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FXR activation remodels hepatic and intestinal transcriptional landscapes in non-alcoholic steatohepatitis

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Abstract

Abstract

The progression from simple steatosis to non-alcoholic steatohepatitis (NASH) has become a significant health concern. FXR activation shows promise in combating this transition and its harmful consequences. However, the specific alterations within the NASH-associated transcriptional network remain elusive, hindering the development of more precise and effective therapeutic strategies. Through comprehensive analysis of liver RNA-seq data from human and mouse NASH samples, we identified core perturbations in the NASH-associated transcriptional network, including impaired cellular metabolism and mitochondrial function, decreased tissue repair capacity, and increased inflammation and fibrosis, thereby elucidating the complex molecular mechanisms underlying NASH progression. Through integrated transcriptomic analysis employing mice treated with different FXR agonists, liver-specific FXR knockout mice, and publicly available human datasets, we determined that hepatic FXR activation effectively ameliorates NASH by reversing the dysregulation of metabolic and inflammatory networks involved in NASH pathogenesis. This ameliorative effect encompasses reversal of fibrosis, reduction of immune cell infiltration, and creation of an immune microenvironment that reflects positive trends in clinical disease progression. By dissecting the FXR core regulatory network directly associated with disease severity and therapeutic response, we identified approximately one-third of patients who may benefit from FXR agonist therapy. A similar analysis of intestinal RNA-seq data from mice treated with FXR agonists and intestine-specific FXR knockout mice revealed that intestinal FXR

activation can alleviate intestinal inflammation and has potential in mitigating hepatic inflammation and fibrosis. Overall, our study reveals the complex pathophysiological characteristics of NASH at the transcriptional level and highlights the intricate interplay between FXR activation and NASH progression and regression. These findings facilitate precise drug development, application, and efficacy evaluation, ultimately aiming to improve patient outcomes.

Full Text

Preamble

FXR Activation Remodels Hepatic and Intestinal Transcriptional Landscapes in Non-Alcoholic Steatohepatitis

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Y.W., G.Z., M.Z., Y.Z., D.W., J.S., Y.L., H.W., R.C., D.Z., and X.D. performed the experiments. Y.W. and Z.Z. analyzed the RNA-seq data. M.Z. assisted with luciferase reporter gene experiments. Y.W., G.Z., Z.Z., H.X., and C.X. were responsible for study concept and design. Y.W. and C.X. wrote the manuscript. C.X., H.X., and J.F. supervised the study.

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Abstract

The progression from simple steatosis to non-alcoholic steatohepatitis (NASH) has emerged as a significant global health concern. Activation of the farnesoid X receptor (FXR) shows promise in counteracting this transition and its detrimental consequences. However, the specific alterations within the NASH-related transcriptional network remain elusive, hindering the development of more precise and effective therapeutic strategies. Through comprehensive analysis of liver RNA-seq data from human and mouse NASH samples, we identified central perturbations within the NASH-associated transcriptional network, including disrupted cellular metabolism and mitochondrial function, decreased tissue repair capability, and increased inflammation and fibrosis, thus shedding light on the complex molecular mechanisms underlying NASH progression. By employing integrated transcriptome profiling of diverse FXR agonist-treated mice, FXR liver-specific knockout mice, and publicly available human datasets, we determined that hepatic FXR activation effectively ameliorated NASH by reversing the dysregulated metabolic and inflammatory networks implicated in NASH pathogenesis. This mitigation encompassed resolving fibrosis, reducing immune infiltration, and creating an immune microenvironment that mirrors positive trends observed in clinical disease regression. By understanding the core regulatory network of FXR, which is directly correlated with disease severity and treatment response, we identified approximately one-third of patients who could potentially benefit from FXR agonist therapy. A similar analysis involving intestinal RNA-seq data from FXR agonist-treated mice and FXR

intestine-specific knockout mice revealed that intestinal FXR activation attenuates intestinal inflammation and shows promise in reducing hepatic inflammation and fibrosis. Collectively, our study uncovers the intricate pathophysiological features of NASH at a transcriptional level and highlights the complex interplay between FXR activation and both NASH progression and regression. These findings contribute to precise drug development, utilization, and efficacy evaluation, ultimately aiming to improve patient outcomes.

Keywords: NAFLD/NASH; FXR; Transcriptome; Gut-liver axis; Agonist

1. Introduction

Non-alcoholic fatty liver disease (NAFLD) has become the most common chronic liver disease, with a global prevalence of approximately 25% over the past four decades. This increasing prevalence will significantly elevate the economic burden on healthcare systems. NAFLD encompasses a broad spectrum of metabolic stress-related liver disorders ranging from simple steatosis to its more severe form, non-alcoholic steatohepatitis (NASH), which is characterized by excessive fat accumulation, cellular damage, and inflammation with varying degrees of fibrosis. NAFLD/NASH is now considered the fastest-growing cause of end-stage liver diseases, including cirrhosis, liver cancer, and liver failure. The exact etiology of NASH remains unclear. The multiple-hit hypothesis provides a more accurate and widely accepted view of NASH pathogenesis, proposing that insulin resistance, obesity, ectopic fat deposition, mitochondrial dysfunction, endoplasmic reticulum stress, oxidative stress, inflammation, gut dysbiosis, and genetic factors can all contribute to NASH development and progression, with different pathogenic drivers occurring in parallel. However, the mechanisms governing the transition from steatotic liver to advanced NASH and fibrosis are not fully understood.

Despite growing therapeutic needs, there are currently no approved drugs for NASH treatment. Current therapeutic targets primarily involve pathways mediating lipid, glucose, and bile acid metabolism, as well as inflammation and fibrosis. FXR is a bile acid-activated nuclear receptor mainly expressed in the liver and intestine that regulates numerous biological processes, including bile acid homeostasis and lipid and glucose metabolism. In multiple preclinical models, FXR agonists have led to NASH resolution through metabolic and anti-inflammatory effects. The steroidal FXR agonist obeticholic acid (OCA), licensed as second-line therapy for primary biliary cholangitis, demonstrated the ability to reduce liver fibrosis without worsening NASH in phase 3 trials and was considered a potential first approval for NASH. However, due to side effects including pruritus and elevated LDL cholesterol levels, the FDA did not approve OCA for this indication. Novel non-steroidal FXR agonists such as tropifexor (LJN452) and TERN101 have been developed but exhibit similar side effects. These adverse effects may be related to FXR action itself, suggesting that our understanding of the overall FXR regulatory network remains inadequate. The lack of systematic studies on the signaling network generated after FXR activa-

tion likely contributes to the limited success in FXR agonist development.

Deciphering transcriptomic signatures through high-throughput sequencing enables identification of critical regulatory nodes involved in essential biological functions across diverse tissues and diseases. NASH development arises from intricate interactions among metabolic and stress pathways, coinciding with significant alterations in liver transcripts. Several studies have indicated that NASH progression is accompanied by increased inflammation and fibrogenesis, in addition to metabolic pathway perturbations, but that FXR activation reverses these phenotypes in mice and humans. Therefore, a comprehensive transcriptomic investigation of FXR activation is imperative.

Herein, we performed transcriptomic analysis of both human and mouse NASH samples and discovered critical changes in the regulatory network during the transition from healthy to NASH conditions, including dysregulated cellular metabolism, mitochondrial function, suppressed tissue repair capability in response to damage, and enhanced inflammation and fibrosis. Notably, we found that hepatic FXR activation effectively reversed all dysregulated networks that propel NASH progression, while intestinal FXR activation exhibited a positive correlation with diminished hepatic inflammation and fibrosis. Additionally, we predicted that approximately one-third of NASH patients are sensitive to FXR agonist therapy using machine learning. Our study broadens understanding of FXR activation' s impact on the gut-liver transcriptional regulatory network in NASH and provides insights into precision therapy with FXR agonists.

Materials and Methods

Animal Studies

Male C57BL/6J mice were purchased from HuaFukang BioScience Company and Vital River Laboratory Animal Technology Co., Ltd. Mice were bred in a specific pathogen-free (SPF) environment and housed under a 12-hour light/dark cycle with ad libitum access to water and food. All experiments were performed according to the Institute of Laboratory Animal Resources guidelines and approved by the Institute Animal Care and Use Committees at Shanghai Institute of Materia Medica or Cascade Pharmaceuticals, Inc.

Male mice aged 7-8 weeks were fed either a low-fat diet (LFD, 10% fat) or a Gubra-Amylin NASH (GAN) diet (40% kcal fat, 20% kcal fructose, and 2% cholesterol; D09100310 from Research Diet Inc., USA) for 6 weeks, or GAN diet plus CCl_4 (CCl_4:corn oil = 1:19, 2 l/g body weight) together with vehicle or FXR agonists (30 mg/kg OCA, 0.1 mg/kg LJN452, and 100 mg/kg TERN101) for an additional 4 weeks. Mice were sacrificed 48 hours after the final CCl_4 injection, and samples were collected for subsequent analysis.

Intestinal FXR-null (FxrΔIE), hepatic FXR-null (FxrΔHep), and control Fxrf/f mice were described previously. Male 7-8-week-old Fxrf/f and FxrΔHep mice on chow diet were treated with OCA (20 mg/kg/day) 2, 24, and 48 hours before

sacrifice.

Liver and Serum Analysis

Livers were collected, rapidly frozen in liquid nitrogen, and stored at -80°C for analysis. Serum was collected and centrifuged at 3,500 rpm for 15 minutes, and the supernatant was stored at -80°C. Liver triglycerides, cholesterol, and hydroxyproline (HYP), as well as serum triglycerides, total cholesterol, HDL-C, LDL-C, ALT, AST, TBIL, and DBIL were measured using commercially available kits (Jiancheng, Nanjing, China) according to the manufacturer' s protocols.

Histological Analysis

Liver sections fixed in 10% formaldehyde aqueous solution and embedded in paraffin were stained with hematoxylin and eosin (H&E) and Sirius red for collagen deposition. Frozen sections were stained with Oil Red O. Blind histological scoring of liver sections was performed according to established criteria including steatosis, lobular inflammation, and ballooning. Sections stained with Sirius red were analyzed to obtain fibrosis scores according to standard criteria. Images were captured with an upright microscope, and quantitative data were obtained using ImageJ software (v1.53).

Quantitative Real-time PCR

Mouse liver and ileum were frozen in liquid nitrogen and stored at -80°C. Total RNA from frozen tissues was isolated and extracted using standard phenol-chloroform extraction with Trizol reagent. cDNA was synthesized from 2 g of total RNA using PrimeScript reverse transcriptase (Takara Bio). Quantitative real-time polymerase chain reactions were performed with SYBR Premix Ex Taq (Takara Bio) and a real-time PCR detection system. Gene expression levels were calculated using the comparative method (2^-ΔΔCt) and presented as relative expression. Real-time PCR primer sequences are summarized in Supporting Table S1.

Public Human Datasets

The publicly available dataset E-MAXP-3291 was downloaded from ArrayExpress (https://www.ebi.ac.uk/arrayexpress/), comprising clinically defined patients: Normal (n = 19), Steatosis (n = 10), and NASH (n = 16). Dataset GSE48452 was downloaded from the GEO database (https://www.ncbi.nlm.nih.gov/geo/). This study assayed genome-wide mRNA levels in hepatic tissue samples using Affymetrix GeneChip® Human 1.0ST arrays. In our study, differential gene expression analysis was performed between control, steatosis (n = 6), and NASH (n = 3) groups.

Dataset GSE135251 was screened and downloaded from the GEO database to

evaluate inflammation and fibrosis status after treatment with three FXR agonists. This dataset contains high-throughput sequencing expression profiles from 216 snap-frozen liver biopsy samples: controls (n = 10), NAFL (n = 51), and NASH with fibrosis stages F0/1 (n = 34), F2 (n = 53), F3 (n = 54), and F4 (n = 14). Normalized and logarithmically converted expression values from different disease biopsies were calculated using the limma relative log expression normalization method. Outliers with abnormal expression levels were removed, and batch effects were corrected using the R package "sva".

RNA Isolation and Library Preparation for RNA-seq

RNA purification, reverse transcription, library construction, and sequencing were performed by Shanghai Majorbio Bio-pharm Biotechnology Co., Ltd. (Shanghai, China) according to the manufacturer' s instructions (Illumina, San Diego, CA).

Quality Control and Read Mapping

Raw paired-end reads were trimmed and quality-controlled using fastp with default parameters. Clean reads were then separately aligned to the reference genome in orientation mode using HISAT2 software. Mapped reads from each sample were assembled using StringTie in a reference-based approach. Probes were transformed, and duplicate genes were removed.

RNA-seq Data Analysis

Mouse gene expression values (FPKM) were normalized and logarithmically converted using the limma relative log expression normalization method. Principal component analysis (PCA) was performed to demonstrate significant differentiation between groups. Normalized RNA-seq data were used for gene expression heatmaps (https://www.omicstudio.cn/home). Software R (version 4.1.2) and the limma R package were used for differential gene expression analysis. Differentially expressed genes (DEGs) were defined using thresholds of $p < 0.05$ and $|\log2(\text{fold-change})| > 0$.

Enrichment Analysis

DEGs identified by the limma package were used as input for Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses using the clusterProfiler R package. A strict cutoff of P values and false discovery rate (FDR) both lower than 0.05 was applied. Top GO and KEGG terms were displayed.

Protein-Protein Interaction Network Analysis

Protein-protein interactions (PPIs) were analyzed using STRING (https://string-db.org/), an online database of known and predicted protein-protein interac-

tions. PPI information derived from STRING was uploaded into Cytoscape (version 3.8.2) for visualization and network construction. The betweenness algorithm identified the top 30 genes with strong interactions and numerous connections to neighboring nodes, while node color shades indicated protein interaction abundance.

Gene Set Analysis

Gene Set Enrichment Analysis (GSEA) was performed on limma-normalized and log-transformed expression values from human and mouse liver NASH samples. To evaluate fibrosis improvement after treatment with four FXR agonists, homozygous gene conversion was performed on mouse RNA-seq data, and gene expression profiles were compared with 216 human liver biopsy samples grouped by fibrosis stage. Pathway and gene analysis involved in fibrosis was performed using the Nanostring nCounter Fibrosis v2 Panel. Normalized Enrichment Score (NES) was calculated to enable comparison between human and mouse pathway enrichment. Mouse and human data used their respective healthy groups as control phenotypes. Additionally, single-sample GSEA (ssGSEA) was performed using normalized and log-transformed human and mouse gene expression data after batch effect removal for comparison. Inflammatory pathways associated with fibrosis from the Nanostring nCounter Fibrosis v2 Panel were used to explore inter-sample correlation using Euclidean distance and Pearson correlation.

Immune Cell Infiltration Analysis

Immune infiltration in the liver was analyzed using ssGSEA based on non-overlapping gene sets representing 28 immune infiltrating cell types, with negative values removed and group infiltration levels calculated as mean values. t-tests were performed comparing three FXR agonist groups with the NASH group, with $P < 0.05$ considered significant. A total of 22 immune infiltrating cell types were evaluated. CIBERSORT code and the standard immune cell expression file "LM22.txt" were obtained from the official website (https://cibersort.stanford.edu/). The proportion of immune cell subsets in mixed cell populations was assessed using stacked diagrams.

Cell Communication Analysis by NicheNet

We first performed single-cell localization analysis of FXR core downregulated genes to determine which receptors are distributed on non-parenchymal cells (NPCs) and identified corresponding ligand information for these receptors. Ligand-receptor interactions were obtained from lr_{network}.rds. NicheNet (v0.1.0) was used to rank ligands based on their accuracy in predicting whether a gene belonged to a gene set of interest compared to a background gene set. A ligand-receptor potential score was calculated to reflect interaction weight in the weighted ligand signaling network from weighted_{networks}.rds.

Statistics

Statistical analyses were performed using GraphPad Prism (version 9.0.0, GraphPad). All experimental values are presented as mean ± SEM. Statistical significance between two groups was determined using a two-tailed Student' s t-test. One-way ANOVA followed by Tukey' s post hoc correction was applied for multi-group comparisons. $P < 0.05$ was considered significant. Correlation analysis was performed using Pearson' s test (two-sided).

Results

Characterization of Global Transcriptomic Features Across Humans and Mice During NASH Progression

To explore which critical biological processes are altered during NASH progression, we analyzed transcriptional changes using two public RNA-seq datasets from NAFLD patients (GSE48452 and GSE130970). Principal component analysis (PCA) showed that livers from healthy controls, simple steatosis, and NASH patients separated along PC1. K-means clustering of genes differentially expressed in both human datasets revealed four clusters (C1, C3, C5, and C7) that were upregulated and four clusters (C2, C4, C6, and C8) that were downregulated in NASH patients. Both KEGG and GO pathway analyses demonstrated that genes induced in clusters C1 and C5 were assigned to inflammation-related pathways such as cytokine-cytokine receptor interaction, toll-like receptor signaling, NF- B signaling, immune cell migration, chemotaxis, and response to external stimulus. Meanwhile, genes induced in clusters C3 and C7 were related to tissue injury and fibrogenesis, including DNA replication, extracellular matrix (ECM) receptor interaction, focal adhesion, ECM organization and assembly, and autophagy.

Most genes repressed in NASH (C2, C4, C6, and C8) were assigned to small molecule catabolic processes, mitochondrial respiration-related pathways, and tissue regeneration-related signaling and processes such as membrane potential, cell specification, Notch and Wnt signaling, and RNA processing and splicing. These results revealed that core pathogenetic factors in NASH patients include increased tissue injury, inflammation, and fibrogenesis, alongside dysregulated small molecule and energy metabolism, impaired mitochondrial function, and suppressed tissue repair.

To explore the conservation of pathway variation in NASH between humans and mice, we established two different NASH mouse models: Gubra-Amylin NASH (GAN) diet-induced NAFL and NASH, and GAN diet plus CCl_4-induced NASH with advanced fibrosis. Mice in both NASH models showed more severe lipid accumulation, inflammation, and fibrosis. RNA-seq of livers from these models showed that control, NAFL, and NASH mice from both models were clearly separated along PC1. K-means clustering of differentially expressed genes revealed three upregulated clusters (C1, C3, and C5) and three downregulated clusters (C2, C4, and C6) in both NASH models. Pathway analysis demonstrated that

genes specifically induced in NASH (C1, C3, and C5) were mainly enriched in focal adhesion, chemokine signaling pathway, ECM receptor interaction, ECM organization, actin filament organization, leukocyte migration, and platelet activation—all related to inflammation and fibrosis. Genes repressed in NASH (C2, C4, and C6) were attributed to small molecule catabolic processes, mitochondrial function, and tissue repair-related pathways such as Wnt signaling, RNA processing, epigenetic modification, and cell cycle.

Overall, pathway analysis revealed that cellular metabolism and mitochondrial function were obstructed and repair function in response to cell damage was hindered during NASH progression in mice, accompanied by inflammation and fibrosis development. Cellular metabolism, mitochondrial function, and tissue repair-related pathways were enriched in both mouse and human downregulated clusters, while upregulated pathways were enriched in immune regulation and fibrogenesis and showed characteristic upregulation when NAFL progressed to NASH. These data suggest that mouse NASH models can recapitulate human NASH pathology. Therefore, intervention targeting these disrupted pathways (defined as the core NASH transcriptional network) in both humans and mice could represent a potential therapeutic strategy for NASH, and mouse studies can be used to interpret the pharmacological consequences of FXR agonists on human NASH.

FXR Agonist Treatment Ameliorates NASH Development

In recent years, research and development of FXR agonists has entered a period of rapid growth, but limited clinical benefits and serious side effects have restricted their applications. Therefore, further understanding the core regulatory network of FXR agonists in NASH is crucial. OCA is the first steroidal FXR agonist to reach clinical endpoints. LJN452 and TERN101 are non-steroidal compounds developed to circumvent hormone-like side effects of OCA. Using different FXR agonists can effectively eliminate off-target effects of the compounds themselves, facilitating better systematic investigation of the core FXR regulatory network in NASH.

We evaluated the therapeutic effects of OCA, LJN452, and TERN101 on GAN diet plus CCl_4-induced NASH with advanced fibrosis. There was no significant difference in body weight between NASH mice and FXR agonist-treated mice, but FXR agonist treatment increased liver weights. Liver triglyceride and HYP levels, but not cholesterol, were significantly reduced after FXR agonist treatment. Treatment with LJN452 and TERN101 reduced serum triglyceride, cholesterol, HDL-C, and LDL-C levels. Additionally, FXR agonists decreased TBIL, DBIL, and liver injury markers ALT and AST. qRT-PCR analysis of FXR target gene expression showed that FXR agonists significantly reduced hepatic Cyp7a1 and Cyp8b1 expression while increasing intestinal Fgf15 and Shp expression, confirming activation of both hepatic and ileal FXR signaling. Liver histology analysis revealed significant reductions in steatosis, inflammation, and fibrosis. These results confirm that FXR agonist treatment ameliorates NASH

as previously reported.

FXR Activation Reverses the Dysregulated Core NASH Transcriptional Network

To elucidate the hepatic FXR-activated transcriptional network in NASH, we performed RNA sequencing on livers from NASH mice treated with FXR agonists (OCA, LJN452, and TERN101). To accurately identify FXR-influenced transcripts, we conducted parallel analysis on livers from hepatocyte-specific FXR-deficient mice (FxrΔHep) and control counterparts (Fxrf/f), both subjected to OCA treatment to augment FXR-dependent signaling. PCA displayed clear separation between liver transcripts from vehicle-treated NASH mice and FXR agonist-treated mice; similarly, distinct separation was observed between OCA-treated Fxrf/f and FxrΔHep mice. Through rigorous DEG analysis, we identified four sets of differentially expressed genes. Intersection of these sets revealed two groups of FXR-dependent genes: 210 consistently upregulated genes and 218 downregulated genes in response to FXR activation.

Pathway enrichment analysis of the 210 upregulated genes, along with Gene Set Enrichment Analysis (GSEA) of entire transcriptomes, revealed significant upregulation of cellular metabolism and mitochondrial function-related pathways following FXR agonist treatment—pathways that were dysfunctional in core NASH networks. Conversely, pathway enrichment analysis of the 218 downregulated genes and GSEA results demonstrated marked reduction in inflammation and fibrosis-related pathways, which represent pathogenic signatures in NASH progression. Notably, FXR agonist treatment appears to exert dual effects by promoting upregulation of beneficial pathways linked to cellular metabolism, mitochondrial function, and RNA processing while concurrently dampening detrimental inflammation and fibrosis-related pathways in the core NASH transcriptional network. Comparison between steroidal FXR agonist OCA and non-steroidal agonists revealed that non-steroidal agonists (LJN452 and TERN101) showed more significant effects on cholesterol metabolism but weaker effects on lipid metabolism compared to OCA.

Collectively, our data demonstrate that hepatic FXR activation can reverse the dysregulated core NASH transcriptional network. This intricate modulation underscores FXR' s potential as a versatile therapeutic target relevant across the spectrum of core pathways implicated in NASH pathogenesis.

Machine Learning Predicts Patient Response to FXR Agonist Therapy

The substantial heterogeneity of NASH significantly influences FXR agonist efficacy, with patients at the same NASH stage responding differently to therapy. We hypothesized that patient responses to FXR agonist therapy could be predicted based on gene expression heterogeneity. Protein-Protein Interaction (PPI) analysis was performed to discover the core gene network regulated by

FXR. The PPI network focusing on the top 30 FXR core upregulated genes highlighted strong negative correlations between Upb1, Mt-Md3, Aox3, Mcee, Dpp4, and Maob with liver triglycerides, indicating involvement of these genes in FXR-mediated steatosis resolution. Furthermore, the top 30 FXR core downregulated genes showed strong positive correlations with ALT and AST, confirming FXR agonists' ability to ameliorate NASH-associated liver injury.

To validate whether these 60 FXR-regulated genes are associated with NASH progression in humans, three machine learning algorithms were applied to dataset GSE135251: random forest (RF), support vector machine (SVM), and logistic regression (Logi). Receiver operating characteristic (ROC) curves revealed that these FXR core-regulated genes could successfully distinguish NASH patients from NAFLD patients. To narrow down the NASH-related and FXR core-regulated gene set and identify NASH patients sensitive to FXR agonist therapy, least absolute shrinkage and selection operator (LASSO) regression analysis was employed. Features with higher importance scores indicate greater gene contribution to the prediction model. Genes were ranked by importance (impurity) scores, with the top 11 and top 16 genes displayed. NASH patients showing opposite expression patterns of these 27 genes are thought to potentially respond better to FXR agonist treatment. ssGSEA was used to differentiate NASH patients based on normalized enrichment scores (NES) of the corresponding FXR core-regulated gene set. ROC curves showed excellent predictive properties of these 27 genes for disease classification. The intersection revealed that approximately 28.64% of NASH patients exhibited more significant alterations in the FXR core-regulated network and thus might be more sensitive to FXR treatment. These results suggest that FXR agonist efficacy may be suboptimal in early NASH stages but might act more effectively in later fibrosis stages, highlighting the need for more precise drug development, utilization, and efficacy assessment.

FXR Activation Mitigates Liver Inflammation and Fibrosis Mainly via Liver Non-Parenchymal Cells

NAFLD is a pro-inflammatory disorder wherein liver inflammation with chronic hepatic injury and resulting fibrosis play pivotal roles in orchestrating the transition from NAFL to NASH. To elucidate the clinical impact of transcriptional modifications following FXR agonist treatment, we conducted comparative analysis between RNA-seq data from experimental mice and orthologous genes from 216 human liver biopsy samples categorized by fibrotic stage. Hierarchical clustering of human and mouse liver samples revealed two distinct groups showing differential enrichment for pre-fibrotic or fibrotic disease stages. PCA highlighted that progression from NAFL to F4 stage in humans and from control to NASH in mice is primarily orchestrated by PC1. Moreover, livers from FXR agonist-treated mice clustered closely around the center of the F2 ellipsoid.

To determine similarity in fibrosis-related pathways between mice and humans, we computed normalized enrichment scores. Analysis of FXR core downregu-

lated pathways revealed suppression of fibrosis-related processes following FXR agonist treatment, including cell-substrate adhesion, collagen fibril organization, collagen metabolic process, and fibroblast proliferation. GSEA of genes involved in fibrosis pathways using the Nanostring nCounter Fibrosis v2 Panel showed that among 17 significantly altered pathways in FXR agonist-treated mice, those in cluster II were predominantly repressed by FXR activation, including critical processes integral to NASH pathophysiology such as focal adhesion kinase, PDGF signaling, ECM synthesis, and collagen biosynthesis and modification. Both Pearson correlation and Manhattan distance analyses indicated that FXR agonist-treated mice were more similar to F0-F1 biopsies, distinct from other fibrosis stages and most dissimilar to F4 stage biopsies.

Regarding specific fibrosis pathways, single-sample GSEA (ssGSEA) showed downregulation of pathways in clusters II and III compared to NASH mice or individuals at the most advanced human NASH stage (F4). Heatmaps of gene expression underscored that FXR agonist treatment significantly curtailed expression of several fibrosis and NASH progression markers (COL1A1, COL1A2, COL3A1, COL4A21, COL4A2, COL5A1, COL5A3, COL6A3, COL14A1, COL16A1, MMP2, and TGFB1) to levels comparable to or below those observed in control diet-fed mice and NAFL patients. These data provide valuable insights into cross-species similarities in fibrogenesis and common therapeutic responses to FXR agonists.

The immune microenvironment undergoes crucial alterations during NASH development, with inflammation-induced liver damage contributing to fibrosis progression. To explore shifts in the immune infiltration landscape following FXR agonist treatment, we performed GSEA on inflammatory pathways closely intertwined with fibrosis using the Nanostring nCounter Fibrosis v2 Panel. Upon FXR agonist treatment, several pro-inflammatory pathways were repressed, most notably type I and II interferon responses, phagocytosis, chemokine and cytokine signaling, granulocyte degranulation, adenosine pathway, and TLR signaling. Investigation of specific immune cell population alterations revealed that central memory CD4+ T cells, immature dendritic cells, and natural killer cells exhibited significant downregulation following FXR agonist treatment. CIBERSORT analysis of immune infiltration deconvolution scores showed that in NASH mice, the fraction of resting dendritic cells, monocytes, and regulatory T cells increased, while naive B cells and plasma cells decreased compared to healthy mice. These findings elucidate the intricate immunomodulatory effects of FXR agonist treatment in NASH. By downregulating pro-inflammatory pathways and modulating specific immune cell subsets, FXR activation demonstrates potential in reshaping the immune landscape, further contributing to fibrosis alleviation and inflammation mitigation.

We next investigated the cellular localization of 180 core downregulated genes at the single-cell level and identified nine genes primarily expressed in liver non-parenchymal cells (NPCs) such as macrophages, T cells, and fibroblasts. Some have been established as causal factors in fibrosis progression, including vascular

cell adhesion molecule-1 (Vcam1), AXL receptor tyrosine kinase (Axl), complement C3a receptor 1 (C3ar1), and integrin subunit beta 2 (Itgb2). NicheNet analysis incorporating ligand-target gene interaction data was conducted to depict their roles in intra-hepatic crosstalk between hepatocytes and NPCs. The nine FXR agonist-induced genes as receptors in NPCs were predicted to have several ligand genes in hepatocytes. Pathway enrichment analysis showed that some genes are involved in fibrosis-related processes such as cell-matrix adhesion (Fga, Agt, and Vegfa) and blood coagulation (Plg, Apoe, Fga, F2, Kng1, C3, and Pros1). These results highlight the importance of hepatic NPCs in mediating FXR agonist-induced effects on inflammation and fibrosis. The intricate interplay between these genes, hepatocyte-derived ligands, and the hepatokine network offers a novel perspective on the multifaceted impact of FXR activation.

Thus, suppression of key inflammation and fibrosis-related processes, along with modulation of different cells in the microenvironment, substantiates the potential of FXR agonists in alleviating NASH.

Intestinal FXR Activation Alleviates NASH Through Gut-Liver Crosstalk

To discover the intestinal transcriptional network activated by FXR in NASH, we performed RNA-seq on samples from NASH mice treated with FXR agonists and analyzed samples from OCA-treated enterocyte-specific FXR-deficient (FxrΔIE) and control (Fxrf/f) mice to identify transcripts influenced by intestinal FXR. PCA distinctly segregated NASH mice and FXR agonist-treated mice along PC1, as well as OCA-treated Fxrf/f and FxrΔIE mice. We obtained four sets of DEGs, and the recognized FXR target gene Fgf15 among the corresponding upregulated or downregulated genes confirmed RNA-seq data reliability. Intersection of these sets identified 55 FXR-dependent upregulated genes and 143 downregulated genes.

Pathway enrichment analysis of the 55 upregulated genes indicated increased lipid biosynthesis and metabolism, bile acid secretion, and transport in the intestine. Conversely, analysis of the 143 downregulated genes pointed to significant downregulation of inflammation-related pathways, including B cell activation, proliferation and differentiation, and leukocyte, lymphocyte, and mononuclear cell proliferation, implying a marked shift in intestinal immunity. To better depict intestinal ecology in NASH and understand FXR activation effects, we performed GSEA to compare transcriptional profiles of NASH mice with healthy mice and FXR agonist-treated mice. Compared to healthy controls, bile acid-related pathways were downregulated while inflammation-related pathways were upregulated in NASH mice. Moreover, FXR agonists reversed these NASH-induced dysregulated pathways in the intestine. Our data support the notion of improved intestinal ecology following FXR activation.

We next investigated FXR agonist influence on the liver via the gut-liver axis in NASH mice. DEG analysis was performed for livers from Fxrf/f vs. FxrΔIE,

Control vs. NASH (GAN diet), and Control vs. NASH (GAN diet + CCl_4). These transcriptional alterations in the liver following intestinal FXR deficiency or activation are closely associated with NASH progression. Both FXR activation and inhibition in the intestine have been reported to produce metabolic benefits in NAFL or NASH. We adopted four intersection strategies to uncover potential benefits of intestinal FXR agonists or inhibitors. Pathway analysis of 35 and 23 genes (FXR inhibitor benefit) demonstrated that FXR inhibition increased autophagy and reduced DNA damage and cell death. Additionally, pathway analysis of 158 and 86 genes (FXR agonist benefit) revealed that intestinal FXR activation leads to decreased inflammation and fibrosis and enhanced energy metabolism. In conclusion, intestinal FXR activation may work independently of the regulatory network centered on intestinal FXR inhibition, but both contribute to NASH resolution through the gut-liver axis.

Discussion

NASH is recognized as a major contributor to hepatitis, cirrhosis, hepatocellular carcinoma, and cardiovascular events, underscoring the urgent need to understand NASH mechanisms and develop effective treatments. FXR belongs to the nuclear receptor superfamily and is highly expressed in the liver and intestine. Targeting FXR represents a promising therapeutic approach for NASH. Recent studies have implicated metabolic benefits of FXR activation in NASH, and multiple FXR agonists have entered clinical trials, including OCA, LJN452, and TERN101. However, mechanisms underlying FXR agonist beneficial effects remain incompletely elucidated. Therefore, understanding the FXR-centered regulatory network in the liver and intestine, as well as gut-liver crosstalk, is imperative. Our study demonstrates that hepatic FXR agonists alleviate NASH-associated phenotypes and produce wide-ranging metabolic benefits, including improved cellular metabolism of amino acids, fatty acids, and carboxylic acids, enhanced mitochondrial function, and reduced inflammation and fibrosis. Alterations in these FXR-centered pathway networks in the liver play critical roles in NASH remission. Machine learning results further suggest that the population sensitive to FXR agonist therapy comprises approximately one-third of total NASH patients. Additionally, FXR is highly expressed in the intestine, where it works with hepatic FXR to control enterohepatic bile acid circulation and maintain metabolic homeostasis. These results expand our understanding of FXR agonist mechanisms of action and applications.

Using different RNA-seq datasets, we demonstrate that NASH mice and humans share similar core NASH transcriptional networks. Unsupervised clustering was performed on transcriptomic data from two batches of NASH mice and two datasets representing three NAFLD stages (healthy, steatosis, and NASH) in humans. Early in NASH, DNA replication and signal transduction are enhanced to repair tissue damage. However, RNA processing is inhibited, suggesting that transcriptional and translational processes are blocked, accompanied by dysregulated small molecule and energy metabolism and mitochondrial dysfunction.

Prolonged tissue damage increases immune cell infiltration and fibrocollagenous tissue, further exacerbating liver injury. This vicious cycle drives NASH progression.

Activation of FXR by different agonists—OCA, LJN452, and TERN101—similarly reverses the dysregulated NASH transcriptional network. However, PCA results indicated strong separation due to structural differences between these FXR agonists, suggesting different modes of action between agonist types (steroidal vs. non-steroidal structure, and even among non-steroidal compounds). The side effects of these FXR agonists remain unresolved and require further study. Nonetheless, the ameliorative effects of these FXR agonists on hepatic lipid accumulation, injury, inflammation, and fibrosis in NASH are definite.

A characteristic feature of NASH is hepatic inflammation, which drives fibrosis development. FXR agonist treatment downregulates inflammation-related pathways, including interferon production, phagocytic cell function, TLR signaling, and neutrophil degranulation, all known to be involved in NASH pathogenesis. Subsequent GSEA analyses reveal downregulated pathways involving collagen biosynthesis, ECM synthesis, and focal adhesion kinase, leading to significant reductions in fibrosis markers comparable to control mice or healthy human livers. These data suggest that hepatic FXR activation remodels the hepatic immune microenvironment and may directly affect immune cells beyond hepatocytes. Further examination of FXR core downregulated genes showed that receptors regulated by FXR agonists, such as those on macrophages, T cells, and fibroblasts, were found on NPCs. Notably, AXL, VCAM1, ITGB2, and C3AR1 have been associated with inflammation and fibrosis.

Intestinal FXR activation has been reported to improve hepatic glucolipid metabolism. However, several studies showed that specific knockout or selective inhibition of intestinal FXR improved obesity-related metabolic dysfunction, including NASH. Therefore, intestinal FXR may act as a bidirectional regulator of liver metabolism and function. Our study reveals that intestinal FXR activation is associated with decreased hepatic inflammation and fibrosis, while intestinal FXR antagonism is linked to enhanced autophagy and ameliorated cell death in the liver.

A recent 18-month clinical trial showed that 22.4% of participants receiving once-daily oral OCA 25 mg achieved $ $1 stage fibrosis improvement without NASH worsening, similar to our predicted proportion of the population sensitive to FXR agonist treatment. In early NASH stages, the proportion of the population susceptible to FXR agonist treatment is low, possibly because FXR agonists produce broad metabolic benefits but have nonspecific effects on lipid accumulation and inflammation. These results suggest that using FXR agonists in early NASH may not be appropriate. FXR has numerous target genes in the organism, some of which remain to be discovered, while the specific pathways through which FXR acts on some target genes are unclear. The core regulatory network of FXR we have identified deserves further investigation and may

provide new targets for NASH drug development.

In summary, given the complex physiological effects of FXR activation, including various intractable side effects, exploring the core regulatory network can facilitate more precise drug development, utilization, and efficacy evaluation. The identification and development of new targets may provide novel insights into NASH treatment. Meanwhile, visualization of the coregulatory network also provides a new transcriptomic perspective for evaluating new drug efficacy.

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Supporting Information

Supporting Fig. S1. Transcriptional characterization of human and mouse NASH at different stages. (A) Liver TG and TC levels in mice fed GAN diet (n = 5-10/group) or GAN diet plus CCl_4 (n = 6/group). (B) GSEA of pathways from Figures 1 and 2. Pathways were split into three clusters by Euclidean clustering. Experimental values are presented as mean ± SEM. *p < 0.05,* ***p < 0.01, and*** p < 0.001; ns, non-significant.

Supporting Fig. S2. Transcriptomic data from livers of FXR agonist-treated mice. (A) PCA of RNA-seq data from mouse livers. (B) Volcano diagram of gene expression from mice treated under different conditions. (C) Heatmap of differentially expressed genes.

Supporting Fig. S3. FXR activation effects on molecular signatures of inflammation and fibrosis. (A) Heatmap showing Pearson correlation matrix between human and mouse at different NASH stages. (B) Violin plots

of ssGSEA scores with accompanying heatmap showing gene expression of indicated pathways in mice and humans. Medians, quartiles, maxima, and minima are denoted by box plots. (C) ssGSEA heatmap of immune infiltration. (D) Immune infiltration analysis by CIBERSORT showing immune cell subset proportions. Expression heatmap of FXR agonist-regulated NPC genes. Experimental values are presented as mean ± SEM. *$p < 0.05$,* ***$p < 0.01$, and*** $p < 0.001$; ns, non-significant.

Supporting Fig. S4. Transcriptomic data from intestine of FXR agonist-treated mice. (A) PCA of RNA-seq data from mouse intestine. (B) Volcano diagram of gene expression from mice treated under different conditions. (C) Heatmap of 55 FXR core upregulated gene expression. (D) KEGG enrichment analysis of genes representing FXR agonist benefit.

Tables

Mouse primers

Gene	Forward sequence	Reverse sequence
Cyp7a1	AACAACCTGCCAGTACTAGATAGC	GTGTAGAGTGAAGTCCTCCTTAGC
Cyp8b1	CTAGGGCCTAAAGGTTCGAGT	GTAGCCGAATAAGCTCAGGAAG
Fgf15	GCCATCAAGGACGTCAGCA	CTTCCTCCGAGTAGCGAATCAG

Figure Legends

FIG. 1. Transcriptomics profiling of livers in humans identifies core NASH transcriptional network. (A) PCA of RNA-seq data from livers of NASH patients (GSE48452: Control, n = 14/group; Steatosis, n = 14/group; Mild (F0-F1), n = 14/group; Advanced (F2-F4), n = 4/group; GSE130970: Control, n = 6/group; Mild (F0-F1), n = 45/group; Advanced (F2-F4), n = 25/group). (B-C) K-means clustering of row-scaled log2FC in expression of hepatocyte genes differentially expressed ($p < 0.05$) between control, steatosis, mild (F0-F1), and advanced (F2-F4) NASH patients. KEGG enrichment analysis for indicated clusters. (D-E) GO enrichment analysis of upregulated clusters (clusters 1, 3, 5, and 7) and downregulated clusters in NASH patients (clusters 2, 4, 6, and 8).

FIG. 2. Transcriptomics profiling of livers in mice identifies core NASH transcriptional network. (A) Overview of experimental design for mouse NASH models. (B) Representative H&E and Sirius red stainings of mice fed GAN diet or GAN diet plus CCl_4. Scale bars, 200 m. (C) PCA of RNA-seq data from livers of mice from two different NASH models (n = 3-4/group). (D) K-means clustering of row-scaled log2FC in expression of hepatocyte genes differentially expressed ($p < 0.05$) between control, NAFLD, and NASH mice. KEGG enrichment analysis for indicated clusters. (E-F) GO enrichment analysis of upregulated clusters (cluster 1, cluster 3, and cluster 5) and downregulated clusters in NASH mice (cluster 2, cluster 4, and cluster 6).

FIG. 3. Treatment with FXR agonists alleviates NASH-associated phenotypes. (A) Experimental design. (B) Body weight and liver weight. (C) Liver TG, TC, and HYP levels. (D) Serum TG, TC, HDL-C, LDL-C, ALT, AST, TBIL, and DBIL levels. (E) Expression of FXR target genes in liver and intestine. (F-G) Representative H&E and Sirius red staining of liver sections. Scale bars, 200 m. Fibrosis score, Sirius red-positive area (%), and NAFLD activity score. Colored bars indicate mean ± SEM (n = 5-6/group). Different condition groups were compared with NASH+Vehicle group. *$p < 0.05$,* ***$p < 0.01$, and*** $p < 0.001$.

FIG. 4. FXR activation reverses the dysregulated core NASH transcriptional network. (A) Schematic diagram of hepatic RNA sequencing experiment and analysis procedure. (B) PCA of RNA-seq data from mouse livers. (C) Heatmap of differentially expressed genes. (D) Interactive Venn diagram. (E) GO enrichment analysis of DEGs. (F) GSEA heatmap of energy metabolism, cell fate, inflammation, and fibrosis.

FIG. 5. Machine learning predicts patients' response to FXR agonist treatment. (A) PPI network analysis of FXR core upregulated intersection genes. Top 30 hub genes are displayed. (B) Correlation heatmap between top 30 FXR core upregulated genes and biochemical indicators of NASH. (C) PPI network analysis of FXR core downregulated intersection genes. Top 30 hub genes are displayed. (D) Correlation heatmap between top 30 FXR core downregulated genes and biochemical indicators of NASH. (E) Receiver operating characteristic (ROC) curves for NASH stage prediction by three different machine learning methods. (F) Penalty plot of top 30 FXR core upregulated and downregulated genes in the LASSO model; error bars represent standard error. (G) Top prediction features selected by random forest impurity measurements. (H-I) ssGSEA of NASH-related and FXR core regulated gene sets using the median as dividing line (Low, n = 103/group; High, n = 103/group). (J) ROC curves for NASH stage prediction by three different machine learning methods. (K) Interactive Venn diagram. (L) Proportion of patients tolerant and sensitive to FXR agonist therapy. *$p < 0.05$,* ***$p < 0.01$, and*** $p < 0.001$.

FIG. 6. Activation of FXR reduces molecular signatures of inflammation and fibrosis. (A) Gene expression heatmap of 17 orthologous genes identified from signature genes characterizing NASH severity in humans. (B) PCA coordinates disease progression in humans and mice according to average signature gene expression values. (C) GSEA heatmap of FXR core downregulated pathways. (D) GSEA heatmap of nCounter Fibrosis v2 Panel pathways between mouse cohorts compared to NAFL-F4 stage patients. Pathways are clustered according to Pearson correlation and grouped into clusters I-III. (E) Violin plots showing ssGSEA scores with accompanying heatmaps showing gene expression of indicated pathways in mice and humans. Medians, quartiles, maxima, and minima are denoted by box plots. (F) GSEA heatmap of inflammatory pathways associated with fibrosis between mouse cohorts. (G) Circle plot showing predicted interaction links between hepatokines (bordeaux) and FXR

agonist-regulated NPC receptors in macrophages (blue), T cells (orange), and fibroblasts (green).

FIG. 7. Activation of intestinal FXR mitigates NASH through the gut-liver axis. (A) Schematic diagram of intestinal RNA sequencing experiment and analysis procedure. (B) Interactive Venn diagram. (C) GO enrichment analysis of intestinal FXR core upregulated genes. (D) Heatmap of DEG expression and GO enrichment analysis of intestinal FXR core downregulated genes. (E) GSEA heatmap of bile acid and inflammation pathways. (F) Volcano plot showing DEGs from liver (Fxrf/f vs. FxrΔIE, Control vs. NASH [GAN diet], and Control vs. NASH [GAN diet + CCl_4]; $p < 0.05$). (G) Interactive Venn diagram showing genes representing FXR agonist benefit and FXR inhibitor benefit. (H) GO enrichment analysis of genes representing FXR inhibitor benefit. (I) GO enrichment analysis of genes representing FXR agonist benefit.

Note: Figure translations are in progress. See original paper for figures.

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