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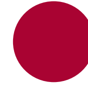

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Original Article

Identification of two microRNA nodes as potential cooperative modulators of liver metabolism

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Aim: Hepatic insulin resistance is a hallmark of type 2 diabetes and non-alcoholic fatty liver disease. Dysregulation of microRNA (miRNA) expression in insulin-resistant livers might coordinate impaired hepatic metabolic function. Here, we aimed to discover miRNAs and their downstream targets involved in hepatic insulin resistance.

Methods: We determined miRNA expression profiles by small RNA sequencing of two mouse models of impaired hepatic insulin action: high-fat diet-induced obesity and liver-specific insulin receptor knockout. Conversely, we assessed the hepatic miRNA expression profile after treatment with the antidiabetic hormone, fibroblast growth factor 21 (FGF21). Ontology analysis of predicted miRNA gene targets was performed to identify regulated gene pathways. Target enrichment analysis and miRNA mimic overexpression *in vitro* were used to identify unified protein targets of nodes of regulated miRNAs.

Results: We identified an array of miRNA species regulated by impaired liver insulin action or after fibroblast growth factor 21 treatment. Ontology analysis of predicted miRNA gene targets identified pathways controlling hepatic energy metabolism and insulin sensitivity. We identified a node of two miRNAs downregulated in the livers of liver-specific insulin receptor knockout mice, miR-883b and miR-205, which positively regulate the expression of transcription factor zinc finger E-box-binding homeobox 1 (ZBED1). We found another node of two miRNAs upregulated in the livers of fibroblast growth factor 21-treated mice, miR-155-3p and miR-1968-5p, which canonically downregulates the caveola component, polymerase I and transcript release factor (PTRF), a gene previously implicated in hepatic energy metabolism.

Conclusions: This study identifies two nodes of coregulated miRNAs that might coordinately control hepatic energy metabolism in states of insulin resistance.

Key words: FGF21, high-fat diet, insulin receptor, insulin resistance, liver, microRNA

INTRODUCTION

HEPATIC INSULIN RESISTANCE is a hallmark of metabolic syndrome and type 2 diabetes. The insulin resistant liver is characterized by excessive lipogenesis and accumulation of lipids in hepatocytes, which forms the pathological basis for non-alcoholic fatty liver disease (NAFLD).[1,2] Currently, the molecular mechanisms driving the development of hepatic insulin resistance and NAFLD are incompletely defined, yet liver insulin resistance and NAFLD appear to be tightly linked.[2,3]

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Fibroblast growth factor 21 (FGF21) is a peptide hormone produced by several organs functioning to regulate fasted- and fed-state whole-body energy metabolism. FGF21 is a promising target for treatment of human metabolic disease because of its beneficial pharmacological effects on energy homeostasis in rodent and non-human primate models of obesity and type 2 diabetes. FGF21 administration induces weight loss, normalizes hyperglycemia and hyperinsulinemia, and restores insulin sensitivity. In the liver, FGF21 increases lipid turnover to reduce hepatic steatosis and improve insulin sensitivity.[4–6]

MicroRNAs (miRNAs) are small non-coding RNA molecules controlling gene expression. Although the canonical view is that miRNAs are negative regulators that destabilize target mRNA molecules and/or inhibit their translation into protein,[7] miRNAs can also regulate target gene expression by several alternative mechanisms,[8,9] including gene upregulation through direct activation of translation.[9]

MicroRNAs regulate various physiological processes, including energy metabolism in skeletal muscle, adipose tissue and in the liver,[10] and are implicated in different pathophysiological states of the liver, for instance hepatitis B and C infection, and liver fibrosis.[11] Hepatic miRNA expression patterns are altered in states of insulin resistance, and these alterations may coordinate an impairment of liver metabolic function. Studies have identified a number of individual miRNA species that are altered in the insulin-resistant liver and have characterized their downstream targets. For instance, miR-802 is upregulated in livers of obese mice and humans, and impairs hepatic metabolic function by targeting hepatocyte nuclear factor 1 beta.[12] In contrast, miR-206 expression is reduced in the livers of obese mice and in fatty acid-treated primary human hepatocytes, and was suggested to enhance liver metabolism by simultaneously de-repressing insulin signaling and inhibiting lipogenesis through its target protein tyrosine phosphatase, non-receptor type 1.[13] However, miRNAs often function in concert, where nodes of several miRNA species cooperatively target common downstream genes and pathways.[14–16]

Here, we used small RNA sequencing (RNA-seq) to identify miRNAs regulated in two mouse models of impaired hepatic insulin action and upon improvement of liver metabolism by FGF21 treatment. We identified several miRNA species showing an opposite regulation with impairment of insulin action and improved metabolic function. We also identified candidate transcription factors for the control of miRNA regulation, and downstream target genes and pathways through which the regulated miRNAs could affect hepatic energy homeostasis. The present results identify, using varying models of insulin sensitivity, two miRNA nodes that potentially participate in the regulation of liver metabolic function.

METHODS

ADDITIONAL INFORMATION CAN be found in Supplemental Experimental Procedures.

Animals

We analyzed miRNAs in livers harvested from a cohort of mice from a previously published study.[17] The cohort was used in the original study for generating tissue gene expression and signaling data, liver triglyceride, and glycogen data, as well as plasma cholesterol, free fatty acid, and triglyceride data. The cohort consisted of eight groups of mice comprising two genotypes, two diets, and pharmacological treatment versus control (67 mice in total, $n = 5–11$): male control (IRlox) and liver-insulin receptor knockout (LIRKO) mice were fed a chow or high-fat diet (HFD) for 7 weeks starting at 7 ± 1 weeks-of-age and treated with subcutaneous infusions of saline or recombinant human FGF21 (1 mg/kg/day) for the last 2 weeks of the diet. Mouse livers were harvested after 2 h of fasting.[17]

Small RNA-sequencing

RNA-seq was performed for five randomly selected mice in each of the eight experimental groups ($n = 5$). Total RNA (10 μg) was used as input for each library prepared using the NEBNext Multiplex Small RNA Library Prep Set for Illumina (New England Biolabs, Ipswich, MA, USA), where acrylamide gel electrophoresis was used for selection of small ncRNA molecules of approximately 15–40 nucleotides in length. Libraries were size-checked and quantified using the Agilent Bioanalyzer 2100 system with DNA 1000 chips (Agilent Technologies, Santa Clara, CA, USA) and the Qubit dsDNA high-sensitivity assay (Life Technologies, Carlsbad, CA, USA). Libraries were subjected to 50-bp single-end sequencing on the HiSeq 2500 sequencer (Illumina, San Diego, CA, USA) at the Danish National High-Throughput DNA Sequencing Center.

MicroRNA mimic transfection

Hepa-1c1c7 mouse hepatoma cells (ATCC CRL-2026) were grown in MEM alpha without nucleosides (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO, USA) and 1% penicillin/streptomycin (Thermo Fisher Scientific). Specific miRNA mimics or negative control (miRCURY LNA miRNA mimics; Qiagen, Hilden, Germany) were introduced into cells by reverse transfection in six-well plates (50 nmol/L mimic, 200 000 cells per well) with Opti-MEM I

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Reduced-Serum Medium and Lipofectamine RNAiMax (Thermo Fisher Scientific). Cells were harvested after 48 h for isolation of total cellular protein as described in Supplemental Experimental Procedures.

Bioinformatics and statistics

Hierarchical clustering analysis

LIRKO-HFD-FGF21, HFD-LIRKO, HFD-FGF21, and LIRKO-FGF21 main contrast intersection sets of differentially regulated miRNAs were selected by hierarchical clustering analysis. Log_2 fold changes were used to generate the matrices, and hierarchical clustering analysis was performed by using the Canberra distance and ward.D2 clustering algorithm.

Functional enrichment analysis for miRNA target genes

For the enrichment analysis of Gene Ontology terms associated with targets of the differentially expressed miRNAs, we used a method of "integrated gene set analysis for microRNA studies"[18] and focused on experimentally validated miRNA-target interactions with the strongest level of evidence from the miRTarBase database (Release 7.0).[19]

MicroRNA target enrichment analysis

Inferred downstream targets of miRNAs differentially expressed in each of the three main contrasts (HFD vs. chow, LIRKO vs. control, and FGF21 vs. saline) were extracted from the RNA-protein Association and Interaction Network database.[20] Each individual target gene was then tested for enrichment among the regulated miRNAs in each contrast in comparison with the non-differentially expressed miRNAs using the geometric test with the Benjamini–Hochberg correction. Enrichment tests were conducted in parallel for up- and downregulated miRNAs.

Promoter motif enrichment analysis

The miRNA gene promoter site was defined as flanking windows (500 or 2000 nucleotides) around the transcription start site of the precursor (for intergenic miRNAs: the 5′ end of the precursor, for intragenic miRNAs: transcription start site of host gene). Promoter motifs enriched among the differentially expressed miRNAs were identified using MEME-ChIP from the MEME suite v.4.11.2.[21] The two flanking window sizes were used as trade-offs between sensitivity and specificity in the motif search. The TOMTOM tool was used for identifying transcription factor binding motifs matching the enriched miRNA promoter motifs.[22]

Statistical analysis

To test for main effects of HFD, insulin receptor KO, and FGF21 treatment on liver zinc finger E-box binding homeobox 1(ZEB1) and polymerase I and transcript release factor (PTRF) mRNA and protein levels, quantitative reverse transcription polymerase chain reaction (qRT–PCR) and western blotting data were subjected to linear model fitting and selection with the step function in R followed by ANOVA. For PTRF protein levels, ANOVA was followed by Tukey's multiple comparison testing. For further details, see Supplemental Experimental Procedures.

MicroRNA mimic transfection experiments were analyzed in Prism 8 (GraphPad, La Jolla, CA, USA) using a mixed-effects analysis (repeated measures 1-way ANOVA equivalent) followed by multiple comparison testing (each specific miRNA mimic vs. negative control) with Dunnett's correction.

For all qRT–PCR and western blotting analyses, $P < 0.05$ was used as significance cut-off, and individual data points were plotted together with 95% confidence intervals (CI).

RESULTS

MICRORNA SIGNATURE OF hepatic insulin resistance and FGF21-improved metabolism

Dysregulation of liver miRNA expression may participate in altered liver function and whole-body insulin-resistance. To determine the miRNA expression profile of the insulin-resistant liver, we used two mouse models of impaired hepatic insulin action previously described by our group:[17] (i) HFD-induced obesity, which mimics hepatic insulin resistance in metabolic syndrome and type 2 diabetes; and (ii) LIRKO.[23] Additionally, the effects of treatment with the antidiabetic hormone, FGF21, which improves liver energy metabolism, on miRNA expression profiles were assessed in each of the models. In total, eight groups of mice were included, comprising all combinations of the two genotypes, the two diets, and the two treatments (experimental setup described in Fig. 1).

We subjected total liver RNA to small RNA-seq, and specifically focused on miRNA expression levels. Multidimensional scaling analysis showed separation of the eight groups of mice into two main clusters of control and LIKRO mice, with no distinct separation of mice based on either diet or treatment (Fig. 2). The observed genotype effect on miRNA expression levels was supported by hierarchical clustering analysis (Fig. S1).

We examined the effects of HFD feeding, lack of liver insulin receptor expression, and FGF21 treatment on hepatic miRNA expression. For this, differentially expressed miRNA species were identified in each of the three

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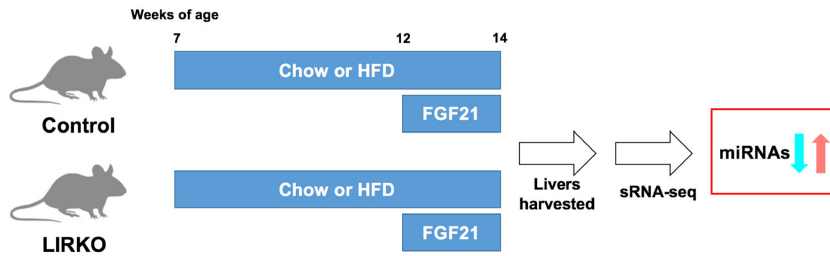

Figure 1 Schematic overview of study design. MicroRNAs (miRNAs) were examined in livers harvested from a cohort of mice used in a previously published study by our group. Control (IRlox) or liver-insulin receptor knockout (LIRKO) mice were fed a chow or high-fat diet (HFD) for 7 weeks and treated with subcutaneous infusions of saline or 1 mg/kg/day human recombinant fibroblast growth factor 21 (FGF21) for the last 1 week of the diet, after which livers were harvested. Total RNA was extracted from the liver and prepared libraries for small RNA sequencing (RNA-seq) to assess regulation of miRNA expression with HFD- or LIRKO-induced hepatic insulin resistance or with FGF21 treatment. [Color figure can be viewed at wileyonlinelibrary.com]

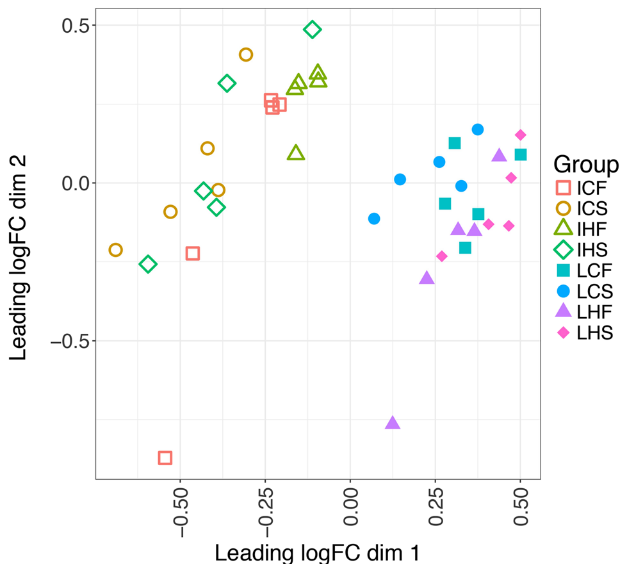

Figure 2 Hepatic microRNA expression signature of insulin resistance and after fibroblast growth factor 21 (FGF21) treatment. Multidimensional scaling analysis of microRNA expression data showing segregation of the microRNA samples from each group of mice. The distances correspond to leading $\log_2$ fold change (FC), which is defined as the root mean square of the largest 500 $\log_2$ FCs between each pair of samples. Groups of mice: ICF: control (IRlox)/Chow/FGF21; ICS: IRlox/Chow/Saline; IHF: IRlox/High-fat diet (HFD)/FGF21; IHS: IRlox/HFD/Saline; LCF: Liver-insulin receptor knockout (LIRKO)/Chow/FGF21; LCS: LIRKO/Chow/Saline; LHF: LIRKO/HFD/FGF21; LHS: LIRKO/HFD/Saline. [Color figure can be viewed at wileyonlinelibrary.com]

paradigms: (i) HFD feeding, irrespective of genotype and FGF21 treatment (HFD vs. chow); (ii) lack of insulin receptor signaling, irrespective of diet and FGF21 treatment (LIRKO vs. control); and (iii) FGF21 treatment irrespective of genotype and diet (FGF21 vs. saline). Across the three paradigms, a total of 386 unique, mature miRNA species were differentially expressed. Validation of the sequencing data was performed by qRT–PCR on a set of miRNAs (sequences of primers are provided in Table S1), showing a range of relative expression levels (logCPM) and differential expression in at least one of the three paradigms with an absolute $\log_2$ fold change ≥0.5. A strong correlation between small RNA-seq- and qRT–PCR-derived results for HFD versus chow ($r = 0.92$; $P = 0.026$), LIRKO versus control ($r = 0.98$; $P = 0.003$), and FGF21 versus saline ($r = 0.94$; $P = 0.016$) validated our results (Fig. S2).

We identified 167 and 171 miRNAs to be up- and downregulated, respectively, in LIRKO versus control mice. In contrast, just 12 miRNAs were upregulated and 12 downregulated by HFD feeding, and 43 upregulated and 49 downregulated by FGF21 treatment (Fig. 3a; Table S2), indicating that the lack of insulin receptor expression in the liver had a stronger overall effect on miRNA expression than HFD feeding or FGF21 treatment. This notion was supported by the multidimensional scaling analysis (Fig. 2) and by the hierarchical clustering analysis (Fig. S1).

Hierarchical clustering analysis was then used to assess which miRNAs were regulated across the models of impaired hepatic insulin action and with improved energy metabolism induced by FGF21 treatment. Seven miRNA species were found differentially expressed in both models of impaired hepatic insulin action and with FGF21

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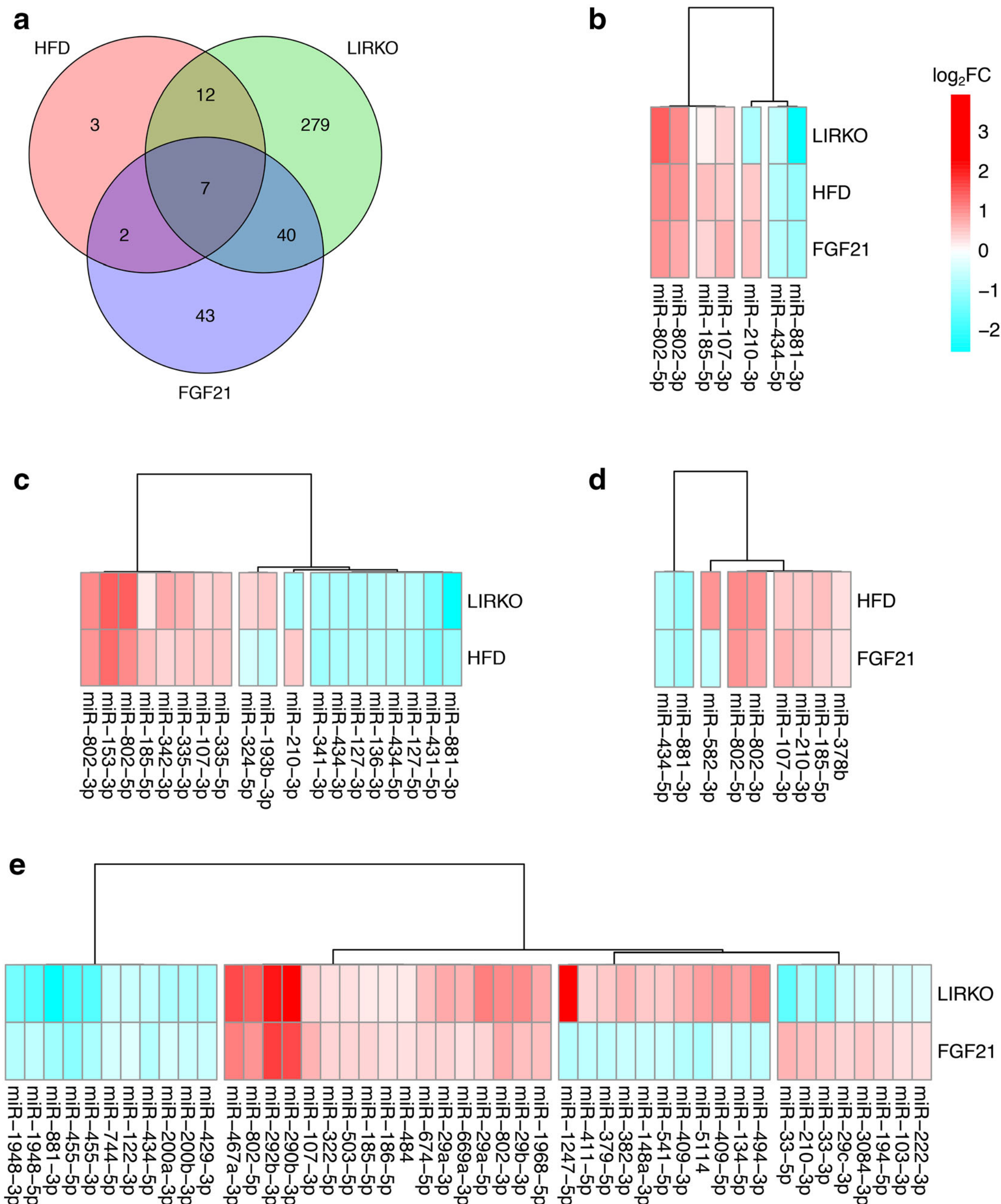

Figure 3 Identification of liver microRNAs (miRNAs) differentially expressed across conditions. (a) Venn diagram showing the number of differentially expressed miRNAs in the three paradigms of high-fat diet (HFD) feeding, liver-insulin receptor knockout (LIRKO), and fibroblast growth factor 21 (FGF21) treatment, and the intersections according to the small RNA sequencing data. (b–e) Heatmaps based on hierarchical clustering of miRNAs differentially expressed in (b) all of the three paradigms or (c) miRNAs showing differential expression both with HFD feeding and in LIRKO control mice (d) or both with FGF21 treatment and in HFD- versus chow-fed mice, (e) or in LIRKO versus control mice. [Color figure can be viewed at wileyonlinelibrary.com]

treatment, of which six miRNAs showed the same direction of regulation in all three paradigms (Fig. 3a,b). Of the 19 miRNAs regulated by both HFD feeding and in LIRKO versus control mice, eight miRNAs were upregulated and eight miRNAs downregulated. In contrast, three miRNAs, miR-210-3p, miR-193b-3p, and miR-324-5p, showed opposite directions of regulation in these two models (Fig. 3a,c). Eight miRNAs that were regulated in the same direction

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and a single miRNA species, miR-582-3p, showed opposite regulation with HFD feeding and FGF21 treatment (Fig. 3a, d). A total of 47 differentially expressed miRNAs were shared between LIRKO versus control mice and FGF21- versus saline-treated mice, of which 28 miRNA species showed the same and 19 miRNAs showed opposite directions of regulation in the two models (Fig. 3a,e).

Upstream factors controlling regulation of miRNAs of hepatic insulin resistance

Knowledge on the upstream mechanisms driving miRNA expression, in particular in hepatic energy metabolism and insulin resistance, is sparse. To identify upstream factors that could be responsible for controlling miRNA expression in hepatic insulin resistance and after FGF21 treatment, we searched for enriched transcription factor binding motifs in the promoter regions of the miRNAs differentially expressed with HFD feeding, LIRKO, or FGF21 treatment using the MEME and DREME motif discovery tools.[21,24] The identified motifs were used to search for potential binding transcription factors using the MEME suite tool, TOMTOM.[22] We discovered two motifs significantly enriched in the promoters of miRNAs regulated in HFD-fed mice (Figs 4,S3a). Two transcription factors, RREB1 and EPAS1, were found as potential binders of the long, AC-rich motif. Of note, despite an extensive match between the consensus DNA binding motifs for these factors and the identified enriched motif, false discovery rate for binding probability exceeded 10%. However, because of the link between RREB1 and hyperinsulinemia and hyperglycemia,[25] and the importance of EPAS1 for hepatic mitochondrial function,[26] these transcription factors could indeed function to regulate hepatic energy metabolism through control of miRNA expression. Several both long (MEME) and short (DREME) motifs were found to be enriched in the promoter regions of miRNAs regulated in LIRKO versus control mouse livers (Figs 4,S3b) or miRNAs regulated by FGF21 treatment (Figs4,S4). Many transcription factors appeared as potential binders of the G-rich, long motif most highly enriched among miRNAs upregulated in LIRKO versus control mouse livers, including several transcription factors and families that control hepatic lipid metabolism or have been implicated in hepatic insulin resistance and NAFLD. These included the SP family, which regulate hepatic gene expression in response to insulin signaling,[27] and Krüppel-like factor 15, a protein suggested to be part of the transcription factor network driving NAFLD development and progression.[28] The most highly enriched A-rich, long motif among FGF21-upregulated miRNA promoters is highly similar to the reverse complement of the T-rich long motif identified for the group of FGF21-downregulated miRNAs (Fig. 4), suggesting that common pathways mediate the miRNA response to FGF21. In line with this, many of the same transcription factors, including several forkhead box (FOX) family members and interferon regulatory factor proteins, are potential binders of the motifs. Interestingly, FOXQ1 and FOXO proteins are key regulators of hepatic energy metabolism, and FOXOs protect against diet-induced fatty liver disease.[29,30] Interferon regulatory factor proteins are implicated in obesity-associated inflammation and insulin resistance. The known functions of the identified transcription factors potentially controlling the expression of the sets of FGF21-regulated miRNAs are thus consistent with the role of FGF21 as a positive modulator of hepatic energy metabolism.[17] Taken together, the present results identified potential upstream regulators of hepatic energy metabolism through a coordinated transcriptional regulation of miRNA expression.

Regulated miRNAs target energy metabolism and inflammation

To identify downstream gene pathways affected by altered miRNA expression in hepatic insulin resistance or after FGF21 treatment, we performed a functional enrichment analysis of Gene Ontology terms for already experimentally validated miRNA targets.[19] For this, we used a newly developed methodology for "integrated gene set analysis for microRNA studies" to take into account the possibility that several differentially expressed miRNAs might target the same gene.[18] Of interest, we observed that miRNAs regulated both with HFD feeding or in LIRKO versus control mice targeted genes and signaling pathways relevant for hepatic energy metabolism and insulin resistance, including pathways related to glucose and lipid metabolism, mitochondrial function, oxidative stress/ER stress, and inflammation (Fig. 5a,b; Table S3). Strikingly, although showing lower enrichment levels, biological processes enriched among the miRNAs regulated by FGF21 treatment were generally of the same categories as those identified for miRNAs regulated by HFD feeding or in LIRKO versus control mice (Fig. 5c; Table S3). Taken together, the present results show that hepatic miRNA expression changes after HFD, or with a lack of insulin receptor expression or FGF21 treatment affect key pathways in liver energy metabolism, and suggest that differentially expressed miRNAs contribute to liver dysfunction in metabolic syndrome and type 2 diabetes.

Identification of microRNA nodes sharing downstream targets

MicroRNAs can function in nodes where several miRNA species cooperate to target common downstream

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Groups of regulated miRNAs	Motif logo	TF	*p*-value	FDR
Upregulated by HFD	E-value: 4.1e-11	RREB1	1.75e-04	1.50e-01
		EPAS1	6.14e-04	2.62e-01
Upregulated in LIRKO *vs.* control	E-value: 6.3e-149	MAZ	5.60e-09	4.72e-06
		SP3	1.27e-07	5.35e-05
		SP4	1.51e-06	4.24e-04
		SP1	2.13e-06	4.48e-04
		ZN148	4.34e-06	7.31e-04
		E2F4	2.08e-04	2.93e-02
		SREBP2	3.05e-04	3.67e-02
		E2F6	4.29e-04	4.51e-02
		KLF15	1.02e-03	8.59e-02
		PURA	1.14e-03	8.76e-02
Upregulated by FGF21 treatment	E-value: 3.3e-018	FOXO1	8.48e-07	4.05e-04
		FOXJ3	9.72e-07	4.05e-04
		FUBP1	7.30e-05	1.23e-02
		FOXD3	7.61e-05	1.23e-02
		FOXM1	8.87e-05	1.23e-02
		SRY	1.54e-04	1.83e-02
		IRF4	1.94e-04	2.02e-02
		FOXO4	2.40e-04	2.22e-02
		FOXJ2	3.09e-04	2.42e-02
		FOXF1	3.19e-04	2.42e-02
		ONEC2	3.95e-04	2.75e-02
		FOXA3	4.90e-04	3.14e-02
		FOXQ1	8.57e-04	5.10e-02
Downregulated by FGF21 treatment	E-value: 7.2e-044	HNF6	4.18e-06	1.78e-03
		ONEC2	4.31e-06	1.78e-03
		FOXJ3	7.91e-06	2.18e-03
		FOXQ1	6.52e-05	1.08e-02
		FOXO4	2.14e-04	2.94e-02
		HXC6	2.64e-04	3.12e-02
		HXB7	3.97e-04	3.50e-02
		SRY	4.06e-04	3.50e-02
		FOXO1	4.82e-04	3.50e-02
		FOXM1	4.92e-04	3.50e-02
		NR2E3	5.08e-04	3.50e-02
		IRF5	7.71e-04	4.75e-02
		FOXF1	8.31e-04	4.75e-02
		ARI3A	8.62e-04	4.75e-02
		IRF4	9.73e-04	5.02e-02
		FUBP1	1.06e-03	5.14e-02
		FOXJ2	1.57e-03	7.21e-02

Figure 4 Transcription factor binding motifs enriched in promoters of regulated microRNAs (miRNAs). Consensus motifs most highly enriched in the promoter regions of miRNAs upregulated with high-fat diet (HFD), upregulated in liver-insulin receptor knockout (LIRKO) versus control livers or up- or downregulated by fibroblast growth factor 21 (FGF21) treatment. Motifs were identified using MEME. For each motif, the E-value of the enrichment statistics is shown, and listed are the transcription factors (TF) identified as statistically significant potential binders (false discovery rate <0.1). Note that for the motif enriched in promoters of miRNAs upregulated by HFD, the false discovery rate (FDR) for the listed transcription factors exceeds 0.1. [Color figure can be viewed at wileyonlinelibrary.com]

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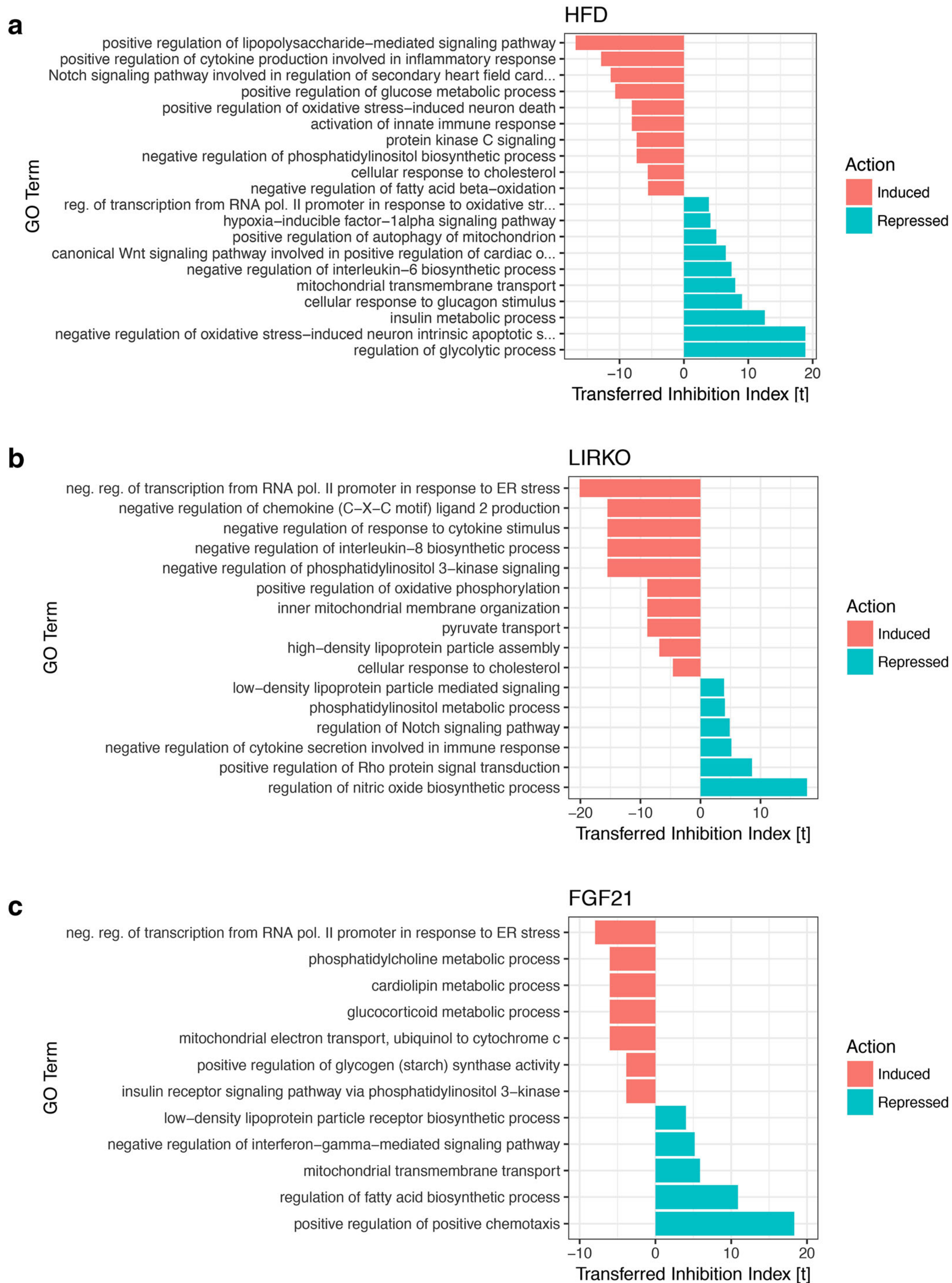

Figure 5 Biological processes associated with hepatic microRNA (miRNA) expression changes. Biological pathways represented as biological process Gene Ontology (GO) terms either induced or repressed by miRNAs differentially expressed in the three paradigms of (a) high-fat diet (HFD)- or (b) liver-insulin receptor knockout (LIRKO)-induced impairment of liver insulin action or (c) fibroblast growth factor 21 (FGF21) treatment. A transferred inhibition index, *t*, was calculated for each GO terms based on experimentally validated miRNA–target interactions and represents the extent of miRNA-mediated repression (positive *t*) or induction (negative *t*) of the process in question. Shown are selected enriched GO terms. All enriched GO terms are listed in Table S3. [Color figure can be viewed at wileyonlinelibrary.com]

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proteins and pathways.[14–16] Accordingly, even small changes in levels of each individual miRNA species in such a node can translate into significant effects on target protein levels and activity of the biological process in question. We aimed to identify groups of miRNAs that could coordinately regulate the same protein targets. First, we predicted the target genes of the sets of miRNAs differentially expressed in the models of impaired hepatic insulin action or with FGF21 treatment using the RNA-protein Association and Interaction Network database. RNA-protein Association and Interaction Network integrates four sources of ncRNA-protein interaction, including *in silico* prediction data from several prediction algorithms and published experimental validation.[20] Then, to identify shared miRNA targets, we tested each protein target identified in the initial prediction step for enrichment among the individual sets of regulated miRNAs compared with the entire group of detected miRNA species. From this analysis, two proteins, ZEB1 and PTRF, were found to be significantly enriched (false discovery rate <0.012 and 0.098, respectively) among targets of two groups of coregulated miRNAs.

Zeb1 is a potential unified miRNA target in LIRKO livers

Target enrichment analysis identified a group of nine miRNA species downregulated in livers of LIRKO mice to all target ZEB1 (Fig. 6a–c). ZEB1 is a transcription factor best known for its role as a driver of epithelial to mesenchymal transition during cancer metastasis and in a number of developmental processes.[31] Interestingly, ZEB1 has been associated with type 2 diabetes through its link with insulin signaling and adipocyte differentiation.[32–34] To examine the regulation of ZEB1 expression in LIRKO mouse livers, we measured *Zeb1* mRNA and protein levels with qRT–PCR and western blotting, respectively. Although *Zeb1* mRNA levels were only slightly reduced (Fig. 6d), protein levels were significantly decreased in LIRKO versus control mouse livers. Strikingly, this alteration was of the same direction as the regulation of the nine ZEB1-targeting miRNAs (average fold change = 0.6; $P = 0.00049$; Figs 6e,S5a), suggesting a role of these miRNA species as positive post-transcriptional regulators of ZEB1 protein expression.

miR-883b and miR-205 upregulate ZEB1 expression in liver cells *in vitro*

To examine the potential of the nine identified miRNAs to target ZEB1, we transfected cultured mouse hepatoma cells with the respective miRNA mimics. After 48 h, ZEB1 protein levels were changed by some of the tested miRNA mimics. Although miR-429 caused a borderline significant reduction in ZEB1 protein levels ($P = 0.063$), both miR-883b and miR-205 increased ZEB1 protein levels (Fig. 7f,g), supporting a role of miR-883b and miR-205 as positive post-transcriptional regulators of hepatic ZEB1 expression.

PTRF is a potential target of three FGF21-induced hepatic miRNAs

In livers treated with FGF21, target enrichment analysis identified a group of three upregulated miRNAs, miR-155-3p, miR-292b-3p, and miR-1968-5p, to all potentially target polymerase I and transcript release factor (PTRF, alternatively named Cavin-1; Fig. 7a–c). PTRF is involved in RNA-polymerase I-catalyzed rRNA transcription,[35,36] and it is essential for the formation of caveolae, a type of lipid rafts found at the plasma membrane of many cell types, including hepatocytes.[37–39] Interestingly, studies in *Ptrf*$^{-/-}$ mice have implicated PTRF in the control of hepatic lipid metabolism.[40,41] Although *Ptrf* mRNA levels were unchanged between livers of FGF21- versus saline-treated mice (Fig. 7d), there was a clear effect of FGF21 treatment on PTRF protein levels, but only in control mice ($P = 0.03$ for the interaction between genotype and FGF21 treatment). FGF21 treatment resulted in a marked upregulation of PTRF levels in livers of FGF21-treated control mice on chow diet (median fold change = 4). We found a similar, though reduced, increase in PTRF in HFD-fed control mice in response to FGF21 treatment (median fold change = 1.9; (adjusted $P = 0.04$ for FGF21 vs. saline in control mice, irrespective of diet). Strikingly, this effect of FGF21 was absent in LIRKO mice (Figs 7e,S5b). These results suggest that PTRF could be a new target of FGF21 action in the liver controlled by the three miRNAs miR-155, miR-292b, and miR-1968. As PTRF was regulated in the same way by FGF21 treatment as the three miRNAs, the present results suggest that these miRNAs act as positive post-transcriptional regulators of hepatic PTRF expression.

miR-155 and miR-1968 downregulate PTRF protein in liver cells *in vitro*

To examine the potential of miR-155, miR-292b, and miR-1968 to regulate liver PTRF expression, we transfected cultured mouse hepatoma cells with the respective miRNA mimics. Although miR-292b-3p mimics had no effect on

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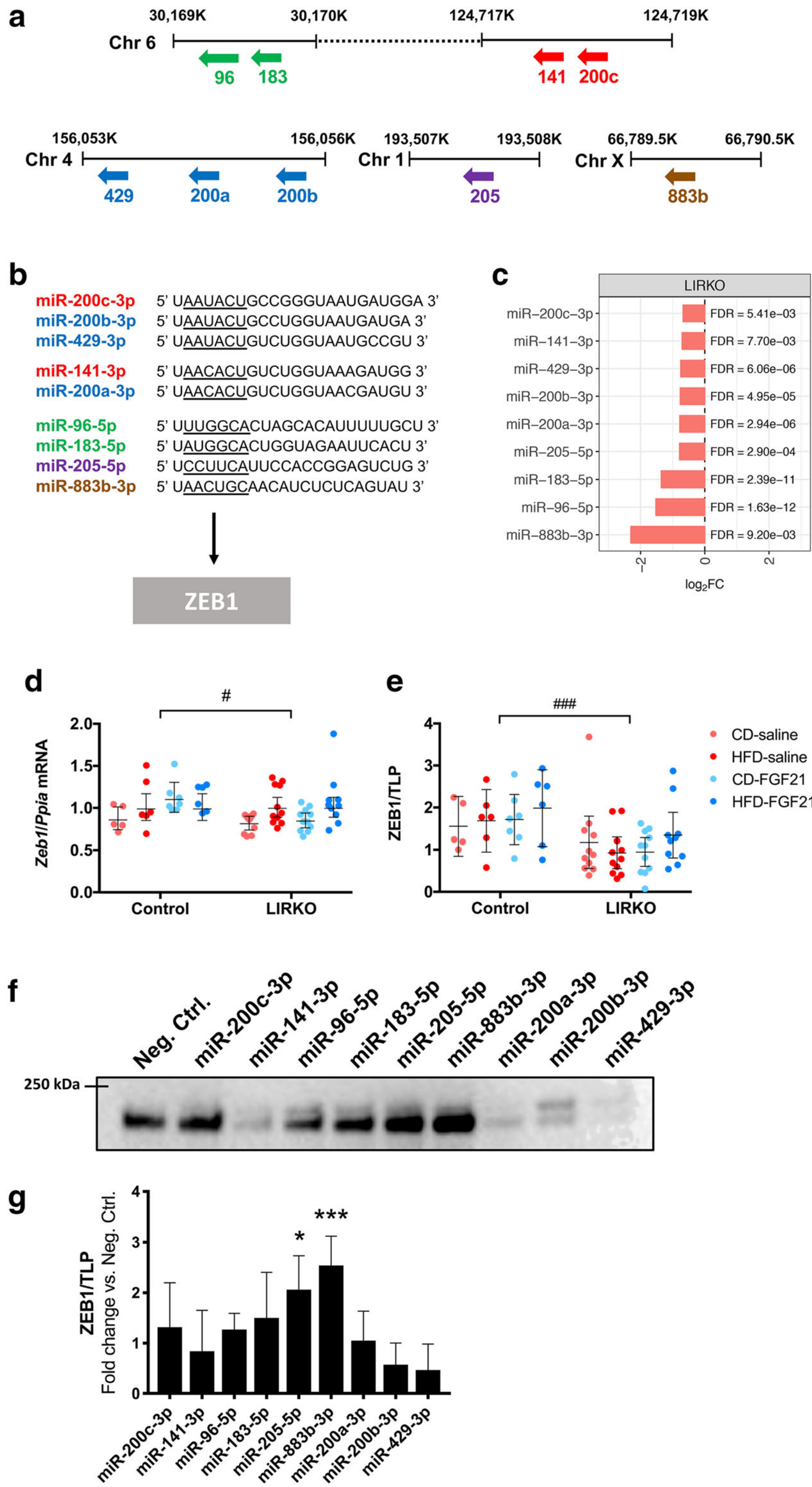

Figure 6 MicroRNAs (miRNAs) downregulated in liver-insulin receptor knockout (LIRKO) mouse livers target the transcription factor Zinc finger E-box-binding homeobox 1 (ZEB1). (a) Chromosomal locations (mm10 assembly) of the genes encoding the nine miRNAs identified as potential ZEB1-targeting molecules. (b) Sequence alignment of the nine miRNAs. Nucleotides 2–7 are underlined, representing the seed sequence through which the miRNAs are predicted or experimentally validated to bind the *Zeb1* 3′ UTR. (c) Regulation of each of the nine Zeb1-targeting miRNAs in the livers of LIRKO versus control mice calculated from the small RNA sequencing data. (d) *Zeb1* mRNA levels normalized to levels of *Ppia* mRNA, as measured by quantitative reverse transcription polymerase chain reaction and (e) ZEB1 protein levels normalized to total lane protein (TLP) in the eight groups of mice calculated by quantification of western blots (Fig. S5a). $^{\#}P < 0.05$ and $^{\#\#\#}P < 0.0005$ between genotypes, $n = 5–11$ animals per group. Note that not indicated in (D) is a significant main effect of high-fat diet (HFD) on *Zeb1* mRNA expression ($P < 0.005$ between diets). (d) The 95% CIs were calculated based on the group means of transformed *Zeb1*/*Ppia* data. (f,g) ZEB1 protein levels in Hepa-1c1c7 cells after 48 h of miRNA mimic or negative control transfection (50 nmol/L) shown with a representative blot of one out of five independent experiments quantified as ZEB1/TLP in. $*P < 0.05$ and $***P < 0.0005$ versus negative control. (e–g) Both bands were quantified in lanes showing a double ZEB1 band. [Color figure can be viewed at wileyonlinelibrary.com]

PTRF protein levels, mimics of both miR-155-3p and miR-1968-5p caused a reduction in cellular PTRF protein expression after 48 h of transfection ($P = 0.067$ for miR-1968; Fig. 7f,g). These results do not support a role of the three examined miRNAs as positive regulators of PTRF expression *in vivo*. Instead, our data suggest that

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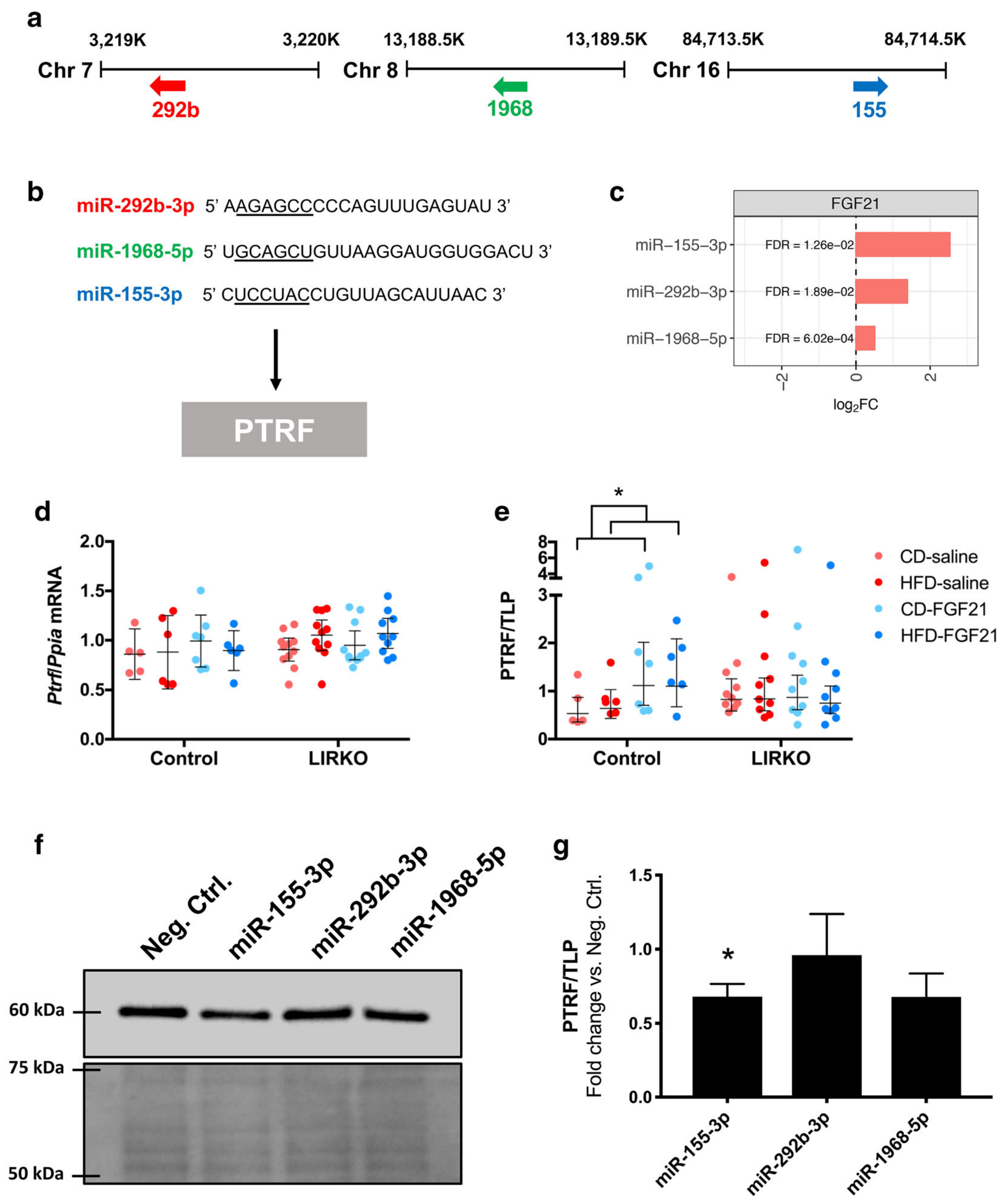

Figure 7 MicroRNAs (miRNAs) upregulated by fibroblast growth factor 21 (FGF21) potentially target polymerase I and transcript release factor (PTRF). (a) Chromosomal locations (mm10 assembly) of the genes encoding the three miRNAs identified as potential PTRF-targeting molecules. (b) Sequence alignment of the three miRNAs. Nucleotides 2–7 are underlined, representing their seed sequence through which they are predicted to bind the *Ptrf* 3′UTR. (c) Regulation of each of the three PTRF-targeting miRNAs in the livers of FGF21- versus saline-treated mice calculated from the small RNA sequencing data. (d) *Ptrf* mRNA levels normalized to levels of *Ppia* mRNA as measured by quantitative reverse transcription polymerase chain reaction, and (e) PTRF protein levels normalized to total lane protein (TLP) in the eight groups of mice calculated by quantification of western blots (Fig. S5b). $*P < 0.05$ between treatments, (d) $n = 5–11$ and(e) 5–10 animals per group. (e) The 95% CIs were calculated based on the group means of transformed PTRF/TLP data. (f,g) PTRF protein levels in Hepa-1c1c7 cells after 48 h of miRNA mimic or negative control transfection (50 nmol/L) shown with (f) a representative blot of one out of three independent experiments (g) quantified as PTRF/TLP. $*P < 0.05$ versus negative control. (f) Shown below the PTRF blot in is part of the stain-free picture used for quantification of TLP of the specific experiment. [Color figure can be viewed at wileyonlinelibrary.com]

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miR-155 and miR-1968 downregulate PTRF protein levels in liver cells.

DISCUSSION

HERE, WE PERFORMED small RNA-seq in the livers from two mouse models of impaired hepatic insulin action and mice treated with the antidiabetic hormone FGF21, and discovered miRNAs regulated by HFD- or LIRKO-induced impairment of liver insulin action or with improvement of liver metabolic function induced by FGF21 treatment. Our promoter sequence analysis identified DNA-binding motifs and putative transcription factors as candidates for upstream control of miRNA expression in hepatic energy metabolism. Functional enrichment analysis of predicted targets identified downstream biological processes likely affected by the observed changes in miRNA expression profiles. We identified the transcription factor ZEB1 and the caveola component PTRF as targets of two different nodes of miRNAs regulated in liver insulin resistance or with FGF21 treatment, respectively.

A total of 16 miRNAs were regulated in the same direction with HFD feeding and in LIRKO versus control mice (Fig. 3c). Among these are miRNA species previously shown to be upregulated in states of hepatic insulin resistance in both mice and humans; for example, miR-802 and miR-107.[12,42] In contrast, the hepatic function of most of the remaining miRNA species regulated in the same direction with HFD feeding and in LIRKO appears vastly unknown. However, many of these miRNAs are conserved in mammals.[43] Thus, it would be interesting to address their role in human insulin resistance, focusing on the concerted actions of the clusters of coregulated miRNA species on liver metabolic function.

We identified 19 miRNAs with opposite regulation in LIRKO versus control mice and with FGF21 treatment (Fig. 3e). In contrast, only miR-582-3p was differentially expressed with opposite directions of regulation after HFD feeding and FGF21 treatment (Fig. 3d). Differences between the two comparisons might reflect the fact that FGF21 has partly distinct actions in LIRKO versus control mice. Although FGF21 reduces total bodyweight and fat mass, and normalizes glycemia regardless of genotype, its beneficial effects on plasma cholesterol and liver triglyceride levels observed in HFD-fed control mice is completely abolished in LIRKO mice.[17] We speculate that the set of 19 miRNAs oppositely regulated with LIRKO-induced lack of hepatic insulin signaling and FGF21 treatment could be of particular importance in the control of hepatic lipid metabolism and its dysregulation in states of insulin resistance.

Target enrichment analysis identified nine downregulated miRNA species as potential targeting molecules for the transcription factor ZEB1 in LIRKO mouse livers (Fig. 6a–c). We found that hepatic ZEB1 protein levels were regulated in the same direction as the nine miRNA-species (Fig. 6d,e), suggesting that these miRNAs could act as positive regulators of hepatic ZEB1 expression. In line with these findings, our miRNA mimic experiments showed that miR-883b (the most downregulated of the nine miRNA species) and miR-205 both upregulate ZEB1 protein levels in mouse liver cells *in vitro* (Fig. 6f,g). These results could be due to indirect effects of miR-883b and miR-205 on ZEB1 protein levels through downregulation of other targets. However, since an initial report from 2007 of miR-369-3p acting either as an activator or a repressor of tumor necrosis factor-α expression depending on the cell cycle state,[44] many studies have shown how specific miRNAs can function as direct activators of target gene translation by interacting with (partially) complementary sites in the 3′ or 5′ UTR of the target transcript.[45–53] Not much is known about the mechanism of miRNA-mediated translational activation, but similar to miRNA-mediated repression, it appears to take place through AGO2-containing micro-ribonucleoprotein complexes.[44,49,52,54,55] Indeed, the 3′UTR of the *Zeb1* mRNA molecule contains two predicted binding sites for miR-883b-3p and one site for miR-205-5p binding (Fig. S6a, b). By binding to these sites, miR-883b and miR-205 could upregulate hepatic ZEB1 expression by activating *Zeb1* mRNA translation into protein.

To the best of our knowledge, we are the first to identify a downstream target for miR-883b. Most studies on miR-205 deal with its role as a driver of epithelial to mesenchymal transition in cancer, where miR-205 functions as a negative regulator of ZEB1 expression.[56] In contrast, just a few studies have examined its role in liver energy metabolism. Langlet *et al*. reported recently that hepatic miR-205-5p expression is increased in liver-specific *Foxo1*, *-3a*, *-4* triple-knockout mice.[57] miR-205 gain- and loss-of-function experiments showed that miR-205 targets components of the insulin signaling pathway to enhance insulin sensitivity and modulate hepatocyte glucose production.[57] The effects of miR-205-5p mimics on Zeb1 expression in primary mouse hepatocytes was also assessed and no effects were observed (Suppl. Data in Langlet *et al*.[57]). However, only *Zeb1* mRNA levels were measured, and in the present study, we observed no effects of the miRNA mimics on *Zeb1* mRNA expression levels (not shown), and only a slight reduction in *Zeb1* mRNA, but a

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more pronounced decrease in ZEB1 protein, expression in LIRKO versus control mouse livers (Fig. 6d,e). We speculate that miR-205, as well as miR-883b, could function to control hepatic energy metabolism through preferential regulation of ZEB1 protein levels.

Literature supports a role of ZEB1 in regulating liver energy metabolism. ZEB1 is a novel pro-adipogenic transcription factor and a central, upstream component of the gene regulatory network driving both mouse and human fat cell differentiation, a network comprising several transcription factors also controlling liver energy metabolism.[32] Recently, it was shown in cancer cells that ZEB1 induces expression of nicotinamide N-methyltransferase (NNMT), an enzyme of NAD metabolism.[58] Interestingly, hepatic NNMT expression correlates with various metabolic parameters in both mice and humans, and NNMT functions as a regulator of liver glucose, lipid, and cholesterol metabolism through stabilization of sirtuin 1 protein.[59] ZEB1 expression has also been linked directly to insulin signaling. Consistent with our findings of reduced ZEB1 levels in the LIRKO liver, insulin receptor silencing in cancer cells implanted in hyperinsulinemic mice decreases ZEB1 expression levels.[34] Thus, loss of insulin signaling in LIRKO livers could be hypothesized to downregulate the identified miRNA species. These miRNAs, in turn, could act as positive regulators of ZEB1 protein abundance and, through this regulation, contribute to altered glucose and lipid metabolism in the liver by targeting pathways, such as the NNMT/sirtuin 1 pathway.

Three miRNAs upregulated in the livers of FGF21- versus saline-treated mice, miR-155-3p, miR-292b-3p, and miR-1968-5p, were identified by target enrichment analysis to potentially target the protein PTRF (Fig. 7b). Western blotting showed that PTRF protein levels were increased in livers of FGF21-treated control mice (Fig. 7e), suggesting that the three miRNA-species function as positive post-transcriptional regulators of PTRF protein expression. However, our *in vitro* miRNA mimic experiments support a function of miR-155 and miR-1968 to downregulate PTRF protein levels in mouse liver cells. Thus, together with the *in vitro* miRNA mimic experiments, our *in vivo* data showing a net upregulation of hepatic PTRF after FGF21 exposure, despite the concomitant upregulation of miR-155 and miR-1968, suggest that these miRNAs do not drive PTRF upregulation. Instead, miR-155 and miR-1968 could act as counterregulatory molecules to fine-tune hepatic PTRF expression as part of the metabolic response to FGF21 treatment. *In vivo* studies with miRNA loss-of-function combined with FGF21 treatment of mice will be required to test if miR-155 and miR-1968 are indeed involved in the control of hepatic PTRF protein expression as part of the metabolic response to FGF21 treatment.

PTRF may be a novel intrahepatic mediator of the metabolic actions of FGF21. Indeed, studies link PTRF to regulation of hepatic energy metabolism. *Ptrf*$^{-/-}$ mouse livers are characterized by mild steatosis and increased expression of lipogenesis genes, even when mice are fed a chow diet.[40] Furthermore, these mice show an impaired hepatic response to prolonged fasting being unable to activate peroxisome proliferator-activated receptor-α and its target genes of fatty acid oxidation and ketogenesis.[41] Interestingly, our data show that the regulation of PTRF protein levels by FGF21 requires an intact hepatic insulin signaling pathway. PTRF protein levels were only increased in control, not in LIRKO, mice treated with FGF21. Furthermore, HFD-fed control mice showed a diminished response to FGF21 treatment compared with chow-fed control mice (Fig. 7e). In line with these observations, we previously showed that intact intrahepatic insulin actions are required for FGF21 to control lipid metabolism in these mice.[17] Future studies should address whether PTRF is required for FGF21 to enhance hepatic oxidative metabolism and normalize intrahepatic lipid levels.

In conclusion, states of impaired insulin action are characterized by alterations in the expression patterns of a large number of miRNAs, of which several miRNA species appear to be counterregulated by treatment with the antidiabetic hormone FGF21. The present results identified potential mechanisms by which a coordinated miRNA expression change controls energy metabolism in the liver. Targeting the upstream factors regulating these miRNA networks may be used therapeutically to ameliorate hepatic function in metabolic disease.

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SUPPORTING INFORMATION

ADDITIONAL SUPPORTING INFORMATION may be found online in the Supporting Information section at the end of the article.

Table S1. Sequence of quantitative reverse transcription polymerase chain reaction primers

Table S2. Small RNA sequencing data for differential expression analysis of microRNAs.

Table S3. Biological processes associated with hepatic microRNA expression changes.

Figure S1. Hierarchical clustering of microRNAs.

Figure S2. Quantitative reverse transcription polymerase chain reaction validation of sequencing data.

Figure S3. Transcription factor (TF) binding motifs enriched in promoters of microRNAs regulated by high-fat diet or in liver-specific insulin receptor knockout versus control mice

Figure S4. Transcription factor (TF) binding motifs enriched in promoters of microRNAs regulated with fibroblast growth factor 21 treatment.

Figure S5. Western blots for quantification of zinc finger E-box-binding homeobox 1 and polymerase I and transcript release factor protein in mouse livers.

Figure S6. Predicted binding sites for miR-883b-3p and miR-205-5p in the Zeb1 3′UTR.

Figure S7. Selection of reference microRNAs for relative quantitative reverse transcription polymerase chain reaction analysis.

Figure S8. Zinc finger E-box-binding homeobox 1 antibody testing by Zeb1 knockdown in C2C12 myoblasts.

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