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Hepatic miR-192-3p reactivation alleviates steatosis by targeting glucocorticoid receptor

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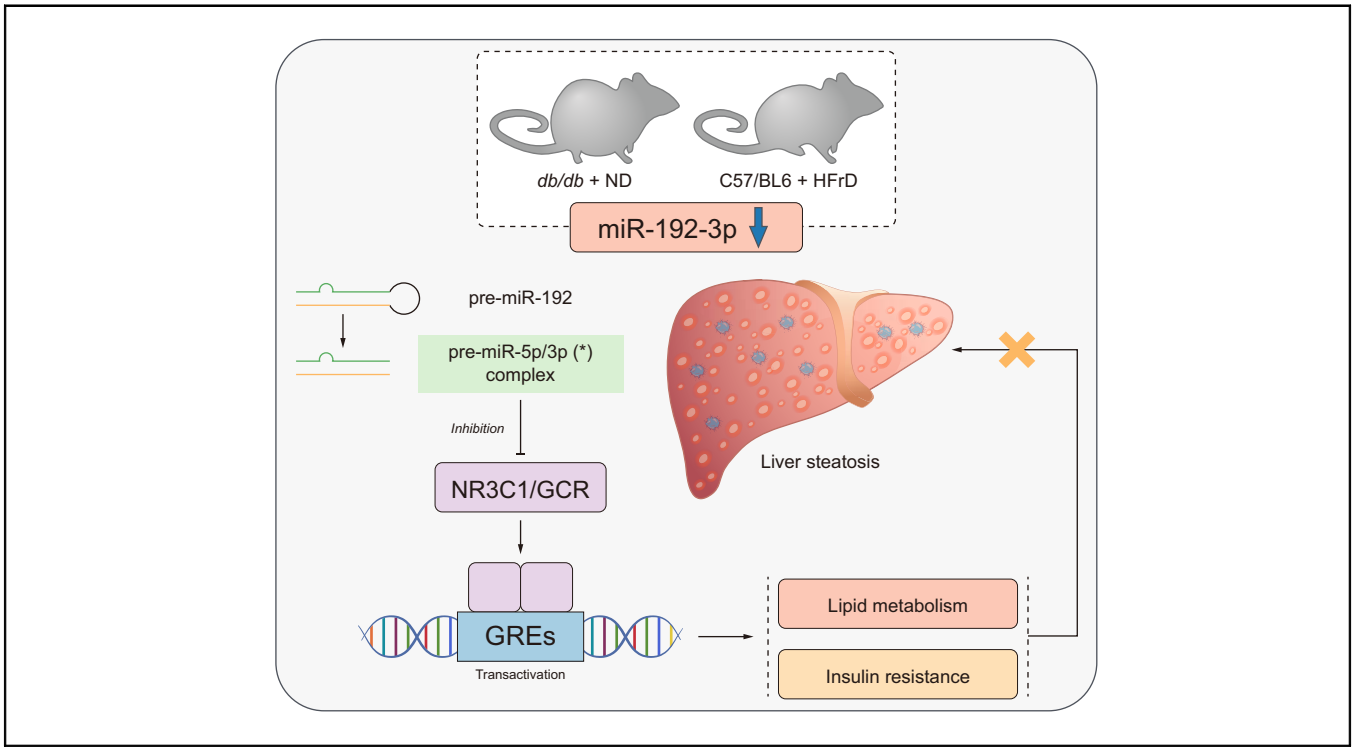
Graphical abstract



Highlights

- Liver-specific knockdown of miR-192-3p recapitulated functional loss of the miRNA as in mice with diabetes.
- This knockdown was characterised by pronounced hepatic micro-vesicular steatosis coupled to insulin resistance.
- *In vivo* overexpression of miR-192-3p alleviated hepatic steatosis in mice with diabetes and wild-type mice with excessive fructose consumption.
- Glucocorticoid receptor (also known as NR3C1) was discovered as the immediate target of miR-192-3p in regulating hepatic lipid turnover and storage.

Lay summary

The potential regulatory activity of star strand microRNA (miRNA) species has been substantially underestimated. In this study, we investigate the role and mechanism of an overlooked star strand miRNA (miR-192-3p) in regulating hepatic steatosis and insulin signalling in the livers of mice with diabetes and mice under excessive carbohydrate consumption.

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Research article

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Hepatic miR-192-3p reactivation alleviates steatosis by targeting glucocorticoid receptor

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Background & Aims: The paradox of hepatic insulin resistance describes the inability for liver to respond to bioenergetics hormones in suppressing gluconeogenesis whilst maintaining lipid synthesis. Here, we report the deficiency of miR-192-3p in the livers of mice with diabetes and its role in alleviating hepatic steatosis.
Methods: As conventional pre-microRNA (miRNA) stem-loop overexpression only boosts guiding strand (*i.e.* miR-192-5p) expression, we adopted an artificial AAV(DJ)-directed, RNA Pol III promoter-driven miRNA hairpin construct for star-strand-specific overexpression in the liver. Liver steatosis and insulin resistance markers were evaluated in primary hepatocytes, mice with diabetes, and mice with excessive carbohydrate consumption.
Results: Functional loss of miR-192-3p in liver exacerbated hepatic micro-vesicular steatosis and insulin resistance in either mice with diabetes or wild-type mice with excessive fructose consumption. Liver-specific overexpression of miR-192-3p effectively halted hepatic steatosis and ameliorated insulin resistance in these mice models. Likewise, hepatocytes overexpressing miR-192-3p exhibited improved lipid accumulation, accompanied with decreases in lipogenesis and lipid-accumulation-related transcripts. Mechanistically, glucocorticoid receptor (GCR, also known as nuclear receptor subfamily 3, group C, member 1 [NR3C1]) was demonstrated to be negatively regulated by miR-192-3p. The effect of miR-192-3p on mitigating micro-vesicular steatosis was ablated by the reactivation of NR3C1.
Conclusions: The star strand miR-192-3p was an undermined glycerolipid regulator involved in controlling fat accumulation and insulin sensitivity in liver through blockade of hepatic GCR signalling; this miRNA may serve as a potential therapeutic option for the common co-mobility of diabetic mellitus and fatty liver disease.

Introduction

Liver, muscle, and adipose tissues are 3 major peripheral effector tissues in response to insulin—the essential bioenergetics endocrine that governs the entry and storage of glucose in normal starved-fed cycles.[1,2] Unlike the other 2 insulin-responsive tissues, liver is a central hub for nutrient homeostasis, which includes synthesising glycogen from glucose after a meal or effectively converting excessive glucose to fatty acids (FAs) as precursor for triglyceride (TAG) synthesis via *de novo* lipogenesis (DNL).[3] Liver is therefore critical for its unique niche in energy homeostasis and the *de facto* effector in balancing blood glucose levels.

Liver steatosis is a sign of lipid overload in the organ either when there is an oversupply of lipid or when there is a failure in lipid breakdown, frequently associated with impaired insulin signalling.[4] Lipid overload manifests in a blend of 2 major forms (*i.e.* macro- and micro-vesicular steatoses).[5] The former relates to a benign, largely asymptomatic form of steatotic liver. In contrast, the latter is adversely associated with metabolic disorder, inflammation, and hepatic fibrosis.[6] Intriguingly, there is an overt linkage between non-alcoholic fatty liver disease (NAFLD) and diabetes, in which NAFLD is prevalent in up to 70% of patients with diabetes,[7] and the reported prevalence of non-alcoholic steatohepatitis in patients with type 2 diabetes mellitus (T2DM) is up to 20%.[8]

Even with the clear close ties, many morbidly obese individuals remain non-diabetic. In line with this clinical observation, leptin-deficient obese (ob/ob) mice usually exhibited a benign hepatic steatosis, whereas leptin-receptor-defective (db/db) mice with diabetes commonly developed inflammation upon

Keywords: Hepatic steatosis; Diabetes mellitus; MicroRNA; Glucocorticoid receptor; Transcription repressor; High carbohydrate consumption.
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consumption of high carbohydrates.[9] It is concretely confirmed that ob/ob mice developed macro-vesicular, while that of db/db mice is predominantly micro-vesicular steatosis instead. Indeed, patients with diabetes manifest hepatic micro-vesicular steatosis with exacerbated neurovascular complications.[10]

MicroRNAs (miRNAs) are naturally occurring non-coding RNAs that bind to the 3′-untranslated regions (3′-UTR) of target mRNAs, leading to either their degradation or translational repression.[11] Given their critical roles exemplified in metabolism control, miRNAs are frequently studied as a target for disease interventions (*i.e.* human cancers[11] and metabolic diseases[12]). Of note, some miRNAs are regulators of liver metabolism (*e.g.* miR-206, which inhibits lipid and glucose production by facilitating insulin signalling[13]). In addition, miR-192 and miR-193b were presumed as proxy diabetes biomarkers, where their abundance was found significantly increased in glucose-intolerant mice and in people with prediabetes.[14] Despite the circulating levels of miR-192 (5p, the guiding strand) were refuted both as a reliable marker for liver damage[15] and diabetic nephropathy,[16] its linkage to diabetic liver condition remains unclear.

In the current study, we found that mice with diabetes with apparent micro-vesicular nature exhibited a substantial loss of the star strand of miR-192 (*i.e.* miR-192-3p). Overexpression (OE) of the sponge against the star strand mimicked the *de facto* functional loss of miR-192-3p-aggravated liver steatosis of micro-vesicular nature in wild-type mice given excessive fructose consumption, recapitulating conditions of impaired insulin response. Alternatively, we adopted an unconventional short hairpin RNA (shRNA) stem-loop construct for the specific OE of the star strand miRNA alone. Indeed, liver-specific OE of miR-192-3p significantly alleviated hepatic steatosis and improved insulin resistance in both mice with diabetes and those given excessive carbohydrates. Finally, our study pinpointed nuclear receptor subfamily 3, group C, member 1 (NR3C1), the glucocorticoid receptor (GCR), as a direct target of miR-192-3p to alleviate liver steatosis and insulin resistance.

Materials and methods

Full details of methods can be found in the supplementary information.

Human serum sample collection

Human serum samples were obtained from the Prince of Wales Hospital, Hong Kong Special Administrative Region, China. Details of patients are listed in Table S1. Studies using human serum were approved by the joint Chinese University of Hong Kong/New Territories East Cluster Clinical Research Ethics Committee. Informed consent was obtained from all participants.

Animal, treatment, and sample collection

All animals were supplied by the Laboratory Animal Service Centre at the Chinese University of Hong Kong. The animals were housed with controlled temperature (25 ± 1°C), a 12-h light–dark cycle, and 55 ± 5% humidity. All procedures were approved by the Animal Experimentation Ethics Committee, Chinese University of Hong Kong under the regulations of the Hong Kong Special Administrative Region government.

A mice fatty liver disease model was established by feeding male mice either with a high-fat diet (HFD; rodent diet with 45 kcal% fat; D12451; Research Diets, New Brunswick, NJ, USA) for 8∼12 weeks starting at the age of 4 weeks, or high-fructose drink (HFrD; water supplemented with fructose 30%) for 8∼12 weeks starting at the age of 4 weeks, whereas mice in the control group were maintained on a normal chow diet (2018SX; ENVIGO, USA). Food and water were provided *ad libitum*. The mice were tail vein injected with adeno-associated virus (AAV) of DJ serotype (AAV DJ) expressing miR-192-3p sponge or miR-192-3p OE for different periods, and then serum and different tissues were collected for further analysis.

Liver-specific AAV delivery of transgenes and *in vivo* imaging

To specifically deliver either miR-192-3p or its sponge into mice livers, AAV DJ carrying transgenes of either constructs was tail vein injected, and repeated injections were performed once every 3 weeks. Alternatively, shNR3C1 construct was subcloned into pSicoR-mCherry vector (Addgene, USA; #31845). Lentivirus carrying pSicoR-shNR3C1 was i.v. injected into 4-week-old mice with diabetes, followed by AAV-pgk-Cre (Addgene; #24593) 2 weeks later. These mice were then sacrificed after 2 months. Red fluorescent signal intensity was detected by the In-Vivo Xtreme Imaging System (Bruker, USA).

Statistical analysis

Statistical analysis was performed using GraphPad Prism Software®. Data derived from cell line or mouse experiments were presented as mean ± standard deviation and assessed by a 2-tailed Student's *t* test or one-way analysis of variance test. A *p* value <0.05 was considered statistically significant.

Results

Functional loss of star strand miR-192-3p in the liver manifested as hepatic micro-vesicular steatosis

Each pre-miRNA stem loop can produce up to 2 mature miRNAs on either strand with relative expression biases in different tissues. Our previous work had demonstrated that both mature miR-192 guiding strand (miR-192-5p) and its star strand miR-192-3p (miR-192*) (Fig. 1A) were enriched as top candidate differentiation regulators along the hepatic lineage.[17] Interestingly, we found that miR-192-3p rather than miR-192-5p was lost in the liver of db/db mice with diabetes when compared with heterozygous lean control (Fig. 1A). The deregulated expression of star strand miRNA instead of the guiding strand in liver pinpointed the necessity to showcase their relative expression in different organs of mice with diabetes.

It was noted that the relative expression bias between the strands (ratio of miR-192-3p/miR-192-5p) is within 2-fold. However, there was a noticeable decrease in ratio for both liver and kidney, while the ratio was increased in mesenteric visceral adipose tissues (VATs) (Fig. 1B). Such relative expression biases in liver, kidney, and VATs were in line with previous reports, which discussed the potential functions in either of these strands.[15,18,19] In addition, the expression of miR-192-3p, albeit much less than its guiding counterpart, remained significantly higher in the liver than in other organs (Fig. S1A and B).

Given its described suppressive effect in lipid storage in adipose tissues, we therefore proposed that miR-192-3p loss is related to liver steatosis, a frequent co-morbidity of diabetes. To assay for the effects of functional loss in hepatic miR-192-3p, we transduced miR-192-3p sponge (a construct that overexpressed 3 times perfect complementary sponge repeats linked to enhanced green fluorescent protein) against miR-192-3p by AAV of liver targeting DJ serotype (AAV DJ) *in vivo* (Fig. S1C and D). As

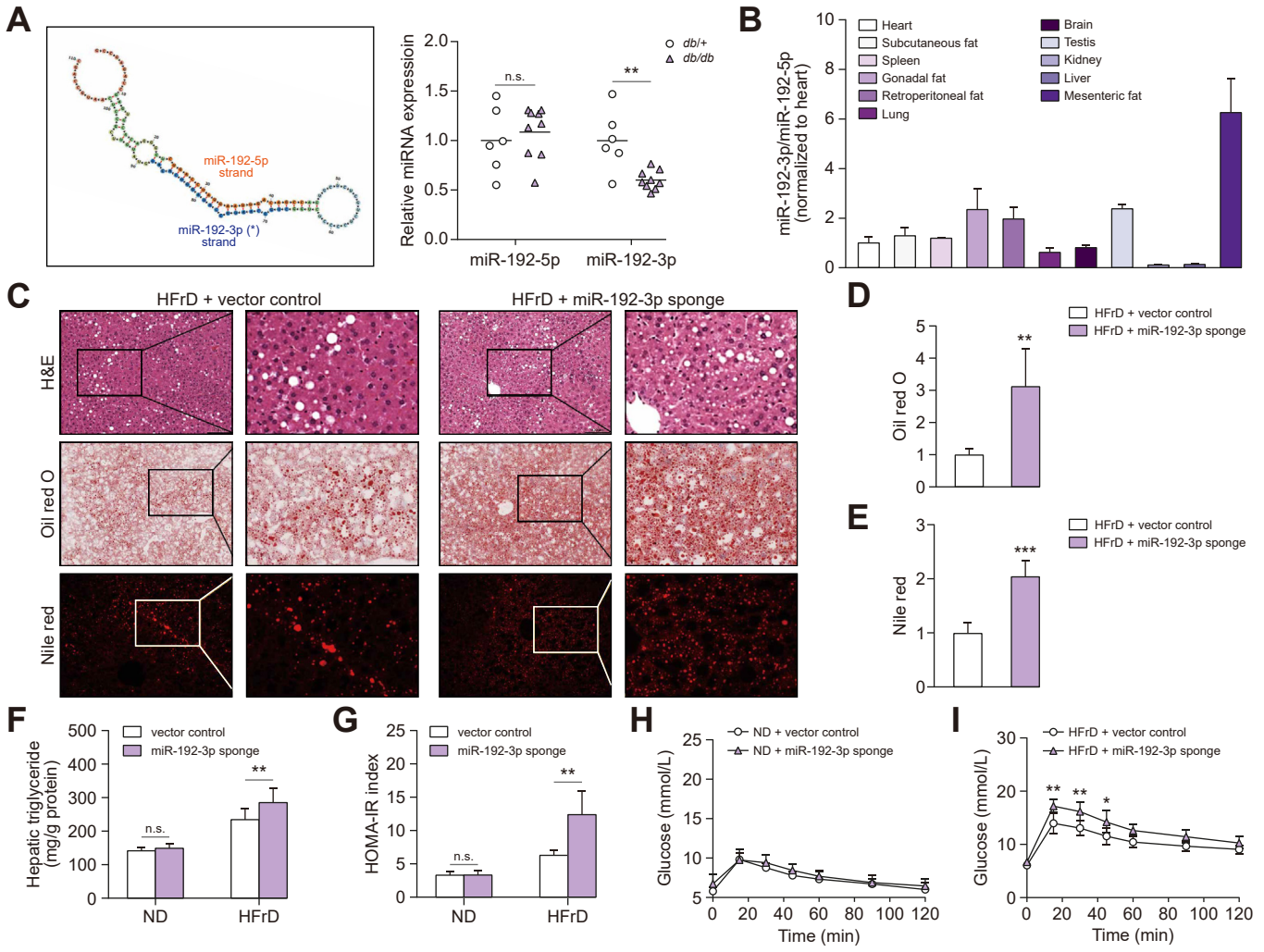

Fig. 1. Hepatic functional loss of miR-192-3p resulted in hepatic micro-vesicular steatosis. Wild-type C57BL/6 mice were fed on ND or HFrD for 8 weeks. (A) Expression of miR-192 and miR-192-3p. (B) Expression ratio of miR-192-3p to miR-192-5p in different tissues of db/db mice. (C–E) Liver histology showed liver-specific loss of miR-192-3p resulted in lipid accumulation and micro-vesicular steatosis in HFrD-fed mice. (F) Hepatic triglyceride levels in the indicated groups. (G–I) Hepatic knockdown of miR-192-3p led to increased insulin resistance, as evidenced by OGTT pattern and HOMA-IR index. Data are represented as mean ± SD; n = 5∼8. $^{*}p$ <0.05, $^{**}p$ <0.01, $^{***}p$ <0.001. Two-tailed Student's *t* test. H&E, haematoxylin and eosin; HFrD, high-fructose drink; HOMA-IR, homeostasis model assessment of insulin resistance; ND, normal diet; OGTT, oral glucose tolerance test.

a precaution, we checked that such liver-specific functional loss of miR-192-3p did not exert visible changes in adiposity of liver tissues nor notable changes in oral glucose tolerance test (OGTT) in wild-type C57/BL6 mice given normal diet (Fig. 1F–H and S1E).

In contrast, wild-type mice given either HFrD (drinking water supplemented with fructose 30%) or HFD (high-calorie diet) demonstrated distinctive hepatic lipid content and OGTT patterns. Hepatic lipid appeared as patchy droplets in those over-expressed with vector control, while animals given miR-192-3p sponge exhibited foamy cytoplasm with minute lipid vesicles (Fig. 1C–E). Knockdown of miR-192-3p also promoted hepatic lipid storage, as evidenced by the increase of TAG levels (Fig. 1F). The homeostatic model assessment of insulin resistance (HOMA-IR) was assayed as the surrogate estimate for peripheral insulin resistance. Functional loss of miR-192-3p demonstrated reduced glucose tolerance, as indicated by the increased HOMA-IR upon miR-192-3p sponge OE (Fig. 1G). OGTT profiles also showed that depletion of miR-192-3p increased the development of glucose intolerance and compensatory hyper-insulinaemia (Fig. 1H and I).

The effect of miR-192-3p loss in mice given HFD was also examined. Despite phenotype not as severe as those given fructose drink, miR-192-3p sponge also aggravated HFD-induced lipid accumulation and insulin resistance (Fig. S1F–I). Taken together, functional loss of miR-192-3p promoted hepatic micro-vesicular steatosis—a detrimental liver lipid overload as that in patients with diabetes, with an aggravated insulin resistance in our mice models.

Overexpressed miR-192-3p altered lipid homeostasis in hepatocytes

As miR-192-3p was implicated in fatty tissue differentiation, loss of this miRNA may impact the role of liver as a key hub for lipid metabolism. We screened the expression of some predicted targets of miR-192-3p in the mice with diabetes, and observed a

remarkably higher gene expression of *Scd* and *Vldlr*, and a modest increase in *Lpin1* in their liver (Fig. S2A). Concurrently, FA translocase *Cd36*/*Scarb3* and DNL enzyme *Fasn* expression were also found higher (Fig. S2A).

Subsequently, hepatic normal human cell line L02 was transfected with this miR-192-3p sponge and stably selected by puromycin to delineate the underlying mechanism of the action of miR-192-3p. Two separate RNA-Seq libraries of non-targeting (NT) and miR-192-3p sponge (S) were constructed. Of the 1,562 genes differentially expressed, more than 400 genes were predicted to be direct miR-192-3p targets or their redundant genes deduced from TargetScan (Fig. 2A). PI3K-Akt signalling, FoxO signalling, adipocytokine signalling, and insulin-resistance-associated pathways were enriched by the Kyoto Encyclopedia of Genes and Genomes catalogue, reinstating the involvement of miR-192-3p in bioenergetics homeostasis (Fig. 2B and C). Gene set enrichment analysis further identified processes associated with lipid, FA, steroid, and monocarboxylic acid metabolism affected by miR-192-3p functional depletion (Fig. 2D and S2B). In addition, lipogenesis- and gluconeogenesis-associated genes were also significantly enriched in the analysis (Fig. 2E).

To validate the role of miR-192-3p in liver steatosis, we designed an artificial construct resembling shRNAs,[20] with miR-192-3p sequence in the anti-sense strand and scrambly manipulated sense strand. This miR-192 stem loop was then transiently transfected into HEK293T cells and lead to a significant 30-fold upregulation of miR-192-3p, without altering the expression of the guiding strand miR-192 (Fig. 2F).

Furthermore, we transfected miR-192-3p mimics into primary mouse hepatocytes and studied genes responsible for FA mobilisation. As expected, genes, such as *Srebf1*, *Fasn*, *Acc*, *Cd36*, and *Ffar4*, were decreased by the mimics (Fig. 2G), suggesting that miR-192-3p might have altered the mobilisation of FAs in hepatocytes.

miR-192-3p inhibited lipid accumulation in human hepatic cell line

Among the major bioenergetic pathways enriched from the panel of differentially expressed genes (DEGs) mentioned previously were mitochondrial aerobic oxidation and pyruvate metabolism, both of which are closely related to FA metabolism. Therefore, we validated the action of miR-192-3p on FA oxidation (FAO) by the Seahorse XF Cell Mito Stress Test. Stable cell clones overexpressing miR-192-3p (miR-192-3p OE) tended to consume more palmitate-bovine serum albumin (PA-BSA) as substrate for FAO when compared with an NT cell line (Fig. 3A). The increase in oxygen consumption rate in miR-192-3p OE cells suggested significantly induced oxidative metabolism, indicating the dissipation of lipids by maximising FAO (Fig. 3B and C). This inductive effect was further blocked by carnitine palmitoyl transferase (CPT) inhibitor etomoxir treatment (Fig. 3B and C).

In addition, miR-192-3p increased the expression of FAO genes encoding CPT1 alpha, long-chain acyl-CoA dehydrogenase, acyl-coenzyme A oxidase 1, hydroxyacyl-CoA dehydrogenase-alpha, and peroxisome-proliferator-activated receptor alpha in cells treated with PA-BSA (Fig. 3D). With reports showing close ties between malfunctioned beta-oxidation to micro-vesicular steatosis,[21] it suggested that miR-192-3p OE shall restore fatty liver in a relatively benign form.

We then examined the role of miR-192-3p in preventing lipid accumulation. OE of miR-192-3p alleviated oleic acid (OA)-induced lipid accumulation in L02 cells (Fig. 3E). Fluorescent staining of neutral lipids showed that lipid spot area per cell was significantly reduced in miR-192-3p OE cells incubated with OA (Fig. 3E), with an obviously reduced intracellular TAG level (Fig. 3F). In addition, overexpressing miR-192-3p also suppressed both mRNA and protein levels of lipogenesis genes, including FA synthase (FASN), CD36, stearoyl-CoA desaturase-1 (SCD1), and SREBP1 (Fig. 3G–I).

Liver-specific expression of miR-192-3p exhibited robust therapeutic effects on liver steatosis in mice

To assess the functional contribution of miR-192-3p to the hepatosteatosis and hyperglycaemia in mice, a liver-specific miR-192-3p expression set-up was generated using AAV DJ. Injection of AAV-miR-192-3p into mice led to high levels of miR-192-3p in liver other than other tissues (Fig. S3A and B). Early adenoviral injection of miR-192-3p to 4-week-old mice with diabetes effectively prevented body weight gain (Fig. 4A and B) and reduced the daily food intake (Fig. S3C–E) when compared with NT control sacrificed 2 months later. Such liver-specific OE of miR-192-3p also attenuated hepatic lipid accumulation and hyperlipidaemia (Fig. 4C), which was further confirmed by the reduction of TAG level in mice treated with miR-192-3p (Fig. 4D). OGTT pattern and HOMA-IR indices were also improved by the OE of miR-192-3p (Fig. 4E and F). Consistently, miR-192-3p significantly suppressed the mRNA and protein levels of genes associated with the synthesis and uptake of FA, alongside induction in those genes responsible for FAO (Fig. 4G–I and S3F).

Delivery of miR-192-3p into HFrD-fed mice also resulted in significant body and liver weight loss (Fig. 4J and K). Consistent with the ability of miR-192-3p in turning over lipid content, H&E and Oil Red O staining showed that liver-specific OE of miR-192-3p reduced hepatic lipid accumulation compared with the NT control (Fig. 4L and M). In addition, hepatic miR-192-3p OE mitigated HFrD-induced glucose intolerance, as evidenced by the OGTT pattern and HOMA-IR index (Fig. 4N and O). The mRNA expression level of lipogenesis-related genes was also found to be downregulated by the long-term treatment of miR-192-3p (Fig. 4P).

miR-192-3p directly targeted GCR in regulating liver glycerolipid homeostasis

To identify potential target genes of miR-192-3p, which may be responsible for its action in liver, we screened the DEGs according to 3 criteria:

- genes must be conserved in the 3′-UTR along predicted domains;
- gene expression should be regulated following miR-192-3p change; and
- the selected genes are implicated in fatty liver diseases.

Fifty-four predicted target genes were enriched from the adopted prediction databases, including TargetScan, miRDB, DIANA, and TargetMiner (Fig. 5A), and then clustered with DEGs from previous RNA-Seq (Fig. 5A). Of note, *NR3C1*, the gene encoding GCR, is of high interest, as GCR signalling was enriched as top candidate pathways by the Ingenuity Pathway Analysis (Fig. S4).

Luciferase report assay in HEK293T showed that miR-192-3p mimics significantly reduced luciferase activity of *NR3C1* 3′-UTR when compared with control mimics (Fig. 5B). The mRNA and protein levels of NR3C1 were also downregulated in human

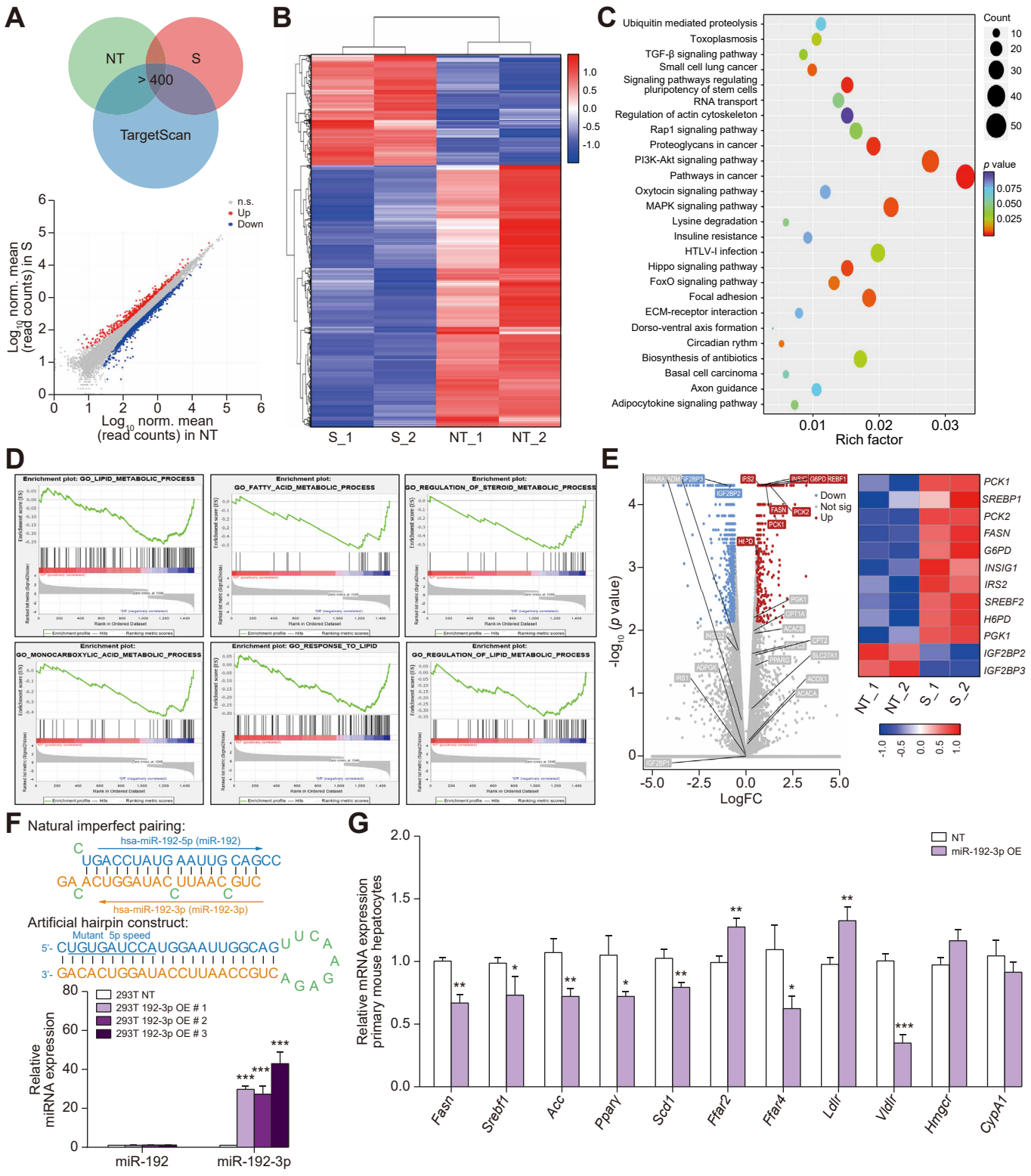

Fig. 2. miR-192-3p altered metabolic homeostasis in hepatocytes. (A) DEGs were overlapped with predicted miR-192-3p targets collected from the TargetScan database in the Venn diagram. The scatter plots indicating relative gene expression profile between groups as indicated, with $\log_2$ (fold change) less than –0.585 and larger than 0.585. (B) Clustering images showing global sample expression profiles and relationships analysed by hierarchical clustering. (C–D) KEGG pathway enrichment analysis and GSEA of DEGs identified lipid-metabolism-associated processes. (E) Volcano plots and expression profile of lipogenesis- and gluconeogenesis-associated genes in the S and NT groups. (F) Artificial hairpin structure of miR-192-3p (miR-192-3p OE) specifically increased the expression of miR-192-3p in HEK293T without altering miR-192 expression. (G) The mRNA expression levels of lipid-metabolism-associated genes in mouse primary hepatocytes transfected with miR-192-3p OE. Data are represented as mean ± SD; n = 3. *p <0.05, **p <0.01, ***p <0.001. Two-tailed Student's t test. DEG, differentially expressed gene; GSEA, gene set enrichment analysis; KEGG, Kyoto Encyclopedia of Genes and Genomes; NT, non-targeting control; OE, overexpression; S, miR-192-3p sponge.

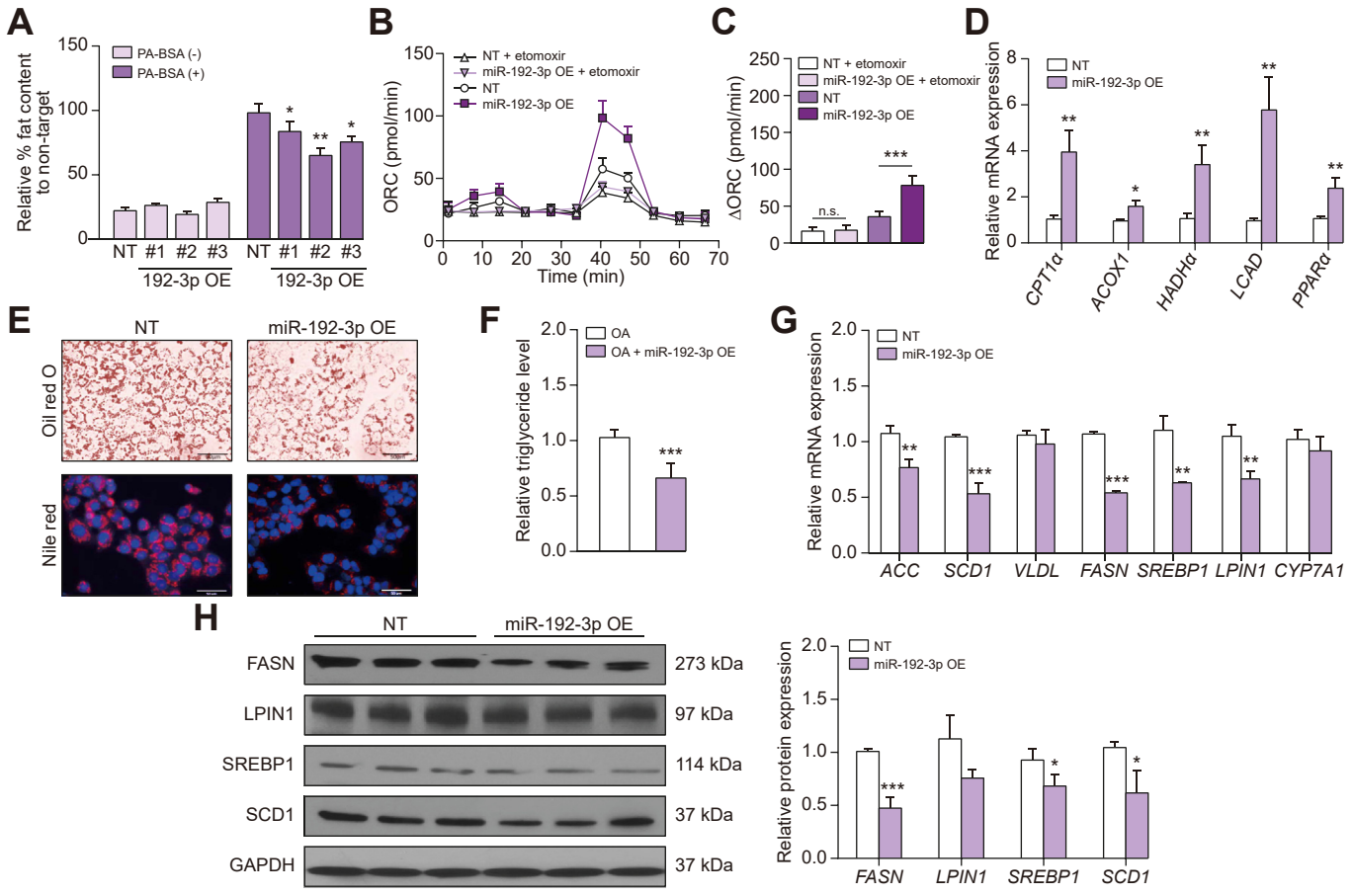

Fig. 3. miR-192-3p negatively regulated lipid accumulation in hepatocytes. L02 cells were stably overexpressed with miR-192-3p (miR-192-3p OE) or NT. (A) Intracellular lipid content in NT and miR-192-3p OE cells using PA-BSA as substrate. (B) Fatty acid oxidation by the Seahorse XF Cell Mito Stress Test with or without CPT inhibitor etomoxir in NT or miR-192-3p OE cells. (C) Quantification of the AUC in each indicated group. (D) The mRNA expression of genes related to fatty acid oxidation in miR-192-3p OE and NT cells. (E) Representative images of Oil Red O and Nile Red staining of NT and miR-192-3p OE cells incubated with OA for 8 h. (F) Intracellular triglyceride levels of NT and miR-192-3p OE cells stimulated by OA for 8 h. (G–I) The relative mRNA and protein expression levels of the indicated molecules in cells of the indicated groups. GAPDH served as controls. Data are represented as mean ± SD. *p <0.05, **p <0.01, ***p <0.001; n = 3. Two-tailed Student's t test. CPT, carnitine palmitoyl transferase; NT, non-targeting control; OA, oleic acid; OE, overexpression; PA-BSA, palmitate-bovine serum albumin.

hepatocytes overexpressed with miR-192-3p (Fig. 5C), reinforcing that miR-192-3p is able to inhibit expression of *NR3C1* by interacting with its 3′-UTR.

As the aberrant glucocorticoid signalling is tightly related to metabolic disorders, including obesity, hyperglycaemia, and insulin resistance, we tested whether *NR3C1* mediates the role of miR-192-3p in regulating liver steatosis. Oil Red O and Nile Red staining of newly synthesised lipid indicated that there is a reduction of lipid accumulation and TAG level under miR-192-3p OE in L02 cells when incubated with OA, which was blocked by forced NR3C1 expression (Fig. 5D and E). Furthermore, such lentiviral OE disrupted the suppressive role of miR-192-3p on both mRNA and protein levels of lipogenesis genes, such as FASN, LIPIN1, SCD1, and SREBP1, suggesting that *NR3C1* mediates the regulatory role of miR-192-3p in lipid homeostasis (Fig. 5F–H).

miR-192-3p target GCR to regulate glycerolipid homeostasis in mice with diabetes

To investigate whether *NR3C1* mediates the inhibitory effects of miR-192-3p on hepatic lipid accumulation, a lentiviral floxed *NR3C1* shRNA was reintroduced to 4-week-old mice with diabetes.[22] Lentiviral OE of the shRNA in pSicoR was eliminated by the AAV(DJ)-pgk-Cre. *In vivo* imager detected substantial fluorescent signal in the liver, lung, and fat, demonstrating an accumulation of lentiviral induction in the body driving the transgene. However, in the presence of liver-targeting Cre, there was a clear reduction of those signals specifically in the liver after Cre recombination (Fig. 6A).

The constitutive knockdown of NR3C1 before Cre activation prevented fat liver accumulation in age-matched mice with diabetes compared with NT control. These mice phenocopied miR-192-3p OE by demonstrating alleviated liver steatosis (Fig. 6B). Subsequently, mice transduced with the shNR3C1 construct were subjected to liver-specific Cre to eliminate gene repression caused by the ectopic shRNA (Fig. S5A and B). The relief in NR3C1 repression had loosened such a layer of control, and therefore restored the hepatic steatotic phenotype manifested with glucose intolerance as in untreated, age-matched mice with diabetes (Fig. 6B and S5C–E), indicating that the interaction between miR-192-3p and NR3C1 contributed to the pathogenesis of hepatosteatosis.

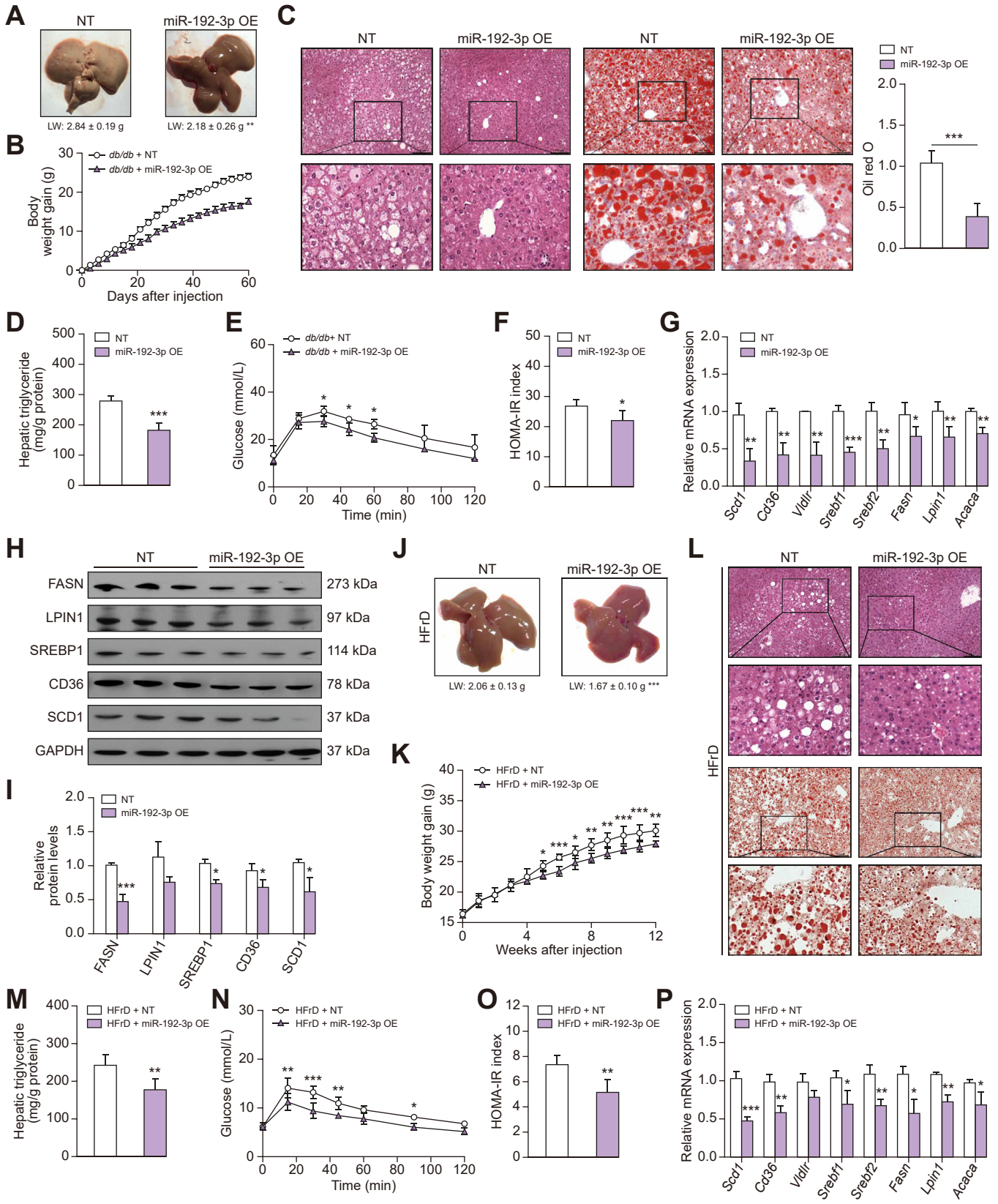

Fig. 4. Delivery of miR-192-3p into livers alleviated hepatic steatosis in mice. Mice with diabetes were injected i.v. with AAV-DJ-miR-192-3p (miR-192-3p OE) or the NT and fed on ND for 8 weeks. C57/BL6 mice were tail i.v. injected with AAV-miR-192-3p OE or NT, and given HFrD for additional 12 weeks. (A–B) Impaired liver and body weight gain of mice with diabetes with miR-192-3p OE. (C) H&E and Oil Red O staining of liver sections showed miR-192-3p OE alleviated hepatic lipid accumulation and steatosis. (D) Hepatic triglyceride levels in the indicated groups. (E–F) OGTT pattern and HOMA-IR index of mice in the indicated groups were profiled by 8 weeks. (G–I) The mRNA and protein levels of lipogenesis-related genes were measured in the mice livers of the 2 groups. (J–K) Liver and body

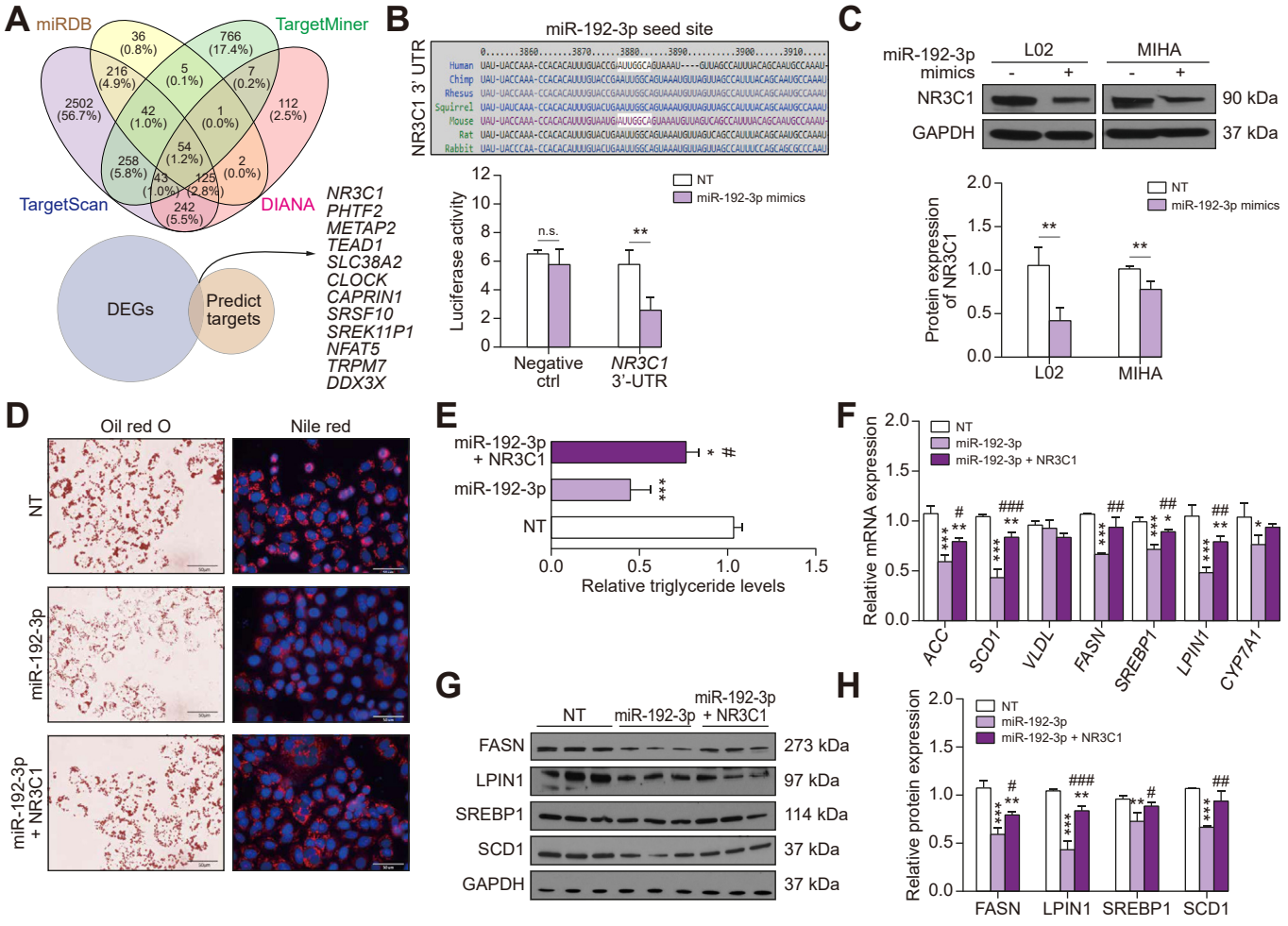

Fig. 5. NR3C1 was identified to be negatively regulated by miR-192-3p in liver steatosis. (A) Combinational analysis of 4 microRNA target prediction databases and the DEGs from the RNAseq experiments identified NR3C1 as top candidate regulated by miR-192-3p. (B) Complimentary sequences to the seed regions of miR-192-3p within the 3′-UTR mRNA of NR3C1 are conserved among different species. Luciferase reporter constructs containing 3′-UTR of human NR3C1 and miR-192-3p OE construct were co-transfected into HEK293T cells; luciferase activity was normalised to the activity of β-galactosidase. (C) Immunoblot analysis of NR3C1 in L02 and MIHA cells overexpressed with miR-192-3p. (D) Oil Red O and Nile Red staining of lipid in L02/miR-192-3p OE cells replenished with NR3C1 construct or not. (E) Hepatic triglyceride levels in the indicated groups. (F–H) Real-time PCR and Western blotting analysis of lipogenesis-related genes in L02/miR-192-3p OE cells transfected with NR3C1 OE construct or not. The cells were all incubated with OA for 8 h. Both the mRNA and protein levels were normalised to GAPDH. Data are represented as mean ± SD; n = 3. $^{*}p$ <0.05, $^{**}p$ <0.01, $^{***}p$ <0.001, NT *vs.* miR-192-3p OE; $^{\#}p$ <0.05, $^{\#\#}p$ <0.01, $^{\#\#\#}p$ <0.001, miR-192-3p OE *vs.* miR-192-3p OE + NR3C1. Student's *t* test and one-way analysis of variance with Tukey's *t* test were used for statistical analyses. DEG, differentially expressed gene; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; NR3C1, nuclear receptor subfamily 3, group C, member 1; OE, overexpression; 3′-UTR, 3′-untranslated region.

To rescue NR3C1 activity in mice injected with AAV-miR-192-3p, mice were given additional intraperitoneal injection of dexamethasone (DEX), an agonist of NR3C1/GCR. The gene components of the NR3C1-regulated lipogenesis in liver of 3 groups (NT, miR-192-3p OE, and miR-192-3p OE + DEX) were analysed (Fig. 6C–F and Fig. S5F–H). The anti-lipogenesis role of miR-192-3p was partially ablated by DEX, as demonstrated by the recovery of hepatic lipid accumulation, TAG level, and insulin resistance (Fig. 6E and F). Livers of mice treated with miR-192-3p OE had significantly reduced lipogenic gene expression while enhancing NR3C1 activity restored their suppressed levels (Fig. 6G–I). Taken together, the results showed that miR-192-3p reduced lipogenesis by interacting with *NR3C1* mRNA, which subsequently alleviated liver steatosis in mice with diabetes.

weight were impaired in HFrD-fed mice after miR-192-3p administration. (L) Histological staining, (M) hepatic triglyceride level, (N) OGTT pattern, and (O) HOMA-IR index were assayed in mice of miR-192-3p OE and NT groups fed on HFrD. (P) The mRNA levels of lipogenesis-related genes were measured in the mice livers of the indicated groups. Data are represented as mean ± SD; n = 5–6. Two-tailed Student's *t* test and one-way analysis of variance were used for statistical analyses. $^{*}p$ <0.05, $^{**}p$ <0.01, $^{***}p$ <0.001. H&E, haematoxylin and eosin; HFrD, high-fructose drink; HOMA-IR, homeostasis model assessment of insulin resistance; ND, normal diet; NT, non-targeting control; OE, overexpression; OGTT, oral glucose tolerance test.

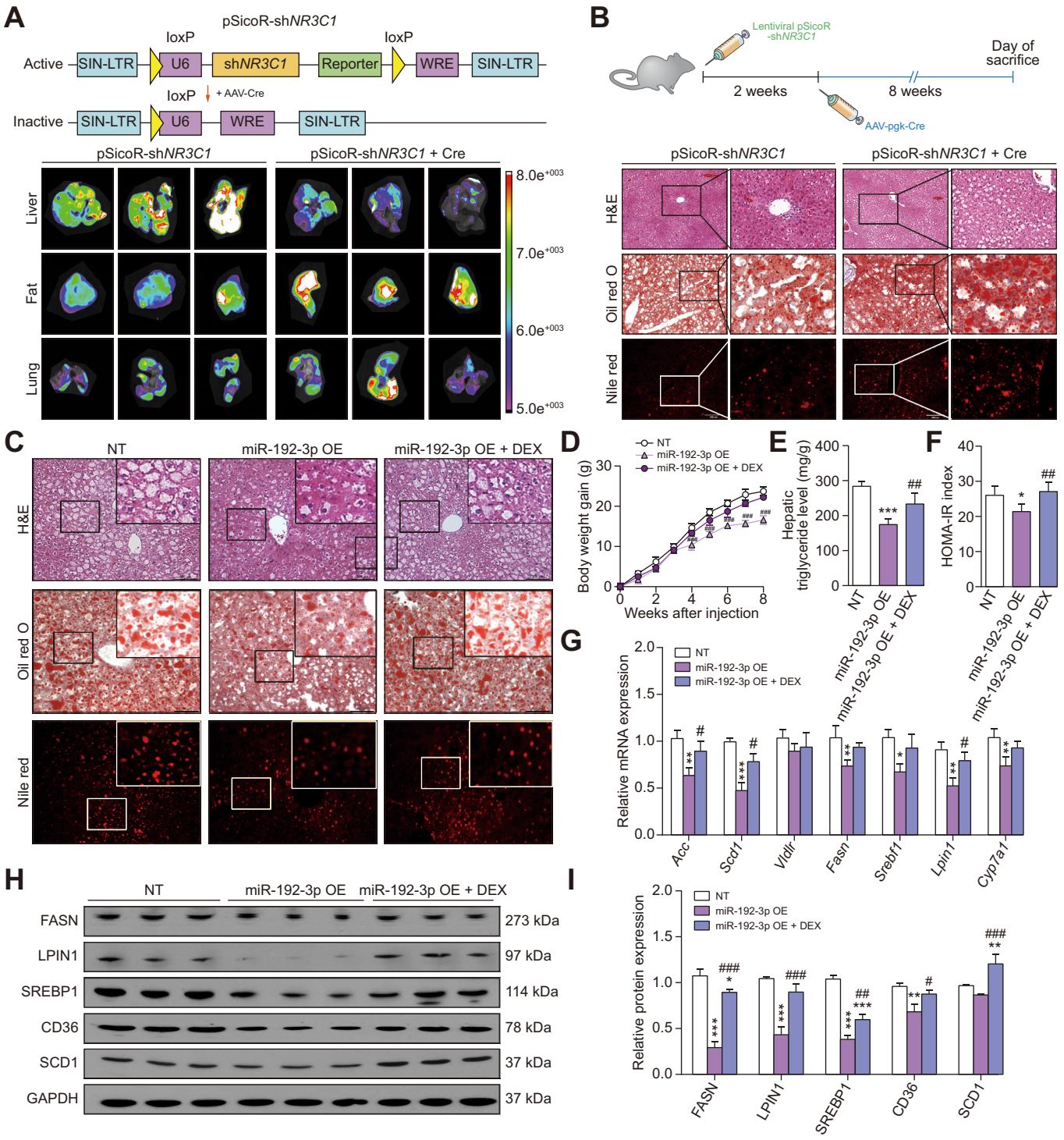

Fig. 6. NR3C1 mediated the inhibitory effects of miR-192-3p on hepatic steatosis in mice. (A) Diagram of liver-specific knockdown of NR3C1 by Cre-LoxP system. Lentiviral of psicoR-shNR3C1 was i.v. injected into 4-week-old mice with diabetes; liver-specific reactivation of NR3C1 was achieved by i.v. injection of AAV-Cre. Fluorescence signalling was shown in different tissues. (B) Representative images of H&E, Oil Red O, and Nile Red staining of liver sections in the indicated groups; n = 4. (C) Histological analysis of liver sections in mice treated with NT, miR-192-3p OE, combinational use of miR-192-3p, and DEX 1 mg/kg/day, twice every week for 2 months. (D–F) Body weight gain, hepatic triglyceride levels, and HOMA-IR index was assayed in the 3 indicated groups. (G–I) Real-time PCR and Western blotting analysis of lipogenesis-associated genes in the 3 indicated groups. Both the mRNA and protein levels were normalised to GAPDH. Data are represented as mean ± SD; n = 5. $^{*}p$ <0.05, $^{**}p$ <0.01, $^{***}p$ <0.001, NT *vs.* miR-192-3p OE; $^{\#}p$ <0.05, $^{\#\#}p$ <0.01, $^{\#\#\#}p$ <0.001, miR-192-3p OE *vs.* miR-192-3p OE + DEX. One-way analysis of variance with Tukey's *t* test. DEX, dexamethasone; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; H&E, haematoxylin and eosin; HOMA-IR, homeostasis model assessment of insulin resistance; NR3C1, nuclear receptor subfamily 3, group C, member 1; NT, non-targeting control; OE, overexpression.

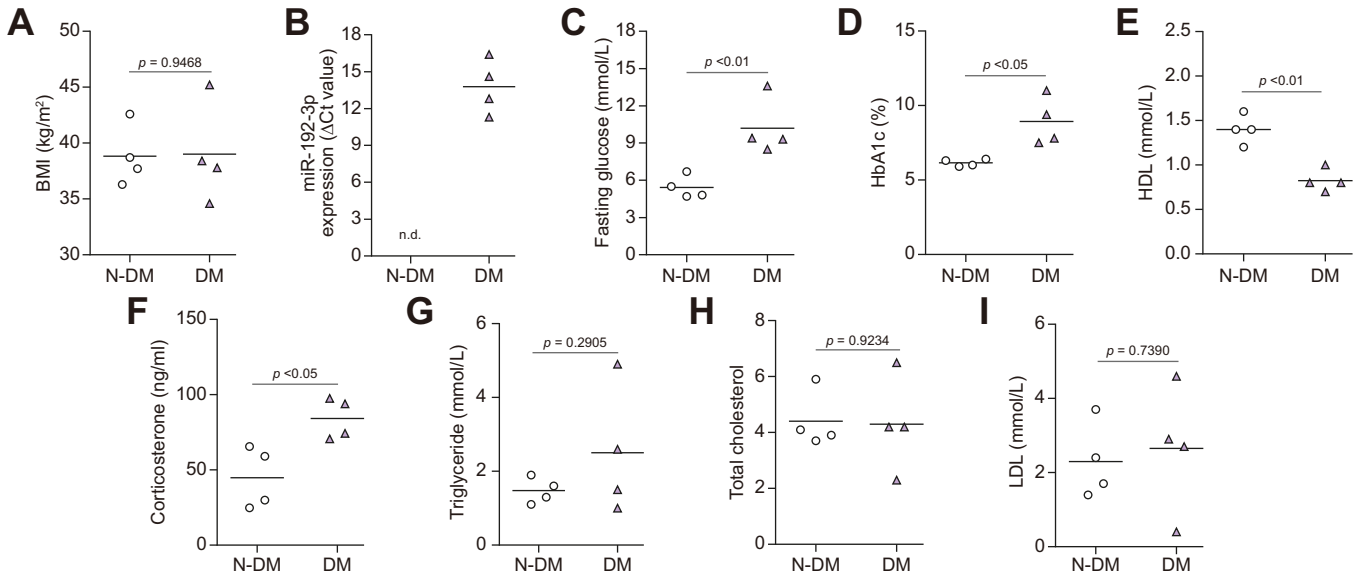

Fig. 7. Serum miR-192-3p is detectable only in patients diagnosed with diabetes mellitus. (A) All human samples exhibited obesity with BMI exceeding normal range. (B) Serum miR-192-3p levels in patients diagnosed with DM or not (N-DM). (C) Fasting glucose, (D) HbA1c, (E) HDL, (F) corticosterone, (G) triglyceride, (H) total cholesterol, and (I) LDL in each group. Data are represented as mean ± SD; n = 4. Two-tailed Student's *t* test. BMI, body mass index; DM, diabetes mellitus; HbA1c, haemoglobin A1c; N-DM, no diabetes mellitus.

Serum miR-192-3p is detected in patients with diabetes mellitus

To correlate circulating miR-192-3p level to clinical features, we quantified the serum miR-192-3p in obese individuals (body mass index >34; Fig. 7A). Among these 8 individuals, only 4 were diagnosed with diabetes mellitus (DM). We then performed TaqMan™ miRNA real-time PCR assay to screen for the presence of the star strand miRNA in their serum samples. Intriguingly, miR-192-3p can be only detected in individual diagnosis with DM, indicating the predictive relevance of miR-192-3p with DM (Fig. 7B). Accordingly, patients with DM exhibited increased levels of fasting glucose (Fig. 7C; p <0.01) and haemoglobin A1c (Fig. 7D; p <0.05), decreased HDL level (Fig. 7E; p <0.01), and also significantly higher corticosterone level (Fig. 7F; p <0.05). The serum TAG, total cholesterol, and LDL levels were also simultaneously examined (Fig. 7G–I).

Discussion

The human body relies on glucose as an immediate energy source to deal with strenuous movements. Being metabolised less efficiently, FAs are the major energy reserve with the prerequisite of a series of oxidation for its release.[23] Liver, muscle, and adipose tissues are the major effector tissues in response to the bioenergetics endocrine via modulating the entry and storage of glucose. Of note, muscle cells are privileged in a lack of glucose-6-phosphatase, therefore refrained from glycogen breakdown to supply glucose to other somatic cells when insulin is low.[24] Adipose tissues respond to insulin by synthesising TAG for compact storage. Liver is therefore the only metabolic hub, which modulates circulating levels of nutrient supply to other tissues. Furthermore, liver is also the major site for cholesterol synthesis in response to metabolic demand tightly regulated by insulin and glucose at the transcription level,[25] highlighting how defective insulin signalling may affect lipid synthesis and storage.

NAFLD is recognised as the predominant form of chronic liver disease associated with metabolic disorders, such as T2DM, obesity, and hyperlipidaemia.[26] NAFLD was caused by the inhability of insulin to suppress hepatic glucose production in obese people with diabetes, with NAFLD further promoting diabetes; paradoxically, fat overproduction by this runaway hepatic TAG synthesis in the liver continues unabated. There is ample evidence to demonstrate impairment in hepatic carbohydrate utilisation leading to lipid dysregulation.[27] Recent efforts have focused on modulating genes along the lipogenic pathway in the liver, which demonstrated marked reduction in hepatic steatosis and even confer resistance to obesity.[28,29]

Our previous study pinpointed that both miR-192-5p and its star strand miR-192-3p (*) were enriched as top regulators along hepatic lineage differentiation.[17] The guiding strand was important in inhibiting transforming growth factor beta signalling in diabetic nephropathy.[18] In our study, only miR-192-3p rather than its guiding strand miR-192-5p was downregulated in the liver of mice with diabetes. This spontaneous loss of miR-192-3p in mice with diabetes further facilitated the runaway hepatic TAG synthesis and promoted hepatic steatosis.

Micro-vesicular steatosis is characterised by functionally defective hepatocytes filled up with foamy cytoplasm and small lipid vesicles, while macro-vesicular steatosis is manifested with a single, large vacuole of lipid that occupies most of the volume in hepatocytes coupled with a displaced nucleus, a detrimental form preceding tissue inflammation.[6,30] Interestingly, our attempts in replenishing miR-192-3p in mice with diabetes not only partially reduced lipid accumulation, but also effectively mediated the storage condition for lipids from the micro-vesicular to the macro-vesicular form. Accordingly, we observed an increase of serum tumour necrosis factor alpha in HFD-fed mice treated with miR-192-3p sponge, implicating the critical role of miR-192-3p along the pathogenesis of liver inflammation (data not shown).

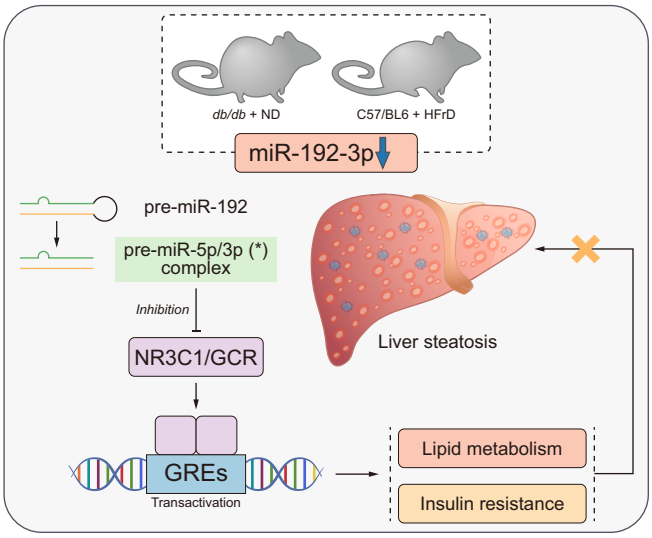

Fig. 8. Schematic representation of miR-192-3p in regulating lipid metabolism and improving liver steatosis. A downregulation of hepatic miR-192-3p was found in mice with diabetes. By post-transcriptional regulation of its target glucocorticoid receptor (GCR/NR3C1), a master regulator of lipogenesis and gluconeogenesis, miR-192-3p significantly alleviates liver steatosis and improves insulin sensitivity, through inhibiting lipogenesis- and gluconeogenesis-associated pathways. GCR, glucocorticoid receptor; HFrD, high-fructose drink; NR3C1, nuclear receptor subfamily 3, group C, member 1.

Conventional miRNA OE relies pre-miRNA stem loop; therefore OE only produces mature guiding strand. Nonetheless, we believed their strand counterparts are useful in targeting an alternative myriad of mRNAs.[31–33] To single out star-strand activation, we adopted the shRNA OE system and successfully overexpressed mature miR-192-3p *in vivo*. Further functional study found that OE of this miR-192-3p effectively prevented hepatic steatosis through restoring insulin sensitivity, which facilitated FAO. To further investigate whether this new OE system is applicable for any mature miRNA strand, we constructed the miR-206, a guiding strand miRNA responsible for lipogenesis,[13] which recapitulated its anti-lipogenesis and anti-gluconeogenesis role in hepatocytes (Fig. S6A–C).

Glucocorticoid signalling was central to modulate the activation of AMPK and represented an important crosstalk to liver responsiveness to insulin.[34] The GCR (also known as NR3C1) was reported to control the metabolism of glucose, protein, and lipid homeostasis, complementing the action of insulin.[35] NR3C1 (Ad-*GcR*$^{-/-}$) knockout mice given HFD exerted attenuated hepatic steatosis and insulin resistance.[36] In our study, we found that manipulating miR-192-3p levels affected the expression of NR3C1, which in turn modulated insulin sensitivity.

In parallel, we observed that shNR3C1 effectively phenocopied the OE of miR-192-3p, while combinational treatment of miR-192-3p and DEX ablated the anti-steatotic actions of miR-192-3p. We therefore propose that the hepato-protective effect of miR-192-3p/NR3C1 axis may arise from both regulation of glucocorticoid signalling and the inter-conversion between these lipid storage forms in hepatocytes, which unleashed a promising lead for the palliative care of the fatty liver disease in patients with diabetes. Notably, miR-192-3p is only detectable in obese patients with diabetes, with heightened corticosterone level in their serum.

In summary, the present study establishes the molecular crosstalk between miR-192-3p and NR3C1 in the regulation of insulin-regulated lipid metabolism and liver steatosis (Fig. 8). Approaches that improve lipid metabolism by manipulating a more distal signal modulator, such as increasing miR-192-3p OE, might rebalance lipid uptake and oxidation through modulating insulin sensitivity, thus making miR-192-3p an attractive drug target for carbohydrate malnutrition and related metabolic disorders, such as liver steatosis, T2DM, and lipogenesis-related diseases.

Abbreviations

AAV, adeno-associated virus; CPT, carnitine palmitoyl transferase; DEG, differentially expressed gene; DEX, dexamethasone; DM, diabetes mellitus; DNL, *de novo* lipogenesis; FA, fatty acid; FAO, fatty acid oxidation; FASN, fatty acid synthase; GCR, glucocorticoid receptor; HFD, high-fat diet; HFrD, high-fructose drink; HOMA-IR, homeostatic model assessment of insulin resistance; miRNA, microRNA; NAFLD, non-alcoholic fatty liver disease; NR3C1, nuclear receptor subfamily 3, group C, member 1; NT, non-targeting; OA, oleic acid; OGTT, oral glucose tolerance test; SCD1, stearoyl-CoA desaturase-1; shRNA, short hairpin RNA; TAG, triacylglyceride/triglyceride; T2DM, type 2 diabetes mellitus; VAT, visceral adipose tissue; 3′-UTR, 3′-untranslated region.

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Conflict of interest

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

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

Authors' contributions

Z.W., K.K.M. contributed to the study concept and design, acquisition and analysis of data, statistical analysis, and drafting of paper. X.Z., A.T.-Y.W. contributed to the acquisition and interpretation of data. H.-H.C., G.L. provided technical support. H.-M.L., A.P.-S.K., J.C.-N.C. provided the human samples. W.-Y.C. contributed to the study concept and design, revision of the paper for important intellectual content, and study supervision.

Data Availability

The RNA-seq raw data are available from the National Center for Biotechnology Information (NCBI) with accession number: PRJNA648925. All other data supporting the findings of this study are available from the corresponding author on reasonable request.

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Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jhepr.2020.100179.

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Author names in bold designate shared co-first authorship

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