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ORIGINAL ARTICLE

MicroRNA-21 is a potential link between non-alcoholic fatty liver disease and hepatocellular carcinoma via modulation of the HBP1-p53-Srebp1c pathway

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ABSTRACT

Background Non-alcoholic fatty liver disease (NAFLD) is a major risk factor for hepatocellular carcinoma (HCC). However, the mechanistic pathways that link both disorders are essentially unknown.

Objective Our study was designed to investigate the role of microRNA-21 in the pathogenesis of NAFLD and its potential involvement in HCC.

Methods Wildtype mice maintained on a high fat diet (HFD) received tail vein injections of microRNA-21-anti-sense oligonucleotide (ASO) or miR-21 mismatched ASO for 4 or 8 weeks. Livers were collected after that time period for lipid content and gene expression analysis. Human hepatoma HepG2 cells incubated with oleate were used to study the role of miR-21 in lipogenesis and analysed with Nile-Red staining. microRNA-21 function in carcinogenesis was determined by soft-agar colony formation, cell cycle analysis and xenograft tumour assay using HepG2 cells.

Results The expression of microRNA-21 was increased in the livers of HFD-treated mice and human HepG2 cells incubated with fatty acid. MicroRNA-21 knockdown in those mice and HepG2 cells impaired lipid accumulation and growth of xenograft tumour. Further studies revealed that *Hbp1* was a novel target of microRNA-21 and a transcriptional activator of *p53*. It is well established that *p53* is a tumour suppressor and an inhibitor of lipogenesis by inhibiting *Srebp1c*. As expected, microRNA-21 knockdown led to increased *HBP1* and *p53* and subsequently reduced lipogenesis and delayed G1/S transition, and the additional treatment of *HBP1*-siRNA antagonised the effect of microRNA-21-ASO, suggesting that *HBP1* mediated the inhibitory effects of microRNA-21-ASO on both hepatic lipid accumulation and hepatocarcinogenesis. Mechanistically, microRNA-21 knockdown induced *p53* transcription, which subsequently reduced expression of genes controlling lipogenesis and cell cycle transition. In contrast, the opposite result was observed with overexpression of microRNA-21, which prevented *p53* transcription.

Conclusions Our findings reveal a novel mechanism by which microRNA-21, in part, promotes hepatic lipid accumulation and cancer progression by interacting with the *Hbp1-p53-Srebp1c* pathway and suggest the potential therapeutic value of microRNA-21-ASO for both disorders.

INTRODUCTION

The incidence of hepatocellular carcinoma (HCC) worldwide nearly matched its mortality,

Significance of this study**What is already known on this subject?**

- miR-21 is upregulated in human hepatocellular carcinoma.
- p53 is a transcriptional repressor of *Srebp1c*.

What are the new findings?

- miR-21 is highly expressed in hepatocytes, and its expression is significantly increased in livers of dietary obese mice and human HepG2 cells incubated with fatty acid.
- Antagonising miR-21 in liver prevents hepatic lipid accumulation and growth of xenograft tumour.
- miR-21 knockdown prevents G1/S transition and cancer cell proliferation.
- *HBP1* is a novel target of miR-21 and a transcriptional activator of *p53*.
- *HBP1* mediates the inhibitory effects of miR-21-anti-sense oligonucleotide on hepatic lipid accumulation and hepatocarcinogenesis.
- miR-21 is a potential association between non-alcoholic fatty liver disease (NAFLD) and hepatocellular carcinoma (HCC) via interacting with the *Hbp1-p53-Srebp1c* pathway.

How might it impact on clinical practice in the foreseeable future?

- Our data suggest that miR-21 is a potential therapeutic target for both NAFLD and HCC.

demonstrating the aggressive nature of this malignancy and limited therapeutic options.¹ Although HBV and HCV are major risk factors of HCC, non-alcoholic fatty liver disease (NAFLD) remains a common underlying pathology to the majority of patients with HCC in the Western world.² The incidence of NAFLD is growing rapidly due to the prevalence of obesity.³ It is estimated that 90% of obese patients have some form of fatty liver, ranging from simple steatosis to more severe forms of non-alcoholic steatohepatitis (NASH) and cirrhosis with its associated high risk of HCC. In addition, given limited effects of chemotherapy and the relative insensitivity of HCC to radiotherapy, complete tumour extirpation represents the only choice for a long-term cure. Unfortunately, the

majority of patients are not eligible for surgical resection because of tumour extent or underlying liver dysfunctions including NAFLD. As described above, despite the strong association between NAFLD and HCC, the underlying mechanisms are largely unknown due in part to their complex nature of disease.

The discovery of a class of naturally occurring small non-coding RNAs, termed microRNAs (miRNAs),^{4,5} has stimulated a new field of research on NAFLD and HCC. Alterations in miRNA expression have been reported in human individuals with NAFLD/NASH and HCC.^{6,7} Reflective of their key roles in lipid metabolism and carcinogenesis,^{5,8} miRNAs have been suggested as novel therapeutic targets for both metabolic diseases and human cancers. However, the miRNAs associated with both NAFLD and its potential sequel HCC are poorly described. Our interest in miR-21 arose initially from hepatocyte-specific miRNA profiling studies in mouse livers, in which we showed that miR-21 is highly expressed in hepatocytes. Furthermore, we observed that high fat diet (HFD) treatment significantly induced expression of miR-21 in livers of mice. By antagonising miR-21 in liver, we were able to prevent hepatic lipid accumulation in dietary obese mice. Consistent with our findings, miR-21 expression was significantly upregulated in human patients with NASH.⁶ It is also known that miR-21 is a potent promoter of HCC and other human cancers.^{7,9} These data led us to hypothesise that miR-21 plays an important role in the pathogenesis of NAFLD and its potential progression to HCC. In the present study, we have investigated the regulatory role of miR-21 in linking NAFLD and HCC in both in vivo and in vitro model systems.

MATERIALS AND METHODS

Bioinformatic analysis

Identification of miR-21 target genes was conducted as previously described with minor revision.¹⁰ In detail, we compiled a list of downregulated genes in livers of patients with NAFLD/NASH by downloading their microarray data from GEO (<http://www.ncbi.nlm.nih.gov/geo/>).¹¹ mRNA profiles of six normal liver samples (male) and eight NAFLD/NASH liver samples (male) were compared using GeneSpring (Agilent Technologies Genomics). Differentially expressed genes were defined by a log-scale ratio ≤ 0.3 between paired samples with a $p < 0.05$. Based on these criteria, we identified 1219 downregulated probes in NAFLD/NASH samples (see online supplementary table S1). To identify genes with binding motifs for miR-21, we downloaded the target gene databases of miR-21 based on TargetScan,¹² Pictar¹³ and Starbase.¹⁴ Only hits from Target or PicTar algorithm that were confirmed by Ago HITS-CLIP (high-throughput sequencing of RNAs isolated by cross-linking immunoprecipitation (HITS-CLIP) from Argonaute protein complex) were selected. These three databases were compared using Microsoft Access 2000, yielding 219 potential targets that have miR-21 binding motif (see online supplementary table S2). We then compared 1219 downregulated probes in livers of patients with NAFLD/NASH with 219 genes that have at least one binding motif for miR-21 using Microsoft Access 2010, which resulted in an overlap of 13 genes between two databases that were considered as potential targets of miR-21 (see online supplementary table S3). Gene ontology (GO) analysis was done using PathwayStudio software (Elsevier).

Animal, diet treatment and sample collection

Male *Dicer1^{fl/fl}* mice on a mixed 129S4, C57Bl/6 strain background¹⁵ were crossed with C57Bl/6 *Alb-Cre^{+/-}* mice¹⁶ to generate *Dicer1^{fl/fl}, Alb-Cre^{+/-}* mice (mice are from Dr Holger

Willenbring's lab at the University of California, San Francisco). To specifically investigate the impact of miRNAs on mature liver function, we initiated Cre recombinase expression in 8-week-old to 10-week-old mice.¹⁷ To restrict Cre expression to hepatocytes, we used a hepatocyte-specific *Transferrin* (*Ttr*) promoter and pseudotyped the vector genome with capsids from AAV8, a serotype that can transduce virtually all hepatocytes in vivo without causing toxicity.^{10,17,18}

To determine the effect of hepatic lipid accumulation on miRNA expression, 8-week-old wildtype male C57Bl/6 mice (Jackson Laboratory, n=6) were maintained on either a normal chow diet (Open Source D12450B: 10% Kcal fat) or an HFD (Open Source D12492: 60% Kcal fat) for 4 weeks as described by Vickers *et al.*¹⁹ After 4 weeks of HFD administration, livers were collected for miRNA and gene expression analysis.

To determine the role of miR-21 in NAFLD, we synthesised locked nucleic acid anti-miR-21 anti-sense oligonucleotide (ASO) (Exiqon) specifically targeting miR-21 and also generated miR-21-mismatched-ASO (miR-21-MM-ASO), a control ASO that differs from miRNAs in four mismatched base pairs. The male C57Bl/6 mice kept on HFD for 4 weeks were divided into two groups; one group (n=8) was treated with miR-21-ASO and the other with miR-21-MM-ASO (control, n=8). Mice received a dose of 25 mg/kg miR-21-ASO or miR-21-MM-ASO (0.9% NaCl) weekly for 4 or 8 weeks via tail vein injection. At those times, the mice were anaesthetised, and blood was collected by way of cardiac puncture. Subsequently, the livers were harvested and immediately frozen in liquid nitrogen and stored at -80°C for gene expression and histological analysis.

Fatty acid treatment of HepG2 cells

HepG2 cells were obtained from Dr Xin Chen's laboratory at the University of California, San Francisco. Sodium oleate was obtained from Sigma-Aldrich and was dissolved in Dulbecco's modified Eagle medium (DMEM) with 1% fatty acid free bovine serum albumin (BSA) (Sigma). Oleate treatment of HepG2 cells was carried out as previously described with minor revision.^{10,20} Specifically, HepG2 cells were plated in four-well chamber slides with DMEM medium supplemented with 10% fetal bovine serum (Invitrogen). After 24 h, HepG2 cells were treated with either control medium (DMEM supplemented with 1% fatty acid free BSA) or medium containing oleate (0.5 mM). The cells were cultured for another 24 h, then lipid accumulation and miR-21 expression were determined by Nile Red Staining (Sigma-Aldrich) and qRT-PCR, respectively (see online supplementary materials and methods for details).

Cell proliferation analysis

HepG2 cells were transfected with miR-21-ASO, scrambled control or miR-21-ASO plus *HBP1*-siRNA using lipofectamine 2000 (Invitrogen). After 48 h, cell proliferation was determined using a MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide) cell proliferation kit (Cell Biolabs) according to the manufacturer's instruction (see online supplementary material and methods for details).

Focus formation assay and flow cytometry analysis

HepG2 cells were used to determine the effect of miR-21 on focus formation and cell cycle progression (see online supplementary material and methods for details).

Xenograft tumour assay

Male BALB/c athymic nude mice (6 weeks old) purchased from Jackson Laboratory were used to study the role of miR-21 in

promoting growth of xenograft tumour from HepG2 cells (see online supplementary material and methods for details).

Histological analysis

Frozen sections of liver were stained with Oil Red-O staining. Paraformaldehyde-fixed, paraffin-embedded sections of liver were stained with H&E (see online supplementary material and methods for details).

Lipid and lipoprotein analysis

Both plasma and hepatic lipid content were enzymatically measured in liver lysates and plasma via a colorimetric assay using a triglyceride assay kit from Roche Diagnostics, according to the manufacturer's protocols (see online supplementary material and methods for details).

Western blot and Q-RT-PCR

Western blot and qRT-PCR were used to determine expression levels of genes. Primers used for quantitative RT-PCR are listed in online supplementary table S4 (see online supplementary material and methods for details).

Statistical analysis

Statistical analysis was performed using GraphPad Prism Software. Data derived from cell-line experiments were presented as mean $\pm$ SD and assessed by a two-tailed Student's *t* test. Statistical difference for cell cycle progression analysis was evaluated using χ^2 test. Mann–Whitney test was used to evaluate the statistical significance for mouse experiments. Each experiment was repeated at least three times; and the error bars represent the SD. $p < 0.05$ was considered to be statistically significant.

RESULTS

miR-21 is robustly induced in livers of mice on HFD and HepG2 cells exposed to high levels of fatty acid

Hepatocytes are the major cells that control lipid metabolism and the primary site of NAFLD and HCC. To investigate the role of miRNAs in both disorders, we compiled hepatocyte-specific miRNA profiles by comparing miRNAs expression of livers of hepatocyte-specific *Dicer1* knockout (*DKO*) and wild-type mice (see online supplementary table S5). We observed that miR-21 was the most significantly downregulated miRNA in livers of *DKO* mice (≥ 39 -fold reduced), indicating that hepatocytes represent a main source of its expression in the liver (figure 1A). To assess the role of miR-21 in NAFLD, we fed wildtype C57Bl/6 mice an HFD (see online supplementary figure S1A–C) and measured its hepatic expression. The results showed that miR-21 had a twofold upregulation in the livers of HFD-treated mice (figure 1B), suggesting its potential role in NAFLD.

We also determined whether fatty acids can increase the expression of miR-21 in human hepatoma HepG2 cells. Oleic acids are the most abundant unsaturated fatty acids in liver triglycerides in both normal subjects and patients with NAFLD.²¹ In this study, HepG2 cells were used because of their increased sensitivity to fat accumulation.²¹ Nile-Red staining revealed that oleic acid treatment led to an increase in intracellular lipids in HepG2 cells (figure 1C, D), which was also associated with upregulation of miR-21 (figure 1E). Taken together, our *in vivo* and *in vitro* studies indicated that both HFD treatment of mice and exposure of HepG2 cells to fatty acid were able to induce expression of miR-21.

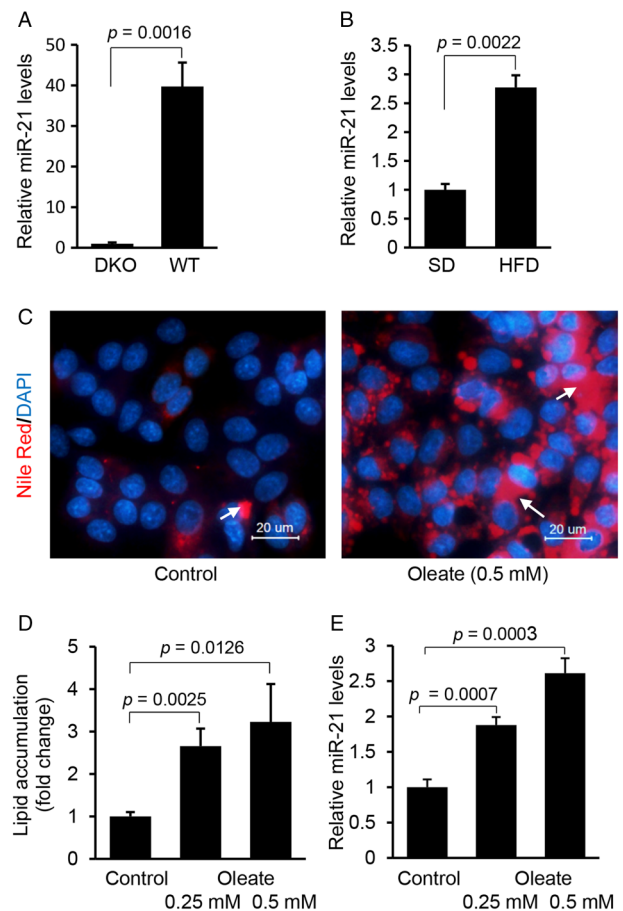


Figure 1 Hepatocyte-specific miR-21 is significantly induced in livers of high fat diet (HFD)-treated mice and HepG2 cells treated with fatty acid. (A) qRT-PCR confirmed that miR-21 was highly expressed in hepatocytes of liver. miR-21 expression in liver was compared between *Dicer1* knockout (*DKO*) ($n=3$) and wildtype mice ($n=3$) using qRT-PCR. Data are presented as mean $\pm$ SD ($p=0.0016$, Student's *t* test). (B) HFD treatment led to higher levels of miR-21 in livers of mice. Briefly, wildtype mice (C57Bl/6) were maintained on HFD for 4 weeks, and then mice were sacrificed and livers collected for miRNA expression analysis. Control mice received standard diet (SD). Data are presented as mean $\pm$ SD ($p=0.0022$, Mann–Whitney test). (C and D) Oleate treatment increased lipid content and subsequently (E) expression of miR-21 in HepG2 cells. HepG2 cells were maintained in Dulbecco's modified Eagle medium containing 0.5 mM oleate. Data are presented as mean $\pm$ SD. In this multiple-groups experiment, we only performed comparison between two groups and Student's *t* test was used for statistical analysis. Lipid droplets in human hepatocytes were labelled with arrows.

To further elucidate the role of miR-21 in hepatic lipid accumulation, we began to identify target genes of miR-21 by combining mRNA profiling of livers of NAFLD individuals with the bioinformatic prediction of miR-21 binding motifs within potential target mRNAs. This led us to identify 13 genes including *HBP1*, *SOX7* and *RHOB* that showed reduced expression in human fatty liver and contained a conserved binding motif for miR-21 (see online supplementary table S3). GO analysis of the above 13 genes revealed that *HBP1* was a potent tumour suppressor by preventing G1/S transition of cell cycle.^{22–23} In addition, our prediction from *in silico* algorithms showed that the 3' UTR of *HBP1* mRNA was 100% complementary to the miR-21 5' seed region, exhibiting the highest prediction score and binding energy (figure 2A). Therefore, we selected *HBP1* as a potential target of miR-21.

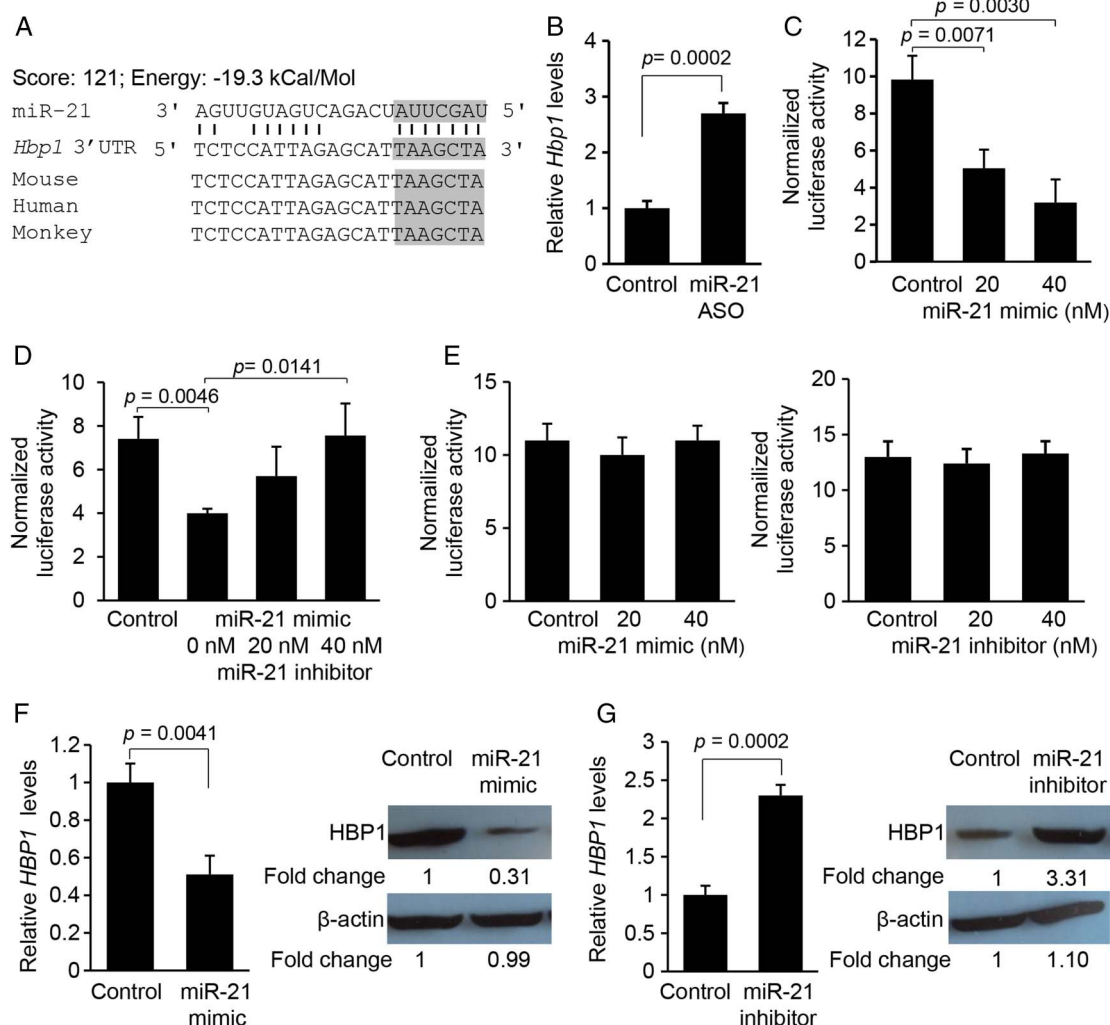


Figure 2 *Hbp1* is a direct target of miR-21. (A) Bioinformatic analysis showing that the seed sequence of miR-21 has a high level of complementarity to *Hbp1* 3' UTR, prediction score and favourable binding energy. Complementary sequences to the seed region of miR-21 within the 3' UTRs of *Hbp1* are conserved between human, mouse and monkey (grey highlight). (B) miR-21 knockdown in high fat diet (HFD)-treated mice led to increased *Hbp1* mRNA levels in liver. C57Bl/6 wildtype mice were kept on normal chow until 8 weeks of age and then maintained on HFD until 16 weeks of age. At 12 weeks of age, the mice were given miR-21-anti-sense oligonucleotide (ASO) (25 mg/kg, tail vein injection) until 16 weeks of age. C57Bl/6 mice maintained on HFD and treated with miR-21-MM-ASO served as controls. The expression levels of *Hbp1* were determined by qRT-PCR. Data are presented as means $\pm$ SD ($p=0.0002$, Mann-Whitney test). (C) miR-21 mimic transfection into Hepa1,6 cells caused dose-dependent inhibition of the activity of a luciferase reporter gene linked to the 3' UTR of mouse *Hbp1*. Data are presented as mean $\pm$ SD. In this multiple-groups experiment, we only performed comparison between two groups among them and Student's *t* test was used for statistical analysis. (D) Conversely, transfection with a miR-21 inhibitor antagonised the binding of miR-21 mimics to the 3' UTR of mouse *Hbp1*, which was reflected by increased luciferase activity. Data are presented as mean $\pm$ SD. Student's *t* test was used for statistical analysis. (E) Mutated binding motif for miR-21 within *Hbp1* 3'UTR impaired miR-21 binding, which was reflected by (i) negligible change of luciferase activity after miR-21 mimics treatment; and (ii) miR-21 inhibitor treatment had no effect on luciferase activity. Data are presented as mean $\pm$ SD ($p\geq 0.1$, Student's *t* test). (F) Transfection of miR-21 mimics into HepG2 cells inhibited expression levels of endogenous *HBP1* as revealed by qRT-PCR and western blot. Data are presented as mean $\pm$ SD ($p=0.0041$, Student's *t* test). (G) miR-21 knockdown by transfecting miR-21 inhibitor into HepG2 cells caused an increase in endogenous *HBP1* at the protein and mRNA levels. Data are presented as mean $\pm$ SD ($p=0.0002$, Student's *t* test).

We next determined its expression in the livers of dietary obese mice treated with miR-21-ASO. It was not surprising that *Hbp1* expression increased more than twofold in the livers of miR-21-ASO-treated mice compared with those treated with miR-21-MM-ASO (figure 2B). Taken together, hepatic expression of miR-21 was increased in dietary obese mice and livers of human patients with NAFLD/NASH, and *Hbp1*, as a potential target of miR-21, showed reduced expression in livers of obese mice and human patients with NAFLD (see online supplementary table S3 and figure S1D). Our findings suggested that the crosstalk between miR-21 and *Hbp1* might play an important role in hepatic lipid accumulation.

Hbp1 is a direct target of miR-21

To establish that miR-21 directly recognises the predicted target site within the 3' UTR of *Hbp1*, the 3' UTR of mouse *Hbp1* mRNA was cloned into a luciferase reporter vector (pMiR-Report) to generate pMiR-*Hbp1*. Mouse Hepa1,6 cells were transfected with pMiR-*Hbp1* and chemically synthesised miR-21 mimic or miR-21 inhibitor. We found that miR-21 mimics significantly downregulated luciferase activity in a dose-dependent fashion (figure 2C). Consistently, miR-21 inhibitor antagonised the inhibitory effect of miR-21 mimics on luciferase activity (figure 2D). Furthermore, we mutated the binding motif for miR-21 within the pMiR-*Hbp1* 3' UTR and found that both

mimics and inhibitors of miR-21 had no effect on luciferase activity (figure 2E), indicating a potentially direct interaction between miR-21 and *Hbp1* mRNA. To further validate that *Hbp1* is a target of miR-21, we increased intracellular levels of miR-21 in HepG2 cells in the absence of fatty acid. qRT-PCR and western blot revealed that miR-21 significantly inhibited expression of *HBP1* (figure 2F). In contrast, miR-21 knockdown led to an increase in mRNA and protein levels of *HBP1* in HepG2 cells (figure 2G). Together, these results confirmed that *Hbp1* is a direct target of miR-21.

HBP1 inhibits expression of *SREBP1C*, *CCND1* and *CCNB1* by activating *p53*

HBP1 is a well-described transcriptional repressor that modulates expression of genes involved in cell cycle progression.²³ Therefore, we overexpressed *HBP1* in HepG2 cells and determined the expression levels of genes involved in cell cycle using Human Cell Cycle RT² Profiler PCR Assay. Interestingly, we observed that *p53* was the most upregulated after overexpression of *HBP1* (figure 3A). *p53* functions as a tumour suppressor and potent inhibitor of lipogenesis by inhibiting transcription of *SREBP1C*,^{24–26} leading to our hypothesis that miR-21 plays roles in both lipogenesis and carcinogenesis by interacting with the HBP1-*p53* pathway.

Overexpression of *HBP1* led to increased mRNA levels of *p53*, implying that *HBP1* might activate transcription of *p53* by binding to its promoter. Therefore, we cloned the *p53* promoter into a luciferase reporter vector (pGL3-Basic) and generated pGL3-*p53*. Hepa1,6 cells were transfected with pGL3-*p53* and *HBP1* expression vector. As expected, overexpression of *HBP1* induced luciferase activity (figure 3B), and *HBP1* knockdown led to decreased luciferase activity (figure 3C). Furthermore, *HBP1* knockdown impaired expression of endogenous *p53* (figure 3D), suggesting that *HBP1* was able to activate transcription of *p53*. HBP1 can function as a transcriptional activator by binding to a specific binding motif (GGGATGGG).²² However, we did not identify this binding motif within the promoter of *p53*, signifying that HBP1 might activate transcription of *p53* by interacting with other transcription factors that have binding sites within the *p53* promoter.

Srebp1c is a transcription factor that activates genes encoding enzymes required for lipid synthesis.^{27–28} Considering the role that *p53* plays in inhibiting lipogenesis by modulating *Srebp1c*,²⁶ we cloned the mouse *Srebp1c* promoter into pGL3-basic vector (pGL3-*Srebp1c*). As expected, co-transfection of *p53* expression vector pGL3-*Srebp1c* and into Hepa1,6 cells significantly reduced luciferase activity in a dose-dependent fashion (see online supplementary figure S2A), and in contrast, *p53* knockdown induced luciferase activity (see online supplementary figure S2B). Furthermore, we observed that overexpression of *p53* led to a decrease in endogenous mRNA levels of *SREBP1C* and its targeted lipogenic genes *SCD1* (stearoyl-CoA desaturase-1), *GPAT* (glycerol 3-phosphate acyltransferase), and *FASN* (fatty acid synthase),²⁹ and genes controlling cell cycle progression including *CCNB1* and *CCND1* in HepG2 cells (figure 3E).³⁰ *p53* knockdown led to the opposite effect (figure 3F), underscoring the central role of *p53* in modulating the expression of genes involved in lipogenesis and cell cycle progression.

To further determine whether *HBP1* prevents transcription of genes associated with lipogenesis and G1/S transition via *p53*-*SREBP1C* pathway, we overexpressed *HBP1* in HepG2 cells and determined expression levels of *p53*, *SREBP1C*, the lipogenic genes and *CCNB1* and *CCND1*. As confirmed by qRT-PCR, *HBP1* overexpression led to increased *p53*, which

subsequently prevented expression of *SREBP1C* and *SCD1*, *FASN* and *GPAT*, as well as *CCNB1* and *CCND1* (figure 3G). In summary, our findings suggested that HBP1 is able to simultaneously inhibit expression of *CCNB1*, *CCND1* and *SREBP1C* by modulating *p53*.

miR-21 prevents expression of *p53* but promotes transcription of *SREBP1C* by modulating *HBP1* expression

We have shown that *HBP1* is a target of miR-21, and HBP1 can activate transcription of *p53*. Meanwhile, *p53* is a transcriptional repressor of *Srebp1c*.^{25–31} Thus, we hypothesised that miR-21 can simultaneously regulate expression of genes involved in lipogenesis and the G1/S transition by modulating the *HBP1*-*p53*-*SREBP1C* pathway. Indeed, overexpression of miR-21 in HepG2 cells inhibited expression of *HBP1*, which subsequently led to a reduction in *p53* and an increase in mRNA levels of *CCND1*, *CCNB1* and *SREBP1C*, as well as its target genes including *SCD1*, *FASN* and *GPAT* (figure 3H), while miR-21 knockdown led to an opposite effect (figure 3I). Our findings indicated that miR-21 is able to modulate expression of genes controlling lipogenesis and cell cycle progression via the *p53*-*SREBP1C* pathway.

miR-21 modulates lipid accumulation in HepG2 cells by interacting with the *HBP1*-*p53* pathway

We then determined whether overexpression of miR-21 can promote lipogenesis. As expected, miR-21 overexpression prevented expression of *HBP1* and *p53* (figure 4A), which subsequently promoted lipid accumulation in HepG2 cells (figure 4B, C).

To determine loss of function for miR-21 in lipid accumulation, we transfected miR-21 inhibitor into oleate-treated HepG2 cells to knock down upregulated miR-21. Antagonising miR-21 led to a significant increase in *HBP1* and *p53* (figure 4D), which subsequently prevented lipid accumulation (figure 4E, F). These data demonstrated that miR-21 was sufficient for the downregulation of *HBP1* and *p53*, which subsequently induced lipid accumulation. To further investigate the role of the interaction between miR-21 and *HBP1* in hepatic lipid accumulation, we mutated the binding motif for miR-21 within the 3' UTR of *Hbp1* in the pMiR-*Hbp1* (referred to as pMiR-*Hbp1*Mu) and introduced the pMiR-*Hbp1* or pMiR-*Hbp1*Mu into oleate-treated HepG2 cells. Since oleate treatment increases miR-21 expression in HepG2 cells, it was expected that it would lead to a decrease of luciferase activity in HepG2 cells transfected with pMiR-*Hbp1* compared with pMiR-*Hbp1*Mu. In fact, oleate treatment of HepG2 cells transfected with pMiR-*Hbp1* resulted in robust repression of luciferase activity compared with pMiR-*Hbp1*-Mu (figure 4G). Together, our results indicated that *HBP1* is a direct target of miR-21 during lipid accumulation in HepG2 cells and the crosstalk of miR-21 with *HBP1* and *p53* plays an important role in hepatic lipid accumulation.

Inhibitory effect of miR-21-ASO on hepatic lipid accumulation is mediated by *HBP1*

To confirm that miR-21 promotes lipogenesis via *HBP1*, we initially antagonised miR-21 by transfecting miR-21-ASO into HepG2 cells to induce expression of *HBP1*, and then knocked down the induced *HBP1* using *HBP1*-siRNA. The results showed that miR-21 knockdown increased *p53* and *HBP1* and reduced lipid content in HepG2 cells, but additional treatment of *HBP1*-siRNA offset the effect of miR-21-ASO (figure 5A–C), which suggested that *HBP1*, in part, mediated the inhibitory effect of miR-21 inhibitor on lipid accumulation. To study the

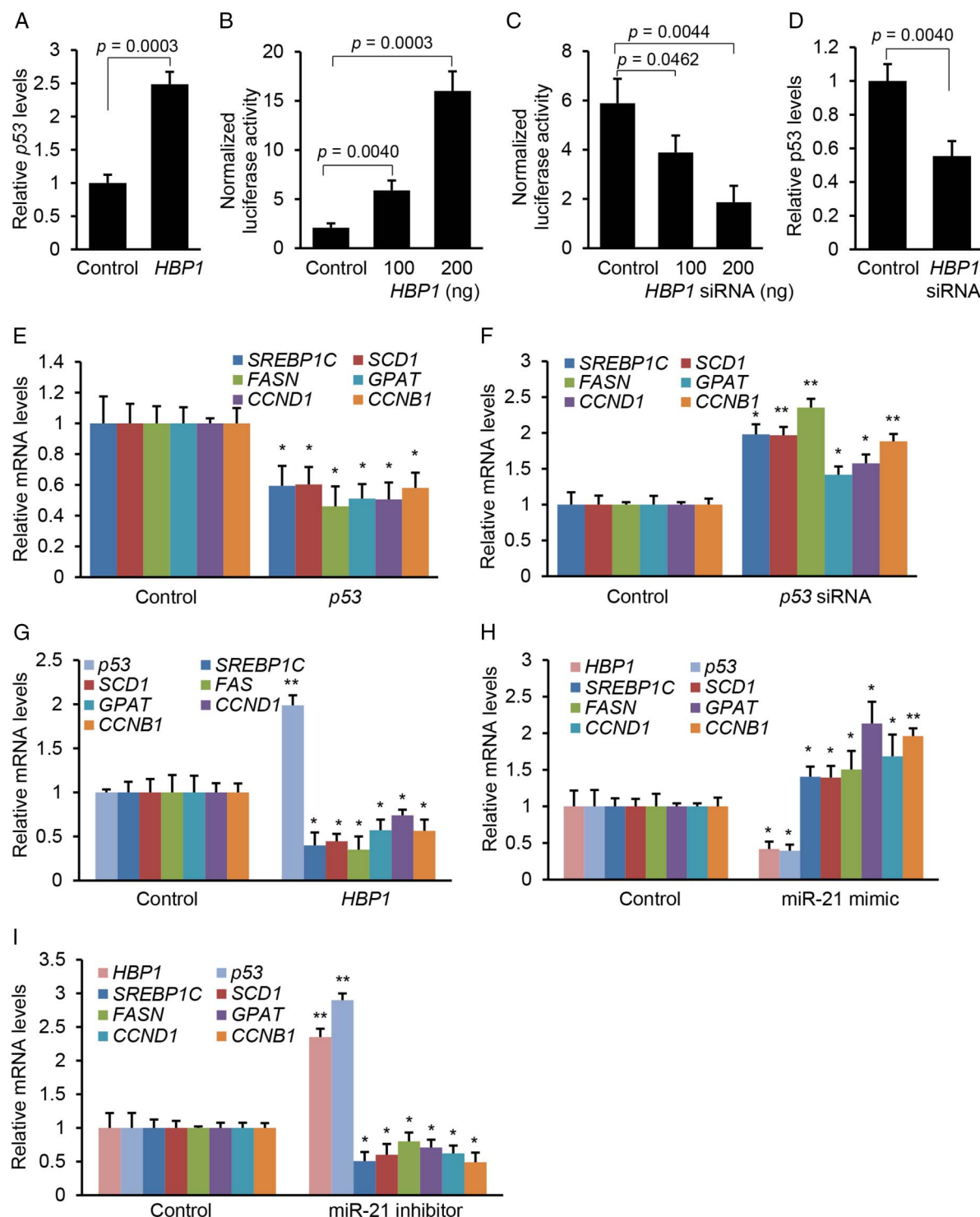
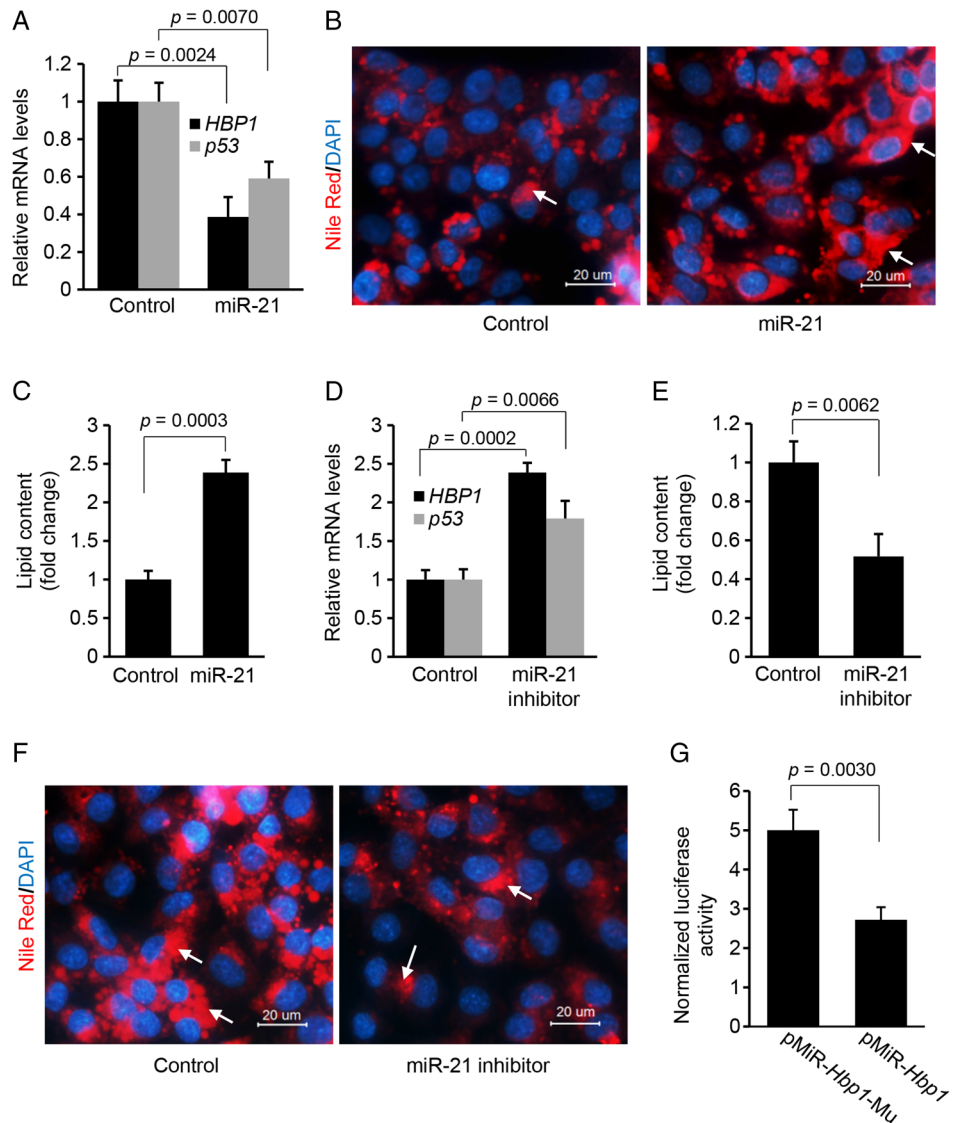


Figure 3 HBP1 is a transcriptional activator of *p53*. (A) Overexpression of *HBP1* increased mRNA levels of *p53* in human HepG2 cells. Data are presented as means $\pm$ SD ($p=0.0003$, Student's *t* test). (B) Overexpression of *HBP1* caused dose-dependent increase of the activity of a luciferase reporter gene linked to the *p53* promoter. Data are presented as mean $\pm$ SD. Student's *t* test was used for statistical analysis. (C) *HBP1* knockdown via its siRNA decreased luciferase activity driven by *p53* promoter. Data are presented as means $\pm$ SD. Student's *t* test was used for statistical analysis. (D) *HBP1* knockdown via its siRNA resulted in decreased mRNA levels of *p53*. Data are presented as mean $\pm$ SD ($*p=0.0040$, Student's *t* test). (E) Overexpression of *p53* led to decreased *SREBP1C*, lipogenic genes *SCD1*, *FASN* and *GPAT* as well as *CCND1* and *CCNB1* in HepG2 cells. Data are presented as mean $\pm$ SD ($*p<0.05$; $**p<0.001$; Student's *t* test). (F) Knockdown of *p53* via its siRNA increased mRNA levels of *SREBP1C*, *FASN*, *SCD1*, *GPAT*, *CCND1* and *CCNB1* in HepG2 cells. Data are presented as mean $\pm$ SD ($*p<0.05$; $**p<0.001$; Student's *t* test). (G) Overexpression of *HBP1* reduced expression of *p53*, which subsequently led to decreased expression of genes involved in lipogenesis and G1/S transition. Data are presented as mean $\pm$ SD ($*p<0.05$; $**p<0.001$; Student's *t* test). (H) Overexpression of miR-21 inhibited *HBP1* and *p53*, which subsequently promoted expression of the lipogenic genes including *SCD1*, *FASN* and *GPAT* and the genes controlling cell cycle progression including *CCNB1* and *CCND1*. Data are presented as mean $\pm$ SD ($*p<0.05$; $**p<0.001$; Student's *t* test). (I) miR-21 knockdown via its inhibitor led to increased *HBP1* and *p53* and decreased expression of the lipogenic and cell cycle-related genes. Data are presented as mean $\pm$ SD ($*p<0.05$; $**p<0.001$; Student's *t* test).

Figure 4 MiR-21 modulates lipid accumulation in HepG2 cells by interacting with the *HBP1*-*p53* pathway. (A) Overexpression of miR-21 inhibited expression of *HBP1* and *p53*, which (B and C) subsequently promoted lipid accumulation in HepG2 cells in the presence of 0.25 mM oleate. Data are presented as mean $\pm$ SD. Student's t test was used for statistical analysis. Lipid droplets were labelled with arrows. (D) miR-21 inhibitor transfection into HepG2 cells cultured with the medium containing 0.5 mM oleate led to an increase in *HBP1* and *p53*, which (E and F) antagonised the effect of upregulated miR-21 on lipid accumulation. Lipid droplets were labelled with arrows. Data are presented as mean $\pm$ SD. Student's t test was used for statistical analysis. (G) Oleate treatment led to a decrease in luciferase activity of pMiR-*Hbp1* as compared with pMiR-*Hbp1*Mu. Data are presented as mean $\pm$ SD ($p=0.0030$, Student's t test).



role of the crosstalk between *p53* and miR-21 in lipogenesis, we transfected oleated-incubated HepG2 cells with miR-21 mimics or a combination of miR-21 mimics and *p53* expression vector. Nile-Red staining and qRT-PCR revealed that miR-21 mimics promoted lipid accumulation in HepG2 cells and the additional *p53* overexpression rescued the effect of miR-21 (see online supplementary figure S3A, B). We further determined whether *p53* deletion could offset the inhibitory effect(s) of miR-21-ASO on lipogenesis. The results showed that miR-21 knockdown led to decreased lipid content and increased *p53*, and additional treatment with *p53* siRNA offset the effects of miR-21-ASO (see online supplementary figure S3C-E). Taken together, our results indicated that miR-21-induced lipid accumulation is, in part, mediated by *HBP1* and *p53*.

***HBP1* mediates the inhibitory effect of miR-21 inhibitor on proliferation, G1/S transition and xenograft tumour from HepG2 cells**

To determine whether *HBP1*, at least in part, mediates the effects of miR-21 on proliferation and G1/S transition, we adopted a similar strategy as described above. MTT and soft agar colony assays revealed that miR-21-ASO administration caused suppression of cellular proliferation in HepG2 cells, but additional treatment of *HBP1*-siRNA counteracted the effects of

miR-21-ASO (figure 5D, E). Cell cycle phase distribution of HepG2 cells further showed that miR-21 knockdown led to a significant increase in G1 phase cells and G1/S arrest, but additional treatment of *HBP1*-siRNA antagonised this effect of miR-21-ASO (figure 5F). Consistent with our in vitro findings, miR-21 knockdown inhibited growth of xenograft tumours in nude mice, and *HBP1*-siRNA treatment counteracted the effect of miR-21-ASO (figure 5G).

As we proposed, *p53* is an important mediator of the miR-21-*Hbp1*-*p53* axis. To determine whether *p53* mediates the inductive effect of miR-21 on proliferation, we treated HepG2 cells with miR-21 mimic or a combination of miR-21 and *p53* expression vector. MTT assay, soft agar colony formation assay and cell cycle analysis revealed that miR-21 promoted proliferation and cell cycle progression, and additional overexpression of *p53* offset the inductive effects of miR-21 (see online supplementary figure S4A-E). Furthermore, miR-21-ASO treatment led to reduced proliferation, delayed G1/S transition and repressed growth of xenograft tumour, and the additional treatment of *p53* siRNA rescued the inhibitory effects of miR-21-ASO (see online supplementary figure S4F-K). In summary, our findings indicated that *HBP1* mediates, at least in part, the inhibitory effects of miR-21-ASO on G1/S transition, proliferation and growth of xenograft tumour.

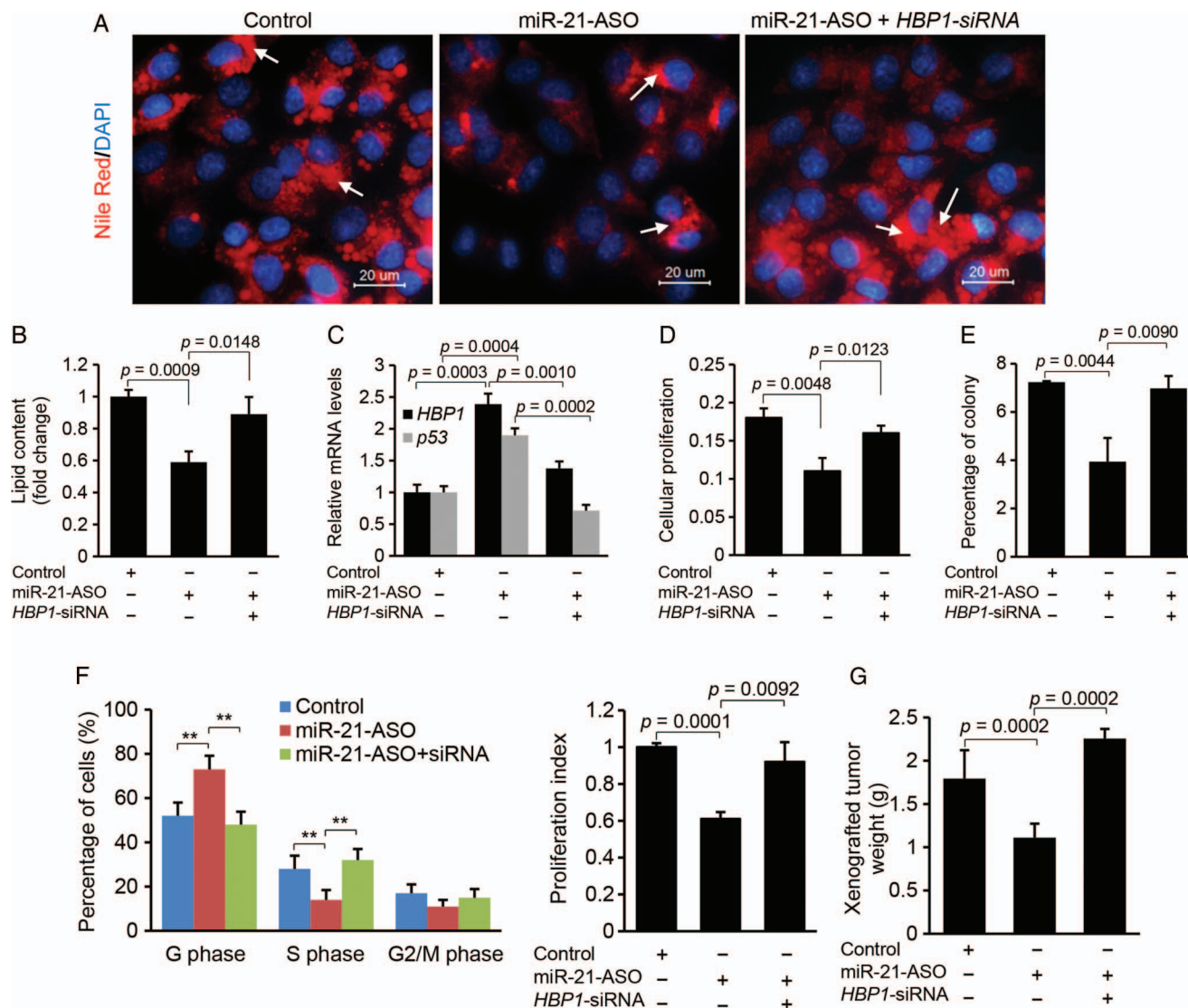


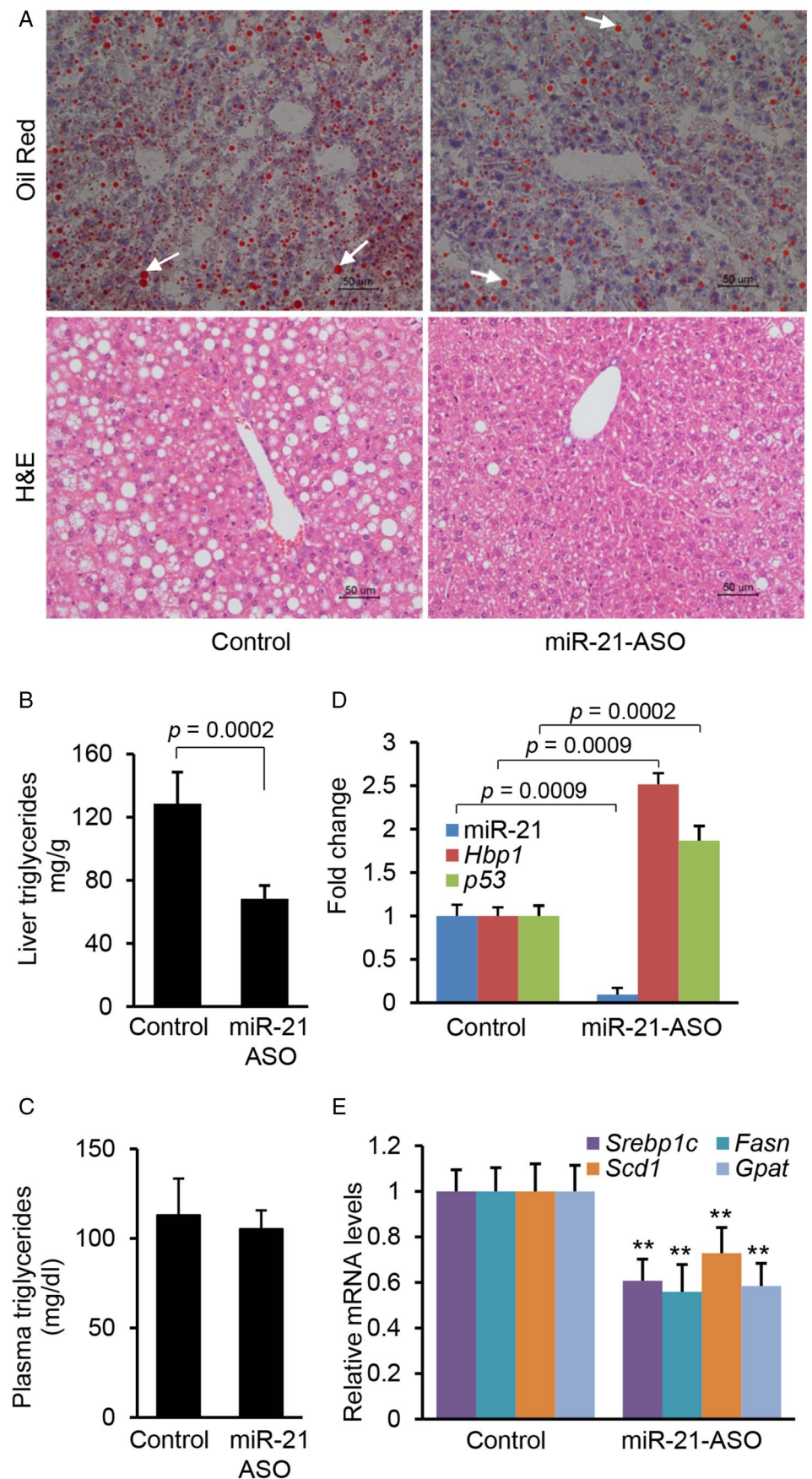
Figure 5 The inhibitory effects of miR-21-anti-sense oligonucleotide (ASO) on lipogenesis, G1/S transition and proliferation are mediated by *HBP1*. (A and B) miR-21 inhibitor transfection into HepG2 cells reduced lipid content, and additional treatment of *HBP1*-siRNA restored lipid levels. Lipid droplets were labelled with arrows. Data are presented as mean $\pm$ SD. Student's t test was used for statistical analysis. (C) miR-21 inhibitor treatment induced expression of *HBP1* and *p53*, and additional knockdown of induced *HBP1* with its siRNA inhibited expression of *HBP1* and *p53*. Specifically, HepG2 cells were treated with oleate (0.5 mM) to induce miR-21, and then miR-21-ASO was transfected into HepG2 cells to knock down upregulated miR-21. Levels of miR-21, *HBP1* and *p53* were determined by qRT-PCR. Data are presented as mean $\pm$ SD. Student's t test was used for statistical analysis. (D) MTT assay revealed that antagonising miR-21 via miR-21 inhibitor caused reduced cellular proliferation in HepG2 cells, and additional knockdown of *HBP1* by its siRNA rescued the effect of miR-21 inhibitor. Data are presented as mean $\pm$ SD. Student's t test was used for statistical analysis. (E) Soft agar colony formation assay revealed that miR-21 knockdown inhibited the growth of HepG2 cells, and the additional treatment of *HBP1*-siRNA antagonised the effect of miR-21 inhibitor. Data are presented as mean $\pm$ SD. Student's t test was used for statistical analysis. (F) miR-21-ASO treatment increased the number of cells in the G1 phase but decreased the number of cells in the S phase, and additional knockdown of upregulated *HBP1* by its siRNA antagonised the effect of miR-21 inhibitor. Quantification of the cell cycle phase distribution was analysed by flow cytometry. The proliferation index was reduced in the miR-21 inhibitor treated HepG2 cells and the additional treatment of *HBP1*-siRNA offset the effect of miR-21 inhibitor. Data are presented as mean $\pm$ SD (* p <0.05, ** p <0.001, χ^2 test). (G) miR-21-ASO inhibited subcutaneous tumours from HepG2 cells in nude mice, and additional treatment of *HBP1*-siRNA counteracted the effect of miR-21-ASO. HepG2 cells treated with miR-21-MM-ASO, miR-21-ASO or a combination of miR-21-ASO and *HBP1*-siRNA were injected subcutaneously into different groups of nude mice. Data are presented as mean $\pm$ SD. Student's t test was used for statistical analysis.

Antagonising miR-21 in dietary obese mice prevents hepatic lipid accumulation

Next, we assessed the functional contribution of increased *Hbp1* and *p53* expression to hepatic lipid accumulation by reducing miR-21 expression in dietary obese mice. C57Bl/6 mice, which had been on an HFD, were injected with either miR-21-ASO or miR-21-MM-ASO for 4 weeks. We observed that antagonising miR-21 significantly reduced levels of triglycerides in livers of

HFD-treated animals (figure 6A, B), in contrast to plasma triglyceride levels (figure 6C). On the other hand, miR-21-ASO treatment had no effect on body and liver weight (see online supplementary table S6). As expected, we also observed a 91% reduction of hepatic miR-21 expression in mice that received miR-21-ASO compared with miR-21-MM-ASO and a twofold increase of *Hbp1* and *p53* (figure 6D). Four-week treatment of miR-21-ASO showed a strong inhibitory effect on

Figure 6 Antagonising miR-21 prevented hepatic lipid accumulation in high fat diet (HFD)-treated mice. (A and B) miR-21 knockdown inhibited lipid accumulation in livers of HFD-fed mice injected with miR-21-anti-sense oligonucleotide (ASO). Representative images are shown. Lipid droplets in livers are labelled with arrows. Cellular triglyceride content was measured by Oil Red staining and triglyceride content was measured with a triglyceride estimation kit. Data are presented as mean $\pm$ SD ($p < 0.0002$, Mann–Whitney test). (C) miR-21 knockdown had no effect on plasma triglyceride in HFD-treated mice. Data are presented as mean $\pm$ SD ($p \geq 0.1$, Mann–Whitney test). (D) miR-21-ASO injection into dietary obese mice resulted in downregulated miR-21 and increased *Hbp1* and *p53* expression. Data are presented as mean $\pm$ SD. Mann–Whitney test was used for statistical analysis. (E) qRT-PCR revealed that HFD-treated mice with decreased levels of miR-21 also retained reduced expression of *Scd1*, *Fasn* and *Gpat* after miR-21-ASO injection. C57BL/6 mice at 8 weeks of age were kept on HFD for an additional 8 weeks. At 12 weeks of age, mice received a dose of 25 mg/kg miR-21-ASO or miR-21-MM-ASO (0.9% NaCl) weekly for 4 weeks via tail vein injection. Data are presented as mean $\pm$ SD. Mann–Whitney test was used for statistical analysis.



hepatosteatosis, but there were no differences in liver and body weight. Therefore, we increased miR-21-ASO treatment time to 8 weeks, which resulted in decreases in both liver weight (see online supplementary table S7) and hepatic lipid content (see online supplementary figure S5B). However, no difference in body weight (see online supplementary table S7), serum free fatty acid and glycerol still was observed (see online supplementary figure S6A, B). In addition, we also observed that miR-21-ASO treatment had no effect on plasma liver enzymes (see online supplementary table S8). These findings indicated that the crosstalk of miR-21 with *Hbp1* and *p53* plays an important role in hepatosteatosis.

We further compared expression levels of *Srebp1c* and lipogenic genes in livers of miR-21-ASO and miR-21-MM-ASO treated mice. As expected, miR-21-ASO treatment led to a significant reduction in *Srebp1c* in the livers of HFD-treated mice (figure 6E). Reduction of *Srebp1c* due to miR-21 knockdown should impair expression of the lipogenic genes. Indeed, in the miR-21-ASO treated group, the mRNA of three enzymes including *Scd1*, *Fasn* and *Gpat* was downregulated at least 1.5 times those of controls (figure 6E).²⁹ Thus, the reduction of *Srebp1c* was associated with a dramatic reduction in the expression of the target enzymes responsible for lipogenesis, which prevented hepatic lipid accumulation. qRT-PCR also revealed that miR-21 knockdown had no effect on β -oxidation-related genes including *Cpt1a*, *Acc2* and *PGC1a* (see online supplementary figure S6C).^{32 33}

In summary, our data have shown that HFD treatment led to increased miR-21, decreased *Hbp1* and *p53*, which subsequently promoted hepatic lipid accumulation and the potential for HCC, whereas antagonising miR-21 led to the opposite and more therapeutic effect. *Hbp1* is inhibited with increased miR-21 levels and its knockdown impaired transcription of *p53* by *Hbp1*, which led to reduced *p53*. The loss of *p53* then resulted in increased transcription of *Srebp1c*, *CCNB1* and *CCND1*, which promoted both lipogenesis and cell replication (figure 7). Our findings indicate the miR-21 plays an important role in linking NAFLD to HCC by interacting with the *Hbp1-p53-Srebp1c* pathway.

DISCUSSION

Our study addresses a potentially important role for miR-21 in the development of NAFLD and HCC and defines a novel mechanism by which miR-21 contributes to lipogenesis and

carcinogenesis via the *Hbp1-p53-Srebp1c* pathway. The observation that antagonising miR-21 in dietary obese mice potentially improves these metabolic parameters clearly indicates a functional role for increased miR-21 expression in the development of NAFLD. In addition, we also observed that miR-21 knockdown prevented an in situ model of tumorigenesis by targeting *HBP1*. Increased miR-21 expression is not restricted to murine obesity models of NAFLD and HCC,^{19 34} but is also detected in human patients with NASH and HCC.^{6 34 35} Thus, miR-21-ASO may act as a unique potential therapeutic approach for the treatment of both disorders.

Despite its putative role in carcinogenesis,³⁶ the mechanism by which miR-21 regulates NAFLD is unknown. In this study, we observed that HFD administration resulted in increased miR-21 and its knockdown prevented hepatic lipid accumulation. In addition, we have functionally validated *Hbp1* as a bona fide target of miR-21, and *Hbp1* is a transcriptional activator of *p53*. It is known that *p53* acts as both a tumour suppressor and inhibitor of lipogenesis by inhibiting *Srebp1c*.^{24 25 31} Our findings, combined with those of others, indicate that miR-21 plays critical roles in the pathogenesis of NAFLD and HCC. In addition, miR-21 deletion in *p53* knockout mice reduced the incidence of liver cancer,³⁷ and *p53* deletion alone promoted hepatosteatosis,²⁵ further supporting the unique role for miR-21 in linking NAFLD to HCC via the *Hbp1-p53-Srebp1c* pathway. miR-21 significantly inhibits expression of *PTEN*,³⁴ a negative regulator of NASH in mice,³⁸ suggesting that it might, in part, mediate the inhibitory effect of miR-21 on hepatosteatosis. In this study, we found that *Hbp1* is a direct target of miR-21 and confirmed that *Hbp1* modulates the inhibitory function of miR-21-ASO on hepatosteatosis and carcinogenesis simultaneously.

We have shown that miR-21 is highly expressed in hepatocytes (figure 1A). Meanwhile, miR-21 is increased but its target *Hbp1* is reduced in livers of HFD-treated mice and human individuals with NAFLD and HCC. All these features of miR-21 lead us to focus on its role in linking NAFLD to HCC. Although other miRNAs might contribute to the development of NAFLD and HCC, few other hepatocyte-specific miRNAs meet the above criteria like miR-21. These include increased expression in both NAFLD and HCC of human and mouse, reduced expression of their target genes in mouse models and human patients, and high expression in hepatocytes. We demonstrated that miR-21 knockdown led to upregulated *Hbp1* and *p53*, downregulated *Srebp1c* and decreased expression of hepatic *Scd1*, *Gpat* and *Fasn*. These findings are consistent with earlier reports that *p53* knockout promoted *Srebp1c* transcription and in turn increased expression of *Scd1*, *Gpat* and *Fasn*, and subsequently hepatic lipogenesis,^{25 28} suggesting that miR-21-dependent *Hbp1-p53* pathway inhibition of *Srebp1c* transcription represents a candidate pathway to cause NAFLD.

There is evidence to suggest that inhibition of *p53* attenuates steatosis and liver injury,^{39 40} which is inconsistent with our findings and those of others.^{25 26 41} One possible explanation is that *p53* is a negative regulator of hepatic lipid accumulation in the early stages of NAFLD, but *p53* is highly expressed at the advanced stages of NAFLD, which may contribute to the high level of apoptosis associated with NASH. In patients with liver steatosis without inflammation, *p53* expression was significantly lower than in steatohepatitis,⁴² further suggesting that *p53* plays different roles in the various developmental stages of NAFLD. Although the mechanism(s) by which miR-21 controls lipogenesis and carcinogenesis in liver clearly requires further investigation, our study has identified an important role for the interaction of miR-21 with *Hbp1* in obesity-induced NAFLD and HCC.

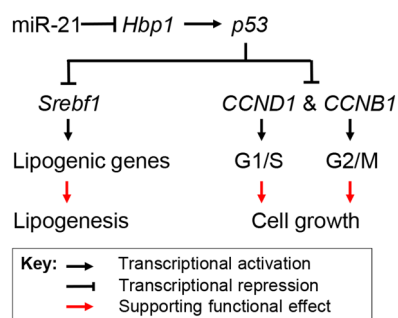


Figure 7 Proposed mechanism by which miR-21 links hepatic lipid accumulation and the development of cancer. *Hbp1* is a transcriptional activator of *p53*, a suppressor of cell cycle progression and inhibitor of lipogenesis by inhibiting transcription of *Srebp1c*. By directly inhibiting *Hbp1* expression, miR-21 prevents expression of *p53*, which facilitates transcription of genes that are required for lipogenesis and the G1/S transition of cell cycle. As a result, increased miR-21 promotes both hepatic lipid accumulation and potentially carcinogenesis.

Contributors HW and RN: acquisition of data. XC and CJS: analysis and interpretation of data. GS: obtaining funding, study supervision, study concept and design and drafting of the manuscript.

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Competing interests None declared.

Ethics approval All procedures involving mice were approved by the Institutional Animal Care Committee at the University of Minnesota, University of California San Francisco, and the Agency for Science Technology and Research Singapore.

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