Cellular Physiology and Biochemistry

Cell Physiol Biochem 2018;45:1487-1505
DOI: 10.1159/000487575
Published online: February 21, 2018
Accepted: January 16, 2018

Original Paper

Metformin-Induced Changes of the Coding Transcriptome and Non-Coding RNAs in the Livers of Non-Alcoholic Fatty Liver Disease Mice

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Key Words
Metformin • Transcriptome • Non-coding RNAs • Non-alcoholic fatty liver disease

Abstract
Background/Aims: Recent studies have suggested that changes in non-coding mRNA play a key role in the progression of non-alcoholic fatty liver disease (NAFLD). Metformin is now recommended and effective for the treatment of NAFLD. We hope the current analyses of the non-coding mRNA transcriptome will provide a better presentation of the potential roles of mRNAs and long non-coding RNAs (lncRNAs) that underlie NAFLD and metformin intervention. ***Methods:*** The present study mainly analysed changes in the coding transcriptome and non-coding RNAs after the application of a five-week metformin intervention. Liver samples from three groups of mice were harvested for transcriptome profiling, which covered mRNA, lncRNA, microRNA (miRNA) and circular RNA (circRNA), using a microarray technique. ***Results:*** A systematic alleviation of high-fat diet (HFD)-induced transcriptome alterations by metformin was observed. The metformin treatment largely reversed the correlations with diabetes-related pathways. Our analysis also suggested interaction networks between differentially expressed lncRNAs and known hepatic disease genes and interactions between circRNA and their disease-related miRNA partners. Eight HFD-responsive lncRNAs and three metformin-responsive lncRNAs were noted due to their widespread associations with disease genes. Moreover, seven miRNAs that interacted with multiple differentially expressed circRNAs were highlighted because they were likely to be associated with metabolic or liver diseases. ***Conclusions:*** The present study identified novel changes in the coding transcriptome and non-coding RNAs in the livers of NAFLD mice after metformin treatment that might shed light on the underlying mechanism by which metformin impedes the progression of NAFLD.

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Introduction

Non-alcoholic fatty liver disease (NAFLD) is a condition that is characterized by excessive fat accumulation in the form of triglycerides and involves a complex interaction of risk factors, including central obesity, dyslipidaemia, hypertension and insulin resistance [1, 2]. A large proportion of NAFLD patients may further develop non-alcoholic steatohepatitis (NASH), fibrosis, cirrhosis, or even hepatocellular carcinoma (HCC) [3, 4]. In recent years, the prevalence of NAFLD has been increasing [5], and NAFLD has become the leading cause of liver disorders worldwide [6, 7]. NAFLD is the most prevalent chronic liver disease, and its pathogenesis is complicated and multi-faceted. According to the double-hit theory, the first hit of NAFLD is the accumulation of abnormal triglycerides in hepatocytes, which results in the second hit in which inflammatory mediators further lead to liver injury, inflammation, and fibrosis [8, 9]. Metformin is a hypoglycaemic drug that has been clinically applied for half a century and not only improves insulin resistance and hyperinsulinaemia but can also directly influence insulin target cells, particularly by increasing insulin sensitivity via post-receptor mechanisms [10, 11]. Thus, metformin is recommended and effective for the treatment of NAFLD [12].

Nevertheless, the detailed mechanism underlying the effect of the metformin intervention for NAFLD is not clear. Specifically, whether and how non-coding RNAs are involved in the progression of NAFLD and the mechanisms of action of metformin treatment are poorly understood. Previous studies have demonstrated transcriptome changes after metformin interventions [13, 14]. For example, Kita et al. profiled the transcriptome of a closely related but not identical NASH mouse model (a HFD plus MCD diet) [13]. Another work by Salomaki et al. focused on the transcriptomic effect of maternal metformin plus HFD exposure on the offspring's' livers [14]. Moreover, metformin treatment elicits transcriptome changes that mimic the calorie restriction transcriptome and thus improving physical performance and increases insulin sensitivity [15]. However, few of these mRNA transcriptomes were published with concerned about the transcriptomic effect of metformin intervention against NAFLD progression.

Furthermore, the key role of miRNAs in the progression of NAFLD has been extensively identified, and scholars have explored the changes in miRNAs after metformin treatment in different diseases [16-18]. Kato et al. and Fujimori et al. analysed the differentially expressed miRNAs after metformin treatment that contribute to metformin's anti-tumour effects against gastric cancer [16] and cholangiocarcinoma [17], respectively. Based on examinations of the miRNA profile in breast cancer cells, Blandino et al. reported that metformin can modulate DICER, which is a key protein in miRNA biogenesis, to elicit a systematic alteration of miRNA expression and an anticancer effect [18]. Noren Hooten et al. demonstrated that the targeting of DICER by metformin plays a role in cellular senescence [19]. To date, only one miRNA transcriptome dataset regarding liver diseases has been published; Katsura et al. profiled the miRNA expression in a methionine- and choline-deficient (MCD) diet mouse model of NASH [20]. Moreover, lncRNAs are also important regulators of the development of NAFLD. Chen et al. conducted lncRNA microarray analyses in an NAFLD rodent model and identified a new lncRNA, FLRL2, which may be involved in Arntl-mediated NAFLD pathogenesis [21]. LncRNA SRA enhances hepatic steatosis mainly by inhibiting the expression of adipose triglyceride lipase (ATGL) [22]. Additionally, genetic variation in lncRNAs is tightly correlated with the risk of NAFLD [23]. Recently, metformin was reported to be involved in the regulation of lncRNAs [24-26]. For example, metformin has been demonstrated to suppress bladder cancer cell proliferation and glycolysis by regulating the expression of the long non-coding RNA UCA1 [25]. Moreover, metformin has also been reported to decrease lncRNA HULC by inhibiting the expression of specificity protein 1 (Sp1) [26]. Therefore, exploration of the changes in the miRNA and ncRNA networks after metformin treatment may provide novel information about the molecular basis of NAFLD that could be exploited for its treatment.

In the current study, we profiled the coding transcriptome and non-coding RNA changes that occurred in response to a high-fat diet (HFD) treatment and metformin treatment

using the microarray technique. The results might aid the elucidation of the molecular mechanisms of the function of metformin in the treatment of NAFLD. We prepared three groups of liver samples from the following mice: 1) mice from the control group that were fed normal chow for 17 weeks; 2) mice from the HFD group that were fed the HFD diet for 17 weeks and intragastrically treated with saline vehicle for the last 5 weeks; and 3) mice from the metformin group that were also fed the HFD diet for 12 week but were intragastrically treated with 3 mg/kg/day metformin for the last 5 weeks. The liver samples from the three groups of mice were harvested for transcriptome profiling, which covered mRNA, long non-coding RNA (lncRNA), microRNA (miRNA) and circular RNA (circRNA), using a microarray technique. Our study suggests that the potential efficacy of metformin in improving the histological endpoints of NAFLD is mediated through the modulation of the transcriptome and non-coding RNA changes.

Materials and Methods

Animal feeding and liver sample extraction

Male 3-week-old C57BL/6J mice (18-21g) were provided by the Spfanimals Company (Beijing, China). The mice were kept in a temperature- and humidity-controlled (20-24°C, 45-55% humidity) environment with free access to food and water and were housed in groups of 3. The protocol was approved by the Beijing Hospital Animal Ethics Committee.These mice were divided into three groups: the control group, the high fat diet (HFD) group and the metformin group. For the control group, the mice were maintained with chow diet (D12450B, 10% kcal from fat, 3.85 kcal/g, Research Diet, USA, http://www.researchdiets.com/opensource-diets/stock-diets) for 17 weeks. For the HFD group and metformin group, the mice were fed a HFD (D12451, 45% kcal from fat, Research Diet, 4.73 kcal/g, USA, http://www.researchdiets.com/opensource-diets/stock-diets) for 12 weeks. Starting at the 15 weeks old of age (i.e. after 12 weeks of HFD feeding), the HFD and metformin group were differentially treated: the mice from metformin group were intragastrically by oral gavage treated with 3 mg/kg/day metformin for 5 weeks, while those from the HFD group were treated with saline vehicle for 5 weeks. Changes in the body weight of each group were determined at 2~4 p.m each week. To measure the amount of daily food intake, the mice were provided with 50 mg food each week. Food intake was measured daily between 3 and 5 PM for a week. Then, the rest food was discarded and new food of 50 mg was added. Daily energy intake per mouse was calculated as follows: energy intake (kcal) = Food intake (g/day) × Energy density (kcal/g) × days. Finally, the liver tissues from different groups of mice were collected respectively at the 20 weeks old of age. It is noteworthy that we prepared three biological replicates for each group, and each biological replicate contains a mixed liver tissue samples collected from four mice of the same group.

Triglyceride measurement

Extraction and measurement of triglycerides in liver tissues or cells were performed as previously reported [27, 28] using a triglyceride enzymatic assay kit (ShenSuoYouFu Medical Diagnostic Products Co., Ltd., Shanghai, China).

Oil Red O staining

Frozen sections of liver specimens were fixed in paraformaldehyde. Then, the slides were stained with Oil Red O as previously described [29, 30].

Hematoxylin and Eosin (H&E) staining

For H&E staining, the slides were first incubated with hematoxylin for 5 min and then washed with 1% ethanol hydrochloride for 3 sec. After washing with water, the slides were stained with eosin for 3 min and dehydrated with an alcohol gradient. Vacuoles were considered the lipid droplets [30].

Immunohistochemical (IHC) staining

Frozen liver sections (7 μm) were stained for $CD68^+$ resident macrophages (CD68 marker, FA11) and infiltrated macrophages (macrophage marker, Mac-1) as described previously [31]. $CD68^+$ pictures were analysed by quantifying the area fraction using imaging software in a blinded fashion (ImageJ).

Western blot

Liver tissues extracts (20 μg of proteins) were separated by 10% SDS-PAGE, transferred to PVDF membrane (Millipore), blocked with 8% nonfat dry milk, and then incubated with a primary antibody at 4°C overnight. The blot was incubated with HRP-conjugated anti-IgG, followed by detection with ECL (Millipore). Antibodies against ACC1 (ab45174), phosphorylated ACC1 (ab68191), INSIG1 (ab70784), S100A4 (ab41532), FABP2 (ab128860), GAPDH (ab181602) were purchased from Abcam. AMPK (#5831) and phosphorylated AMPK (Thr^{172}) (#2531) were obtained from Cell Signaling Technology. An antibody to SERPINA12 was purchased from Bioss (bs-7536R-HRP).

RNA isolation

Total 15 mg of liver tissues were collected and flash frozen by liquid nitrogen. Then the total RNA was extracted by using the Trizol (Invitrogen, USA) following the manufacturer's recommendations. The miRNA, however, was extracted using the miRNeasy Serum/Plasma kit (Qiagen; Valencia, CA) according to the manufacturer's protocol.

Microarray analysis

The transcriptomes of the liver samples were analyzed by three microarray platforms targeting different types of RNAs. The transcriptome for coding genes and lncRNAs was analyzed by CapitalBio mouse lncRNA+mRNA v1.0 microarray (CapitalBio; Beijing, China), while the miRNA and circRNA transcriptome was analyzed by Agilent mouse miRNA 21.0 microarray (Agilent, USA) and CapitalBio mouse circRNA v1.0 microarray (CapitalBio; Beijing, China), respectively. The total RNA samples were Cy3 labeled by using CapitalBio cRNA Amplification and Labeling Kit, while the miRNA samples were Cy3 labeled by using the Agilent miRNA Complete Labeling and Hyb Kit, according to the manufacturer's guidelines. The microarray hybridization, washing and scanning were performed following the standard pipeline. Agilent Feature Extraction software (version 10.7) was used to analyze the acquired array images. Quantile normalization and subsequent data processing were performed using the GeneSpring GX software (Agilent Technologies). The differentially expressed coding genes or non-coding RNAs were obtained by using a combine threshold of fold change>1.5 and t-test P-value<0.05 for all transcriptome comparisons. We noted that the expression of different transcripts of the same non-coding gene was highly varied and hard to be summarized. Therefore, we used the gene-wise normalized fold changes for coding genes, but used the most significantly changed transcript as the representative for the non-coding genes.

The transcriptome data was submitted to GEO database with accession GSE94844.

Correlation with other transcriptome alterations

We manually curated the disease-related gene expression profiles of mouse liver tissues from the GEO database [32]. For each gene expression profile, we calculated the case vs. control fold changes of gene expression. The correlation of fold changes was measured by Spearman correlation coefficient, and we only considered highly significant correlation (P-value<1e-20) for further investigation. Since Spearman correlation coefficient requires two fold change vectors of the same length, we used fold changes of the shared genes from the compared two gene expression profiles to calculate the Spearman correlation coefficient.

LncRNA-gene, miRNA-target and circRNA-miRNA interaction analysis

For each differentially expressed lncRNA, its expression correlations with the mRNAs in our dataset were measured by Spearman correlation coefficient. Among the significant co-expressed lncRNA-mRNA pairs (Spearman correlation coefficient ρ>0.8 and p-value<0.01), two kinds of interactions were obtained. First, if the lncRNA gene was settled within 100 kb from its co-expressed mRNA coding gene, this lncRNA-mRNA pair was deemed as a cis-interacting pair. Second, if there is a predicted physical interaction between the co-expressed lncRNA and mRNA, this lncRNA-mRNA pair was deemed as a trans-interacting pair. Such physical interaction was predicted by the LncTar software [33] with the moderate stringency threshold. For clarity, the interactions between one lncRNA and different mRNAs of the same gene were merged into single lncRNA-gene interacting pair.

The miRNA-target interactions were predicted by the CapitalBio miRNA microarray analysis protocol. Briefly, this protocol incorporated 10 state-of-the-art miRNA target prediction tools including miRWalk,

Microt4, miRanda, mirbridge, miRDB, miRMap, miRNAMap, Pictar2 PITA, RNA22, RNAhybrid and Targetscan. Only the consensus targets supported by at least 8 out of 10 prediction tools were retained.

The experientially identified circRNA-miRNA interactions were obtained from the StarBase (http://starbase.sysu.edu.cn) [34]. To further expand the scope of circRNA-miRNA interactions, we also collected mRNA-miRNA interactions from the StarBase. If the genomic location of one circRNA covers the target site of a specific miRNA, this circRNA-miRNA pair was also deemed interacting.

Functional enrichment analysis and gene set enrichment analysis (GSEA)

The function and pathway enrichment for differentially expressed coding genes, the target genes of differentially expressed miRNAs and the host genes of differentially expressed circRNAs were respectively analyzed by gProfileR online tool (http:// biit.cs.ut.ee/gprofiler) with default parameters and threshold [35]. To reduce the redundancy of the resulted terms, we applied "best per parent" filtration provided by the gProfileR tool to the enriched terms. As for the functional enrichment analysis for the differentially expressed lncRNAs, we first downloaded Gene Ontology (GO) functional annotations of mouse lncRNAs from the NONCODE database (http://www.noncode.org) [36]. In line with the common functional enrichment analysis protocols [37], the enrichment of GO terms were tested by Fisher exact test followed by the Benjamini-Hochberg correction. The terms with a corrected P-value<0.05 were denoted as the significantly enriched GO terms. Note that the GO terms which are too general (i.e. terms associated with more than 1000 lncRNAs) or too specific (i.e. terms associated with less than three lncRNAs) were discarded before analysis.

The GSEA was performed by using Broad Institute GSEA software (http://software.broadinstitute.org/gsea) [38] with default parameters except the gene set perturbation was used. The gene set perturbation is suitable for the gene expression dataset with small number of biological replicates. We compared expression profiles in our dataset with the MSigDB hallmark gene sets (http://software.broadinstitute.org/gsea/msigdb/index.jsp) [39] and applied a rigorous false discover rate (FDR) threshold of <5% to find the significantly enriched gene sets, as recommend in the software's user manual.

Results

Metformin treatment improves lipid accumulation in the livers of HFD mice

Compared with the control group (NC), the body weights of the HFD mice were significantly increased, but metformin treatment for five weeks significantly reduced the body weight by 10.8% (Fig. 1A). Moreover, metformin had a tendency to reduce the energy intake of the mice (Fig. 1B). Compared with the NC group, the levels of total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), triglycerides (TG), high-density lipoprotein cholesterol (HDL-C), glucose (GLU), gamma-glutamyl transpeptidase (gamma-GT), alanine transaminase (ALT) and aspartate transaminase (AST) were significantly increased in the HFD group (Table 1). Compared with the HFD group, the TC, LDL-C, TG, GLU, ALT and AST levels were significantly decreased, and the HDL-C level was significantly increased after 5 weeks of metformin intervention (Table 1). Additionally, the lipid accumulation, liver weight, and liver index of the HFD mice were significantly elevated compared to the controls, but the five-week metformin intervention significantly abolished these effects (Fig. 1C, 1D, 1E and 1F). Moreover, we quantified the TG and TC contents of the livers. Our data demonstrated that the hepatic TG and TC contents were markedly increased in the HFD group but were reduced by the metformin intervention (Fig. 1G and 1H). As presented in Table 1, the levels of ALT and AST were elevated in the livers of the HFD mice. Therefore, we examined whether there was any histological evidence of steatohepatitis, i.e., hepatic inflammatory infiltration. As evidenced by IHC staining for $CD68^+$, obvious hepatic inflammatory infiltration was identified in the livers of the HFD mice, and these histological changes were greatly improved after metformin treatment (Fig. 1I). Additionally, quantification of the $CD68^+$ IHC staining indicated the macrophage content was increased to 3.57%±0.46% in the livers of HFD mice, whereas this value was 0.35%±0.21% in the C57 mice; moreover, metformin treatment reduced the macrophage content to 1.34%±0.36%, which indicated that metformin impeded NAFLD progression in HFD mice (Fig. 1I). In line with the elevated serum GLU level in the

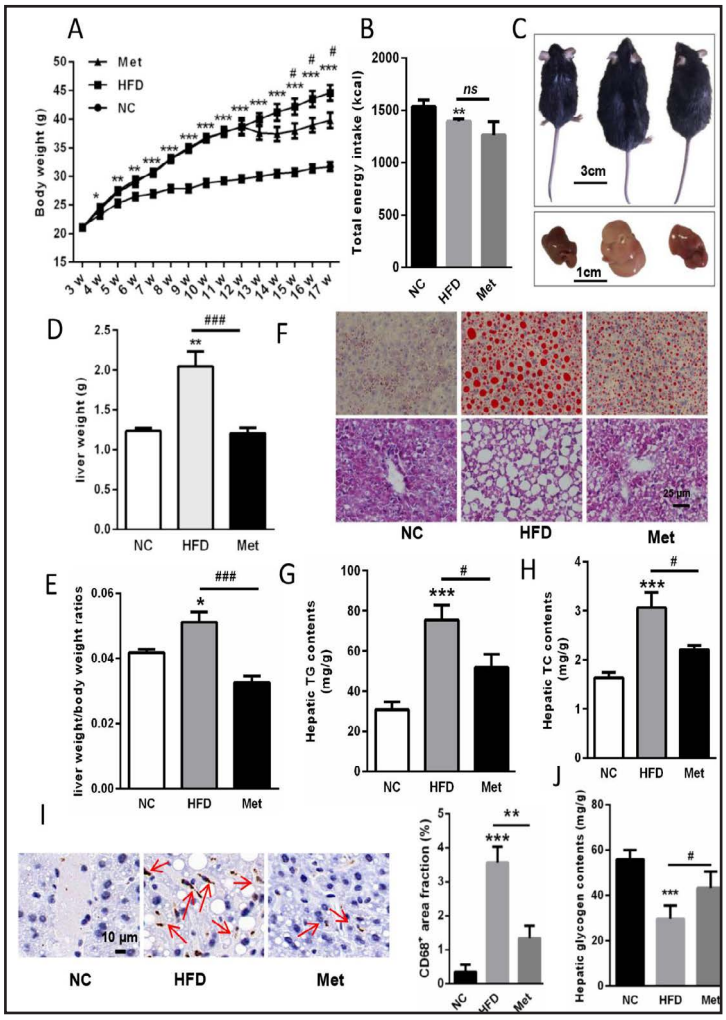

Fig. 1. Metformin treatment improves the lipid accumulation in the livers of HFD mice. (A) Metformin treatment significantly decreased HFD-induced body weight enhancement. (B) Metformin has a tendency to reduce energy intake in mice. (C) Representative images of mice livers. (D) Liver weight and (E) liver index were reduced by metformin treatment. (F) H&E and Oil Red O staining. Hepatic TG (G) and TC (H) contents were markedly increased in HFD group, but were decreased by metformin intervention. (I) IHC staining of $CD68^{+}$ (Red arrow indicated positive staining of CD68). (J) Hepatic glycogen contents were increased in the HFD mice, but was decreased after metformin treatment. n=5 mice in each group. ***P-value<0.01 vs. NC, $^{\#}$P-value<0.05 vs. HFD.

Table 1. Comparison of biochemical indexes between three groups of mice after 5 weeks intervention with metformin. *P-value<0.05, **P-value<0.01, ***P-value<0.001 vs. NC, $^{\#}$P-value<0.05, $^{\#\#\#}$P-value<0.001 vs. HFD

Groups	TG (mmol/L)	TC (mmol/L)	LDL-C (mmol/L)	HDL-C (mmol/L)	GLU	γ-GT (U/L)	ALT (U/L)	AST (U/L)
NC	0.59±0.06	2.38±0.10	1.06±0.06	2.25±0.05	5.2±0.75	38±3	40±4	129±12
HFD	1.03±0.04***	4.04±0.26***	1.75±0.16***	3.21±0.19***	13.9±2.27	64±5*	68±7**	195±15*
Metformin	0.77±0.03$^{\#\#\#}$	3.28±0.18$^{\#}$	1.26±0.09$^{\#}$	3.34±0.15	8.0±0.94	45±4$^{\#}$	46±2$^{\#}$	150±7$^{\#}$

livers of the HFD mice, the hepatic glycogen content was decreased in the HFD mice, which indicated a state of insulin resistance. Unsurprisingly, the metformin treatment decreased the serum GLU level and enhanced hepatic glycogen accumulation, which was suggestive of an improvement in insulin resistance in the HFD mice (Table 1 and Fig. 1H). These data indicated the protective role of metformin in the treatment of NAFLD in mice.

The coding transcriptome alterations recapitulate the liver disease-related genes and disease-induced gene expression changes

Here, we considered two types of comparisons between transcriptomes, i.e., the "HFD group vs. the control group" and the "metformin group vs. the HFD group". For conciseness, the differentially expressed genes based on the "HFD group vs. control group" comparison

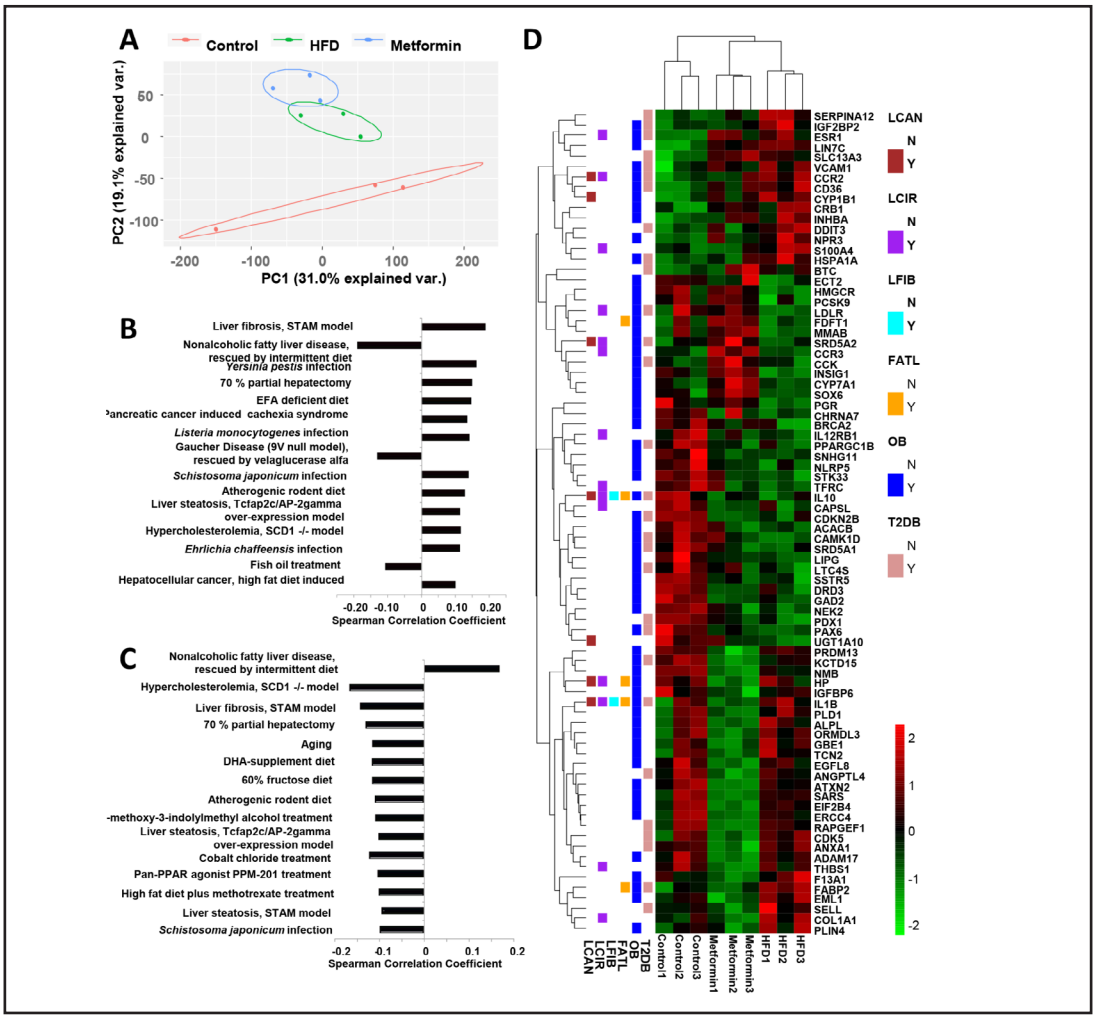

Fig. 2. Transcriptome changes in response to HFD and metformin treatment. (A) The principle component analysis bi-plot of the transcriptome profiles using first two principle components (PC1 and PC2), where the samples from the same group were clustered. (B) The barplot showing the correlations between the gene expression fold changes induced by HFD treatment (HFD vs. control) and those under other situations. (C) The barplot showing the correlations between the gene expression fold changes induced by metformin treatment (metformin vs. HFD) and those under other situations. (D) The heatmap depicting the gene expression alteration of the disease-associated differentially expressed genes. The disease associations of the genes were indicated by the colored sidebar on the left of heatmap: T2DB, type 2 diabetes; OB, obesity; FATL, fatty liver; LFIB, liver fibrosis; LCIR, liver cirrhosis; LCAN, liver cancer.

are referred to as "HFD-responsive genes", and those from the "metformin group vs. HFD group" comparison are referred to as "metformin-responsive genes". We first focused on the expression changes of the coding genes. The transcriptomes of the coding genes were profiled using a CapitalBio mouse lncRNA+mRNA v1.0 microarray. We applied principle component analysis (PCA) to these transcriptome profiles of coding genes to test whether the different sample groups could be clearly separated by their transcriptome signatures. As illustrated in Fig. 2A, the three groups of samples could be clearly separated by the first two principle components (PC1 and PC2), which explained 31.0% and 19.1% of the variation, respectively. Moreover, the samples from the HFD group were closely clustered in the PCA plot as were the samples from the metformin group. We then analysed the genes that were differentially expressed in response to the HFD and metformin treatments. In total, 540 HFD-responsive genes (178 up-regulated and 362 down-regulated) and 390 metformin-responsive genes

(109 up-regulated and 281 down-regulated) were obtained. These results indicated that the HFD and metformin treatments induced prominent and consistent transcriptome alterations. To test whether such transcriptome alterations recapitulated the changes in the liver transcriptomes in disease conditions, chemical component treatment conditions or life-style intervention situations, we calculated the Spearman correlation coefficients (SCCs) of the fold changes in gene expression after the HFD and metformin treatments in our dataset with the fold changes in other situations as reported in public gene expression datasets [32]. A positive SCC indicated that two situations induced similar alterations of in the liver transcriptome, while a negative SCC indicated that one situation could partly reverse the alterations caused by another situation. Fig. 2B displays the top 15 situations that correlated with the HFD-induced transcriptome changes. The positively correlated situations included not only known HFD-induced pathological conditions, such as HFD-induced hepatocellular cancer and atherogenic rodent diet feeding but also other metabolic disorders, such as EFA deficiency and hypercholesterolaemia. The HFD-induced transcriptome changes also positively correlated with other liver diseases or injuries, including liver fibrosis, liver steatosis, 70% partial hepatectomy and infections with several pathogens. Among the top 15 correlated situations, only three exhibited negative correlations, and all of these were conditions of drug or lifestyle interventions against liver diseases, which indicated that the HFD treatment could counteract such beneficial effects. Unlike HFD, the metformin treatment-induced transcriptome changes were negatively correlated with multiple liver disease and injury situations (Fig. 2C). The metformin-induced transcriptome changes were also negatively correlated with treatments with some drugs that are hazardous to the liver, including 1-methoxy-3-indolylmethyl [40], the pan-PPAR agonist PPM-201 [41] and cobalt chloride [42] (Fig. 2D). Only one positive correlation was observed among the top 15 correlated situations, i.e., the intermittent diet intervention, which rescued NAFLD. In all, these results suggested that the HFD treatment induced adverse transcriptome alterations that were similar to those induced by liver diseases and injury, whereas the metformin treatment could reverse such adverse changes and presumably elicit protective effects against liver diseases and injury.

Next, we focused on the differentially expressed coding genes. AMP-activated protein kinase (AMPK) is a well-known downstream target of metformin in hepatocytes. To prove the effect of metformin, we examined the levels of p-AMPK and the phosphorylation of acetyl-CoA carboxylase (p-ACC) in the livers of all the three groups. As illustrated in Fig. 3A, the levels of p-AMPK and p-ACC were reduced in the livers of the HFD mice, but metformin treatment significantly increased the phosphorylation of AMPK and ACC. The activation of AMPK by metformin indicated that metformin was effective in the livers of then HFD mice. Among these differently expressed genes, we noticed the existence of known disease genes for diabetes, obesity and other liver diseases. The disease genes for type 2 diabetes,

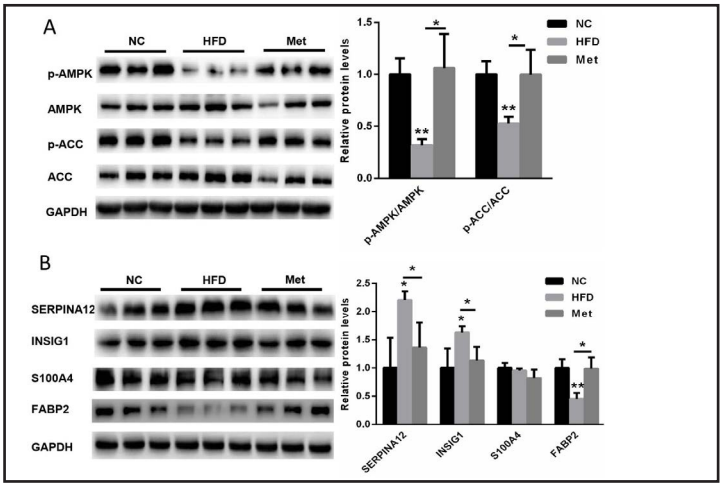

Fig. 3. Protein expression changes in the livers of control, HFD and metformin-treated mice. (A) The levels of p-AMPK and p-ACC were reduced in the livers of HFD mice, but metformin treatment significantly increased the phosphorylation of AMPK and ACC. **P-value<0.05 vs. NC, #P-value<0.05 vs. HFD. (B) Western blot analysis of SERPINA12, INSIG1, FABP2 and S100A4 expression in the livers of control, HFD and HFD plus metformin group. **P-value<0.05 vs. NC, #P-value<0.05 vs. HFD.

obesity, fatty liver, liver fibrosis, liver cirrhosis and liver cancer were retrieved from the GAD database (https://geneticassociationdb.nih.gov/) [43] using corresponding disease keywords. Among the 540 HFD-responsive genes, 42 genes were associated with at least one of the aforementioned six diseases. Similarly, 42 of the 390 metformin-responsive genes were also disease-associated. The gene expression changes and disease associations of these genes are summarized in Fig. 3D. Among these changes and associations, 4 disease genes were both HFD-responsive and metformin-responsive. *SERPINA12* is a diabetes-associated gene, and *INSIG1* is an obesity-associated gene. These two genes were up-regulated by the HFD and down-regulated by the metformin treatment. *S100A4* is a liver cirrhosis-associated gene, and *FABP2* is associated with diabetes, obesity and fatty liver. These two genes were down-regulated by HFD and up-regulated by metformin treatment. We also examined the expression levels of these genes in the livers of the control, HFD and HFD plus metformin groups. As presented in Fig. 3B, the protein levels of SERPINA12 and INSIG1 were increased by HFD and decreased by metformin treatment, whereas the FABP2 level was lower in HFD group and higher after the metformin treatment. However, no significant change in S100A4 was identified in the livers of the HFD or metformin groups (Fig. 3B), which may have been due to post-transcriptional regulation.

Functional analysis of the differentially expressed long non-coding RNAs

To date, there are some lncRNAs that have been reported to be associated with metabolic or liver diseases (mostly liver cancer). We curated these disease-related lncRNAs from the lncRNADisease database [44] and the literature and found 19 lncRNAs that have homologs in the mouse genome. The expression patterns of these lncRNA in our transcriptome profile can be roughly classified into four classes. The first class contains lncRNAs that are induced (at least 10% expression change) in response to HFD but repressed (at least 10% expression change) in response to metformin treatment. Intuitively, this expression pattern implies that these lncRNAs may contribute to the beneficial effects of metformin interventions because their HFD-induced expressions could be reversed by metformin. Three lncRNAs belonged to this class: AIRN, H19, and FTX (Fig. 4A). Interestingly, AIRN is one of the two conserved lncRNAs that has been demonstrated to be involved in fatty liver disease [45] (the other is NEAT1). This lncRNA is also related to liver cancer [45]. H19 is essential for tumour growth and is over-expressed in hepatocellular carcinomas [46]. Additionally, H19-related aberrant

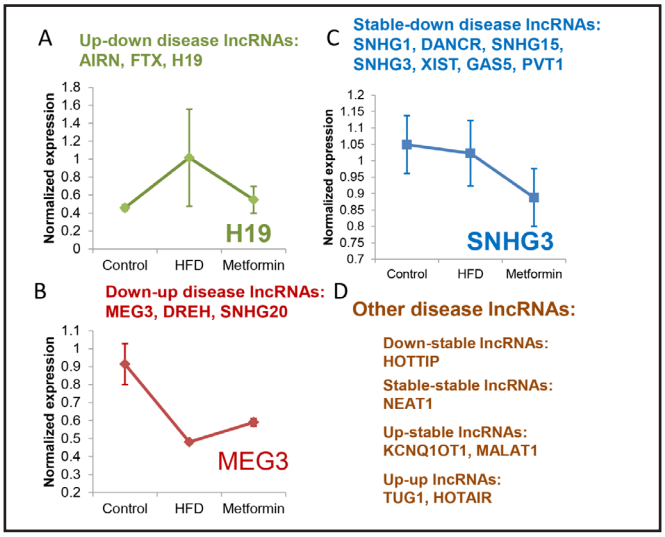

Fig. 4. The expression pattern of the conserved liver or metabolic disease-associated lncRNAs. According to the expression pattern in response to HFD and metformin, these disease-associated lncRNAs are roughly classified into four classes: (A) lncRNAs that are induced by HFD but repressed by metformin; (B) lncRNAs that are repressed by HFD but induced by metformin; (C) lncRNAs whose expression levels do not response to HFD but are reduced by metformin; (D) lncRNAs that show other expression pattern. Note that because most of these lncRNA did not exhibit prominent expression changes, we assigned lncRNAs that show at least 10% expression alteration as the nominal induced/repressed lncRNAs here. For the first three classes, one typical expression pattern is shown with error bars that indicate standard error.

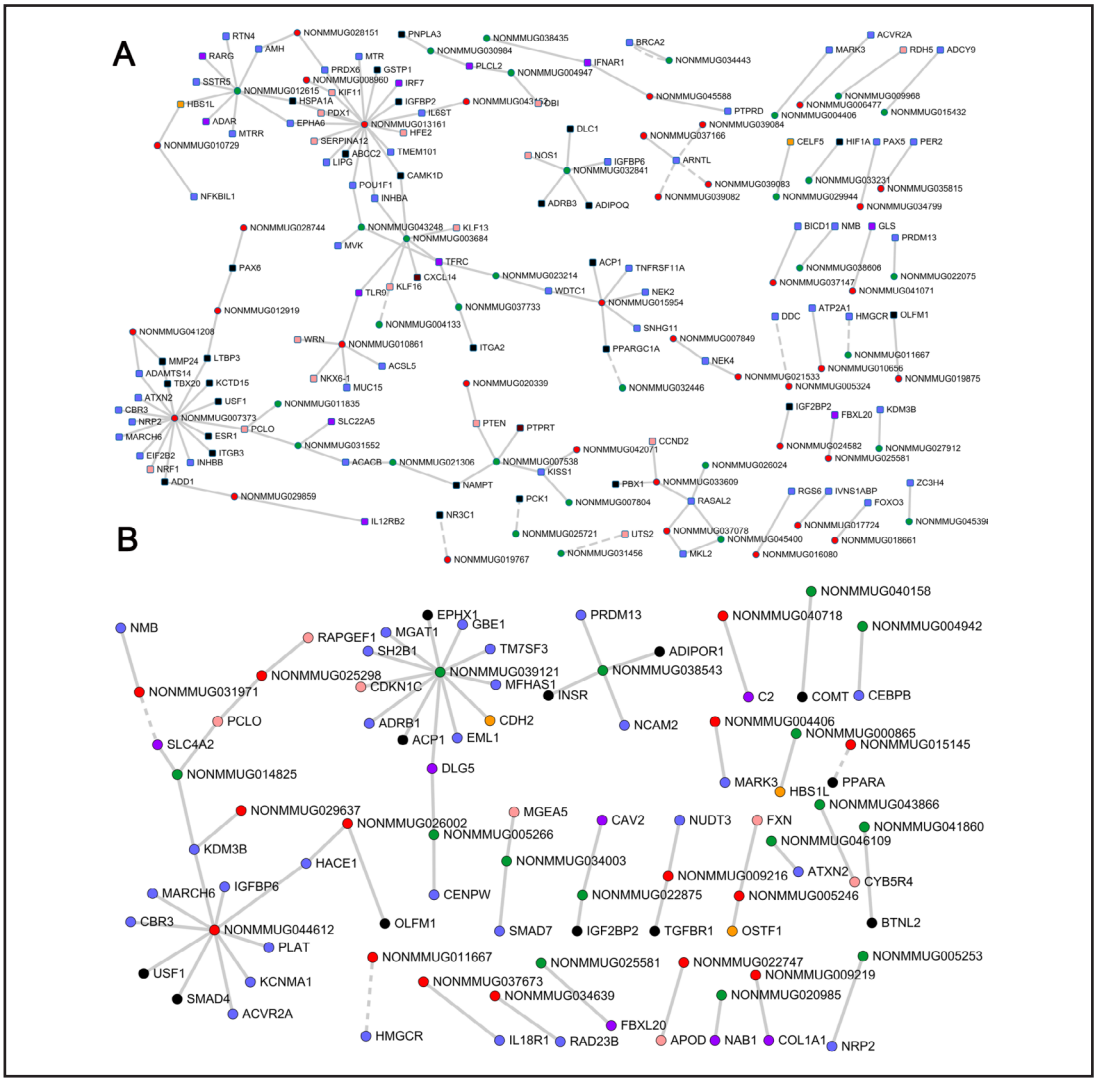

Fig. 5. The predicted interactions between differentially expressed lncRNAs and the disease associated genes. The interactions of HFD-responsive lncRNAs and metformin-responsive lncRNAs were shown in panels (A) and (B), respectively. The cis-interactions, where the lncRNA and disease-associated gene were located closely on the genome, were indicated by dashed lines. The trans-interactions, where the lncRNA and the mRNAs of the disease-associated genes had at least one predicted physical interaction, were indicated by solid lines. The up-regulated lncRNAs were indicated as red rounds, while the down-regulated lncRNAs were indicated as green rounds. The genes associated with type 2 diabetes, obesity, fatty liver, liver fibrosis, liver cirrhosis and liver cancer were denoted as pink, blue, orange, cyan, violet and brown boxes, respectively. Genes associated with more than one of the aforementioned disease were denoted as black boxes.

DNA methylation has been found to be associated with childhood obesity [47]. FTX is also involved in liver cancer, but its role is more complicated. On the one hand, FTX can bind MCM2 and miR-374a to inhibit tumour cell proliferation [48]. On the other hand, FTX can produce miR-545, which promotes tumour cell proliferation [49]. The second class exhibited the mirrored expression pattern, i.e., they were repressed by the HFD but induced by metformin (Fig. 4B). Among these lncRNAs, DREH is a known suppressor of hepatocellular carcinoma metastasis [50]. Similarly, the reduced expression of MEG3 is associated with tumour cell proliferation in liver cancer [51]. This lncRNA can also inhibit liver fibrosis [52]. The re-activation of the expressions of these lncRNAs by metformin might be related

to the protective effect of metformin against liver diseases. In contrast, SNHG20 is related to tumour growth [53]. Its re-activation by metformin may imply a potentially damaging effect. Nevertheless, because we did not profile the transcriptome changes in a cancer condition, the actual relationship between SNHG20 and metformin interventions in liver cancer conditions is not clear. Overall, the V-shaped expression pattern of the lncRNAs from the first two classes could be, at least partly, related to the beneficial effects of metformin. The third class of lncRNAs did not respond to the HFD treatment (<10% expression change) but were more or less down-regulated by metformin (Fig. 4C). Because these lncRNAs are frequently associated with liver or metabolic diseases, their reduced expressions in response to metformin may also be beneficial. For example, DANCR, SNHG3 and SNHG1 are lncRNAs that promote tumour cell proliferation in liver cancer and imply poor prognoses [54-56]; moreover, these lncRNAs exhibited tendencies towards reduced expression in response to metformin treatment in our transcriptome profile. Another lncRNA, GAS5, is positively correlated with the incidence of type 2 diabetes [57], and its down-regulation by metformin might imply a potential improvement of the metabolic condition by metformin. However, the inhibition of this lncRNA has also been demonstrated to be involved in the progression of liver fibrosis and liver cancer [58, 59]. This example again emphasizes that some lncRNAs might play discrepant roles in different disease stages, so their actual responses to metformin in conditions of liver fibrosis and different liver cancer stages should be tested using mouse models other than the NAFLD model used here. The fourth class includes all of the lncRNAs (6 of 19) that exhibited other expression patterns and therefore have no intuitive implications regarding the beneficial role of metformin (Fig. 4D). Nevertheless, because most of these lncRNAs are associated with cancer, their responses to metformin interventions in cancer circumstances should be verified in the future.

We further attempted to identify additional functional clues about the differentially expressed lncRNAs based on their co-expressed coding genes. However, more than 3000 co-expressed mRNAs (Spearman correlation coefficients $\rho>0.8$ and P-values<0.01) were found, and the resulting co-expressed gene sets were not significantly enriched for any functional terms. If a more rigorous threshold (e.g., $\rho>0.95$ and P-value<0.001) was applied, only a few unspecific functional terms, such as oxidoreductase activity and cellular metabolic process, were enriched. Therefore, we attempted to narrow down the scope of the co-expressed genes by predicting potential lncRNA-gene interactions among the co-expressed lncRNA-gene pairs. Two types of interactions were considered. First, if the co-expressed lncRNA and gene were proximal to each other on the genome, they were denoted as a cis-interacting pair. Second, if a lncRNA had a predicted physical interaction with an mRNA (as predicted with the LncTar software [33]), the lncRNA and the gene producing this mRNA were predicted to have a trans-interaction. We found that this procedure indeed prominently reduced the number of lncRNA-gene pairs. In total, 69 cis-interactions and 2154 trans-interactions were found among the 264 HFD-responsive lncRNAs and 1529 coding genes, and 37 cis-interactions and 872 trans-interactions were found among the 111 metformin-responsive lncRNAs and 793 coding genes. Indeed, there were some predicted interactions between the differentially expressed lncRNAs and the metabolic or liver disease-associated coding genes as exemplified in Fig. 5. We will discuss these interactions in the Discussion section.

Differentially expressed microRNAs and their targets

The miRNA transcriptomes were profiled with an Agilent mouse miRNA 21.0 microarray. By Comparisons of the miRNA transcriptomes from the different groups of miRNA samples identified seven HFD-responsive miRNAs (three up-regulated and four down-regulated after HFD treatment) and four metformin-responsive miRNAs (two up-regulated and two down-regulated after HFD treatment; Table 2). We attempted to analyse the functions of these differentially expressed miRNAs by performing functional enrichment analysis on their target genes. By extracting the consensus predictions from 8 of the 10 state-of-the-art miRNA target prediction tools, we obtained 2894 and 705 high-confident target genes for HFD-responsive miRNAs and metformin-responsive miRNAs, respectively. We further

tested whether the differentially expressed miRNAs were enriched for disease-related miRNA sets using the TAM tool [60]. However, no significant over-representations of specific disease terms were obtained. Therefore, we manually checked the disease associations of the differentially expressed miRNAs by querying the HMDD database [61] and the miR2Disease [62] database. The results are summarized in Table 2. Among the differentially expressed miRNAs, the miR-34a, which is a HFD-responsive miRNA, exhibited a wide spectrum of disease associations that included but was not limited to, diabetes, hepatocellular carcinoma, non-alcoholic fatty liver disease, colorectal cancer, melanoma, neuroblastoma, pancreatic cancer, prostate cancer, glioblastoma, glioma, medulloblastoma, chronic lymphocytic leukaemia, non-small cell lung cancer and retinoblastoma [61, 62]. Similar to miR-34a, miR-185 was also up-regulated after HFD treatment. This miRNA was associated with multiple cancer types including hepatocellular carcinoma. Two metformin-responsive miRNAs, i.e., miR-199b and miR-451, were also found to be associated with hepatocellular carcinoma or NAFLD.

Differentially expressed circular RNAs and their interactions with microRNAs

Finally, we analysed the transcriptome changes related to a relatively novel class of non-coding RNA, i.e., circular RNAs (circRNAs). CircRNAs are mostly derived from protein coding genes, but unlike mRNAs, they form a circular topology via a back-splicing process and act as miRNA sponges (i.e., miRNA-competing transcripts [63, 64]). We probed the circRNA transcriptome using the novel CapitalBio mouse circRNA v1.0 microarray. Three hundred ninety-six HFD-responsive circRNAs (231 up-regulated and 165 down-regulated circRNAs) and 222 metformin-responsive circRNAs (126 up-regulated and 96 down-regulated circRNAs) were identified. We first analysed the enriched functions of the host genes of these differentially expressed circRNAs. We noted that the functional enrichment analysis of circRNAs did not signify the importance of the circRNAs for metabolism and liver diseases. Therefore, we at-

Table 2. The differentially expressed miRNAs and their disease association. * The disease associations of miRNAs were retrieved from the HMDD and the miR2Disease databases. LCAN, liver cancer; OCAN, other cancer. ** Whether this miRNA showed an expression change consistent with the expression changes observed by Katsura et al. [20] More specifically, if the expression of an HFD-responsive miRNA was also consistently changed after methionine- and choline-deficient diet induced non-alcoholic steatohepatitis, this miRNA was classified as a consistently deregulated miRNAs. Similarly, if the expression of metformin-responsive miRNA was consistently changed after metformin treatment in Katsura et al.'s study, this miRNA was also classified as a consistently deregulated miRNAs [20]

miRNA	Regulation	Fold Change	P-value	Disease Association*				Consistently deregulated?**
				Diabetes	FATL	LCAN	OCAN	
HFD-responsive miRNAs								
mmu-miR-185-5p	up	1.65	0.044	NO	NO	YES	YES	NO
mmu-miR-455-3p	up	13.59	0.001	NO	NO	NO	NO	NO
mmu-miR-34a-5p	up	1.99	0.038	YES	YES	YES	YES	YES
mmu-miR-3102-5p	down	0.62	0.002	NO	NO	NO	NO	YES
mmu-miR-3960	down	0.66	0.041	NO	NO	NO	NO	NO
mmu-miR-6538	down	0.57	0.019	NO	NO	NO	NO	NO
mmu-miR-3963	down	0.59	0.048	NO	NO	NO	NO	YES
Metformin-responsive miRNAs								
mmu-miR-702-3p	up	29.57	0.001	NO	NO	NO	NO	NO
mmu-miR-7085-3p	up	15.17	0.002	NO	NO	NO	NO	NO
mmu-miR-199b-5p	down	0.60	0.047	NO	NO	YES	YES	NO
mmu-miR-451a	down	0.48	0.009	NO	YES	NO	YES	NO

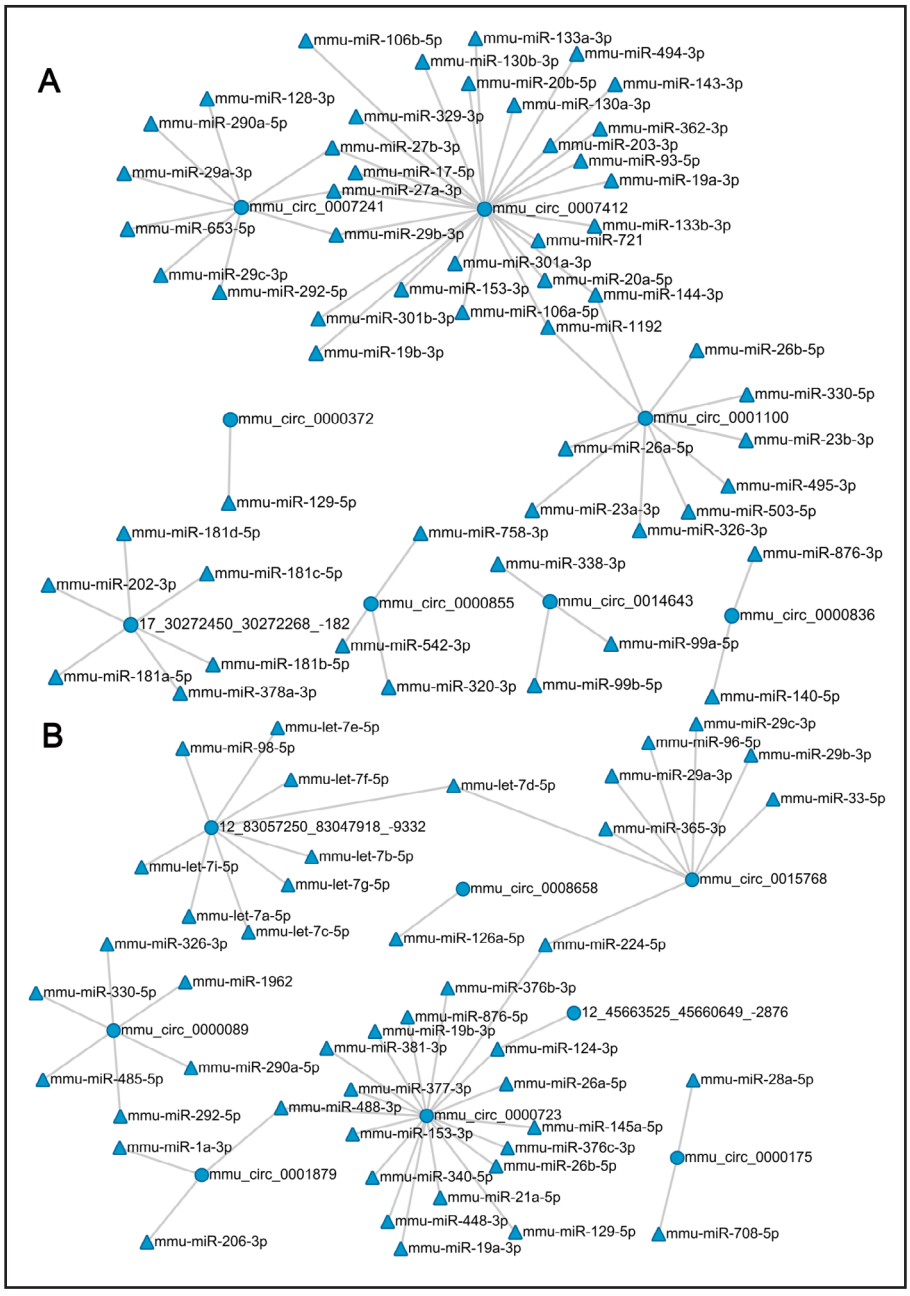

Fig. 6. Experimentally identified interactions between the differentially expressed circRNAs and the miRNAs. The interactions of HFD-responsive lncRNAs and metformin-responsive lncRNAs were shown in panels (A) and (B), respectively.

tempted to focus on another primary function of circRNA, i.e., their actions as miRNA sponges [64]. The interactions between the differentially expressed circRNAs and miRNAs were derived from the datasets of StarBase [34] and are illustrated in Fig. 6. We noted that some of the miRNAs that interacted with multiple circRNAs tended to be involved in disease, and this involvement will be discussed in detail in the Discussion section.

Discussion

NAFLD is now thought to be the most important metabolic syndrome of the liver [65, 66]. Patients with NAFLD often have many accompanying clinical disorders including obesity, impaired glucose tolerance, type 2 diabetes, hypertension and hyperlipidaemia [67]. These diseases that are associated with NAFLD may increase the risk of cardiovascular disease and further aggravate the liver damage [68]. Therefore, the key to the treatment of NAFLD is the improvement of the metabolic disturbances associated with insulin resistance [69]. Insulin-sensitizing drugs, such as metformin, may correct some of these metabolic disorders

[70]. Additionally, the cardiovascular protective effect of metformin in patients with diabetes cannot be attributed only to reduced blood glucose but may also be correlated with the protective role of metformin in lipid metabolism and vascular endothelial function [11, 71]. Studies also suggest that the use of metformin by obese patients with or without diabetes can significantly reduce systemic and visceral fat [72, 73]. Metformin treatment-induced weight loss might be related to the inhibition of net caloric intake due to reduced appetite [74]. In accordance with these finding, we found that energy intake was reduced after the HFD mice were treated with metformin.

In western countries, a significant amount NAFLD is caused by beverages containing high glucose and fructose contents, and HFDs exacerbate this situation, which subsequently initiates the classic spectrum of liver disease [75]. It has been reported that the coaction of ethanol and fructose with a high-fat diet worsens dyslipidaemia and insulin resistance-accompanied liver damage [76]. Moreover, a fructose diet has been demonstrated to increase hepatic lipocalin-2 (LCN-2) expression and thereby induce oxidative stress and mitochondrial dysfunction [77]. In the current study, the serum TG, TC, LDL-c, HDL-c, GLU, γ-GT, ALT, and AST levels were increased in the livers of the HFD mice compared with those of the normal controls. Furthermore, we demonstrated reductions in the glycogen contents and increased inflammatory infiltration in the livers of the HFD mice compared with those of the control mice, which suggests the development of hepatic damage, insulin resistance, and metabolic syndrome. Interestingly, the metformin treatment reversed these parameters in the livers of the HFD mice, which indicates the protective role of metformin against the development of hepatic steatosis and damage. Based on these findings, it will be interesting to explore the potential role of metformin in the progression of fructose-enriched diet-induced NAFLD.

We first focused on the differentially expressed coding genes. The GO functional terms and KEGG pathways analysis indicated that the HFD-responsive gene alterations might be reversed by metformin treatment; the relevant genes include those involved in the steroid biosynthetic process function and the steroid biosynthesis pathway. Additionally, we also noted that few gene sets were specifically correlated with metformin treatment. For example, the gene set related to androgen response was enriched in the metformin-treated transcriptomes but showed no significant correlation with HFD. Indeed, the metformin targeting of the androgen signalling pathway has been implicated in the non-diabetes or metabolism disorder-related use of metformin in the treatment polycystic ovary syndrome [78].

Next, the effects of metformin on non-coding RNAs, including lncRNAs, miRNAs and circRNAs, were evaluated in the current study. Although metformin-induced lncRNA and circRNA expressions have not been systematically analysed by transcriptome profiling to the extent of our knowledge, there are indeed a few transcriptome studies about metformin-induced miRNA expression alterations (i.e., GSE71905, GSE55523, GSE37038, GSE30289, and GSE73393). Among the five relevant studies, two studies profiled miRNAs in livers with distinct healthy or diseased statuses, and three studies focused on the effects on metformin interventions on tumours or tumour cell lines. We compared the metformin-induced miRNAs in other tissues with the metformin-responsive miRNAs identified in our study. In most cases, the HFD-responsive miRNAs and the metformin-responsive miRNAs did not exhibit significant changes their expressions in other metformin interventions contexts. Indeed, we also noted that the overlap between the lists of differentially expressed miRNAs from these five studies is also very limited. Therefore, the miRNAs involved in the mechanisms of metformin intervention seemed to be context-dependent; thus, transcriptome profiling of miRNAs across different tissues and disease statuses is continuously required for the investigation of the mechanisms of metformin interventions. We also note that one miRNA, i.e., miR-34a, seems to exhibit consistent expression changes in different contexts. According to the records of the HMDD database [17] and the miR2Disease database [18], this miRNA is associated with diabetes, NAFLD and multiple cancers. Therefore, this miRNA could play an important role in metformin interventions across various conditions and could be a potential therapeutic target that requires further investigation.

We further investigated the interactions between the differentially expressed lncRNAs and gene known to be related to the aforementioned six typical liver or metabolism diseases. The interactions between the HFD-responsive lncRNAs and disease genes are illustrated in Fig. 5A. Although most of these lncRNAs interacted with one or two disease genes, eight of the HFD-responsive lncRNAs were noted because of their wide associations with other disease genes (i.e., interactions with more than three disease genes) and included four up-regulated lncRNAs (NONMMUG013161, NONMMUG010861, NONMMUG015954, and NONMMUG007373) and four down-regulated lncRNAs (NONMMUG012615, NONMMUG003684, NONMMUG032841, and NONMMUG007538). Similarly, as illustrated in Fig. 5B, three metformin-responsive lncRNAs, including one up-regulated lncRNA (NONMMUG044612) and two down-regulated lncRNAs (NONMMUG039121, NONMMUG038543), were predicted to interact with more than three disease genes. Due to their predicted wide associations with metabolism or liver disease genes, these lncRNAs would be interesting targets for further investigations.

Comparisons of the miRNA transcriptomes from different groups of miRNA samples identified seven HFD-responsive miRNAs (three up-regulated and four down-regulated after HFD treatment) and four metformin-responsive miRNAs (two up-regulated and two down-regulated after HFD treatment; Table 2). We note that Katsura *et al.* has also analysed miRNA transcriptome changes under conditions related to those in our study. In their study, NAFLD was induced in mice with a methionine- and choline-deficient diet (MCD), and metformin treatment was applied to relieve the disease situation [20]. Because the HFD treatment in our study also induced NAFLD, we attempted to determine whether any consensus regarding the differentially expressed miRNAs could be obtained. Indeed, miR-34a was up-regulated in response to both the HFD and MCD treatments (Table 2), which indicates miR-34a could be a hallmark miRNA for NAFLD. In contrast, miR-3102 and miR-3963 were consistently down-regulated in the two fatty liver-inducing situations. The function of these two miRNAs in liver metabolism and disease are currently unknown, but these miRNAs should be interesting candidates for further studies. Unfortunately, however, we did not identify any overlap between the miRNAs that were differentially expressed after metformin treatment in two studies. To determine whether this lack of overlap was a simple result of the threshold settings utilised in our study, we obtained the top 5 up-regulated and top 5 down-regulated miRNAs after metformin treatment from Katsura *et al.*'s results. None of these miRNAs exhibited a fold change larger than 1.1 in our dataset, which indicates that metformin may induce the expressions of different miRNAs in different disease models.

Finally, we noted that the functional enrichment analysis of circRNA reported in the above section did not signify the importance of the circRNAs in metabolism and liver diseases. Therefore, we focused on the primary function of circRNA, i.e., acting as miRNA sponges [64]. As illustrated in Fig. 6, each circRNA can interact with five or six miRNAs. In contrast, most miRNAs could only interact with one of the differentially expressed circRNAs. Nevertheless, there were a few miRNAs that could interact with two circRNAs and therefore bridge two circRNAs. Interestingly, these bridging miRNAs could be associated with liver diseases according to the HMDD and miR2Disease databases [61, 62]. Among these miRNAs, miR-27a, miR-27b, miR-29b, and let-7d were associated with NAFLD, miR-27a and let-7d were associated with hepatocellular carcinoma, miR-27b was also associated with obesity, and miR-29b was also associated with diabetes. These results suggested that differentially expressed circRNAs could regulate the disease-related pathway by competitively interacting with important miRNAs underlying the pathogeneses of diabetes, fatty liver and other diseases.

Conclusion

In summary, our study identified changes in the coding and non-coding transcriptomes in the livers of HFD mice without and with metformin treatment, and this information may enable a better presentation of the potential roles of ncRNAs that underlie NAFLD and

metformin intervention. In future studies, deep explorations of the corresponding roles and molecular mechanisms of these non-coding RNAs will be required.

Acknowledgements

We thank Mrs. Suwei Zhang from Capital Bio, Beijing, China for microarray technical supports.

This work was supported by 973 program grants from the National Basic Research Program of China [2014CB910503 to J.L.], the National Natural Science Foundation of China [81570789 to J. L., 81700765 to J.G.].

Disclosure Statement

These authors declare no conflicts of financial interest.

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