Research Article

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Bromodomain and extraterminal (BET) proteins regulate biliary-driven liver regeneration

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Background & Aims: During liver regeneration, hepatocytes are derived from pre-existing hepatocytes. However, if hepatocyte proliferation is compromised, biliary epithelial cells (BECs) become the source of new hepatocytes. We recently reported on a zebrafish liver regeneration model in which BECs extensively contribute to hepatocytes. Using this model, we performed a targeted chemical screen to identify important factors that regulate BEC-driven liver regeneration, the mechanisms of which remain largely unknown.

Methods: Using *Tg(fabp10a:CFP-NTR)* zebrafish, we examined the effects of 44 selected compounds on BEC-driven liver regeneration. Liver size was assessed by *fabp10a:*DsRed expression; liver marker expression was analyzed by immunostaining, *in situ* hybridization and quantitative PCR. Proliferation and apoptosis were also examined. Moreover, we used a mouse liver injury model, choline-deficient, ethionine-supplemented (CDE) diet.

Results: We identified 10 compounds that affected regenerating liver size. Among them, only bromodomain and extraterminal domain (BET) inhibitors, JQ1 and iBET151, blocked both Prox1 and Hnf4a induction in BECs. BET inhibition during hepatocyte ablation blocked BEC dedifferentiation into hepatoblast-like cells (HB-LCs). Intriguingly, after JQ1 washout, liver regeneration resumed, indicating temporal, but not permanent, perturbation of liver regeneration by BET inhibition. BET inhibition after hepatocyte ablation suppressed the proliferation of newly generated hepatocytes and delayed hepatocyte maturation. Importantly, Myca overexpression, in part, rescued the proliferation defect. Furthermore, oval cell numbers in mice fed CDE diet were greatly reduced upon JQ1 administration, supporting the zebrafish findings.

Conclusions: BET proteins regulate BEC-driven liver regeneration at multiple steps: BEC dedifferentiation, HB-LC proliferation, the proliferation of newly generated hepatocytes, and hepatocyte maturation.

Keywords: Liver progenitor cells; Dedifferentiation; JQ1; Oval cells; Zebrafish; BET inhibitors; Liver regeneration.

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Abbreviations: BEC, biliary epithelial cell; CDE, choline-deficient, ethionine-supplemented; BET, bromodomain and extraterminal domain; HB-LCs, hepatoblast-like cells; LPC, liver progenitor cell; Mtz, metronidazole; DMSO, dimethyl sulfoxide; NTR, nitroreductase; CFP, cyan fluorescent protein; dpf, days post-fertilization; FXR, farnesoid X receptor; qPCR, quantitative polymerase chain reaction; EdU, 5-ethynyl-2'-deoxyuridine; WISH, whole-mount in situ hybridization; ALT, alanine aminotransferase.

Introduction

In terms of the origin of regenerated hepatocytes, there are two types of liver regeneration: hepatocyte- and biliary-driven liver regeneration. Upon liver injury or resection, hepatocytes proliferate to recover the lost liver mass. However, if hepatocyte proliferation is compromised, as observed in chronic liver diseases and certain liver toxin injury models, liver progenitor cells (LPCs), also called oval cells, are activated and these proliferative LPCs contribute to regenerated hepatocytes [1,2]. These LPCs appear to be derived from biliary epithelial cells (BECs) in the canals of Hering [1,2]. This BEC-driven liver regeneration has been postulated based on *in vitro* data and marker expression analyses of rodent and human samples. However, recent lineage tracing studies in mice reveal that pre-existing hepatocytes are the main source of regenerated hepatocytes in current oval cell activation models [3,4], raising a controversy about BEC-driven liver regeneration. Detailed marker analyses of human livers with cirrhosis [5] and massive hepatic necrosis [6] strongly suggest BEC contribution to regenerated hepatocytes in the human livers. However, the origin of regenerated hepatocytes in humans is still inconclusive due to a lack of cell-lineage tracing data. Importantly, the Forbes group recently reported the first evidence of robust BEC-driven liver regeneration in mice [7]. Hepatocyte-specific deletion of *Mdm2* causes hepatocyte senescence and subsequent apoptosis, which completely blocks hepatocyte proliferation. In these mice, oval cell activation occurs and LPCs derived from BECs give rise to hepatocytes, thereby resulting in liver recovery. We previously reported that injury severity influences the extent of BEC-driven liver regeneration and that upon

extreme hepatocyte ablation, BECs extensively give rise to hepatocytes in zebrafish [8], a phenomenon further confirmed by two separate groups [9,10].

Liver transplantation is the only definitive treatment for end-stage liver disease; however, the shortage of donor livers makes this therapy extremely limited. Thus, augmenting innate BEC-driven liver regeneration in chronic liver diseases may be an attractive therapeutic alternative for such patients. A better understanding of this process at the molecular level will provide mechanistic insights and may lead to future clinical therapies. Given the extensive contribution of BECs to hepatocytes in the zebrafish regeneration model, we used this model to identify critical players that regulate BEC-driven liver regeneration.

Here, we report a targeted chemical screen that identified two compounds, iBET151 and JQ1, which inhibit the function of bromodomain and extraterminal (BET) proteins, as potent inhibitors of BEC-driven liver regeneration. The BET protein family, consisting of BRDT, BRD2, BRD3, and BRD4 in mammals, shares two highly conserved N-terminal bromodomains and a C-terminal extraterminal domain [11]. The bromodomain is a chromatin interaction module that recognizes acetylated lysine residues on histone tails; the extraterminal domain interacts with other histone modifying proteins. By binding to the acetylated lysine residues and recruiting transcriptional regulator components, BET proteins regulate the transcription of target genes. The recent development of the highly potent, specific BET inhibitors, JQ1 [12], iBET762 [13] and iBET151 [14], led to the explosion of BET protein research in the cancer field because of its potent anti-cancer effect on various tumors in animal models [15–17]. As a result, some BET inhibitors are in clinical trials for patients with T cell lymphoma and multiple myeloma. In contrast to the extensive research on BET proteins in cancers, there are few reports describing their roles in regeneration [18] or liver biology [19]. Moreover, their role in liver regeneration has not been reported yet. Here, using the BET-specific inhibitors, we investigated the roles of BET proteins in BEC-driven liver regeneration and revealed their essential roles in this process.

Materials and methods

Zebrafish studies

Experiments were performed with approval of the Institutional Animal Care and Use Committee (IACUC) at the University of Pittsburgh. Embryos and adult fish were raised and maintained under standard laboratory conditions [20]. We used the following transgenic lines: *Tg(fabp10a:CFP-nfsB)*^{s931} [8], *Tg(fabp10a:DsRed,ela31:EGFP)*^{gz15} [21], *Tg(EPV.TP1-Mmu.Hbb:hist2h2l-mCherry)*^{s939} [22], *Tg(EPV.TP1-Mmu.Hbb:Venus-Mmu.Odc1)*^{s940} [22], *Tg(fabp10a:mAGFP-gmnn,cryaa:ECFP)*^{pt608}, *Tg(fabp10a:CAAX-EGFP)*^{s942} [23], and *Tg(hsp70l:VP16-myca,cryaa:ECFP)*^{pt605} [referred to here as *Tg(fabp10a:CFP-NTR)*, *Tg(fabp10a:DsRed)*, *Tg(Tp1:H2B-mCherry)*, *Tg(Tp1:Venus-PEST)*, *Tg(fabp10a:mAG-zGem)*, *Tg(fabp10a:rasGFP)*, and *Tg(hs:VP16-myca)*, respectively].

Hepatocyte ablation was performed by treating *Tg(fabp10a:CFP-NTR)* larvae with 10 mM metronidazole (Mtz) in egg water supplemented with 0.2% dimethyl sulfoxide (DMSO). For chemical screening, the larvae were treated with 44 compounds from A0 h to R24 h or from R0 h to R48 h, and *fabp10a*:DsRed expression at R24 h or R48 h was imaged to assess liver size using the Leica M205 FA microscope. For BET inhibition, larvae were treated with 3 μM JQ1 or 50 μM iBET151.

Detailed screening procedures, analytic methods, and the methods for mouse studies are described in the Supplementary material.

Results

BET inhibitor treatment impairs BEC-driven liver regeneration

We [8] and others [9,10] recently reported on a zebrafish liver regeneration model in which upon severe hepatocyte loss, regenerating hepatocytes are derived from BECs. In this model, transgenic fish that express nitroreductase (NTR) under the hepatocyte-specific *fabp10a* promoter were used. NTR converts the nontoxic prodrug, Mtz, into a cytotoxic drug; therefore, Mtz treatment results in hepatocyte ablation in the transgenic fish. Using this model, we performed a targeted chemical screen to identify pathways or factors that regulate BEC-driven liver regeneration (Supplementary Fig. 1). We used two transgenic lines for this screen: 1) *Tg(fabp10a:CFP-NTR)* that expresses NTR fused with cyan fluorescent protein (CFP) in hepatocytes, allowing for hepatocyte-specific ablation upon Mtz treatment; and 2) *Tg(fabp10a:DsRed)* that expresses DsRed in hepatocytes, allowing for easy assessment of liver size under an epifluorescence microscope. The double transgenic larvae were treated with Mtz from 3.5 to 5 days post-fertilization (dpf) for 36 h (ablation, A36 h). After Mtz washout (regeneration, R0 h), which is scored as the start of regeneration, the liver size was analyzed at R24 h or R48 h. BEC-driven liver regeneration progresses through several steps, including BEC dedifferentiation into hepatoblast-like cells (HB-LCs), their proliferation and subsequent differentiation into hepatocytes, and the proliferation of newly generated hepatocytes (Supplementary Fig. 2) [8]. To identify the effect of compounds on both early and late steps of the regeneration, each compound was applied during two different time-windows: from A0 h to R24 h and from R0 h to R48 h (Fig. 1A). We screened 44 compounds with known targets to determine if they could regulate liver regeneration; of these, 10 compounds significantly affected liver size (Fig. 1B; Supplementary Table 1). Among these 10 compounds, only JQ1 and iBET151 significantly affected liver size, as assessed by *fabp10a:*DsRed expression, in both A0 h-R24 h and R0 h-R48 h treatments (Fig. 1B, C).

To determine whether the hit compounds could block BEC dedifferentiation into HB-LCs, we examined the expression of both Prox1 and Hnf4a, used as HB-LC markers in combination [8], together with the expression of *Tp1:*H2B-mCherry, driven by Notch-responsive elements [22], which marks BECs and BEC-derived cells. The prolonged stability of H2B-mCherry allows one to trace cell lineages over several cell divisions [22]. JQ1 or iBET151 treatment blocked Prox1 and Hnf4a expression in *Tp1:*H2B-mCherry^{+} cells; GW4064, a farnesoid X receptor (FXR) agonist, reduced Hnf4a, but not Prox1, expression (Supplementary Table 1). Since the two BET inhibitors blocked both Prox1 and Hnf4a expression and BET proteins have not yet been implicated in liver regeneration, we selected these inhibitors for further detailed analyses. In the following analyses, JQ1 was preferably used over iBET151 due to the former's superior efficacy and lower working concentration (Supplementary Fig. 3).

JQ1 specifically inhibits BET protein family members: BRDT, BRD2, BRD3, and BRD4. While *Brdt* is specifically expressed in testis, *Brd2-4* are broadly expressed in mice [24,25]. We examined the expression of their zebrafish orthologs, *brd2a*, *brd2b*, *brd3a*, *brd3b* and *brd4*, in control and regenerating larvae. All of the five genes are expressed in the normal liver and the