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Hdac1 Regulates Differentiation of Bipotent Liver Progenitor Cells During Regeneration via Sox9b and Cdk8

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Abstract

Background & Aims: Upon liver injury in which hepatocyte proliferation is compromised, liver progenitor cells (LPCs), derived from biliary epithelial cells (BECs), differentiate into hepatocytes.

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Little is known about mechanisms of LPC differentiation. We used zebrafish and mouse models of liver injury to study the mechanisms.

Methods: We used transgenic zebrafish, Tg(*fabp10a:CFP-NTR*), to study the effects of compounds that alter epigenetic factors on BEC-mediated liver regeneration. We analyzed zebrafish with disruptions of the histone deacetylase 1 gene (*hdac1*) or exposed to MS-275 (an inhibitor of Hdac1, Hdac2, and Hdac3). We also analyzed zebrafish with mutations in *sox9b*, *fbxw7*, *kdm1a*, and *notch3*. Zebrafish larvae were collected and analyzed by whole-mount immunostaining and in situ hybridization; their liver tissues were collected for quantitative reverse transcription PCR. We studied mice in which hepatocyte-specific deletion of β -catenin (*Ctnnb1^{flox/flox}* mice injected with AAV8-TBG-Cre) induces differentiation of LPCs into hepatocytes following a choline-deficient, ethionine-supplemented (CDE) diet. Liver tissues were collected and analyzed by immunohistochemistry and immunoblots. We performed immunohistochemical analyses of liver tissues from patients with compensated or decompensated cirrhosis or acute on chronic liver failure (n=15).

Results: Loss of Hdac1 activity in zebrafish blocked differentiation of LPCs into hepatocytes by increasing levels of *sox9b* mRNA and reduced differentiation of LPCs into BECs by increasing levels of *cdk8* mRNA, which encodes a negative regulator gene of Notch signaling. We identified Notch3 as the receptor that regulates differentiation of LPCs into BECs. Loss of activity of Kdm1a, a lysine demethylase that forms repressive complexes with Hdac1, produced the same defects in differentiation of LPCs into hepatocytes and BECs as observed in zebrafish with loss of Hdac1 activity. Administration of MS-275 to mice with hepatocyte-specific loss of β -catenin impaired differentiation of LPCs into hepatocytes following the CDE diet. HDAC1 was expressed in reactive ducts and hepatocyte buds of liver tissues from patients with cirrhosis.

Conclusions: Hdac1 regulates differentiation of LPCs into hepatocytes via Sox9b and differentiation of LPCs into BECs via Cdk8, Fbxw7, and Notch3 in zebrafish with severe hepatocyte loss. HDAC1 activity was also required for differentiation of LPCs into hepatocytes in mice with liver injury following the CDE diet. These pathways might be manipulated to induce LPC differentiation for treatment of patients with advanced liver diseases.

Graphical Abstract

Keywords

hepatic; development; CDE diet; signal transduction

Introduction

Upon liver injury, hepatocytes proliferate to recover lost liver mass. However, when hepatocyte proliferation is compromised, a phenomenon observed in advanced liver diseases, biliary epithelial cells (BECs) activate or dedifferentiate into liver progenitor cells (LPCs), also called oval cells or ductular reactions, and then differentiate into hepatocytes^{1, 2}. A correlation between disease severity and LPC number in patients with chronic liver diseases³ suggests that BECs are indeed activated in the diseased liver but that the activated LPCs poorly differentiate into hepatocytes. Although LPCs can give rise to hepatocytes, they also secrete inflammatory cytokines that can cause inflammation and subsequent fibrosis⁴. Therefore, it has been hypothesized that promoting differentiation of LPCs into hepatocytes has a two-fold beneficial effect in liver patients: generating more hepatocytes and reducing inflammation. Despite the potential clinical significance of the mechanistic understanding of LPC differentiation, the molecular mechanisms underlying this process are poorly understood.

We⁵ and others^{6, 7} previously reported that following near-complete hepatocyte ablation in zebrafish, BECs extensively contribute to hepatocytes, thereby leading to a full liver recovery. Recently, a similar BEC-driven liver regeneration was observed in mice in the settings of impaired hepatocyte proliferation^{8, 9} and long-term chronic injury¹⁰. Importantly, in human regressed cirrhotic livers, a large percentage of parenchyma appears to originate from LPCs¹¹, implying the importance of BEC-driven liver regeneration in cirrhosis regression. Given the robust BEC-driven liver regeneration in these zebrafish and mouse liver injury models, these models will help one elucidate the molecular mechanisms underlying liver regeneration in advanced liver diseases (i.e., LPC-driven liver regeneration).

In the zebrafish model, the complete ablation of hepatocytes in larvae leads to the collapse of the entire intrahepatic biliary network, and all preexisting BECs dedifferentiate into LPCs¹². During regeneration, a small subset of LPCs differentiate into BECs, with which the intrahepatic biliary network is reestablished. Thus, this zebrafish model will allow one to investigate differentiation of LPCs into BECs as well as into hepatocytes. Given the contribution of LPCs into BECs in rodent¹³ and human¹⁴ biliary injury settings, understanding the mechanisms underlying LPC differentiation into BECs as well as hepatocytes will lead to the comprehensive understanding of the molecular mechanisms underlying LPC-driven liver regeneration. Given the key roles of epigenetics in cellular reprogramming¹⁵, we focused on epigenetic regulators that can control LPC differentiation, because promoting its differentiation may have a beneficial effect on patients with advanced liver diseases. Here, we show the crucial role of the histone deacetylase Hdac1 in differentiation of LPCs into both hepatocytes and BECs and provide two distinct, novel molecular mechanisms by which Hdac1 regulates this differentiation process during regeneration.

Materials and Methods

Zebrafish and mouse studies

All animals are housed in temperature- and light-controlled facilities and are maintained in accordance with the Guide for Care and Use of Laboratory Animals and the Animal Welfare Act. Experiments were performed with approval of the Institutional Animal Care and Use Committee (IACUC) at the University of Pittsburgh. Zebrafish and mouse lines used in this work, detailed analytic methods and human studies are described in the Supplementary Material.

Results

Identification of small molecules that block LPC-to-hepatocyte differentiation during regeneration

To identify chemical agents that affect LPC-to-hepatocyte differentiation, we performed an *in vivo* chemical screen using our established zebrafish model of BEC-driven liver regeneration⁵. A library of known epigenetic compounds was selected, because our previous chemical screening resulted in the identification of BET proteins as an important regulator of BEC-driven liver regeneration¹², raising a possibility that other epigenetic regulators may also play essential roles in this process. For this screen, we used triple transgenic zebrafish: (1) *Tg(fabp10a:CFP-NTR)* that expresses nitroreductase (NTR) fused with cyan fluorescent protein (CFP) in hepatocytes, allowing for hepatocyte-specific ablation upon metronidazole (Mtz) treatment, (2) *Tg(Tp1:H2B-mCherry)* that expresses histone 2B (H2B) and mCherry fusion proteins strongly in BECs and weakly in BEC-derived hepatocytes, and (3) *Tg(fabp10a:rasGFP)* that expresses the membrane form of GFP strongly in hepatocytes and weakly in LPCs¹². The triple transgenic larvae were treated with Mtz from 3.5 to 5 days post-fertilization (dpf) for 36 hours (ablation, A36h), which results in near-complete hepatocyte ablation, followed by Mtz washout scored as the start of regeneration (regeneration, R0h). We treated the larvae with compounds from A20h, before BEC dedifferentiation occurs, to R24h, at which larvae were harvested for subsequent whole-mount immunostaining (Fig. 1A). To identify a compound that regulates LPC-to-hepatocyte differentiation, we examined the expression of a hepatocyte marker *Bhmt*¹⁶. Through this screening, we identified three compounds that blocked *Bhmt* expression in regenerating livers at R24h: MS-275, trichostatin A (TSA), and OG-L002 (Fig. 1B). In regenerating livers treated with these three compounds, *fabp10a:rasGFP* expression was still detected, albeit lower than in control regenerating livers (Fig. 1B), suggesting that BECs dedifferentiated into LPCs in these compound-treated regenerating livers. MS-275 inhibits HDAC1/2/3^{17, 18} and TSA is a pan-HDAC inhibitor¹⁹. OG-L002 inhibits lysine-specific histone demethylases 1A (KDM1A, also called LSD1)²⁰, which forms the CoREST repressor complex with HDAC1/2²¹. The identification of these compounds suggests that HDAC1/2-containing, histone-modifying repressor complexes regulate LPC-to-hepatocyte differentiation during regeneration. Moreover, we found that *hdac1* and *kdm1a* expression was highly upregulated in regenerating livers at R6h (Fig. 1C–E) and diminished later at R12h and R24h (Fig. S1). Specifically, *Hdac1* expression in BECs was not observed at A18h yet but strongly induced at R6h; as the liver recovers, its expression was reduced at R24h (Fig. S1A, arrows).

Collectively, given the absence of *hdac2* gene in the zebrafish genome, we hypothesized that *hdac1* regulates LPC-to-hepatocyte differentiation during regeneration.

MS-275 treatment impairs LPC differentiation into either hepatocytes or BECs

We next investigated in detail the effect of MS-275 treatment on BEC-driven liver regeneration by examining the expression of additional liver markers at R24h (Fig. 2A). During BEC-driven liver regeneration, Hnf4a, a hepatoblast/hepatocyte marker, expression is induced in LPCs and is maintained in hepatocytes, but not in BECs; BEC marker expression is sustained in LPCs, but disappears from LPCs when these cells differentiate into hepatocytes⁵. In MS-275-treated regenerating livers, Hnf4a was normally induced in BEC-derived cells (Fig. 2B), further supporting the normal dedifferentiation of BECs into LPCs. *Tg(Tp1:VenusPEST)* and *Tg(Tp1:H2B-mCherry)* lines express fluorescent proteins under the same *Tp1* promoter containing the Notch-responsive element²² and reveal BECs in the liver²³. The short half-life of VenusPEST proteins only reveals cells with active Notch signaling, whereas the prolonged stability of H2B-mCherry proteins allows for tracing BEC-derived cells even when Notch signaling is off⁵. Analysis of these BEC markers together with Hnf4a revealed the reduced number of hepatocytes (Hnf4a⁺/VenusPEST⁻) and BECs (Hnf4a⁻/VenusPEST^{strong}) in MS-275-treated regenerating livers at R24h compared to controls (Fig. 2B–D). Intriguingly, undifferentiated or less differentiated cells, defined as Hnf4a⁺/VenusPEST^{weak}, were detected in MS-275-treated, but not control, regenerating livers (Fig. 2B, blue bar), which was further confirmed by analyzing the expression of Alcam, another BEC marker. BECs are Alcam⁺/VenusPEST^{strong} (Fig. 2C, arrows) and hepatocytes are Alcam⁻/VenusPEST⁻. Alcam⁺/VenusPEST^{weak} or ⁻ cells (Fig. 2C, arrowheads) were not detected in control regenerating livers, whereas these cells were abundantly present in MS-275-treated regenerating livers (Fig. 2E). The defect in LPC-to-hepatocyte differentiation was further confirmed by almost no expression of the hepatocyte markers *cp* and *gc* in MS-275-treated regenerating livers at R24h (Fig. 2F). Moreover, the continuous treatment of MS-275 until R48h also blocked Bhmt expression in regenerating livers at R48h (Fig. S2A). However, the washout of MS-275 at A36h/R0h resulted in Bhmt expression in most regenerating livers at R24h, albeit weaker than in controls (Fig. S2B). Importantly, MS-275 treatment from A36h did not affect LPC differentiation into either hepatocytes or BECs (Fig. 2), indicating the A20h-A36h period as the critical time-window of MS-275 effect on LPC differentiation during regeneration.

Upon 70% partial hepatectomy or carbon tetrachloride (CCl₄) injection, in which hepatocytes contribute to regenerated hepatocytes (i.e., hepatocyte-driven liver regeneration), hepatocyte-specific *Hdac1/2* double-knockout mice exhibit reduced hepatocyte proliferation and increased apoptosis²⁴. Given this positive role of HDAC1/2 in hepatocyte-driven liver regeneration, we also examined MS-275 effect on proliferation and cell death during BEC-driven liver regeneration. The proliferation of BEC-derived cells was not significantly different between control and MS-275-treated regenerating livers at R6h (Fig. S3A) and R24h (Fig. S3D) and their number was comparable between DMSO- and MS-275-treated regenerating livers at R0h (Fig. S3G). Unexpectedly, BEC proliferation, as assessed by the percentage of EdU⁺ cells among BECs, was significantly increased in MS-275-treated regenerating livers at R24h compared to controls (Fig. S3E). Few BEC-

derived cells were dying in either control or MS-275-treated regenerating livers at R12h (Fig. S3F). These proliferation and cell death data suggest a distinct role of Hdac1 in BEC-driven liver regeneration compared to hepatocyte-driven liver regeneration.

Hdac1 is required for LPC differentiation during BEC-driven liver regeneration

HDAC1 and HDAC2 usually play redundant roles in mice; therefore, only deletion of both genes causes defects in diverse developmental or regeneration processes^{24, 25}. However, the absence of *hdac2* gene in the zebrafish genome makes it possible to detect a phenotype in *hdac1* mutant zebrafish²⁶. *hdac1* homozygous mutants die around 2–4 dpf and exhibit severe defects in multiple organs, including the liver, but heterozygous mutants normally survive without any noticeable defects²⁶. Thus, we used *hdac1*^{+/-} mutants for our BEC-driven liver regeneration study. BEC-driven liver regeneration appeared normal at R24h in *hdac1*^{+/-} mutants; however, by reducing the dosage of MS-275 from 25 μ M, we found a dosage that resulted in a regeneration defect in only *hdac1*^{+/-}, but not wild-type, larvae. The treatment of 10 μ M MS-275 blocked LPC-to-hepatocyte differentiation in *hdac1*^{+/-} but not wild-type larvae, as assessed by *Bhmt*, *cp*, and *gc* expression at R24h (Fig. 3A–D). Although 10 μ M MS-275 treatment resulted in reduced BEC number in wild-type regenerating livers, the number was further decreased in *hdac1*^{+/-} regenerating livers (Fig. 3E and 3F). Altogether, these mutant data indicate *hdac1* as the key *hdac* gene required for LPC differentiation during regeneration.

Hdac1 regulates LPC-to-hepatocyte differentiation by repressing *sox9b* expression

Given that genes and pathways used during development are often re-utilized during regeneration, to determine the molecular mechanisms by which Hdac1 regulates LPC differentiation, we examined the expression levels of hepatic genes that play important roles in liver development. qPCR analysis revealed that *sox9b* and *foxa3* were highly upregulated in MS-275-treated regenerating livers compared with DMSO-treated regenerating livers at R6h (Fig. 4A). We focused on these genes because both Hdac1 and Kdm1a inhibition impaired LPC-driven liver regeneration (Fig. 1B), suggesting that the CoREST complex, which contains Hdac1 and Kdm1a, controls the regeneration by repressing gene expression. Intriguingly, it was reported that HDAC inhibitor treatment increased *SOX9* expression in human primary fetal hepatocytes²⁷ and clear cell sarcoma cells²⁸. TSA treatment or *HDAC1* knockdown also increased *SOX9* expression in human lung adenocarcinoma cells²⁹. Given the role of SOX9 in maintaining stem cell/progenitor states in liver cancer³⁰ and mammary³¹ stem cells, we hypothesized that the enhanced *sox9b* expression in MS-275-treated regenerating livers prevented LPC differentiation. To test this hypothesis, we lowered *sox9b* expression in regenerating livers using *sox9b* heterozygous mutants. As previously reported, BEC-driven liver regeneration failed to occur in *sox9b*^{-/-} mutants⁷, whereas it did occur in *sox9b*^{+/-} mutants (Fig. S4). In *sox9b*^{+/-} larvae, *Bhmt* was normally expressed in regenerating hepatocytes at R24h (Fig. S4A), but BEC number was significantly reduced at this stage compared to their wild-type siblings (Fig. S4B), indicating the haploinsufficiency of *sox9b* in regulating BEC number during BEC-driven liver regeneration. Importantly, hepatocyte differentiation defect observed in *hdac1*^{+/-} larvae treated with 10 μ M MS-275 was significantly rescued in *sox9b*^{+/-} mutants, as assessed by *Bhmt* expression (Fig. 4B). In

contrast to this hepatocyte differentiation defect, the reduced BEC number phenotype was not rescued in *sox9b*^{+/-} mutants (Fig. S4C).

Next, using a chromatin immunoprecipitation (ChIP)-qPCR assay, we determined whether the enhanced *sox9b* expression in MS-275-treated regenerating livers is mediated by the hyper-acetylation of the *sox9b* genomic locus. To obtain the sufficient amount of liver tissues required for this assay, we used adult zebrafish, as previously reported⁵. The *Tg(fabp10a:CFP-NTR)* adult fish were treated with 5 mM Mtz only for 5 hours and subsequently treated with MS-275 from R19h to R4d. As observed in the larvae, Bmmt expression was greatly reduced and *sox9b* expression was significantly upregulated in MS-275-treated regenerating adult livers compared with control regenerating livers (Fig. 4C and 4D), suggesting LPC-to-hepatocyte differentiation defect. To examine the histone acetylation status of the *sox9b* genomic locus, we assessed the levels of acetylation of histone H3 at lysine 9 (H3K9ac), a marker of active gene promoters, within the -3-kb region from the *sox9b* transcription start site, because such -3-kb region regulates *Sox9* expression in the mouse liver³² and zebrafish³³. We randomly selected 14 genomic loci in this region and further narrowed down to two loci for H3K9ac enrichment analysis. We also selected a region that is located 8.5 kb upstream from the *sox9b* 5' UTR as a negative control. H3K9ac enrichment in the two selected regions was significantly increased in regenerating livers compared with uninjured livers (Fig. 4E), consistent with the enhanced expression of *sox9b* in regenerating livers (Fig. 4D). Importantly, the H3K9ac enrichment was further increased in MS-275-treated regenerating livers compared with the control regenerating livers (Fig. 4E). As a negative control, the level of H3K9ac enrichment in the control region was very low and comparable between the uninjured livers and MS-275-treated regenerating livers (Fig. 4E). Altogether, these *sox9b* mutant and ChIP-qPCR data reveal *sox9b* as the key downstream target gene of Hdac1 that regulates LPC-to-hepatocyte differentiation.

Hdac1 regulates LPC-to-BEC differentiation by repressing Cdk8/Fbxw7-mediated degradation of Notch intracellular domain (NICD)

We next sought to determine the molecular mechanism by which Hdac1 regulates LPC-to-BEC differentiation. During development, Notch signaling is required for differentiation of hepatoblasts into BECs; overactivation of Notch signaling in hepatoblasts makes excessive BECs³². Furthermore, Notch signaling has been considered a key driver that differentiates LPCs into BECs in rodent biliary injury models¹³ and human biliary diseases¹⁴. Thus, we hypothesized that the defect in LPC-to-BEC differentiation observed in MS-275-treated regenerating livers (Fig. 2B–E) might be caused by reduced Notch activity. To test this hypothesis, we treated *hdac1*^{+/-} larvae with a low dose of the Notch inhibitor, LY411575³⁴. The treatment of 0.1 μM LY411575 reduced BEC number greatly in *hdac1*^{+/-}, but moderately in wild-type, regenerating livers (Fig. 5A), suggesting that *hdac1*^{+/-} regenerating livers exhibited reduced Notch activity compared to wild-type. We aimed to rescue the BEC defect observed in MS-275-treated regenerating livers by enhancing Notch activity with the *Tg(hs:N3ICD)* line that expresses Notch3 intracellular domain upon heat-shock³⁵. Although the BEC defect was not rescued, we observed that ectopic Notch activity induced by *hs:N3ICD* expression disappeared much faster in MS-275-treated regenerating livers than in DMSO-treated regenerating livers, as assessed by *Tp1:VenusPEST* expression. At R30h, six

hours after the last heat-shock, weak but noticeable *Tp1*:VenusPEST expression was observed broadly in DMSO-treated regenerating livers, whereas such expression was barely observed in MS-275-treated regenerating livers (Fig. 5B), raising a possibility that MS-275 treatment promotes the degradation of NICD proteins in regenerating livers. NICD is degraded by the ubiquitin/proteasome pathway: it is first phosphorylated by Cdk8 and then phosphorylated NICD is ubiquitinated by an SCF complex containing an E3 ubiquitin ligase substrate adaptor, Fbxw7³⁶. In regenerating livers at R6h, *cdk8*, *fbxw7*, and *skp1*, a core component gene of the SCF complex³⁷, were upregulated (Fig. 5C), and *cdk8* expression was further increased in MS-275-treated regenerating livers (Fig. 5D). Intrigued by this upregulation, we tested whether reducing Cdk8 activity could restore the reduced BEC number phenotype observed in MS-275-treated regenerating livers. Indeed, the treatment of the Cdk8 inhibitor, senexin A³⁸, restored the BEC number to normal levels (Fig. 5E and S5A). Moreover, reducing Fbxw7 level with *fbxw7* heterozygous mutants restored, albeit in part, the reduced BEC number in MS-275-treated *hdac1*^{+/-} mutants (Fig. 5F and S5B). BEC numbers in regenerating livers at R24h were increased in both senexin A-treated larvae and *fbxw7*^{+/-} mutants (Fig. 5E and 5F), further supporting that enhanced Notch activity results in excessive BECs. These increased BEC numbers were also restored to the normal number upon Hdac1 repression (senexin A versus senexin A/MS-275 co-treatment in Fig. 5E; *fbxw7*^{+/-} versus *fbxw7*^{+/-};*hdac1*^{+/-} in Fig. 5F). Altogether, these data indicate that Hdac1 regulates LPC-to-BEC differentiation during regeneration by positively controlling Notch activity through the repression of Cdk8/Fbxw7-mediated degradation of NICD.

Notch3 is required for LPC-to-BEC differentiation during regeneration

We next determined which Notch receptor(s) regulated LPC differentiation during regeneration. Given its biliary-restricted expression³⁹, we examined if *notch3* was implicated in BEC-driven liver regeneration. *notch3*^{-/-} mutants exhibited the normal differentiation of LPCs into hepatocytes in regenerating livers at R24h, as assessed by Bhmt expression (Fig. S6B). By contrast, *notch3*^{-/-} mutants had few BECs in regenerating livers at R24h (Fig. 5G and S6A), indicating Notch3 as the essential Notch receptor for BEC-driven liver regeneration, particularly LPC-to-BEC differentiation. Using *notch3*^{+/-} mutants, which have the normal number of BECs in regenerating livers, we determined whether the treatment of a suboptimal dose of MS-275 could reduce the number of BECs in the regenerating livers of *notch3*^{+/-}, but not wild-type, larvae. The treatment of 1.5 μ M MS-275 did not affect BEC number in wild-type regenerating livers, but significantly reduced its number in *notch3*^{+/-} regenerating livers at R24h (Fig. 5G). Altogether, these *notch3* mutant data reveal the essential role of Notch3 in LPC-to-BEC differentiation during regeneration and further support that Hdac1 regulates this differentiation process by positively controlling Notch signaling.

Kdm1a also regulates LPC differentiation into either hepatocytes or BECs during regeneration

Given that Kdm1a suppression with OG-L002 blocked LPC-to-hepatocyte differentiation (Fig. 1B) and that Kdm1a and Hdac1 often function in the same repressive complexes²¹, we hypothesized that Kdm1a suppression also blocked LPC-to-BEC differentiation as observed upon Hdac1 suppression. Indeed, OG-L002 treatment greatly reduced BEC number in

regenerating livers at R24h (Fig. 6A). Furthermore, *kdm1a*^{-/-} mutants also exhibited the defects in LPC differentiation: in regenerating livers at R24h, *kdm1a*^{-/-} mutants displayed almost no Bhmt expression (Fig. 6B) and significantly reduced BEC number compared with their siblings (Fig. 6C). Altogether, these Kdm1a data suggest that Hdac1 and Kdm1a coregulate LPC differentiation during regeneration.

Evidence in mammals that supports the role of Hdac1 in LPC-to-hepatocyte differentiation

To determine whether the role of Hdac1 in LPC differentiation is conserved in mammals, we examined the effect of MS-275 on LPC-to-hepatocyte differentiation in a new mouse liver injury model, in which a small subset of BEC-derived LPCs contribute to hepatocytes⁴⁰. In this model, hepatocyte-specific deletion of the β -catenin gene, *Ctnnb1*, (KO) near-completely prevents hepatocyte proliferation following choline-deficient, ethionine supplemented (CDE) diet-induced liver injury, permitting β -catenin⁺ BECs to give rise to hepatocytes. KO mice were fed a CDE diet for 2 weeks followed by recovery on a normal chow diet for 7 days; MS-275 or vehicle was intraperitoneally injected daily from R1d during the recovery phase (Fig. 7A). There was no significant difference between DMSO- and MS-275-injected regenerating mice at R7d in body weight, liver weight, and serum liver injury marker levels (Fig. S7), suggesting that MS-275 administration did not exacerbate liver damage. Expectedly, MS-275 administration increased the levels of acetylation of histone H3 at lysine 27 (H3K27ac) and H3K9ac in the liver (Fig. 7E). In DMSO-injected regenerating livers at R7d, a significant number of CK19⁻/HNF4A⁺/ β -catenin⁺ hepatocytes (BEC-derived hepatocytes in the KO) were observed, whereas in MS-275-injected regenerating livers their number was greatly reduced (Fig. 7B, arrows). We found that the proliferation rate of the BEC-derived hepatocytes was comparable between DMSO- and MS-275-injected regenerating livers (Fig. 7C, arrows), ruling out a possibility that the decrease in their number in MS-275-injected regenerating livers is due to reduced proliferation. Moreover, although not significant, overall ductular reactions were slightly increased in MS-275-injected regenerating livers (Fig. 7D). We also found that MS-275 injection increased SOX9 expression in the regenerating livers (Fig. 7E), as observed in zebrafish. Collectively, these mouse data strongly support the findings from zebrafish that Hdac1 regulates LPC-to-hepatocyte differentiation during regeneration.

Next, we investigated HDAC1 and SOX9 expression in human livers with advanced liver diseases by examining 15 patient liver specimens with three types of cirrhosis: compensated, decompensated and acute-on-chronic liver failure (ACLF). SOX9 expression was detected in BECs and most reactive ducts in all measured specimens (Table S6). Although a few hepatocytes inside of hepatocyte buds demonstrated SOX9 expression (Fig. 7F, arrows), most hepatocyte buds did not express SOX9 (Fig. 7F). Among 15 patients, HDAC1 expression was detected in 7 hepatitis B virus (HBV)-associated cirrhotic patients, either compensated or decompensated; however, it was undetectable in alcohol-associated decompensated cirrhotic and ACLF patients (Fig. 7F and Table S6). HDAC1 was expressed in reactive ducts and hepatocytes (Fig. 7F, arrowheads and white arrows, respectively). Intriguingly, HBV-associated cirrhotic livers strongly expressed HDAC1 in hepatocyte buds, while SOX9 expression in these LPC-derived hepatocytes was undetectable (Fig. 7F). It is worth noting that the ACLF patients, who received liver transplantation, did not have the

detectable, hepatic expression of HDAC1 (Fig. 7F). Their livers were full of SOX9⁺ reactive ducts (Fig. 7F), implying a defect in LPC-to-hepatocyte differentiation. Whether ACLF patients who spontaneously recovered express HDAC1 requires further investigation. In addition, in contrast to HBV-associated cirrhosis, 2 alcohol-associated decompensated cirrhotic patients did not express HDAC1 (Table S6). It might explain the previous study showing that LPCs failed to differentiate into hepatocytes in patients with alcoholic hepatitis⁴¹. Collectively, these human data suggest the conserved role of HDAC1 in LPC-to-hepatocyte differentiation.

Discussion

In this study, we provide novel molecular mechanisms by which Hdac1 regulates differentiation of LPCs into either hepatocytes or BECs during regeneration. These fate decisions have usually been studied using LPCs isolated and established from diseased livers *in vitro*. Although such *in vitro* studies have revealed the molecular mechanisms underlying LPC differentiation^{13, 42}, findings from the *in vitro* studies need to be validated *in vivo*. However, lack of *in vivo* models in which LPCs efficiently differentiate into both hepatocytes and BECs during regeneration has prevented such *in vivo* validation. Recently, liver injury models in which BECs, via LPCs, extensively contribute to hepatocytes have been established in zebrafish⁵⁻⁷ and mice⁸⁻¹⁰. Particularly, in the zebrafish model, liver regeneration occurs through differentiation of LPCs into both hepatocytes and BECs; therefore, this model can be used not only to validate findings from *in vitro* studies but also to reveal novel molecular mechanisms underlying LPC differentiation during regeneration. Using the zebrafish model, we here show that (1) Hdac1 represses *sox9b* expression, thereby permitting LPC-to-hepatocyte differentiation and that (2) Hdac1 represses *cdk8* expression, thereby enhancing Notch signaling, which induces LPC-to-BEC differentiation (Fig. 6D). These findings not only confirm the known role of Notch signaling in LPC-to-BEC differentiation^{13, 14, 42}, but they also reveal three crucial genes (*cdk8*, *fbxw7*, and *notch3*) that regulate Notch signaling during liver regeneration, which is further supported by an *in vitro* study showing that *Fbxw7* deficiency skews the differentiation of mouse LPCs toward BECs⁴³. Complementary to these findings from zebrafish, we also provide the evidence of the role of Hdac1 in LPC-to-hepatocyte differentiation in mice and humans.

Despite its wide usage in the liver field as a BEC/LPC marker and a lineage tracing tool⁴⁴, the role of Sox9 in liver injury settings remains largely unknown. In this study, we reveal the repressive role of Sox9b in LPC-to-hepatocyte differentiation. This finding is supported by an *in vitro* study showing the negative role of SOX9 in the differentiation of human LPCs into hepatocytes⁴⁵. This role of Sox9b is rather consistent with the role of mammalian SOX9 in the maintenance of stemness and the inhibition of differentiation in liver cancer³⁰ and mammary³¹ stem cells and kidney progenitor cells⁴⁶. SOX9 is restrictively expressed in BECs in the normal liver; however, its expression is often induced in hepatocytes in various rodent liver injury *Hdac1/2* double-knockout mice²⁵ models⁴⁷ and in human liver diseases⁴⁵. Based on our finding about the role of Sox9b in LPCs, we speculate that SOX9 expression in hepatocytes may make them lose their cellular identity, thereby promoting their dedifferentiation into oval cells (i.e., LPCs) and simultaneously preventing their

differentiation into hepatocytes. It will be interesting to examine in mice if hepatocyte- or BEC-specific deletion of *Sox9* results in any defect in LPC-driven liver regeneration.

It was previously suggested that in hepatocyte injury settings, such as the CDE model, Wnt/ β -catenin signaling activated by macrophage-derived Wnt3a represses Notch activity in LPCs by inducing *Numb* expression, thereby promoting differentiation of LPCs into hepatocytes¹³. It was also suggested that in biliary injury settings, such as the 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) model, Notch signaling activated by myofibroblast-derived Jagged1 promotes LPC differentiation into BECs¹³. However, more recent *in vivo* studies showed that either the activation of Wnt/ β -catenin signaling or the inhibition of Notch signaling in BECs/LPCs was not sufficient to differentiate DDC-induced LPCs into hepatocytes⁴⁸, indicating that additional factors regulate this differentiation process. In our study, reduction of *Sox9b* expression rescued a defect in LPC-to-hepatocyte differentiation observed in *Hdac1*-repressed regenerating livers, suggesting *Sox9* as the additional factor that regulates LPC-to-hepatocyte differentiation. As a key component of multiple transcriptional repressor complexes⁴⁹, *Hdac1* represses the expression of numerous genes by removing acetylation marks on the lysine residues of histones. Therefore, it is surprising that reducing the expression level or the activity of a single gene is sufficient to rescue a defect in LPC differentiation observed in *Hdac1*-repressed larvae: *sox9b* for LPC-to-hepatocyte differentiation and *cdk8* or *fbxw7* for LPC-to-BEC differentiation. Likewise, reducing the expression level of *Bmp4* with *Bmp4*^{+/-} mutants rescued a defect in proximal airway development observed in foregut endoderm-specific *Hdac1/2* double-knockout mice²⁵.

During development, *Sox9* is not required for differentiation of hepatoblasts into BECs in mice⁵⁰ or zebrafish²³, while *Sox9* appears to regulate LPC-to-BEC differentiation during regeneration, as suggested by the reduced BEC number in *sox9b*^{+/-} regenerating livers (Fig. S4B). It was recently reported that both *Sox4* and *Sox9* deletion is required for blocking hepatoblast-to-BEC differentiation⁵¹, indicating that *Sox4* compensates for the absence of *Sox9* in the developing liver. However, during BEC-driven liver regeneration, other genes, such as *Sox4*, do not compensate for the absence of *Sox9*. Likewise, *Notch3* is not required for hepatoblast-to-BEC differentiation during development because of the compensation by *Notch2*⁵². However, the absence of BECs in regenerating livers of *notch3*^{-/-} mutants (Fig. S6A) indicates the failed compensation by other Notch receptors during BEC-driven liver regeneration. It is tempting to speculate that developmental processes have more compensatory mechanisms than regeneration processes, because every animal goes through developmental processes for its survival but only a subset of animals go through regeneration processes for their survival.

In summary, we provide the molecular mechanisms underlying LPC differentiation into either hepatocytes or BECs. Given the potential of promoting innate liver regeneration as therapeutics for advanced human liver diseases, a better understanding of the molecular mechanisms underlying LPC-driven liver regeneration is crucial for developing such a therapy. Not only does our finding that *Hdac1* regulates LPC differentiation via *Sox9b* and *Cdk8-Fbxw7-Notch3* reveal novel molecular mechanisms underlying LPC differentiation,

but it also suggests a means to repress Sox9 expression or function as a potential therapy to promote liver regeneration in patients with advanced liver diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

ACLF	acute-on-chronic liver failure
BEC	biliary epithelial cell
CDE	choline-deficient, ethionine supplemented
CFP	cyan fluorescent protein
ChIP	chromatin immunoprecipitation
DDC	3,5-diethoxycarbonyl-1,4-dihydrocollidine
DMSO	dimethyl sulfoxide
dpf	days post-fertilization
EdU	5-ethynyl-2'-deoxyuridine
HBV	hepatitis B virus
H3K9ac	acetylation of histone H3 at lysine 9
H3K27ac	acetylation of histone H3 at lysine 27
LPC	liver progenitor cell
Mtz	metronidazole
NICD	Notch intracellular domain
NTR	nitroreductase
PTU	1-phenyl-2-thiourea
qPCR	quantitative polymerase chain reaction

WISH

whole-mount in situ hybridization

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Figure 1. Identification of compounds that block LPC-to-hepatocyte differentiation during regeneration.

(A) Experimental scheme illustrating the stages of Mtz and testing compound treatment and analysis (arrow). (B) Single-optical section images showing the expression of *Bhmt*, *fabp10a:rasGFP*, and *Tp1:H2B-mCherry* in regenerating livers at R24h. Numbers indicate the proportion of larvae exhibiting the representative expression shown. (C) Single-optical section images showing the expression of *Hdac1*, *Tp1:H2B-mCherry*, and *fabp10a:CFP-NTR* in the normal liver at 5 dpf and the regenerating liver at R6h. Arrows point to BEC-derived cells expressing *Hdac1*. (D) Whole-mount in situ hybridization (WISH) images showing *hdac1* and *kdm1a* expression in normal livers at 5 dpf and regenerating livers at R6h. Dashed lines outline control livers; arrows point to regenerating livers. (E) qPCR data showing the relative expression levels of *hdac1* and *kdm1a* between 5-dpf control livers and R6h-regenerating livers. Scale bars: 100 μ m; error bars: $\pm$ SEM.

Figure 2. MS-275 treatment impairs LPC differentiation into either hepatocytes or BECs during regeneration.

(A) Experimental scheme illustrating the stages of Mtz and MS-275 treatment and analysis (arrow). (B) Single-optical section images showing the expression of Hnf4a, *Tp1:VenusPEST*, *Tp1:H2B-mCherry*, and *fabp10a:CFP-NTR* in regenerating livers at R24h. Among BEC-derived, H2B-mCherry⁺ cells, Hnf4a⁺/VenusPEST^{strong} cells are BECs (arrows) and Hnf4a⁺/VenusPEST⁻ cells are hepatocytes (open arrows). A third cell type, Hnf4a⁺/VenusPEST^{weak} cells, is present (arrowheads) in MS-275-treated, but not control, regenerating livers. Quantification of the percentage of hepatocytes, BECs, and undifferentiated or less differentiated cells among H2B-mCherry⁺ cells is shown. n indicates the number of larvae examined. (C) Single-optical section images showing the expression of Alcarn, *Tp1:VenusPEST*, *Tp1:H2B-mCherry*, and *fabp10a:CFP-NTR* in regenerating livers at R24h. Arrows point to BECs; arrowheads point to Alcarn⁺/VenusPEST⁺ cells derived from BECs. (D, E) Quantification of the percentage of BECs (D) or Alcarn⁺/

VenusPEST^{weak or -} cells (E) among H2B-mCherry⁺ (BEC-derived) cells, as shown in C. (F) WISH images showing *cp* and *gc* expression in regenerating livers at R24h. Arrows point to regenerating livers. Numbers indicate the proportion of larvae exhibiting the representative expression shown. Scale bars: 100 μ m; error bars: $\pm$ SEM.

Figure 3. Hdac1 is required for LPC differentiation during regeneration.

(A) Experimental scheme illustrating the stages of Mtz and MS-275 treatment and analysis (arrow). (B) Single-optical section images showing Bbmt and *Tp1:H2B-mCherry* expression in regenerating livers at R24h. (C) Quantification of the percentage of Bbmt⁺ hepatocytes among BEC-derived cells, as shown in B. (D) WISH images showing *cp* and *gc* expression in regenerating livers at R24h. Arrows point to regenerating livers. (E) Confocal projection images showing the expression of Alcarn, *Tp1:VenusPEST*, and *Tp1:H2B-mCherry* in regenerating livers at R24h. Arrows point to VenusPEST⁺/Alcarn⁺ cells (i.e., BECs); arrowheads point to VenusPEST⁻/Alcarn⁺ cells. (F) Quantification of BEC number per area, as shown in E. Scale bars: 100 μ m; error bars: $\pm$ SEM.

Figure 4. Hdac1 regulates LPC-to-hepatocyte differentiation by repressing *sox9b* expression. (A) qPCR data showing the relative expression levels of *fabp10a*, *hnf4a*, *sox9b*, *foxa1/2/3*, *epcam*, and *prox1a* between DMSO- and MS-275-treated regenerating livers at R6h. (B) Single-optical section images showing Bhmt and *Tp1*:H2B-mCherry expression in regenerating livers at R24h. Quantification of the percentage of Bhmt⁺ hepatocytes among BEC-derived cells is shown. (C) Single-optical section images showing Bhmt and *Tp1*:H2B-mCherry expression in regenerating livers of adult zebrafish at R4d. (D) qPCR data showing the relative expression levels of *bhmt* and *sox9b* among uninjured control livers, DMSO- and MS-275-treated regenerating livers at R4d. (E) ChIP-qPCR data showing the relative enrichment of the selected *sox9b* promoter regions among uninjured control livers, DMSO- and MS-275-treated regenerating livers at R4d, following immunoprecipitation with H3K9ac antibody. Scheme illustrates the *sox9b* genomic locus. Arrows point to the regions amplified

by qPCR. Green and yellow boxes denote untranslated and coding regions, respectively.
Scale bars: 100 μ m; error bars: $\pm$ SEM.

Figure 5. Hdac1 regulates LPC-to-BEC differentiation via Cdk8, Fbxw7 and Notch3.

(A) Confocal projection images showing the expression of *Tp1:H2B-mCherry*, *Tp1:VenusPEST*, and Alcam in regenerating livers (dashed lines) at R24h. Quantification of VenusPEST⁺/Alcam⁺ cell (i.e., BEC) number is shown. (B) Single-optical section images showing *Tp1:VenusPEST* and *Tp1:H2B-mCherry* expression in regenerating livers (dashed lines) at R30h. *hs:N3ICD* expression was induced by multiple heat-shocks at A24h, R3h, and R24h. (C) WISH images showing *fbwx7*, *cdk8*, and *skp1* expression in normal livers (dashed lines) at 5 dpf and regenerating livers (arrows) at R6h. (D) qPCR data showing the relative expression levels of *fbwx7*, *skp1*, *cdk8*, *her9*, *her2*, and *her15.1* between DMSO- and MS-275-treated regenerating livers at R6h. (E-G) Quantification of BEC number per area, as shown in Fig. S5A, S5B and S6A, respectively. Scale bars: 100 μ m; error bars: $\pm$ SEM.

Figure 6. Kdm1a regulates LPC differentiation during regeneration.

(A, C) Confocal projection images showing the expression of Alcam, *Tp1*:VenusPEST, and *Tp1*:H2B-mCherry in regenerating livers (dashed lines) at R24h. Quantification of BEC number per area is shown. (B) Single-optical section images showing Bhmt and *Tp1*:H2B-mCherry expression in regenerating livers at R24h. Quantification of the percentage of Bhmt⁺ hepatocytes among BEC-derived cells is shown; both strong and weak Bhmt expression were considered Bhmt⁺. (D) Cartoon illustrating the process of BEC-driven liver regeneration upon massive hepatocyte ablation in zebrafish larvae, focusing on the role of Hdac1 in LPC differentiation into hepatocytes and BECs. Scale bars: 100 μ m; error bars: $\pm$ SEM.

Figure 7. Evidence in mammals that supports the role of Hdac1 in LPC-to-hepatocyte differentiation.

(A) Experimental scheme illustrating the period of a CDE diet, AAV8 and MS-275 injection stages, and analysis stage. (B) Section confocal images showing CK19, HNF4A and β -catenin expression in regenerating mouse livers at R7d. Arrows point to BEC-derived hepatocytes (CK19⁻/HNF4A⁺/ β -catenin⁺); arrowheads to BECs (CK19⁺/HNF4A⁻/ β -catenin⁺). (C) Section confocal images showing PCNA, HNF4A and β -catenin expression with DAPI staining in regenerating livers at R7d. Arrows point to PCNA⁺/HNF4A⁺/ β -catenin⁺ hepatocytes. Quantification of the percentage of PCNA⁺ among BEC-derived hepatocytes is shown. (D) Section images showing anti-pan-cytokeratin (panCK) immunostaining. Quantification of panCK⁺ area is shown. (E) Western blot images of whole liver lysate from DMSO- or MS-275-injected regenerating livers at R7d. (F) Human liver section images showing SOX9 and HDAC1 expression. Arrows point to SOX9⁺ hepatocytes; arrowheads to

HDAC1⁺ reactive ducts; white arrows to HDAC1⁺ hepatocytes. Scale bars: 100 μ m (B–D), 50 μ m (F); error bars: $\pm$ SEM.

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