genes involved in hepatic inflammation and necrosis. Among them, 2 genes encode IL6 and IL-1β that are pro-inflammatory factors (Fig. 5C-D, Fig. S5C); and 3 genes encoding Fas-associated protein with death domain (FADD), Fas cell surface death receptor (FAS), and tumor necrosis factor receptor type 1-associated DEATH domain protein (TRADD) are involved in apoptosis and necrosis (Fig. 5E-F, Fig. S5D).[35] In addition to promoting inflammation,[36] IL6 can induce expression of *Tgfb1*, a key cytokine involved in the pathogenesis of fibrosis.[37] Indeed, both protein and mRNA levels of *Tgfb1* and *Timp1* in livers of HFD-treated mice were significantly increased after miR-378 treatment (Fig. 5G-H, Fig. S5E), which was consistent with enhanced fibrosis progression in miR-378 treated mice. However, miR-378 treatment exhibited no effect on expression of lipogenic genes encoding sterol regulatory element-binding protein 1 (Srebp1c), fatty acid synthase (FASN) and glycerol-3-phosphate acyltransferase (GPAT) (Fig. 5I-J, Fig. S5F). In human NASH livers, mRNA levels of *TNFα* were significantly increased (Fig. 5K). Together, the results indicated

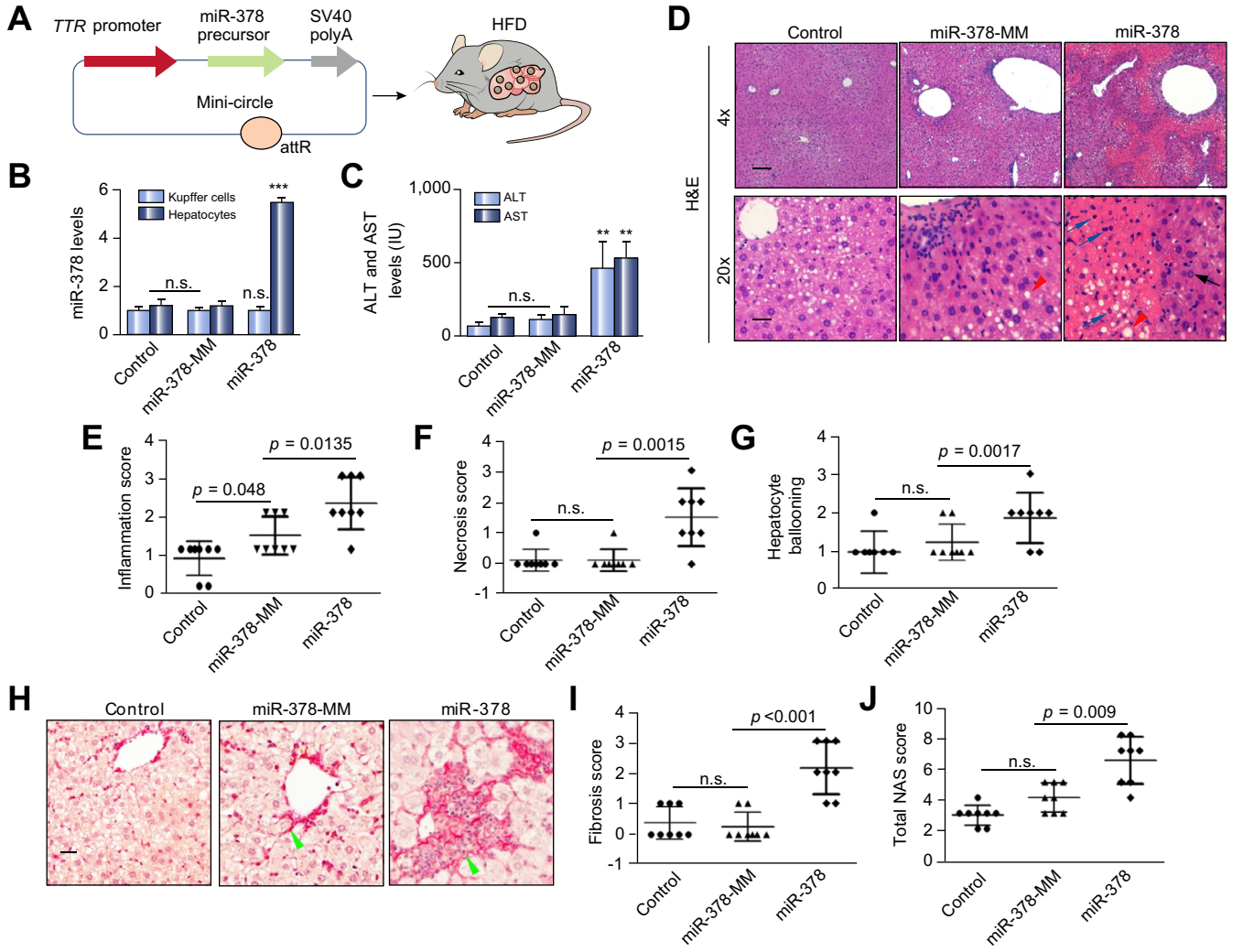

Fig. 4. miR-378 promotes hepatic inflammation and fibrosis in dietary obese mice. (A) Diagram of hepatic-specific miR-378 expression vector construction (MC-*TTR*-miR-378). (B) Levels of miR-378 in hepatocytes and Kupffer cells isolated from mice treated with MC-*TTR*-miR-378 or MC-*TTR*-miR-378-MM. Eight-week-old wild-type male C57Bl/6 mice were maintained on the HFD for 8 weeks. At 16 weeks of age, mice were injected with either MC-*TTR*-miR-378 (n = 8) or MC-*TTR*-miR-378-MM (Control, n = 8) weekly for another 9 weeks. A group of mice before MC-*TTR*-miR-378 injection served as another control. (C) ALT and AST levels in the above 3 groups of mice. (D) miR-378 significantly induced hepatic inflammation, necrosis and apoptosis as revealed by H&E staining of livers from 3 groups of mice. The eosinophilic areas (pink color) are necrotic. Blue arrow is shrunken nucleus of necrosis; and black arrow is normal hepatocyte. (E-F) Increased inflammation score and necrosis score in mice treated with MC-*TTR*-miR-378. (G) Increased hepatocyte ballooning in mice treated with MC-*TTR*-miR-378. (H) Aggravated fibrosis in livers of mice treated with MC-*TTR*-miR-378, as revealed by Sirius staining. Green arrow head is fiber stain. (I) Increased fibrosis score in mice treated with MC-*TTR*-miR-378. (J) Increased NAS in mice treated with MC-*TTR*-miR-378. Data represent mean ± SEM. $^{**}p$ <0.01; $^{***}p$ <0.001 (ANOVA Test). ALT, alanine aminotransferase; AST, aspartate aminotransferase; HFD, high-fat diet; NAFLD, non-alcoholic fatty liver disease; NAS, NAFLD activity score.

that miR-378 activated the NF-κB-TNFα axis in HFD-treated mice, which promoted hepatic inflammation and fibrosis.

TNFα signaling is required for miR-378 to promote hepatic inflammation and fibrosis

To determine whether TNFα signaling is required for miR-378 to trigger the development of NASH and fibrosis, wild-type C57Bl6 mice kept on an HFD for 8 weeks were injected with either MC-*TTR*-miR-378-MM (control), MC-*TTR*-miR-378, *Tnfα* shRNA or a combination of MC-*TTR*-miR-378 and *Tnfα* shRNA for 10 weeks. A group of mice before MC-*TTR*-miR-378 injection served as another control. Such a design allowed us to determine whether ablation of TNFα signaling activated by miR-378 can offset the effect of miR-378 on NASH progression. By 10 weeks post injection, no significant change in liver weight and body weight gain was observed among 4 groups of mice treated with MC-*TTR*-miR-378-MM, MC-*TTR*-miR-378, *Tnfα* shRNA or a combination of MC-*TTR*-miR-378 and *Tnfα* shRNA (Table S4). Serum chemistry showed that miR-378 treatment led to a slight increase in plasma triglycerides and glucose (Table S4). ALT and AST levels were also elevated in miR-378-treated mice (Table S4). We next compared mice treated with miR-378-MM or miR-378. By 10 weeks post injection, miR-378-treated mice exhibited slightly increased hepatosteatosis, with *Tnfα* knockdown reducing hepatosteatosis enhanced by miR-378 (Fig. S6A-B). Manipulation of miR-378 triggered the rapid development of hepatic inflammation and hepatocyte injury in mice, as indicated by increased scores in inflammation, necrosis and hepatocyte ballooning (Fig. 6A–D). Sirius staining and increased fibrosis score confirmed the development of

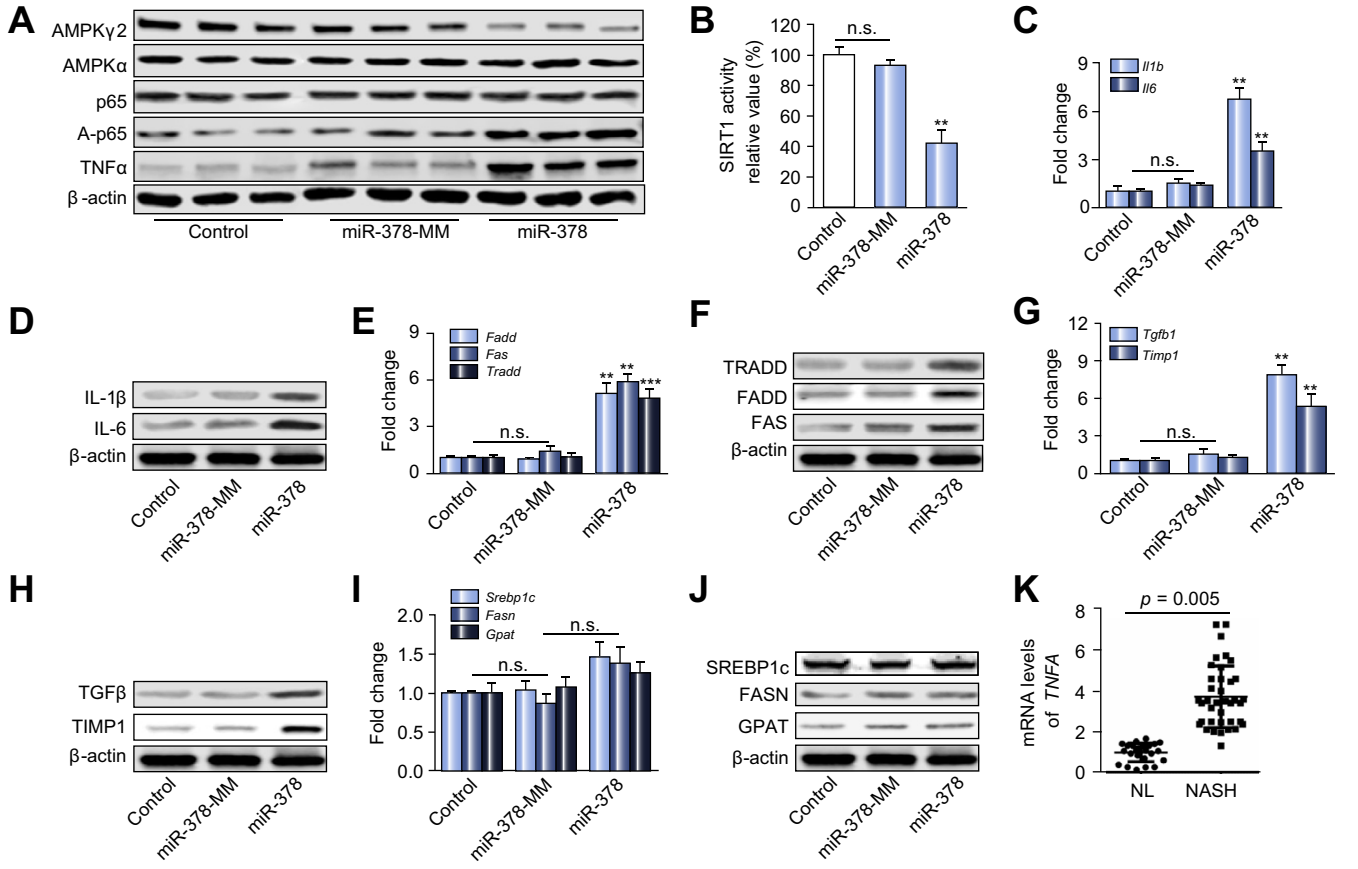

Fig. 5. Liver-specific expression of miR-378 activates NF-κB-TNFα signaling. (A) Protein levels of AMPKγ2, AMPKα, total p65, acetylated p65 and TNFα in livers of 3 groups of mice before MC-*TTR*-miR-378 treatment (n = 8) and treated with MC-*TTR*-miR-378 (n = 8) or MC-*TTR*-miR-378-MM (Control, n = 8). (B) SIRT1 activities in livers of 3 groups of mice. (C-D) Increased mRNA and protein levels of *Il1b* and *Il6* in livers of miR-378-treated mice. (E-F) Increased mRNA and protein levels of genes encoding FADD, FAS and TRADD in livers of miR-378-treated mice. (G-H) Increased mRNA and protein levels of *Timp1* and *Tgfb1* in livers of mice treated with MC-*TTR*-miR-378. (I-J) No significant change in mRNA and protein levels of *Srebp1c*, *Fasn* and *Gpat* in livers of 3 groups of mice. (K) 3.8-fold increase in mRNA levels of *TNFA* in livers of 38 patients with NAFLD/NASH compared to 24 normal individuals. Data represent mean ± SEM. $^{**}p$ <0.01; $^{***}p$ <0.001 (A–J: ANOVA Test; K: Mann-Whitney test). NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis.

fibrosis in miR-378-treated mice (Fig. 6E-F). NAS score indicated that miR-378 was able to induce NASH (Fig. 6G).

Next, we examined whether TNFα is required for miR-378 to promote NASH progression. To this end, we compared 3 groups of mice treated with either *Tnfα* shRNA, miR-378 or a combination of miR-378 and *Tnfα* shRNA. Compared to the miR-378-MM-treated control mice, *Tnfα* shRNA treatment reduced levels of ALT and AST (Table S4). A combined treatment of *Tnfα* shRNA and miR-378 partially reduced high levels of ALT and AST that were induced by miR-378 (Table S4). Remarkably, mice treated with a combination of *Tnfα* shRNA and miR-378 exhibited significantly less inflammation, hepatocyte injury and fibrosis than mice treated with miR-378 (Fig. 6A–E). miR-378 treatment significantly increased NAS, while additional knockdown of *Tnfα* offset the promoting effect of miR-378 on NAS (Fig. 6G). Consistent with activation of the NF-κB-TNFα pathway and the development of NASH/fibrosis, miR-378 treatment significantly increased both mRNA and protein levels of genes related to hepatic inflammation, necrosis, and fibrosis, which include *Il6*, *Il1b*, *Fadd*, *Fas*, *Tradd*, *Tgfb1* and *Timp1*, while additional treatment of *Tnfα* shRNA offset the effect of miR-378 (Fig. S7A–H). These data suggested that TNFα, a liver injury-promoting cytokine, is an important contributing factor to hepatic inflammation, hepatocyte injury and fibrosis induced by miR-378.

Discussion

The transition of hepatosteatosis to NASH/fibrosis and eventually cirrhosis is a key step towards the development of HCC. However, the factors and signaling cascades that actively prevent this pathological process are poorly described. By administering miR-378 to HFD-treated mice, we confirmed that miR-378 is a robust promoter of liver injury and fibrosis.[21] Furthermore, we found that NASH induced by miR-378 depended to a large extent on the associated inflammatory response associated with increased levels of TNFα and IL6. Mechanistically, we delineated a previously unrecognized pathway composed of miR-378, AMPK, SIRT1, NF-κB and TNFα that promotes the development of NASH and fibrosis (Fig. 6H). While signaling pathways in hepatocytes are known to promote hepatic inflammation and fibrosis,[38] both Kupffer cells and hepatic stellate cells play similarly important roles in their pathogenesis. However, our finding showed that hepatocytes represent the main source of miR-378 expression, suggesting that hepatocytes contribute to NASH development in livers of HFD-treated mice, further confirming the important role of hepatocytes in the development of NASH and fibrosis in miR-378-treated mice.

The importance of miR-378 in hepatosteatosis and energy homeostasis has been studied in miR-378 knockout mice.[22,24] miR-378 is a strong promoter of hepatosteatosis by targeting

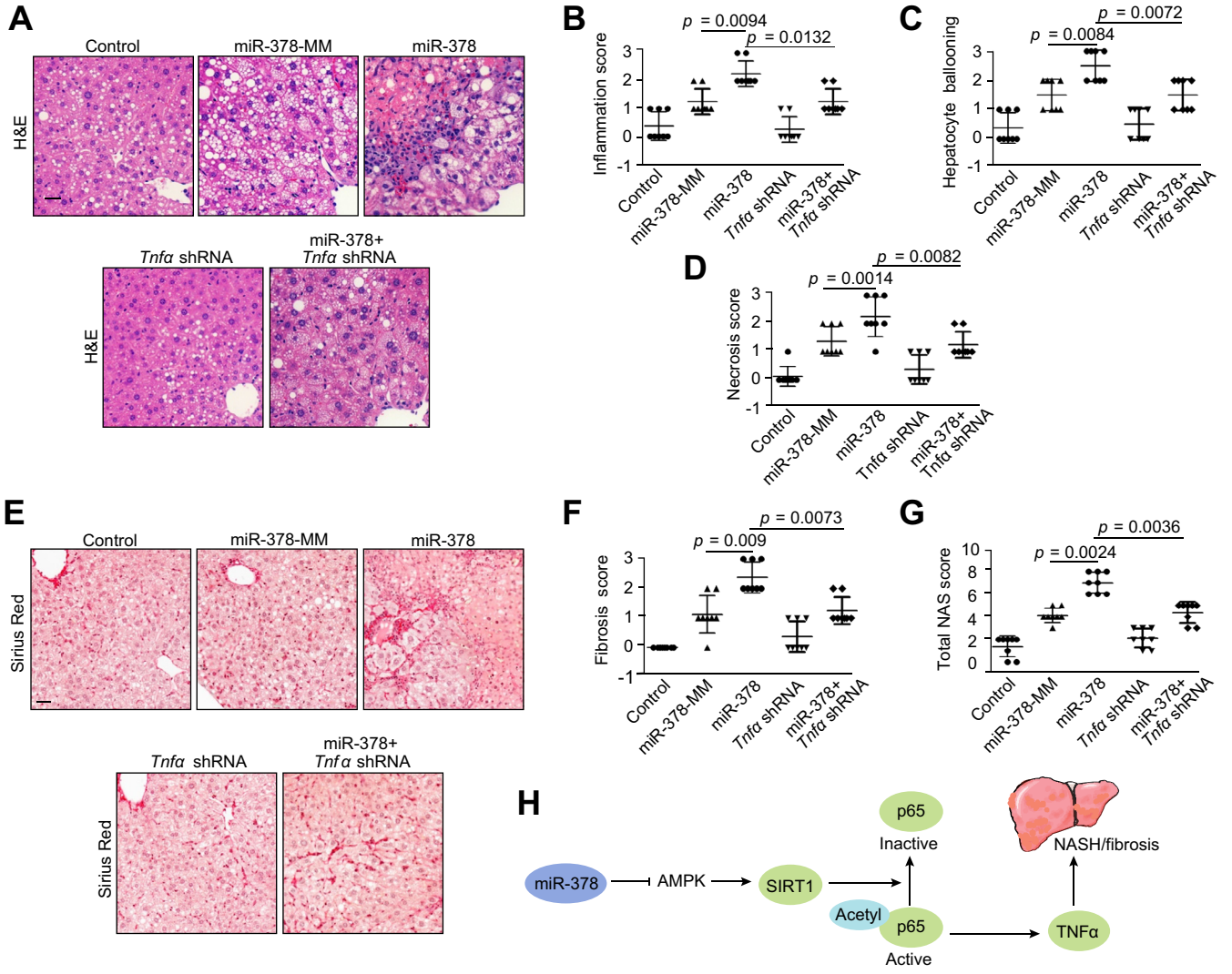

Fig. 6. TNFα signaling is required for miR-378 to promote hepatic inflammation and fibrosis. (A) H&E staining of livers from 4 groups of mice treated with MC-*TTR*-miR-378-MM (Control), MC-*TTR*-miR-378 or a combination of MC-*TTR*-miR-378, MC-*TTR*-Tnfα shRNA. A group of mice before MC-*TTR*-miR-378 treatment served as another control. MC-*TTR*-miR-378 treatment promoted hepatic inflammation and fibrosis, and additional treatment of MC-*TTR*-Tnfα shRNA offset the effects of miR-378. (B) Inflammation score in livers of 5 groups of mice. (C) Hepatocyte ballooning score in livers of 5 groups of mice. (D) Necrosis score in livers of 5 groups of mice. (E) Sirius staining of livers from 5 groups of mice. (F) Fibrosis score in livers of 5 groups of mice. (G) NAS in livers of 5 groups of mice. (H) A proposed mechanism by which miR-378 triggers hepatic inflammation and fibrosis in dietary obese mice. HFD treatment increases miR-378 expression. By targeting *Prkag2*, miR-378 impairs deacetylase activity of SIRT1. Reduced SIRT1 activity fails to inhibit transcription activity of NF-κB by deacetylating p65, which subsequently facilitates TNFα signaling. Activated TNFα signaling triggers the development of hepatic inflammation and fibrosis. Data represent mean ± SEM (A–G: ANOVA Test). HFD, high-fat diet; NAFLD, non-alcoholic fatty liver disease; NAS, NAFLD activity score.

nuclear respiratory factor 1 (*Nrf1*).[21,23] However, the role of miR-378 in regulating NASH and fibrosis remains speculative. Here we determined that miR-378 is a negative regulator of AMPK signaling, thereby facilitating the NF-κB-TNFα axis, and leading to the development of NASH, liver injury and fibrosis. Another important question regarding miR-378 that should be answered is the mechanism of dysregulated miR-378 in fatty livers. By analyzing the promoter of miR-378, we observed that NRF1 is a transcription repressor of miR-378.[21] It is known that Nrf1 is a critical regulator of NASH,[39] suggesting the potential role of the negative feedback loop between miR-378 and *Nrf1* in regulating NASH.

miR-378 inhibition has strong therapeutic potential for NASH. However, effective therapeutic approaches for NAFLD/NASH are lacking. Many types of preclinical models of NAFLD/NASH have been developed, including chemical models, genetic models and dietary models.[20] Each individual model addresses different aspects of the disease spectrum. For example, in the MCD-treated mouse model mice developed NAFLD within a relatively short period. However, the associated weight loss and lack of insulin resistance makes it quite different from human NAFLD.[40] Similar to the MCD diet, choline-deficient L-amino-defined diet treated mice fail to develop insulin resistance.[20,41] Genetic models present certain advantages concerning experimental duration and NAFLD severity.[20] However, it should be noted that these mutations are very rare in humans. The disease conditions induced by gene knockout or chemicals did not mimic the initiation and progression of NASH and fibrosis in patients.[20] Histopathological characteristics of NASH in HFD-treated mice resemble those of human NASH.[19] However, HFD mouse models often produce variable results with regard to the degree of steatosis, inflammation and fibrosis.[19] Plenty of time is needed to induce NASH in HFD-treated mice. miR-378 is a naturally-occurring small molecule in mice and humans.

Notably, the interaction between miR-378 and *Prkag2* is conserved between humans and mice. This finding indicates that mice treated with a combination of miR-378 and HFD are a potentially accurate model to study the pathogenesis of human NASH.

AMPK activity is reduced in fatty livers and increasing its activity may be a viable therapeutic strategy to treat hepatosteatosis.[42] Furthermore, AMPK pathways are involved in the inhibition of NF-κB signaling and suppression of inflammation.[30] However, the role of miRNAs in modulating AMPK signaling is rarely reported. In mouse hepatocytes, our data suggest that miR-378 is an activator of NF-κB-TNFα axis by inhibiting AMPK signaling. Furthermore, activation of the NF-κB-TNFα axis by miR-378 appears to be a key player in the development of hepatic inflammation, liver injury and fibrosis in HFD-treated mice.

In summary, we have employed detailed molecular and cellular approaches and mouse models to demonstrate that miR-378 is a key player in modulating NASH via TNFα signaling. miR-378 acts as an important component of the molecular circuit to induce spontaneous activation of inflammatory genes with potential implications in NASH pathogenesis. The insights obtained from this study advance our understanding of the physiological roles of miR-378 in regulating hepatic inflammation and liver injury, which is important to address the complexity of the development of NASH and fibrosis. In addition, both miR-378 and *Prkag2* and their expression pattern are conserved between humans and mice, suggesting that miR-378-ASO as a potential therapeutic agent can be applied to humans.

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Conflicts of interest

The authors declare no conflicts of interest that pertain to this work.

Please refer to the accompanying ICMJE disclosure forms for further details.

Authors' contributions

G.S., G.Z. conceived the project, G.S. and G.Z supervised the project; G.S., T.Z and J.H designed the experiments; T.Z., J.H., and X. W. performed experiments; X.Z. performed target prediction and database comparison; C.J.S and J.N. analyzed and interpreted data; and G.S. and G.Z wrote the manuscript with input and editing from the authors.

Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jhep.2018.08.026.

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Author names in bold designate shared co-first authorship

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