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ORIGINAL ARTICLE

Alcohol-associated fibrosis in females is mediated by female-specific activation of lysine demethylases KDM5B and KDM5C

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Abstract

Alcohol-associated liver disease is a major cause of alcohol-related mortality. However, the mechanisms underlying disease progression are not fully understood. Recently we found that liver molecular pathways are altered by alcohol consumption differently in males and females. We were able to associate these sex-specific pathways with two upstream regulators: H3K4-specific demethylase enzymes KDM5B and KDM5C. Mice were fed the Lieber-DeCarli alcohol liquid diet for 3 weeks or a combination of a high-fat diet with alcohol in water for 16 weeks (western diet alcohol model [WDA] model). To assess the role of histone demethylases, mice were treated with AAV-shControl, AAV-shKdm5b, and/or AAV-shKdm5c and/or AAV-shAhR vectors. Gene expression and epigenetic changes after *Kdm5b/5c* knockdown were assessed by RNA-sequencing and H3K4me3 chromatin immunoprecipitation analysis. We found that less than 5% of genes affected by *Kdm5b*/*Kdm5c* knockdown were common between males and females. In females, *Kdm5b*/*Kdm5c* knockdown prevented fibrosis development in mice fed the WDA alcohol diet for 16 weeks and decreased fibrosis-associated gene expression in mice fed the Lieber-DeCarli alcohol liquid diet. In contrast, fibrosis was not affected by *Kdm5b*/*Kdm5c* knockdown in males. We found that KDM5B and KDM5C promote fibrosis in females through down-regulation of the aryl hydrocarbon receptor (AhR) pathway components in hepatic stellate cells. *Kdm5b*/*Kdm5c* knockdown resulted in an up-regulation of *Ahr, Arnt*, and *Aip* in female but not in male mice, thus preventing fibrosis development. *Ahr* knockdown in combination with *Kdm5b/Kdm5c* knockdown restored profibrotic gene expression. *Conclusion:* KDM5 demethylases contribute to differences between males and females in the alcohol response in the liver. The KDM5/AhR axis is a female-specific mechanism of fibrosis development in alcohol-fed mice.

INTRODUCTION

Alcohol-associated liver disease (ALD) is a complex disease. It has long been recognized that the consequences of alcohol consumption are different in males and females.[1–5] Females have higher mortality in acute alcohol-associated hepatitis, even though men have lower median platelet counts and higher serum creatinine, alanine aminotransferase, and gamma-glutamyltransferase concentrations.[1] Several studies indicate that alcohol metabolism is different between males and females.[4–6] Other studies have noted differences in expression of heat shock proteins, hepatocyte proliferation, interleukin (IL)–6 signaling pathway, and alcohol-induced hepatocyte apoptosis.[7] Several studies reported that males and females have differences in inflammatory responses to alcohol.[2] Some of the differences were reported to be dependent on the sex-specific hormone milieu of the animal, but others persist even in cells isolated from males and females independently of endogenous hormones.[4]

Several liver-enriched transcription factors are reported to control sex-biased gene expression in the liver, including aryl hydrocarbon receptor (AhR).[8–10] AhR is a xenobiotic receptor that senses various environmental toxins and regulates xenobiotic metabolism.[11] After binding by either exogenous or endogenous ligands, AhR translocates into the nucleus, where it heterodimerizes with the AhR nuclear translocator (ARNT) and regulates the transcription of genes involved in xenobiotic metabolism, inflammatory response, and metabolic reprogramming. Studies using $Ahr^{-/-}$ mice have implicated AhR and its endogenous ligands in tissue development, energy metabolism, gut microbiota, stem cell differentiation, circadian rhythm, and adaptive immunity.[11–18]

In addition to its function as a transcriptional factor, AhR also participates in the proteasome-dependent protein degradation that targets selected proteins in the Cullin4B E3 ligase complex. The substrate proteins for the CUL4B-AhR complex include estrogen receptor α, androgen receptor, and β-catenin.[10,19] This role of AhR in sex steroid metabolism may explain why either the loss of AhR or AhR activation affects sex-biased gene expression. Several reports indicate that AhR activation can diminish the divergence between the sexes of liver-specific genes.[9] On the other hand, Ahr inactivation leads to dysregulation of hepatic growth hormone signaling components and suppression of signal transducer and activator of transcription 5b target genes, resulting in feminization of the liver.[8]

The role of AhR in liver fibrosis is controversial.[12,13,18] Both loss and gain of AhR activity can lead to liver fibrosis. Recent studies showed that *Ahr* is expressed at high levels in quiescent hepatic stellate cells (HSCs), but the expression decreased with HSC activation. Mice lacking *Ahr* in HSCs, but not hepatocytes or Kupffer cells, develop more severe fibrosis, suggesting that HSC AhR plays a role in maintaining HSC quiescence, thus preventing fibrosis development.[13]

In this work we have shown that alcohol-associated liver fibrosis development is regulated differently in males and females. We identified two transcriptional regulators involved in sex differences in fibrosis development. These are the histone lysine demethylases specific to histone H3K4 methylation: KDM5B and KDM5C. KDM5B and KDM5C play a central role in epigenetic gene repression. They were first identified for their role in brain development.[20] In addition, KDM5 demethylases are involved in cancer development,[21–24] inflammation control,[25] and replication stress response.[26] Recently we have shown that they regulate alcohol response in the liver in a sex-specific way.[27]

In this work we found that in alcohol-fed mice, KDM5B and KDM5C mediate female-specific gene-expression changes involved in fibrosis development. We identified the aryl hydrocarbon receptor as one of the targets of KDM5 demethylases and showed that KDM5-mediated AhR inhibition is a female-specific mechanism of fibrosis development. Taken together, these results show that KDM5 demethylases are regulators of sex-specific mechanisms in the hepatic response to alcohol.

METHODS

Mice and feeding procedures

Six 7-week-old C57BL6/J mice were purchased from Jackson Laboratory. All mice were housed in a temperature-controlled, specific pathogen-free environment with 12-hours light–dark cycles. All animal handling procedures were approved by the Institutional Animal Care and Use Committee at the University of Kansas Medical Center (Kansas City, KS).

Lieber-DeCarli liquid diet feeding was performed as previously described.[28] Both male and female mice received control or alcohol liquid diet (4.8% alcohol) for 3–5 weeks. For the previously described western diet alcohol model (WDA),[29] both male and female mice were fed *ad libitum* Western diet (WD; Research Diets, Inc., Cat. No. D12079B, 40% calories from fat [90% milk fat, 10% corn oil], 0.2% cholesterol), and alcohol was given *ad libitum* in water at indicated concentrations. Mice in alcohol groups received progressively increasing amount of alcohol in water (1%, 3%, 10%, 15%, and 20% for 3 days each). After reaching 20%, mice were then alternated between 20% (4 days: Thursday until Monday) and 10% (3 days: Monday until Thursday). Mice were caged in groups of two to five mice per cage, but for individual experiments all mice were caged with the same number of animals per cage.

Vectors

AAV8-U6-m-AhR-shRNA and AAV8-GFP-U6-scrmb-shRNA, and AAV9-U6-m-Kdm5b-shRNA, AAV9-U6-m-Kdm5c-shRNA, and AAV9-U6-scrmb-shRNA were from Vector BioLabs (Malvern, PA).

Human and mouse short hairpin RNA (shRNA) vectors were from Sigma-Aldrich (Cat #TRCN0000014759, TRCN0000379331, TRCN0000295348, and TRCN0000022087).

Antibodies

Anti-KDM5B, Ani-KDM5C, and Anti-H3K4me3 antibodies were from Cell Signaling Technology (Beverly, MA). Anti-β-actin, anti-AhR, anti–α-smooth muscle actin and anti–collagen type I alpha 1 chain (Col1a1) antibodies were from Santa Cruz.

Liver cell isolation

Liver cells were isolated by a modification of the method described by Troutman et al.[30] Mouse livers were digested by retrograde perfusion with liberase through the inferior vena cava. The dissociated cell mixture was placed into a 50-ml conical tube and centrifuged twice at 50*g* for 2 min to pellet hepatocytes. The non-parenchymal cells (NPC)-containing cell supernatant was further used to isolate Kupffer cells (KCs), liver sinusoidal endothelial cells (LSECs), and HSCs. The cell suspension was pelleted by centrifugation (700*g*, 10 min, 4°C) and resuspended in phosphate-buffered saline (PBS) and OptiPrep (Sigma) to a final concentration of 17%. Afterward, 5 ml of the indicated suspension was placed in a 15-ml polystyrene conical centrifuge tube (BD Biosciences) and overlaid with 5 ml of a 9% Optiprep solution followed by 2 ml of PBS. After centrifugation at 1400*g* for 20 min at 4°C with decreased acceleration and without breaks, the various cell types were arranged according to their density. HSCs were enriched in the upper cell layer, whereas KCs and LSECs were separated as a second layer of higher density. Cell fractions were collected separately by pipetting. HSC purity over 99% was confirmed by retinoid-based fluorescence-activated cell sorting.

scRNA sequencing

Single-cell RNA sequencing (scRNA-seq) analysis was performed as previously described.[27] Live hepatocytes and NPC cells were purified using the dead cell removal kit (MiltenyiBiotec). Liver cells were immediately used to generate barcoded complementary DNA cDNA libraries using a 10× Genomics Chromium platform (10× Genomics, Pleasanton, CA) with a total input of 10,000 cells per condition. Single-cell sequencing libraries were constructed using the NovaSeq 6000 Sequencing System (Illumina, Inc., San Diego, CA) targeting 10,000 cells at 25,000 reads per cell. For scRNA-seq computational analyses, raw sequencing data were processed using the 10× Genomics Cell Ranger pipeline (version 1.3). First, cellranger demultiplexed libraries based on sample indices and converted the barcode and read data to FASTQ files. Second, cellranger took FASTQ files and performed alignment, filtering, and unique molecular identifier counting. Generated output files were loaded in the Seurat R toolkit[31] and Loupe Cell Browser for single-cell data visualization and interpretation.

RNA sequencing

For RNA-sequencing (RNA-seq) analysis, total RNA was isolated from liver using the Qiagen RNA isolation kit. Three individual mice per condition were used. Library generation and sequencing was performed by BGI genomics services (BGI, Cambridge, MA). Twenty-four samples were sequenced using the BGISEQ platform, on average generating about 4.57G Gb bases per sample. HISAT was used to align the clean reads to the reference genome. Bowtie2 was used to align the clean reads to the reference genes. The average mapping ratio with a reference genome (GRCm38.p6) was 96.14%, and 16,869 genes were identified. Differential gene expression was identified with DESeq2.

Chromatin immunoprecipitation sequencing

Chromatin immunoprecipitation sequencing (ChIP-seq) was performed by Active Motif (Carlsbad, CA) using H3K4me3 antibodies (Cat # 39159) at 4 μl per chromatin sample. Peaks were called using the MACS 2.1.0 algorithm. MACS cutoff was p value = 1×10^{-7} for narrow peaks and 1×10^{-1} for broad peaks. Peak filtering was performed by removing false ChIP-seq peaks as defined within the ENCODE blacklist. Top differential regions were identified using DESeq2. A differential region motif analysis was performed using HOMER.

ChIP

ChIP was performed as described previously.[32,33] Whole liver cells (1×10^7) were cross-linked by the addition of 1% formaldehyde for 10 min. Cells were lysed with 10 mM Tris–HCl (pH 8.0), 10 mM NaCl, 3 mM $MgCl_2$, and 0.5% NP-40. Nuclei were collected by

centrifugation, resuspended in 1% sodium dodecyl sulfate, 5 mmol/L ethylene diamine tetraacetic acid (EDTA), and 50 mmol/L Tris–HCl (pH 8.0) and sonicated to generate chromatin to an average length of about 100 to 500 bp. Next, samples in 1% Triton X-100, 2 mM EDTA, 20 mM Tris–HCl (pH 8.1), and 150 mM NaCl were immunoprecipitated overnight at 4°C with 4 μg ChIP-grade antibody. A total of 20 μl of magnetic beads (Dynabeads M-280; Invitrogen) were used to purify immunocomplexes. Following purification, cross-links were reverted by incubation at 65°C for 6 hours. Samples were purified with a Qiagen DNA purification kit.

Human samples

Liver tissue microarray containing de-identified human liver cirrhosis samples was purchased from US Biolab.

De-identified liver transplant explant samples were obtained from KUMC Liver Bank and used for whole liver RNA isolation.

Immunohistochemistry/ immunofluorescence

Liver tissue sections (5-μm thick) were prepared from formalin-fixed, paraffin-embedded samples. The quantification of sirius red–stained sections was performed in a blinded manner. Immunostaining on formalin-fixed sections was performed by deparaffinization and rehydration followed by antigen retrieval by heating in a pressure cooker (121°C) for 5 min in 10 mM sodium citrate (pH 6.0) as described previously.[34] Peroxidase activity was blocked by incubation in 3% hydrogen peroxide for 10 min. Sections were rinsed 3 times in PBS/PBS-T (0.1% Tween-20) and incubated in Dako Protein Block (Dako) at room temperature for 1 hours. After removal of blocking solution, slides were placed into a humidified chamber and incubated overnight with a primary antibody, diluted 1:300 in Dako Protein Block at 4°C. Antigen was detected using the SignalStain Boost immunohistochemistry (IHC) detection reagent (catalog #8114; Cell Signaling Technology), developed with diaminobenzidene (Dako, Carpinteria, CA), counterstained with hematoxylin (Sigma-Aldrich), and mounted.

Alternatively, slides were incubated with fluorescent Alexa Flour–conjugated secondary antibodies diluted 1:300 in 0.1 μg/ml 4′,6-diamidino-2-phenylindole in Dako Protein Block for 1 hours at room temperature. Slides were then washed with PBS and mounted with FluorSave Reagent (Calbiochem, La Jolla, CA).

Signal intensity for both IHC and immunofluorescence was analyzed by Aperio ImageScope 12.1. A total of five random field were quantified in a blinded way to obtain average signal intensity.

Real-time polymerase chain reaction

RNA was extracted from livers using the RNeasy Mini Kit (Qiagen). cDNA was generated using the RNA reverse-transcription kit (Applied Biosystems, Cat. #4368814). Quantitative real-time reverse-transcription polymerase chain reaction was performed in a CFX96 Real-Time System (Bio-Rad) using specific sense and antisense primers combined with iQ SYBR Green Supermix (Bio-Rad) for 40 amplification cycles: 5 s at 95°C, 10 s at 57°C, and 30 s at 72°C. Messenger RNA (mRNA) concentrations were calculated relative to *Actb*.

Actb fwd	ATGTCACGCACGATTTCCCT
Actb rvs	CGGGACCTGACAGACTACCT
Col1a1-fwd	TGGCCAAGAAGACATCCCTG
Col1a1-rvs	GGGTTTCCACGTCTCACCAT
Aip fwd	CGGACCTGTTTCGGCAACTC
Aip rvs	AAGCTTGTGCTTTCAAAATGGC
Arnt fwd	GGGGAACTGGCAACACATCTA
Arnt rvs	TCTGCTGCCTCCAAGATCAAA
Ahr fwd	TTCCGCCCGGTCTTCTGTAT
Ahr rvs	TACCTGGGCTTTCAGCAGTC
Kdm5b fwd	AGAGCAAGACCACGGCATTC
Kdm5b rvs	GTACCATTGGGCAGGTAGGC
Kdm5c fwd	TATGGGGCTGACATCCATTCC
Kdm5c rvs	TTAGGTGCCGCTTACTGTCA
Tgfb1-fwd	TACGTCAGACATTCGGGAAGC
Tgfb1-rvs	TTTAATCTCTGCAAGCGCAGC

Statistics

Results are expressed as mean ± SD. The Student *t* test, paired *t* test, Pearson's correlation, or one-way analysis of variance with Bonferroni *post hoc* test was used for statistical analyses. p value < 0.05 was considered significant.

RESULTS

Alcohol-induced fibrosis is prevented by *Kdm5b* and *Kdm5c* knockdown in female but not in male mice

Recently we performed scRNA-seq analysis to identify sex-specific pathways affected by alcohol in individual subsets of liver cells.[27] We found that males and females dramatically differ in their response to alcohol, and we identified KDM5B and KDM5C as regulators of male/female differences, specifically male-specific alcohol response in hepatocytes.[27]

We further assessed the role of KDM5B and KDM5C in other aspects of ALD. We used two models of ALD: short term Lieber-DeCarli alcohol liquid diet feeding, to assess early events in ALD development, and the 16-week Western diet with alcohol in the drinking water (WDA) model, recently described by our group[29] to study more advanced ALD with fibrosis. We used AAV-shRNA vectors to knockdown *Kdm5b*, or both *Kdm5b* and *Kdm5c*, in the livers of mice fed alcohol.

We found that *Kdm5b* and *Kdm5c* knockdown resulted in a significant decrease of sirius red staining in female mice (Figure 1A) fed WDA diet for 16 weeks. Females that received AAV-shKdm5b and AAV-shKdm5c vectors showed no significant fibrosis development compared with mice that did not drink alcohol (Figure 1A). In contrast, *Kdm5b* and *Kdm5c* knockdown did not affect fibrosis development in males (Figure 1A).

Similarly, we found that *Kdm5b* knockdown alone or *Kdm5b* and *Kdm5c* knockdown together resulted in a significant decrease of transforming growth factor b1 (*Tgfb1*) gene expression in the livers of alcohol liquid diet–fed females, but not male mice in both models of ALD (Figure 1B,C).

As a role of KDM5B and KDM5C in fibrosis has not been previously reported, we confirmed that this mechanism is relevant in human liver using the GTEx database. We found a significant correlation between *KDM5B* and *TGFB1* or *COL1A1* gene expression in these data (Figure 1D).

Next, we assessed the correlation between *KDM5B* and *COL1A1* by staining in human liver disease specimens (Figure 1E). We found a significant correlation between *KDM5B* and *COL1A1* in female samples (r = 0.67, p value < 0.05). In contrast, no significant correlation was found in male samples (Figure 1E). In addition, we analyzed correlation between *KDM5B* and *TGFB1* gene expression in samples from male and female transplant explant samples from KUMC Liver Bank. We found a positive correlation between *KDM5B* and *TGFB1* gene expression in samples from female patients (r = 0.61), while no significant correlation was observed in samples from male patients (Figure 1E).

Finally, we used a previously published data set, GSE48452, to assess the correlation between *KDM5B* and fibrosis-related gene expression in the liver. We observed that *KDM5B* gene expression was slightly higher in female patients with fibrosis compared to patients without fibrosis. In addition, expression in female patients was significantly higher than in male patients (Figure 1F, left). Moreover, we found a significant correlation between *KDM5B* and *TGFB1* or *COL3A1* gene expression in samples from female patients (r = 0.38 and 0.37, p value < 0.05), while no significant correlation was observed in samples from male patients (Figure 1F).

Taken together, these data suggest that KDM5 demethylases control alcohol-induced fibrosis development in female but not male mice and humans.

Kdm5b and *Kdm5c* knockdown results in female-specific changes in immune response and fibrosis-related gene expression

Our data suggest that although males and females both develop fibrosis after alcohol exposure, the mechanism of fibrosis development is different.

We analyzed differentially regulated genes in alcohol-fed males and females by whole liver mRNA RNA-seq analysis (GSE195889; Figure 2A,B). To assess functional enrichment in differentially regulated genes, we used the DAVID tool.[35] We found that sex-biased genes (p < 0.01) in alcohol-fed animals were related to immune response signaling (Figure 2B). Most of these genes were up-regulated in females, and several of these genes were previously implicated in HSC activation and fibrosis development, including *Il13ra*, *Cd74*, *Tyrobp*, *Itgb2*, *Lum*, and others.[36–38]

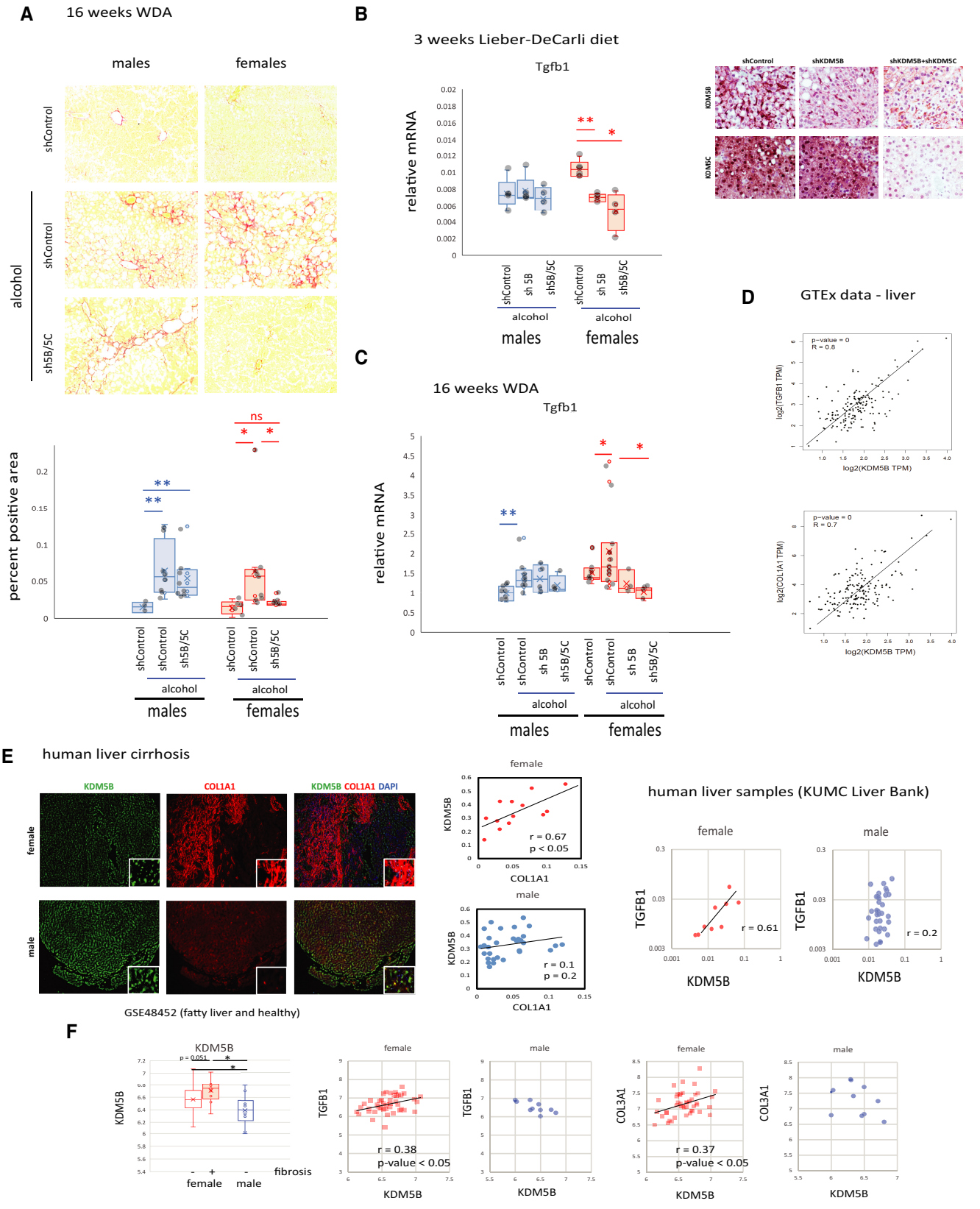
To find the targets of KDM5B/KDM5C demethylases in alcohol-fed females, we performed RNA-seq analysis of whole-liver mRNA after gene knockdown (GSE195889; Figure 2C–E). In agreement with predicted sex differences, *Kdm5b/Kdm5c* knockdown resulted in multiple gene-expression changes in females; however, only a few of these were common between males and females (Figure 2C).

We observed that there is an overlap between genes differentially regulated in males and females fed alcohol and genes affected by *Kdm5b/Kdm5c* knockdown in females (Figure 2D). Moreover, genes up-regulated in females compared with males were down-regulated by the knockdown and vice versa (Figure 2D), suggesting that in alcohol-fed females, KDM5B and KDM5C contribute to sex-biased gene expression.

Top genes affected by *Kdm5b/Kdm5c* knockdown in females included immune response genes (*Il13ra2*, *Tnfrsf1b*, *Cxcl9*, and others), genes associated with fibrosis signaling (e.g., *Lum*, *Itgav*, *Col3a1*), and genes related to proliferation and cell cycle (Figure 2E).

KDM5B and KDM5C regulate a large variety of genes, and their function and gene target set are dependent on context (i.e., cell type, stimulus, or disease state).[22,24,39] AAV-mediated shRNA delivery produced significant *Kdm5b* and *Kdm5c* knockdown in multiple cell types, including hepatocytes, endothelial cells, and HSCs (Figure 3A).

To determine which cell types contributed to gene-expression changes after *Kdm5b/Kdm5c* knockdown, we assessed gene expression of fibrosis and inflammation-associated genes in individual cell clusters using the scRNA-seq data set we previously



obtained.[27] We reclustered cells from females fed alcohol diet and analyzed gene expression in individual clusters. We found that top differentially regulated fibrosis and inflammation associated genes such as *Col3a1*, *Itgav*, *Cfh*, *C1s1*, and *Gabarapl1* were predominantly expressed in HSCs, whereas a few of the others were more specific to macrophages (*Tnfrsf1b*) or endothelial cells (*Lifr*) (Figure 3B). These data suggest that KDM5B

FIGURE 1 *Kdm5b* and *Kdm5c* knockdown prevents alcohol-induced fibrosis in female mice. (A) Male and female mice were fed *ad libitum* Western diet and given either plain water or water containing alcohol (alternating 10% and 20%) for 16 weeks. AAV as indicated was injected intraperitoneally at 10^{11} gc per mouse. Top: Sirius red staining in livers of mice; bottom: percent positive area of sirius red staining. Percent positive area was quantified using Aperio ImageScope 12.1. A total of five random fields were analyzed in a blinded way to obtain average for each of n = 4–6 mice per group (*$p < 0.05$, **$p < 0.01$). (B,C) Male and female mice were fed alcohol Lieber-DeCarli liquid diet for 3 weeks or western diet alcohol model (WDA) diet for 16 weeks (n = 4–6 mice per group). AAV as indicated was injected intraperitoneally at 10^{11} gc per mouse. Relative messenger RNA (mRNA) expression in livers of mice (*$p < 0.05$, **p value < 0.01). (B) Right: Representative images of immunohistochemistry (IHC) staining for KDM5B and KDM5C proteins in livers of mice in indicated groups. (D) Correlation between *KDM5B* and fibrosis-related gene expression in human liver specimens from GTEx database. (E) Human liver-disease tissue sections were stained with anti-KDM5B and anti–collagen type I alpha 1 chain (COL1A1) specific antibodies. Left: Examples of staining in samples from male and female patients. Middle: Correlation between KDM5B and COL1A1 staining intensity in males (n = 50) and females (n = 13). Right: Correlation between *KDM5B* and *TGFB1* gene expression in male (n = 35) and female (n = 10) transplant explants from KUMC liver Bank. (F) Left: *KDM5B* gene expression in liver samples from male (n = 10) and female (n = 44) patients with mild liver disease or healthy controls (GSE48452). Right: Correlation between *KDM5B* and transforming growth factor B1 (*TGFB1*) or *COL3A1* gene expression in males and females. DAPI, 4′,6-diamidino-2-phenylindole; ns, not significant

and KDM5C regulate gene expression in HSCs as well as other cell types.

We further confirmed that KDM5B might play a role in activation of stellate cells by IHC analysis of KDM5B protein expression in females fed alcohol for 16 weeks (Figure 3C). We found that KDM5B expression was elevated in COL1A1-positive cells in mouse liver. To assess the role of *Kdm5b* in HSCs, we isolated mouse HSCs and treated them with shControl or shKdm5b vector. We found that *Kdm5b* knockdown resulted in a decrease in levels of *Col1a1* and *Tgfb1*, suggesting that KDM5B might contribute to HSC activation and collagen production (Figure 3D). In addition, we found that *Kdm5b* knockdown decreased levels of *Col1a1* and *Tgfb1* in HSCs isolated from female but not male mice (Figure 3D).

Taken together, KDM5 demethylases regulate fibrosis-associated gene expression in HSCs in a sex-specific way.

Kdm5b and *Kdm5c* knockdown results in H3K4 methylation changes at AhR-dependent promoters

To further determine the mechanism of KDM5B/KDM5C-dependent regulation of fibrosis-associated genes in the liver, we studied global epigenetic changes induced by *Kdm5b/Kdm5c* knockdown in alcohol-fed mice using ChIP-seq analysis of H3K4me3 modifications.[27] Increased promoter histone methylation after knockdown correlated with increased gene expression in the livers (Figure 4A). Motif analysis of peaks that were increased after *Kdm5b/Kdm5c* knockdown showed an enrichment of a motif similar to the motif for AhR/ARNT binding (Figure 4B). AhR is known to suppress fibrosis in the liver. AhR-null mice have reduced liver size and develop liver fibrosis in part through abnormal levels of active TGFβ.[12,18] These data suggest that KDM5B/KDM5C can suppress AhR-dependent gene expression, thus mediating fibrosis development.

In human liver sample databases, we found a significant negative correlation between *KDM5B* gene expression and expression of AhR target genes such as *CYP1A1* and *CYP1A2* (Figure 4C), suggesting that KDM5B might negatively regulate AhR activation in humans.

Alcohol reduces *Ahr* gene expression in the liver of both male and female mice (Figure 4D). By analyzing protein expression by IHC, we found that, in females, alcohol reduced AhR levels in all cell types, whereas in males, expression in nonparenchymal cells was still present after alcohol feeding (Figure 4D). By using scRNA-seq data, we observed that *Ahr* gene expression was significantly down-regulated in alcohol-fed females compared with males in HSCs and not in other cell types (Figure 4E, left). We confirmed this finding by isolating HSCs from males and females and measuring *Ahr* gene expression. We found that female HSCs have significantly lower levels of *Ahr*, whereas the expression of *Col1a1*, a marker of HSC activation, was increased (Figure 4E, right).

Kdm5b and *Kdm5c* knockdown results in *Ahr* up-regulation in female mice

We found that *Kdm5b/Kdm5c* knockdown in alcohol-fed mice results in a small H3K4 methylation increase at the promoters of *Ahr* itself and the genes encoding AhR-interacting proteins AIP and ARNT (Figure 5A), suggesting that KDM5B can regulate AhR by promoter binding to *Ahr* and other AhR pathway genes.

Using mouse primary cells, we confirmed that *Kdm5b* or *Kdm5c* knockdown results in an up-regulation of these genes in mouse HSCs (Figure 5B). Next, we compared the ability of KDM5B and KDM5C to regulate *Ahr* and *Aip* gene expression in HSCs isolated from male and female mice (Figure 5C). We found that *Kdm5b/Kdm5c* knockdown results in up-regulation of *Ahr* and *Aip* gene expression in cells isolated from females but not males. Moreover, treatment with a selective estrogen receptor

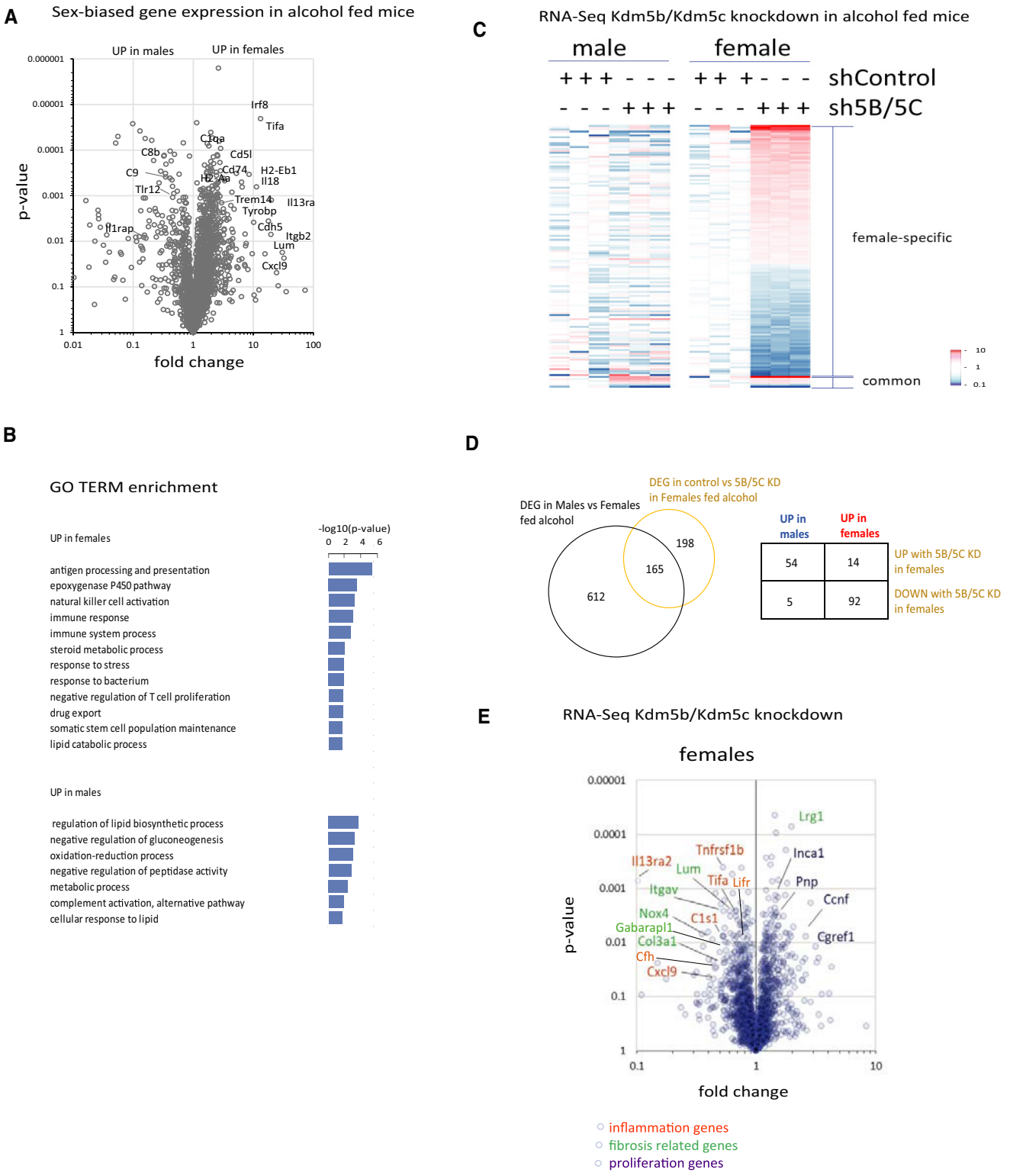


FIGURE 2 KDM5B and KDM5C regulate female-specific gene expression in alcohol-fed mice. (A,B) Male and female mice were fed alcohol Lieber-DeCarli liquid diet for 3 weeks. (A) Volcano plot of differentially regulated genes in male and female mice (n = 3 mice per group). (B) Gene Ontology (GO) TERM enrichment in genes up-regulated in males and females. (C–E) Male and female mice were fed alcohol Lieber-DeCarli liquid diet for 3 weeks. Mice received AAV-shControl at 2×10^{11} gc per mouse or AAV-shKdm5b and AAV-shKdm5c at 1×10^{11} gc of each virus per mouse (n = 3 mice per group). (C) Top differentially expressed genes in female mice. (D) Venn diagrams of differentially regulated genes between males (M) and females (F) fed alcohol and between female mice fed alcohol that received shControl or shKdm5b/shKdm5c vectors (5B/5C KD in females). (E) Volcano plot of differentially regulated genes in female mice fed alcohol that received shControl or shKdm5b/shKdm5c vectors

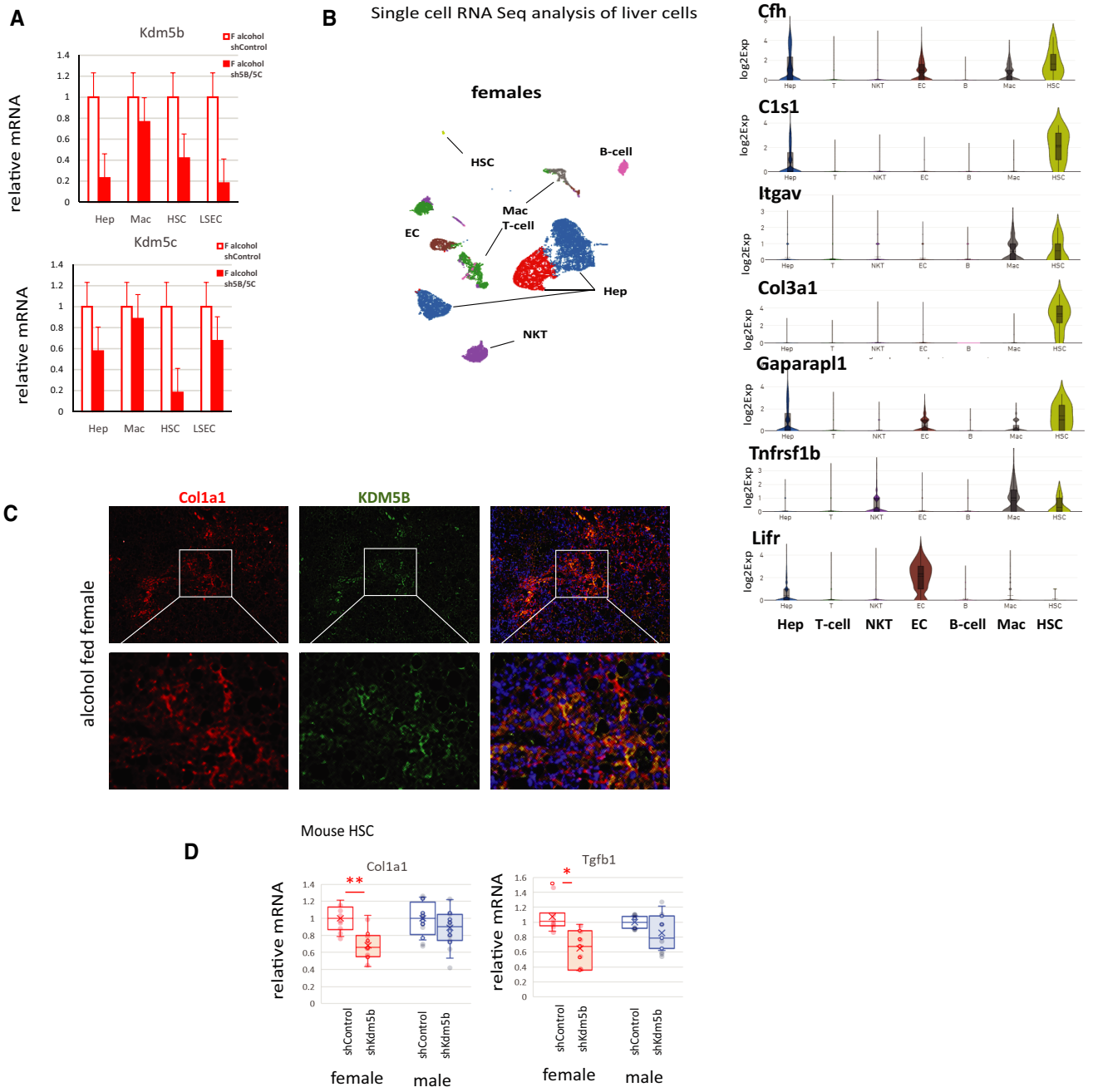


FIGURE 3 KDM5B regulate fibrogenic gene expression in hepatic stellate cells (HSCs). (A) Relative mRNA levels in hepatocytes (hep), macrophages (mac), HSCs, and liver sinusoidal endothelial cells (LSECs) isolated from mice that received AAV-shControl at 2×10^{11} gc per mouse or AAV-shKdm5b and AAV-shKdm5c at 1×10^{11} gc of each virus per mouse (n = 3 mice per group). (B) Single-cell RNA-sequencing (scRNA-seq) analysis of liver cells from female mice fed alcohol Lieber-DeCarli liquid diet. Gene-expression cells from clusters of HSC macrophages (mac), endothelial cells (EC), T cells, B cells, natural killer T cells (NKT), and hepatocytes (hep). (C) Mice were fed WDA diet for 16 weeks. Representative images of immunofluorescence (IF) staining for COL1A1 (red) and KDM5B (green), and merge with DAPI (blue). (D) Relative mRNA expression in HSCs isolated from male and female mice treated with shControl or shKdm5b vectors (n = 4–6 independent experiments; $*p < 0.05$)

modulator (tamoxifen) prevented the ability of KDM5B and KDM5C to regulate *Ahr* and *Aip* (Figure 5B), suggesting that estrogen signaling in females is involved in AhR inhibition through activation of KDM5 demethylases.

Next, we examined KDM5B binding to the promoters of these genes in the presence of alcohol (Figure 5D). We found that alcohol increases KDM5B binding to the promoters in females but not in males, and the knockdown reduces the binding (Figure 5D). As a result, we found that *Kdm5b/Kdm5c* knockdown increased expression of *Ahr*, *Arnt*, and *Aip* specifically in female livers in mice fed WDA for 16 weeks (Figure 5E). In mice fed Lieber-DeCarli diet, we observed similar results

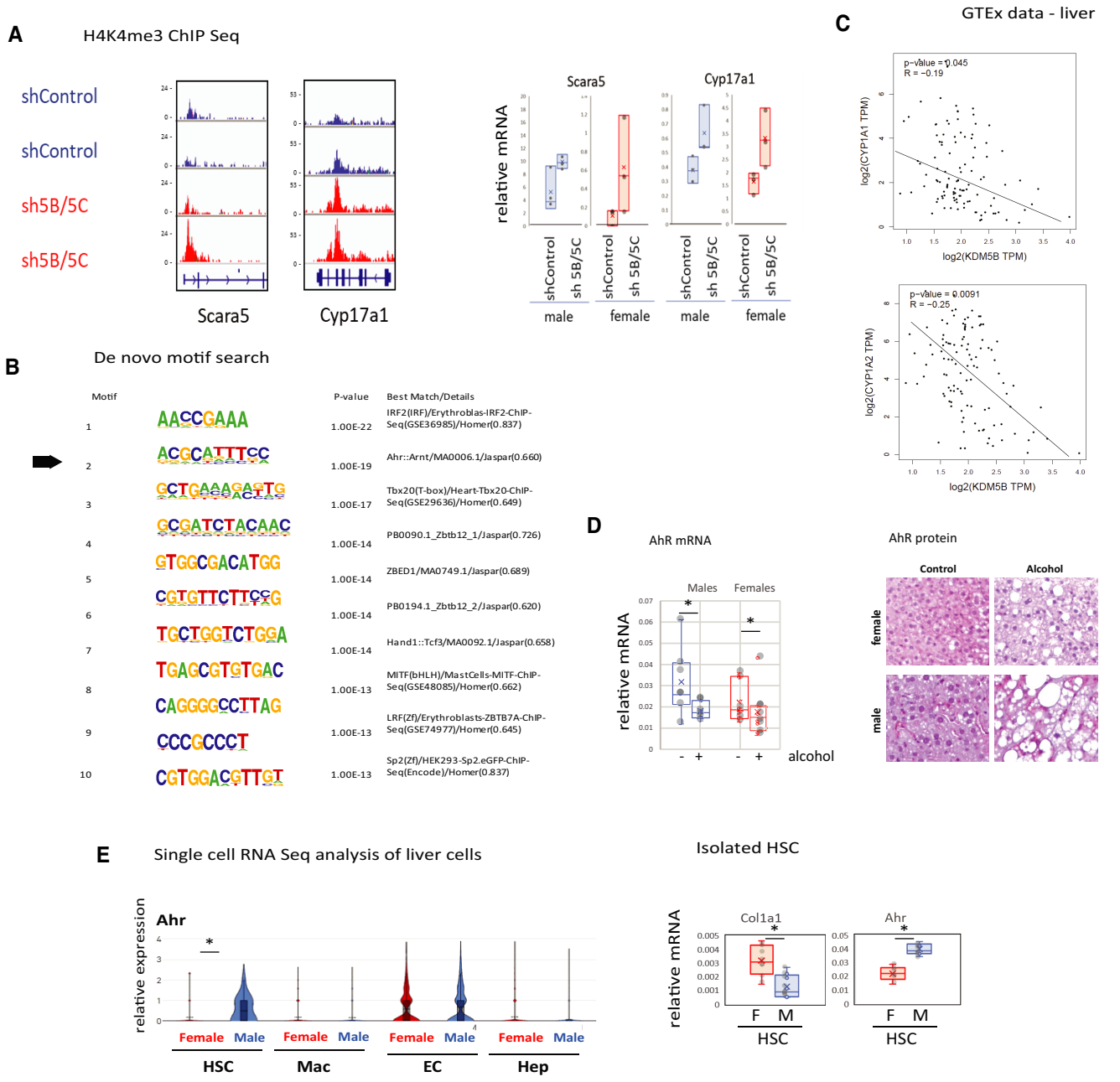


FIGURE 4 KDM5B and KDM5C regulate aryl hydrocarbon receptor (AhR) signaling. (A,B) Chromatin immunoprecipitation sequencing (ChIP-seq) analysis of whole livers from alcohol-fed mice (WDA for 16 weeks). Mice received AAV-shControl at 2×10^{11} gc per mouse or AAV-shKdm5b and AAV-shKdm5c at 1×10^{11} gc of each virus per mouse (n = 3 mice per group). (A) Examples of differentially regulated regions in *Scara5* and *Cyp17a1* genes and mRNA expression changes in alcohol-fed mice after *Kdm5b* and *Kdm5c* knockdown. (B) *De novo* motif search in peaks up-regulated by *Kdm5b/Kdm5c* knockdown. (C) Correlation between KDM5B and AhR-dependent gene expression in human liver specimens from GTEx database. (D) Left: Relative mRNA expression in mice fed alcohol or control diet (WDA model; n = 6–8 per group; $*p < 0.05$). Right: IHC staining in liver section from male and female mice fed alcohol or control diet (WDA model). (E) scRNA-seq analysis of liver cells from mice fed alcohol Lieber-DeCarli liquid diet. Gene expression in male and female cells from clusters of mac, EC, and hep ($*p < 0.05$). Relative mRNA expression in isolated HSCs from male and female mice fed alcohol (n = 4 mice per group; $*p < 0.05$)

for *Arnt* and *Aip* that were up-regulated only in females, whereas *Ahr* was up-regulated in both genders (Figure 5F).

Taken together, KDM5B and KDM5C negatively regulate AhR in female HSCs by increased binding to *Ahr* and other gene promoters in an estrogen receptor–dependent way.

Ahr knockdown abolishes the protective effect of *Kdm5b* and *Kdm5c* knockdown on liver fibrosis in female livers

To assess the role of AhR in fibrosis development in alcohol-fed mice, we used AAV-mediated knockdown of *Ahr* in alcohol-fed mice that either received AAV-shControl

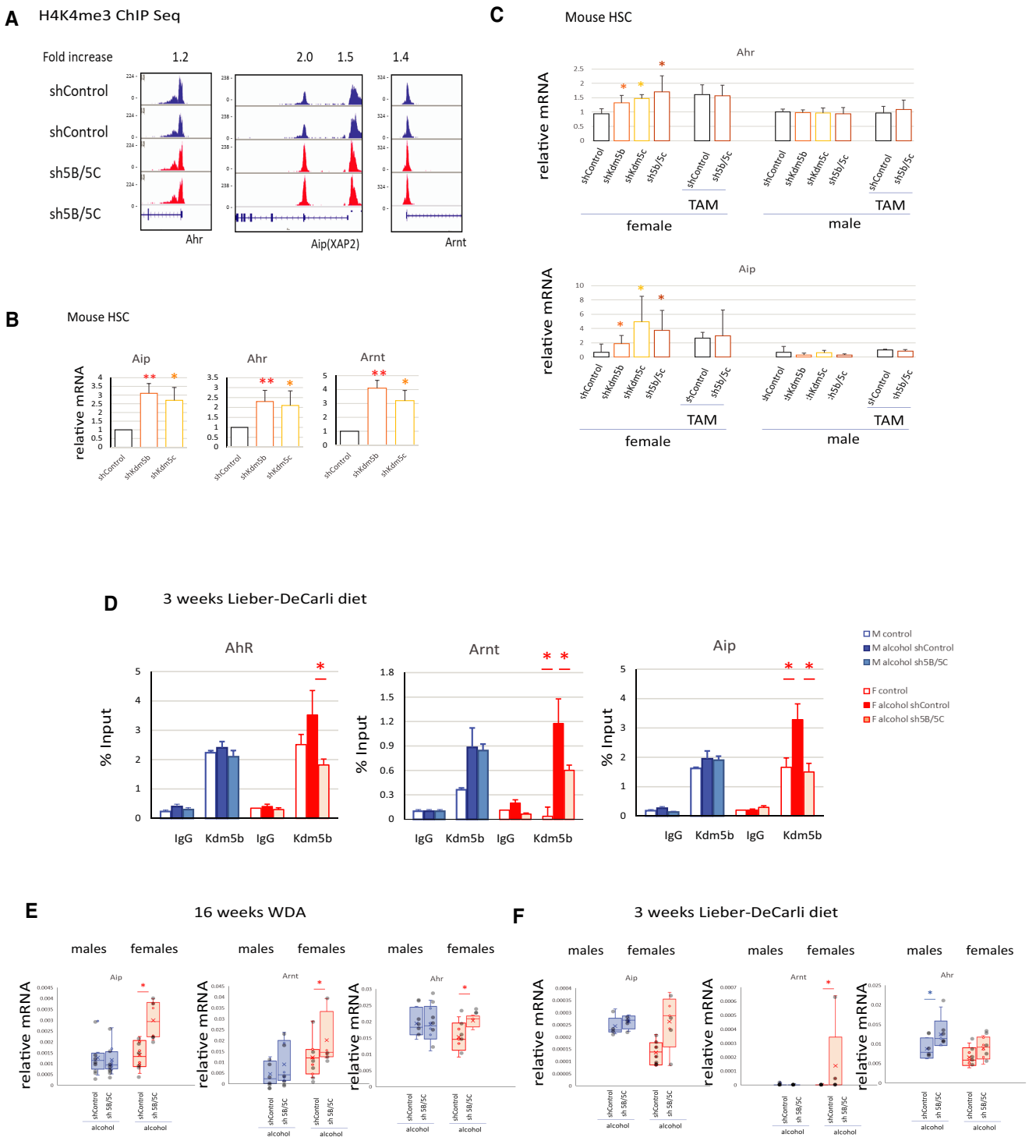


FIGURE 5 KDM5B and KDM5C regulate *Ahr* expression in females. (A) H3K4me3 peaks at the Ahr, AhR nuclear translocator (Arnt), and Aip gene regions. (B) Relative mRNA expression in mouse HSCs that received shControl vector or short hairpin RNA (shRNA) vector specific for *Kdm5b* or *Kdm5c* as indicated (n = 4 independent experiments) (*$p < 0.05$, **$p < 0.01$ compared with shControl). (C) Relative mRNA expression in mouse HSCs that received shControl vector or vector specific for *Kdm5b* or *Kdm5c* or both in the presence of 1 μM tamoxifen where indicated (n = 4 independent experiments; *$p < 0.05$ compared with shControl). (D–F) KDM5B ChIP (D) and relative mRNA expression in livers of mice fed Lieber-DeCarli (E) or WDA (F) diets (n = 4–6 mice per group; *$p < 0.05$)

or AAV-shKdm5b and AAV-shKdm5c (Figure 6A). Gene-expression analysis suggested that in female mice, a subset of genes regulated by *Kdm5b/Kdm5c* knockdown was restored to control levels after *Ahr* knockdown (Figure 6B). These genes were not affected by *Kdm5b/Kdm5c* knockdown or *Ahr* knockdown in males.

Principal component analysis confirmed these observations (Figure 6C). Gene-expression changes

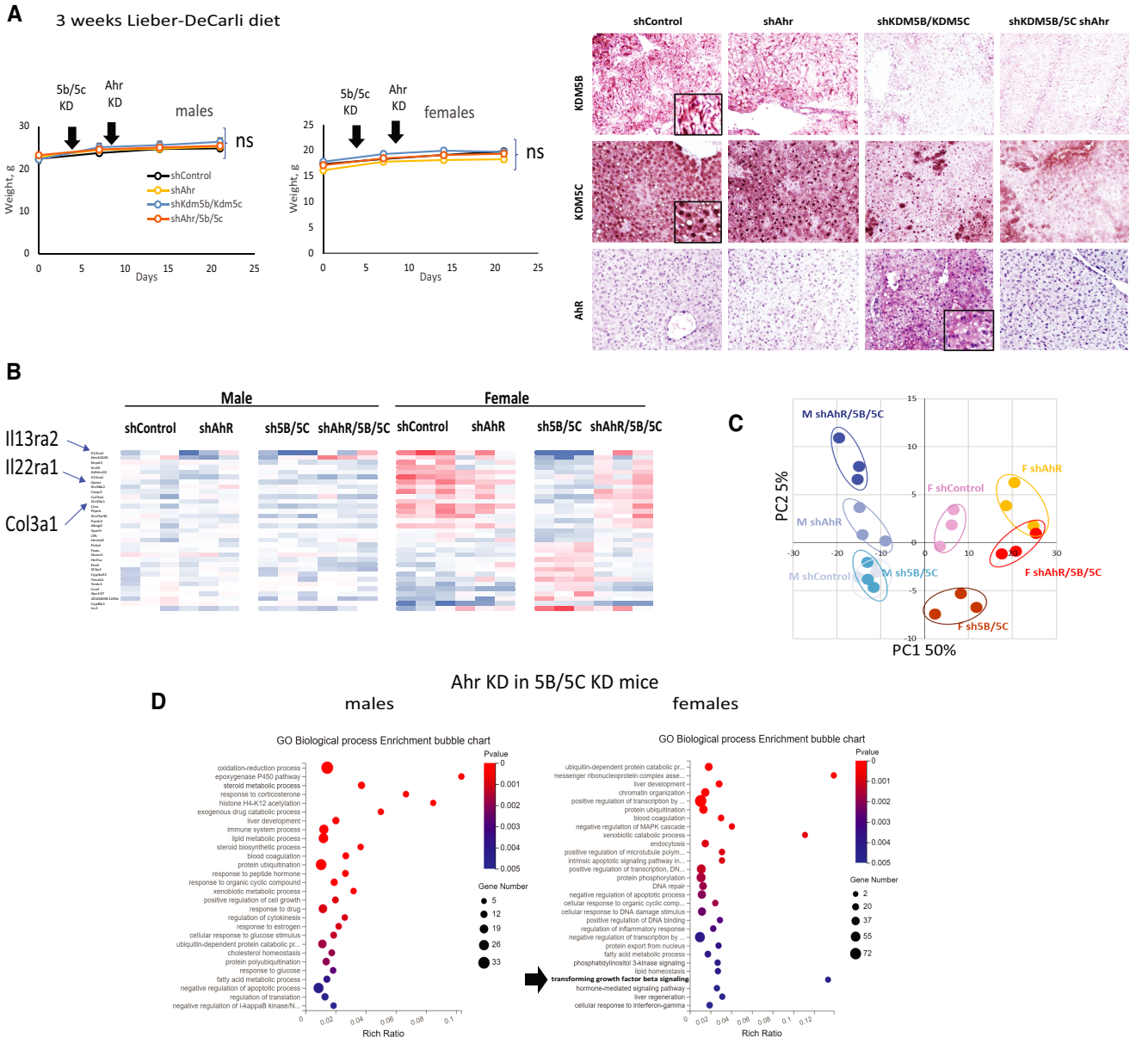


FIGURE 6 *Ahr* knockdown restores gene-expression changes in female mice that received *Kdm5b/Kdm5c*-specific shRNA. Male and female mice were fed alcohol Lieber-DeCarli liquid diet for 3 weeks. Mice received AAV-shControl at 2×10^{11} gc per mouse or AAV-shKdm5b and AAV-shKdm5c at 1×10^{11} gc of each virus per mouse at the first day of alcohol. Three days later, mice received AAV-shControl or AAV-shAhR at 1×10^{11} gc per mouse (n = 5 mice per group). (A) Left: Weight change over time in male and female mice. Right: IHC staining in liver section from male and female mice fed alcohol. (B–D) RNA-sequencing analysis of differentially regulated genes. (B) Relative mRNA expression in eight groups of mice (n = 3 mice per group). (C) Principal component (PC) analysis. (D) GO TERM enrichment of gene differentially regulated by *Ahr* knockdown in males and females that received AAV-shKdm5b and AAV-shKdm5c vectors. MAPK, mitogen-activated protein kinase

induced by *Kdm5b/Kdm5c* knockdown in females represented by the PC2 component were completely reverted to control conditions by *Ahr* knockdown (Figure 6C).

Gene ontology analysis (Dr. Tom tool from BGI Genomics) showed that *Ahr* knockdown in *Kdm5b/Kdm5c* knockdown mice affected genes involved in xenobiotic metabolism both in males and in females (Figure 6D). In contrast, the TGFβ pathway was among the pathways predicted to be regulated by *Ahr* knockdown in *Kdm5b/Kdm5c* knockdown females but not in males (Figure 6D).

Indeed, we found that after *Ahr* knockdown in *Kdm5b/Kdm5c* knockdown mice, there was an increase in gene expression of several fibrosis-related genes that were down-regulated after *Kdm5b/Kdm5c* knockdown (Figure 7A).

Moreover, we found that *Ahr* knockdown restored levels of sirius red–positive staining in alcohol-fed female mice after *Kdm5b/Kdm5c* knockdown (Figure 7B,C).

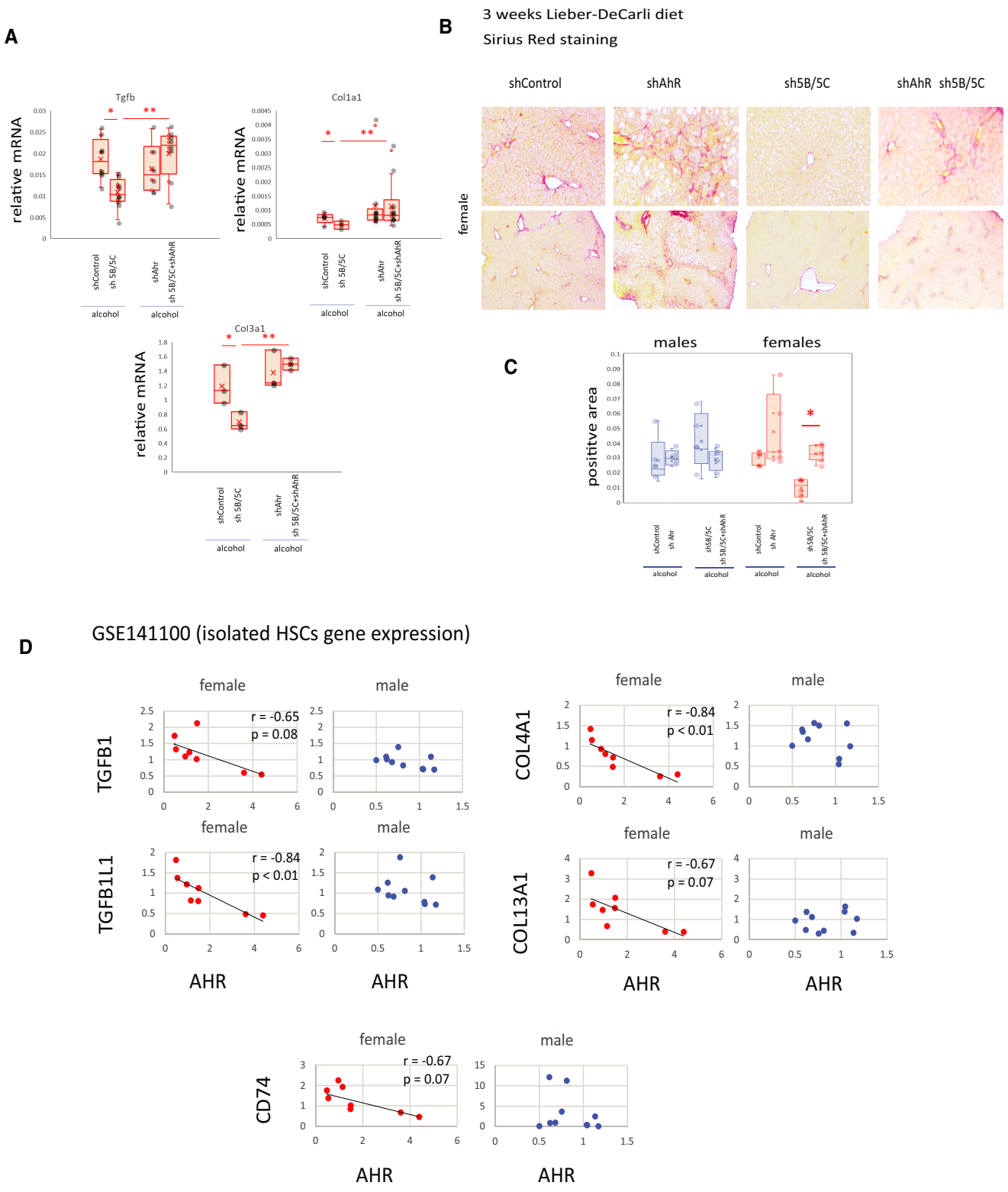


FIGURE 7 *Ahr* knockdown restores fibrosis level in female mice that received *Kdm5b/Kdm5c*-specific shRNA. (A) Relative mRNA expression in livers of female mice as in Figure 6 (n = 3–6 mice per group; *$p < 0.05$, **p value < 0.01). (B) Sirius red staining in livers of female mice. (C) Percent positive area of sirius red staining (n = 5 mice per group; *p value < 0.05). (D) Correlation between *AHR* and fibrosis associated gene expression in HSCs from males (n = 10) and females (n = 8) (GSE 141100)

Neither *Kdm5b/Kdm5c* knockdown nor *Ahr* knockdown affected sirius red–positive staining in alcohol-fed males.

In agreement with these data, we found that *AHR* expression in HSCs (GSE141100) negatively correlates with gene expression of *TGFB1*, and other

fibrosis-associated genes and genes identified to be specifically activated in stellate cells by alcohol, such as *CD74*.[36] This correlation was present in cells isolated from females but not in cells isolated from males.

Taken together, the KDM5/AhR axis is a female-specific mechanism for fibrosis development in the presence of alcohol.

DISCUSSION

ALD is a major cause of alcohol-related mortality.[1,40–42] The mechanisms responsible for ALD development and progression are not fully understood, and there is limited therapy for any stage of ALD.[40,41] Differences between males and females in ALD development are well established. However, the mechanisms of these differences are not fully understood. Sex differences are often attributed to differences in alcohol metabolism.[5–7] However, more recent studies revealed that males and females differ not only in lipid-related hepatocyte apoptosis and oxidative stress pathways, but also in innate and adaptive immunity, fibrosis signaling, growth factor receptor signaling, and other pathways.[3,43–46] Gonadectomy and ovariectomy experiments suggest that these pathways are in part regulated by sex hormone signaling.[44]

In this study we found that liver molecular pathways are altered by alcohol consumption differently in males and females. We identified several differentially regulated pathways in nonparenchymal cells that are associated with inflammation and fibrosis development. We found that H3K4-specific demethylase enzymes are among the top regulators of differentially regulated pathways in males and females, particularly the demethylase KDM5B.

We found that in alcohol-fed mice, KDM5B and another demethylase, KDM5C, have sex-specific roles in the liver after alcohol exposure. Although we were able to show that KDM5B and KDM5C are key regulators of the set of sex-biased alcohol-regulated genes, it is not clear why these demethylases are activated differently in males and females. Only 3% of differentially regulated genes after *Kdm5b/Kdm5c* knockdown were common between males and females (24 of 944 transcripts), and several of these were altered in the opposite direction in males and females.

In female mice, KDM5B and KDM5C are involved in alcohol-induced fibrosis development. They regulate expression of *Tgfb1* and *Col1a1* in mice and *TGFB1* and *COL1A1* in humans in a female-specific way. A role of KDM5B in TGFβ gene expression was previously reported in mammary tissue using whole-body knockout mice.[47] We confirmed a strong correlation between *KDM5B* and *TGFB1* in liver tissue in mice and in human specimens.

Although KDM5C showed primarily nuclear localization, we observed that KDM5B is present in cytosol as well. Previous studies suggested that KDM5B might have targets outside of the nucleus. KDM5B phosphorylation was previously reported to alter KDM5B cytosolic but not nuclear abundance and control its target specificity.[48] Whether cytosolic KDM5B or KDM5B phosphorylation in HSCs is relevant for its function in controlling HSC activation is not clear.

Furthermore, we found that HSC activation requires KDM5B and KDM5C-mediated *Ahr* down-regulation. AhR was previously implicated in liver fibrosis development both directly via suppression of profibrotic gene expression in stellate cells[13] and indirectly via immune-response regulation in hepatocytes and liver immune cells[12,15] or via IL-22 regulation in the gut.[14] We found that *Kdm5b/Kdm5c* knockdown induces *Ahr* and *Arnt* expression in HSCs in the presence of alcohol. *Ahr* knockdown also results in a small increase in fibrosis confirming the role of *Ahr* in ALD fibrosis.

More importantly, *Kdm5b/Kdm5c* knockdown has no effect on profibrotic gene expression in *Ahr* knockdown animals. Taken together, our data suggest that KDM5B and KDM5C in stellate cells are contributing to the KDM5B/KDM5C-dependent fibrosis development through regulation of AhR in HSCs.

Interestingly, we observed alcohol-induced KDM5B/KDM5C-mediated AhR down-regulation only in female mice. This could be due to estrogen receptor signaling in females. Estrogen receptor is known to regulate AhR/ARNT-dependent gene expression,[17,49] and data from *Kdm5b* knockout mice suggest that KDM5B is necessary for estrogen receptor signaling.[47] Several studies suggest that estrogen may be an important pathogenic cofactor in development of alcohol liver injury and alcohol-induced inflammatory cytokine production.[44,46] Estrogen treatment enhances alcohol-induced injury in ovariectomized females.[44] Estrogen receptors are expressed in all liver cells, hepatocytes and nonparenchymal cells, and it has been implicated in KC inflammatory cytokine production and HSC activation. Estrogen regulates signals shown to have crucial roles in pathogenesis of ALD, tumor necrosis factor alpha, TGF-β, and interleukins.[44,45] Our data suggest that estrogen signaling in the presence of alcohol activates KDM5 demethylases to suppress AhR signaling in HSCs and other nonparenchymal cells to promote stellate cell activation and fibrosis development.

Alcohol induces liver fibrosis in both sexes, but the striking finding of our study is that the mechanisms and pathways involved are different in males and females. Male–female differences in fibrosis development are well known. Males are known to be more susceptible to fibrosis development in response to toxins such as TAA or CCl_4. The greater rate of hepatic fibrosis progression seen in men may be due to a higher level

of hepatocellular apoptosis and drug-induced HSC activation. Recent data suggest that sex-biased genetic programs in liver fibrosis are controlled by differences in histone methylation of fibrosis-associated genes.[50] We found that in alcohol-fed mice, females show early activation of a fibrogenic program, which is regulated by histone demethylases KDM5B and KDM5C, in contrast to males, which develop fibrosis by another mechanism.

Overall, our data suggest that the lysine demethylases KDM5B and KDM5C contribute to differences in ALD progression in both males and females. In females, these enzymes are potential therapeutic targets for alcohol-associated fibrosis and cirrhosis. The key message is that different therapeutic approaches may be required in males and females to best prevent and treat ALD. Future studies to identify the mechanisms by which these demethylases are linked to alcohol will be necessary to optimize these approaches.

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CONFLICT OF INTEREST

Nothing to report.

AUTHOR CONTRIBUTIONS

Experiments: Michael Schonfeld and Janice Averilla. *Data analysis:* Michael Schonfeld, Janice Averilla, Steven A. Weinman, and Irina Tikhanovich. *Bioinformatic analysis:* Sumedha Gunewardena. *Study design and manuscript draft:* Steven A. Weinman and Irina Tikhanovich. All authors edited the manuscript and approved the final version.

DATA AVAILABILITY STATEMENT

Raw sequencing data for all reported data sets are available upon request.

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