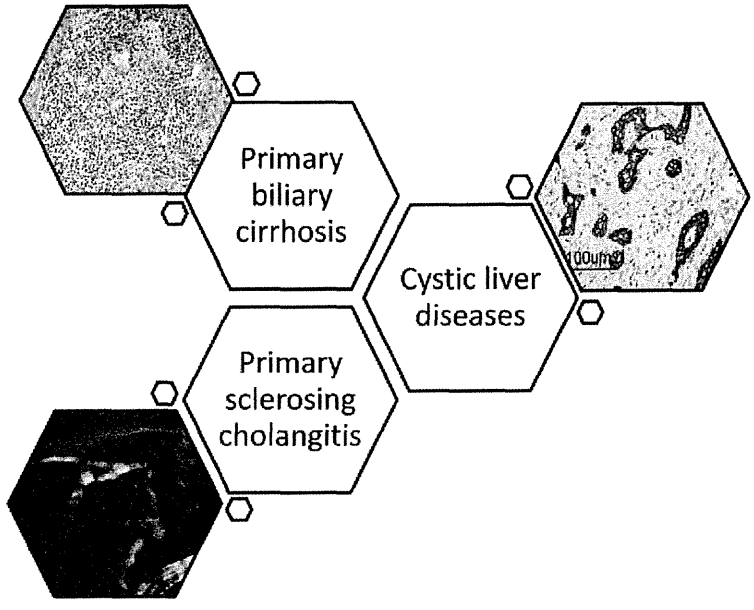


Fig. 3. Diverse groups of cholestatic liver diseases.
The representative human cholestatic liver diseases are listed. The pathogenesis of most of these diseases remains to be unclear.

replicative activity which is advantage for increased number of infected hepatocytes and 2) increased accumulation of virion in host's hepatocytes, leading to increased cytotoxicity. Thus, viral factors seemed to be more important and influential in FHB caused by this specific viral strain. Of course, more factors other than viral factors could be involved in the pathogenesis of FHB, although our experienced viral strain is the first case of reproducible fatale viral strain. Thus, future studies should include more sensitive methods to detect dangerous these viral factors in practice.

## Primary Biliary Cirrhosis (PBC)

PBC is also named as intractable liver disease by Japanese Ministry of Health, Labor, and Welfare. This rare disease could be summarized as chronic chloestatic liver disease, resulting in biliary cirrhosis and finally in liver failure (Ueno et al. 2007) as like other liver diseases such as primary sclerosing cholangitis (Fig. 3). In its early stage, ursodeoxycholic acid (UDCA) was reported to be effective in stand of the prolonged time needed for liver transplantation (Poupon et al. 1997). However, in the advanced stage, only liver transplantation is an effective option for improving the survival. Although the bile duct injury is executed by infiltrating lymphocytes, the application of corticosteroids, usually effective in other immune-mediated collagen diseases, is generally ineffective. Pathologically, this bile duct destruction is observed mainly in interlobular bile ducts, whereas larger (septal) or smaller (ductile) ducts are essentially preserved from injury. Thus, the size of the bile duct is characteristic and important in the pathogenesis of PBC. We have paid most of our attention on this selectivity of the bile duct to understand the pathogenesis of PBC.

First of all, the bile duct epithelial cells or cholangiocytes represent less than 3% of liver parenchymal cells. Until late 1980s, when the isolation and culture of cholangiocytes became possible, their physiological and cellular functions were poorly understood. Subsequently, along with the advancement of molecular techniques, the studies of cholangiocytes have been progressed rapidly. For example, we have demonstrated the expression of aquaporin 1 by cholangiocytes, which are influenced by hepatic inflammation (Roberts et al. 1994; Tamai et al. 2006). We have also proposed the idea of heterogeneity of cholangiocytes, and this idea has been proved physiologically and molecularly (Alpini et al. 1996, 1997). The heterogeneity includes the differences in the mechanism of cell death and intracellular pathway toward choleresis via PKC or cAMP (Alpini et al. 2001). Moreover, like the heterogeneity of renal tubules, large cholangiocytes have the different phenotype regarding molecular or physiological aspects (Lesage et al. 2001; Ueno et al. 2003). These studies about the understanding of the heterogeneity of cholangiocytes will contribute to answering the key question regarding the selective destruction of bile ducts seen in PBC. Recently, we have applied 2-dimension gel electrophoresis to perform proteomics study (Kido et al. 2009). With this sophisticated technique, we found that the expression of annexin A2 was increased in cholangiocytes when exposed to inflammatory cytokines. In addition, the artificial regulation of this molecule could affect choleresis, and thus the modification of the expression of annexin A2 could be a possible therapeutic application for the management of clinical cholestasis.

Apart from these cellular studies, several efforts have been done to establish animal models of PBC (Ueno et al.

1996, 2010; Moritoki et al. 2007, 2009a, 2009b: Chuang et al. 2008). Among them, we have found that CD95, a key antigen to execute apoptosis, plays central role in the pathogenesis of the cholangiocyte injury in both mouse graft versus host disease and NODc3c4 cholangiopathies (Ueno et al. 2000; Nakagome et al. 2007). The inhibition of CD95 also suppressed cytotoxicity in the graft versus host disease model.

More recently, the retrovirus infection was reported as a possible pathogen for pathogenesis of PBC (Xu et al. 2003). However, we could not find these viral derived endogenous genes specifically from serum samples obtained from patients with PBC. Moreover, therapeutic effects of administration of reverse transcriptase inhibitor, lamivudine, were not apparent in UDCA-refractory patients in randomized controlled trial (Fukushima et al. 2010). However, since a part of retroviral gene was found in liver sample from female NODc3c4 mice (Ninomiya et al. 2012), still the involvement of retorovirus in the pathogenesis of PBC remains to be unclear.

## Closing remarks

In this review article, the current endeavors to understand the pathogenesis of intractable liver diseases were introduced using FH and PBC. However, other than these two diseases, there are numbers of other liver diseases, such as liver cirrhosis, non-alcoholic fatty liver diseases, which are expected to increase in next decades (Kakazu et al. 2009; Obara et al. 2010; Kakazu et al. 2011). Obviously, still more efforts to understand these liver diseases are important to improve global healthcare.

## Acknowledgments

The receipt of the 2010 Gold Prize from Tohoku University School of Medicine was possible only with the outstanding mentorship from Professor Tooru Shimosewaga, and outstanding colleagues in hepatologists group at Tohoku University Graduate School of Medicine. Also, we are grateful for numbers of paramedics and technicians for supporting our experiments, especially staffs in experimental animal facility and cell culture laboratory.

## Conflict of Interest

The author does not have potential conflict of interest regarding this review article.

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*Am J Physiol Gastrointest Liver Physiol* 302: G182–G193, 2012.
First published October 6, 2011; doi:10.1152/ajpgi.00205.2011.

# Suppression of the HPA axis during extrahepatic biliary obstruction induces cholangiocyte proliferation in the rat

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Submitted 26 May 2011; accepted in final form 30 September 2011

**Quinn M, Ueno Y, Pae HY, Huang L, Frampton G, Galindo C, Francis H, Horvat D, McMillin M, DeMorrow S.** Suppression of the HPA axis during extrahepatic biliary obstruction induces cholangiocyte proliferation in the rat. *Am J Physiol Gastrointest Liver Physiol* 302: G182–G193, 2012. First published October 6, 2011; doi:10.1152/ajpgi.00205.2011.—Cholestatic patients often present with clinical features suggestive of adrenal insufficiency. In the bile duct-ligated (BDL) model of cholestasis, the hypothalamic-pituitary-adrenal (HPA) axis is suppressed. The consequences of this suppression on cholangiocyte proliferation are unknown. We evaluated *1*) HPA axis activity in various rat models of cholestasis and *2*) effects of HPA axis modulation on cholangiocyte proliferation. Expression of regulatory molecules of the HPA axis was determined after BDL, partial BDL, and α-naphthylisothiocyanate (ANIT) intoxication. The HPA axis was suppressed by inhibition of hypothalamic corticotropin-releasing hormone (CRH) expression by central administration of CRH-specific Vivo-morpholinos or by adrenalectomy. After BDL, the HPA axis was reactivated by *1*) central administration of CRH, *2*) systemic ACTH treatment, or *3*) treatment with cortisol or corticosterone for 7 days postsurgery. There was decreased expression of *1*) hypothalamic CRH, *2*) pituitary ACTH, and *3*) key glucocorticoid synthesis enzymes in the adrenal glands. Serum corticosterone and cortisol remained low after BDL (but not partial BDL) compared with sham surgery and after 2 wk of ANIT feeding. Experimental suppression of the HPA axis increased cholangiocyte proliferation, shown by increased cytokeratin-19- and proliferating cell nuclear antigen-positive cholangiocytes. Conversely, restoration of HPA axis activity inhibited BDL-induced cholangiocyte proliferation. Suppression of the HPA axis is an early event following BDL and induces cholangiocyte proliferation. Knowledge of the role of the HPA axis during cholestasis may lead to development of innovative treatment paradigms for chronic liver disease.

corticotropin-releasing hormone; glucocorticoids; biliary epithelium; adrenocorticotropic hormone

CHOLANGIOCYTES ARE EPITHELIAL cells that line the intra- and extrahepatic bile ducts. They are constitutively mitotically dormant but possess marked proliferative capacity (2), which is apparent during experimental conditions, such as cholestasis induced by bile duct ligation (BDL) or α-naphthylisothiocyanate (ANIT) intoxication (1), as well as in human cholangiopathies (2). In humans, cholangiocyte proliferation occurs in extrahepatic biliary obstruction, in the course of chronic cholestatic liver diseases (e.g., primary sclerosing cholangitis, primary biliary cirrhosis, liver allograft rejection, and graft-vs.-host disease) (2), and in many forms of liver injury (e.g., in response to alcohol, toxin, or drugs) (2, 43).

The hypothalamic-pituitary-adrenal (HPA) axis describes a complex set of positive- and negative-feedback influences between the hypothalamus, pituitary gland, and adrenal gland (25). These feedforward and feedback mechanisms work in a neuroendocrine manner to modulate a number of physiological processes, such as immunity (32), digestion (30), and the body's response to stress (30). In addition, the HPA axis has been shown to have an influence on human psychology (7, 53).

The mechanism by which the HPA axis remains in homeostasis depends widely on the release and uptake of several key regulatory molecules. The hypothalamus contains neuroendocrine neurons that secrete corticotropin-releasing hormone (CRH), among other humoral agents. CRH will, in turn, act on the pituitary gland to stimulate the production of ACTH, which is derived from the proteolytic cleavage of proopiomelanocortin (POMC) to release ACTH, as well as other peptide fragments such as β-lipotropin (44), into the circulation. Circulating ACTH then induces the adrenal gland to synthesize and release circulating corticosteroids, such as cortisol and corticosterone. These circulating corticosteroids then modulate the vast array of physiological processes influenced by the HPA axis and are also responsible for initiating a negative-feedback loop to shut down corticosteroid production. There are several mechanisms by which the HPA axis can be suppressed. The hippocampus, which is the major controlling organ of the HPA axis, is the initial location in which the HPA axis can be suppressed. However, every region involved in the HPA axis has a negative-feedback mechanism, all leading to reduced circulating corticosteroid levels (6, 8, 24, 26, 27, 29).

Cholestatic patients exhibit clinical features suggestive of adrenal insufficiency, such as hypovolemia, hypotension, and renal failure (55, 57). Furthermore, patients with congenital hypopituitarism or glucocorticoid deficiency often exhibit cholestatic hepatitis (5, 20, 28). In the BDL rat model of cholestasis, there is a general suppression of HPA axis responsiveness to stress (48), as well as a defective CRH-mediated response (46). However, the implications of HPA axis suppression and the subsequent effects of central CRH administration on cholangiocyte proliferation and function have not been studied. Therefore, the aims of the present study were to elucidate the connection between the HPA axis and the liver, in particular how the HPA axis can affect cholangiocyte proliferation.

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## MATERIALS AND METHODS

### *Materials*

Unless otherwise indicated, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO) and were of the highest grade available. Antibodies against proliferating cell nuclear antigen (PCNA), cytochrome *P*-450 11b1 (Cyp11b1), hydroxysteroid dehydrogenase 3β (HSD-3β), and CRH were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The specific cytokeratin-19 (CK-19) antibody was purchased from Vector Laboratories (Burlingame, CA), and the ACTH-specific antibody was purchased from Abcam (Cambridge, MA). All primers were purchased from SABiosciences (Frederick, MD). The CRH Vivo-morpholino (5′-ACCAGCAGCCGCAGCCGCATGTTTA) and corresponding mismatched control sequence (5′-ACgAcCAGCgGCAGCCcCATcTTTA) were purchased from Gene Tools (Philomath, OR). Recombinant rat ACTH and the enzyme immunoassay (EIA) kits for detecting CRH and ACTH were obtained from Phoenix Pharmaceuticals (Burlingame, CA). Cortisol and corticosterone EIA kits were purchased from Cayman Chemical (Ann Arbor, MI).

### *Animal Treatment*

Male Sprague-Dawley rats (150–175 g body wt) were purchased from Charles River and maintained in a temperature-controlled environment (20–22°C) with a 12:12-h light-dark cycle. Animals had free access to drinking water and standard rat chow. All animal experiments were performed in accordance with the guidelines of the Scott and White Institutional Animal Care and Use Committee, which approved the procotol. Rats underwent surgery to ligate the common bile duct (BDL) or a single lobe (partial BDL), as described previously (18). For the ANIT intoxication studies, rats were fed a diet containing 0.1% ANIT or the control diet (AIN 76, Dyets, Bethlehem, PA), as described elsewhere (34). A description of the experimental groups is outlined in Table 1. On the days indicated postsurgery, tissue and serum were collected for further analysis between 8 and 9 AM to minimize the circadian variations in glucocorticoid levels (11).

### *Cholangiocyte Proliferation*

Cholangiocyte proliferation was assessed in liver sections from the treatment groups by *1*) immunohistochemical staining for CK-19 to assess intrahepatic biliary mass and *2*) PCNA immunoreactivity as a marker of proliferative capacity using the method described elsewhere (14, 34). After they were stained, the sections were counterstained with hematoxylin and examined with a microscope (model BX 40, Olympus Optical). Over 100 cholangiocytes were counted in a random, blinded fashion in 3 different fields for each group of animals. Data are expressed as number of CK-19- and PCNA-positive cholangiocytes per portal tract.

### *Assessment of HPA Axis Activity*

The circulating glucocorticoids represent the functional output of the HPA axis (41). Circulating cortisol and corticosterone levels in serum from normal and BDL rats were assessed using commercially available EIA kits according to the manufacturer's instructions.

*CRH levels in the hypothalamus.* CRH expression and secretion were assessed at various times after BDL by *1*) real-time PCR (9), *2*) immunoblotting (10), *3*) immunofluorescence (10, 21), and *4*) CRH-specific EIA. Briefly, rats underwent BDL surgery, and at various times postsurgery the animals were euthanized and the brain was removed. The hypothalamus was dissected and rapidly snap-frozen in liquid nitrogen. For the real-time PCR analysis, RNA was extracted using the RNeasy Mini kit (Qiagen, Valencia, CA) according to the instructions provided by the vendor and reverse-transcribed using the Reaction Ready First Strand cDNA synthesis kit (SABiosciences-Qiagen, Frederick, MD). These reactions were used as templates for the PCR assays using a SYBR Green PCR Master Mix (SABiosciences-Qiagen) in the real-time thermal cycler (Mx3005P sequence detection system, Agilent) and specific primers against rat CRH (SABiosciences-Qiagen; Ref Seq NM_031019, product size 108 bp, reference position 650 bp). A cycle threshold ($\Delta\Delta C_T$) analysis was performed (9, 35) using the normal tissue as the control sample. Data are expressed as relative mRNA levels (means ± SE, $n = 4$).

CRH protein expression was assessed in hypothalamic protein lysates using immunoblotting, as described previously (10), and a CRH-specific antibody (C-20, Santa Cruz Biotechnology). The intensity of the bands was determined by scanning video densitometry using a PhosphorImager (Storm 860, Amersham Biosciences, Piscataway, NJ) and ImageQuant TLV 2003.02 software. Furthermore, CRH immunoreactivity was assessed in brain sections by immunofluorescence. Normal or BDL rats were transcardially perfused with 4% paraformalydehyde. The brains were removed and postfixed for a further 24 h in 4% paraformalydehyde and then cryoprotected in 30% sucrose (wt/vol in 1× PBS). Free-floating immunofluorescence staining of CRH was performed in brain sections (20 μm) via the method described previously (10, 21). Negative controls were stained using preimmune serum in the place of the primary antibody. Images were taken on an inverted confocal microscope (model IX71, Olympus).

Table 1. *Summary of animal treatment groups*

| | Treatment | Tissue Collection |
|---|---|---|
| BDL | None | 1, 3, 5, and 7 days |
| Sham BDL | | 1, 3, 5, and 7 days |
| Partial BDL | | 7 days |
| 0.1% ANIT intoxication | | 7 and 14 days |
| ANIT control diet | | 7 and 14 days |
| Adrenalectomy | | 7 days |
| | CRH morpholino (4 μg·rat$^{-1}$·day$^{-1}$ icv) | 7 days |
| | CRH mismatched morpholino (4 μg·rat$^{-1}$·day$^{-1}$ icv) | 7 days |
| BDL | ACTH (100 μg·kg$^{-1}$·day$^{-1}$ ip) | 7 days |
| Sham BDL | ACTH (100 μg·kg$^{-1}$·day$^{-1}$ ip) | 7 days |
| BDL | CRH (5 μg·rat$^{-1}$·day$^{-1}$ icv) | 7 days |
| Sham BDL | CRH (5 μg·rat$^{-1}$·day$^{-1}$ icv) | 7 days |
| BDL | Cortisol (1 mg·kg$^{-1}$·day$^{-1}$ ip) | 7 days |
| Sham BDL | Cortisol (1 mg·kg$^{-1}$·day$^{-1}$ ip) | 7 days |
| BDL | Corticosterone (1 mg·kg$^{-1}$·day$^{-1}$ ip) | 7 days |
| Sham BDL | Corticosterone (1 mg·kg$^{-1}$·day$^{-1}$ ip) | 7 days |

Intraperitoneal treatments were administered via implanted osmotic minipump. BDL, bile duct ligation; ANIT, α-naphthylisothiocyanate; CRH, corticotropin-releasing hormone.

In parallel, circulating (in serum) and tissue (in hypothalamic protein lysates) levels of CRH in normal and BDL rats were assessed using a CRH-specific EIA (Phoenix Pharmaceuticals) following the manufacturer's instructions. Tissue CRH levels are expressed as the ratio of CRH to total protein in the sample, whereas circulating CRH levels are expressed as the amount of CRH per milliliter of serum.

*ACTH levels in the pituitary.* ACTH expression and secretion were assessed at various times after BDL by *1*) real-time PCR (9), *2*) immunoblotting (10), and *3*) ACTH-specific EIA. Briefly, rats underwent BDL surgery, and at various times postsurgery the animals were euthanized and the pituitaries were removed and snap-frozen. Primers specific for rat POMC (SABiosciences-Qiagen; Ref Seq NM_13926,

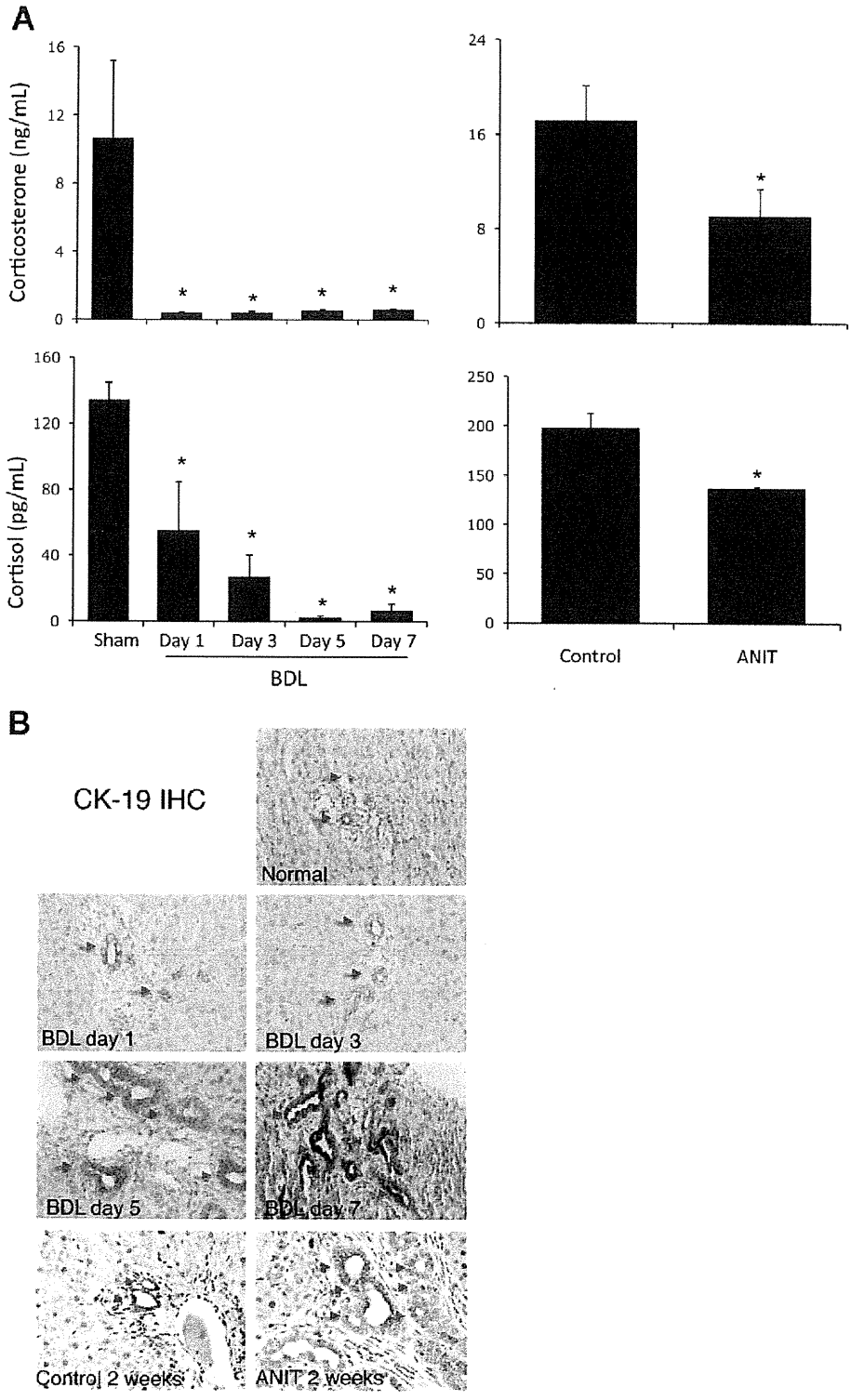


Fig. 1. Circulating glucocorticoid levels are reduced as an early event after bile duct ligation (BDL) and after 2 wk of α-naphthylisothiocyanate (ANIT) intoxication. *A*: cortisol and corticosterone in serum from rats after BDL surgery or ANIT feeding. Values are means ± SE; $n = 5$. *$P < 0.05$ vs. sham. *B*: cholangiocyte proliferation at various times after BDL surgery or ANIT feeding assessed by cytokeratin-19 (CK-19) immunohistochemistry (IHC). Original magnification ×20.

product size 160 bp, reference position 673 bp) were used in the real-time PCR analysis as a reflection of ACTH mRNA expression. Immunoblotting for ACTH was performed as outlined above using an antibody directed against amino acids 1–20 of ACTH (clone B427, Abcam). In parallel, circulating (in serum) and tissue (in pituitary protein lysates) levels of ACTH in normal and BDL rats were assessed using an ACTH-specific EIA (Phoenix Pharmaceuticals) following the manufacturer's instructions.

*Expression of steroidogenic enzymes in the adrenal glands.* The expression levels of two key enzymes in the synthesis of glucocorticoids, HSD-3β and Cyp11b1, were assessed in the adrenal glands by real-time PCR and immunoblotting. Specifically, rats underwent BDL surgery, and at various times postsurgery the animals were euthanized and the adrenal glands were removed and snap-frozen. Specific primers for HSD-3β (SABiosciences-Qiagen; Ref Seq NM_001007719, product size 96 bp, reference position 1,462 bp) and Cyp11b1 (SABiosciences-Qiagen; Ref Seq NM_012537, product size 84 bp, reference position 1,472 bp) were used in the real-time PCR analysis, as described above. Immunoblotting was also performed using specific antibodies against HSD-3β (Santa Cruz Biotechnology) and Cyp11b1 (Santa Cruz Biotechnology).

## RESULTS

### *HPA Axis Is Suppressed in Experimental Models of Cholestasis*

Circulating levels of cortisol and corticosterone were dramatically decreased from 1 to 7 days after BDL surgery compared with sham-operated rats (Fig. 1*A*) but were not suppressed after partial single-lobe BDL (data not shown). Using another well-characterized model of cholestasis involving the feeding of ANIT to rats, we observed a decrease in circulating levels of cortisol and corticosterone after 2 wk on the ANIT diet (Fig. 1*A*), but not at 1 wk (data not shown). To put this into a temporal context, we assessed cholangiocyte proliferation in parallel and found a slight increase in biliary mass 1 and 3 days after surgery and a more significant increase 5 and 7 days after surgery (Fig. 1*B*). In the ANIT model of cholestasis, biliary mass did not change significantly after 1 wk (data not shown) but increased dramatically at 2 wk (Fig. 1*B*). Because the more dramatic effects on the HPA axis were found

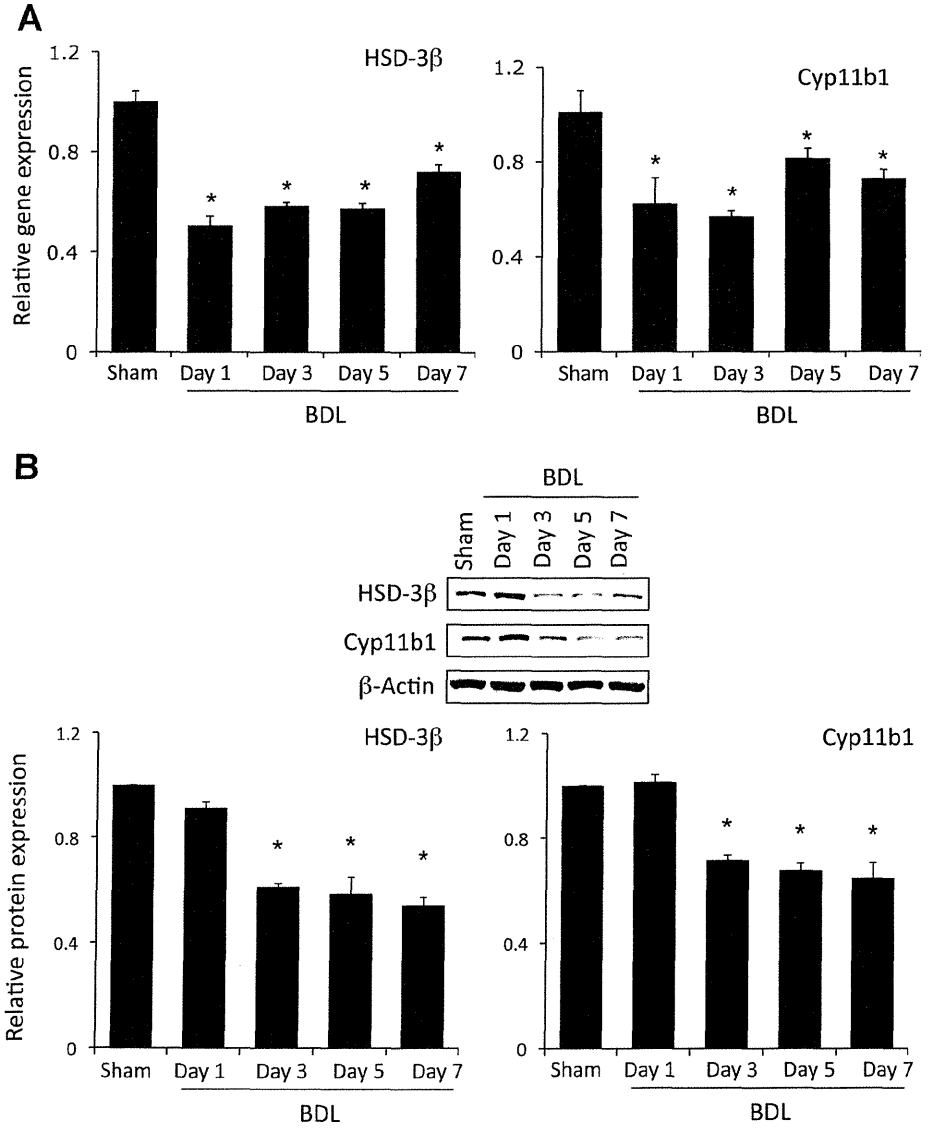


Fig. 2. Key glucocorticoid synthesis enzymes are downregulated in adrenal glands after BDL. Rats underwent BDL surgery, and adrenal glands were dissected at various times postsurgery. Expression of hydroxysteroid dehydrogenase 3β (HSD-3β) and cytochrome *P*-450 11b1 (Cyp11b1) was assessed by real-time PCR (*A*) and immunoblotting (*B*). Values are means ± SE; $n = 4$. *$P < 0.05$ vs. sham.

using the BDL model of cholestasis, subsequent experiments were performed using this model.

To assess if the reduced circulating glucocorticoid levels are a result of decreased steroidogenesis and to further pinpoint the precise level at which suppression of the HPA axis occurs during cholestasis, expression levels of two of the key regulatory enzymes in the steroidogenic pathway were assessed in the adrenal gland. HSD-3β is an enzyme that is involved in the early stages of conversion of cholesterol to glucocorticoids and is responsible for the enzymatic conversion of pregnenolone and its derivatives to progesterone derivatives (52). The mRNA and protein expression of HSD-3β is suppressed as an early event (1 day) after BDL surgery, an effect that continues up to 7 days (Fig. 2).

One of the downstream events in steroid biosynthesis is conversion of deoxycorticosterone and 11-deoxycortisol to corticosterone and cortisol, respectively. Both of these reactions are catalyzed by Cyp11b1 (54), the expression of which is also significantly reduced 1–7 days after BDL surgery (Fig. 2). Taken together, our data suggest that the decrease in circulating glucocorticoid levels after BDL is probably due to a decrease in the expression of key regulatory enzymes in the steroidogenesis pathway.

The expression and activity of the steroidogenesis enzymes is regulated by the secretion of ACTH from the anterior pituitary into the bloodstream (40). Therefore, we wished to determine if the reduced expression of the steroidogenesis enzymes is a result of decreased ACTH levels in the pituitary. As mentioned previously, ACTH is derived from the proteolytic cleavage of POMC to release the active ACTH peptide (44). POMC mRNA expression was decreased after BDL (Fig. 3*A*), suggesting a suppressed expression of the ACTH precursor. In addition, ACTH protein expression and content in the pituitary were decreased significantly after BDL surgery compared with sham-treated animals (Fig. 3, *B* and *C*). This suppressed expression also translated to a reduced level of circulating ACTH in the serum of BDL rats from 1 day postsurgery (Fig. 3*D*).

ACTH expression and secretion in the pituitary are under the direct control of CRH released from the hypothalamus (45). Given that we see decreased ACTH expression and release from the pituitary, we also wanted to evaluate if there was a decrease in hypothalamic CRH expression and secretion after BDL. CRH mRNA and protein expression were significantly decreased from 1 to 7 days after BDL surgery (Fig. 4, *A* and *B*). Furthermore, CRH was strongly expressed in the paraventricular nucleus of the hypothalamus of sham-operated animals but suppressed after BDL surgery (Fig. 4*C*). In addition, the CRH content of the hypothalamus decreased significantly 1 day after surgery, an effect that continued up to 7 days (Fig. 4*D*). Taken together, these data suggest that the hypothalamic CRH expression is suppressed as an early event after BDL surgery. To determine if this also translates to a decrease in CRH secretion, circulating CRH levels were assessed in the serum. Indeed, a decrease in CRH amounts

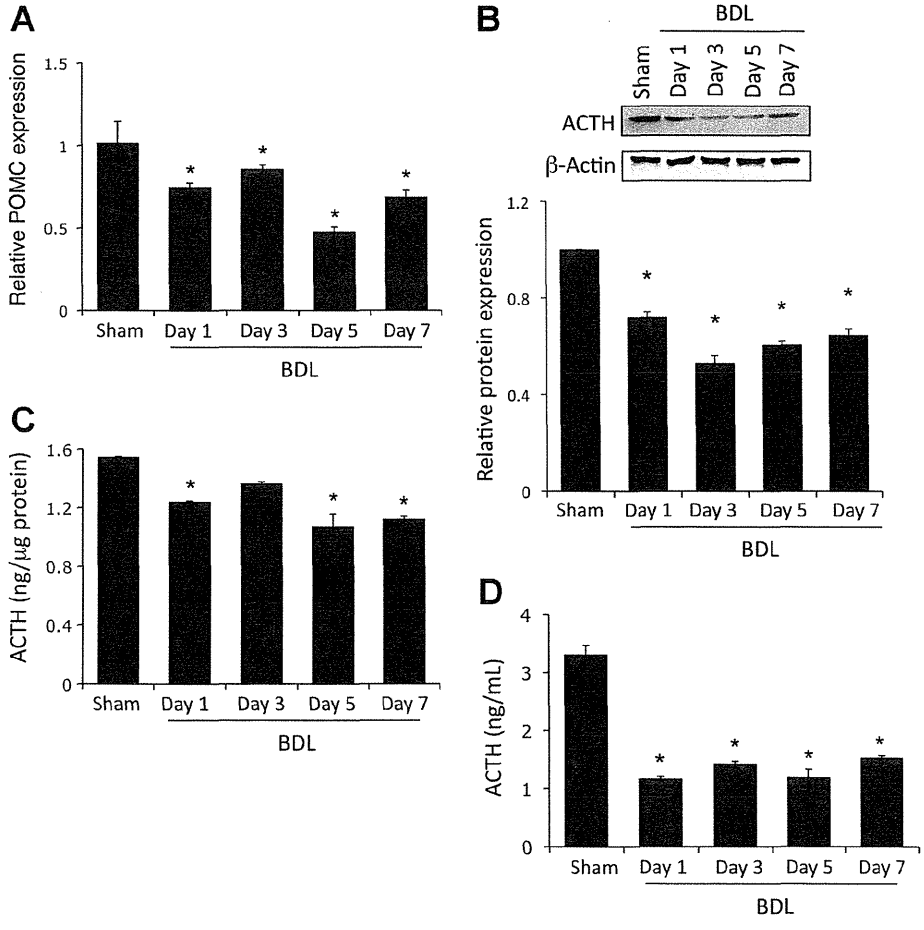


Fig. 3. ACTH expression is downregulated in the pituitary after BDL. Rats underwent BDL surgery, and the pituitary was dissected at various times postsurgery. *A*: expression of the precursor peptide proopiomelanocortin (POMC) assessed by real-time PCR. *B*: ACTH protein expression assessed by immunoblotting. Values are means ± SE; $n = 4$. *$P < 0.05$ vs. sham. *C* and *D*: ACTH levels in pituitary lysates and serum, respectively, assessed using commercially available enzyme immunoassay (EIA). Values are means ± SE; $n = 5$. *$P < 0.05$ vs. sham.

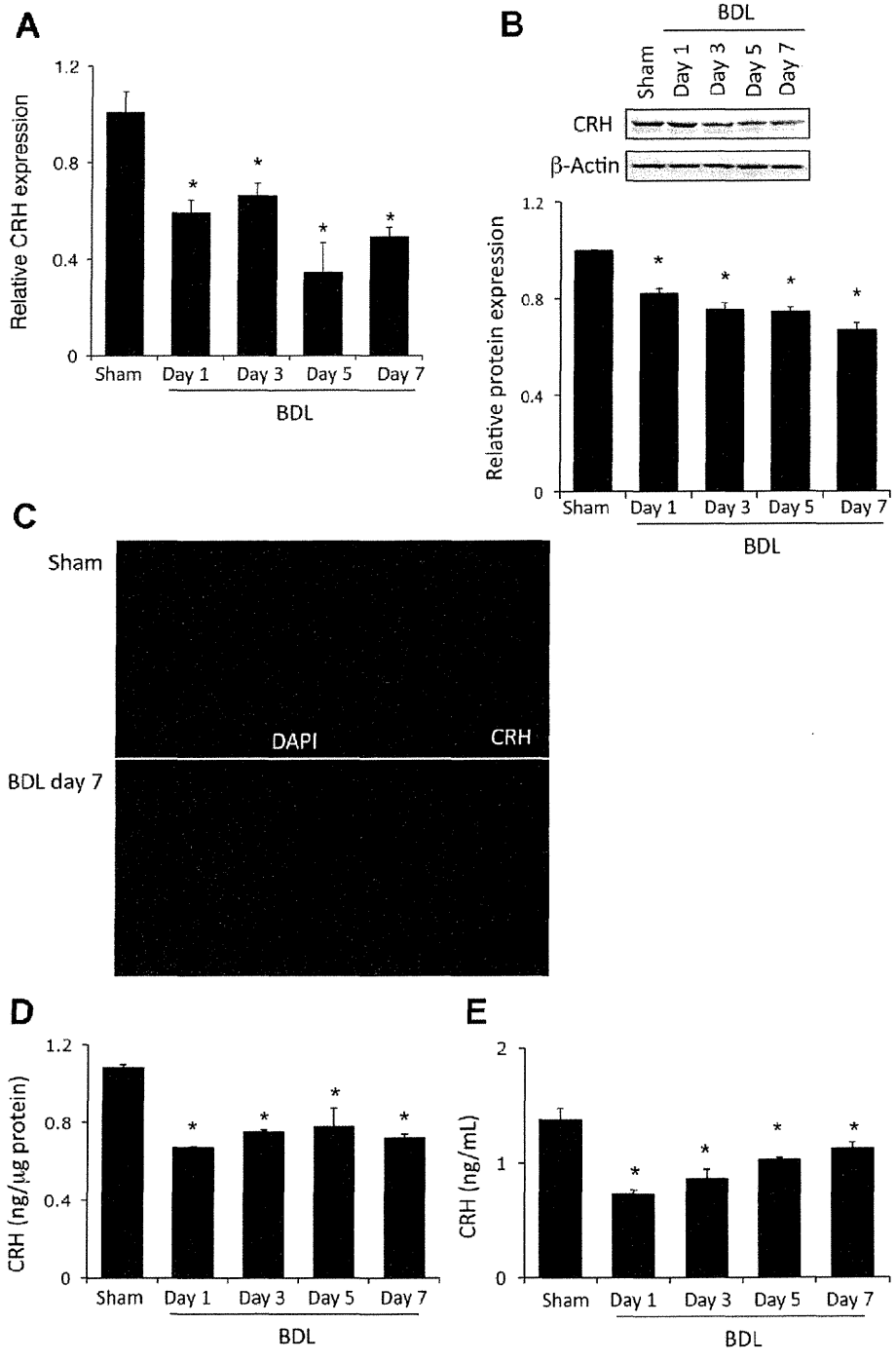


Fig. 4. Corticotropin-releasing hormone (CRH) expression is downregulated in the hypothalamus after BDL. Rats underwent BDL surgery, and the hypothalamus was dissected at various times postsurgery. Expression of CRH was assessed by real-time PCR (*A*) and immunoblotting (*B*). Values are means ± SE; $n = 4$. *$P < 0.05$. *C*: hypothalamic CRH immunoreactivity assessed by immunofluorescence (red) in whole brain sections 7 days after BDL or sham surgery. Sections were counterstained with 4′,6-diaminido-2-phenylindole (DAPI, blue). Original magnification ×40. *D* and *E*: CRH levels in pituitary lysates and serum, respectively, assessed by commercially available EIA. Values are means ± SE; $n = 5$. *$P <$ 0.05 vs. sham.

in the serum was observed 1 day after BDL, and this decrease continued over the time course studied (Fig. 4*E*). Taken together, our data indicate that all regulatory humoral factors regulating the HPA axis are suppressed after BDL, although the precise mechanism by which this occurs is largely unknown.

### *Suppression of the HPA Axis Increased Biliary Mass by Inducing Cholangiocyte Proliferation*

To determine the consequences (if any) of reduced glucocorticoid levels caused by suppression of the HPA axis on cholangiocyte proliferation, we took a two-tiered approach. First, HPA axis activity was suppressed by surgical removal of the adrenal glands. As expected, adrenalectomy significantly reduced the serum levels of corticosterone (Fig. 5*A*). Suppression of the HPA axis in this manner significantly increased bile duct mass 7 days after surgery, as demonstrated by CK-19 immunoreactivity (Fig. 5*B*). Furthermore, the percentage of PCNA-positive cholangiocytes increased after BDL and adrenalectomy (Fig. 5*C*), suggesting that bile duct mass is increased by increasing the proliferative capacity of cholangiocytes.

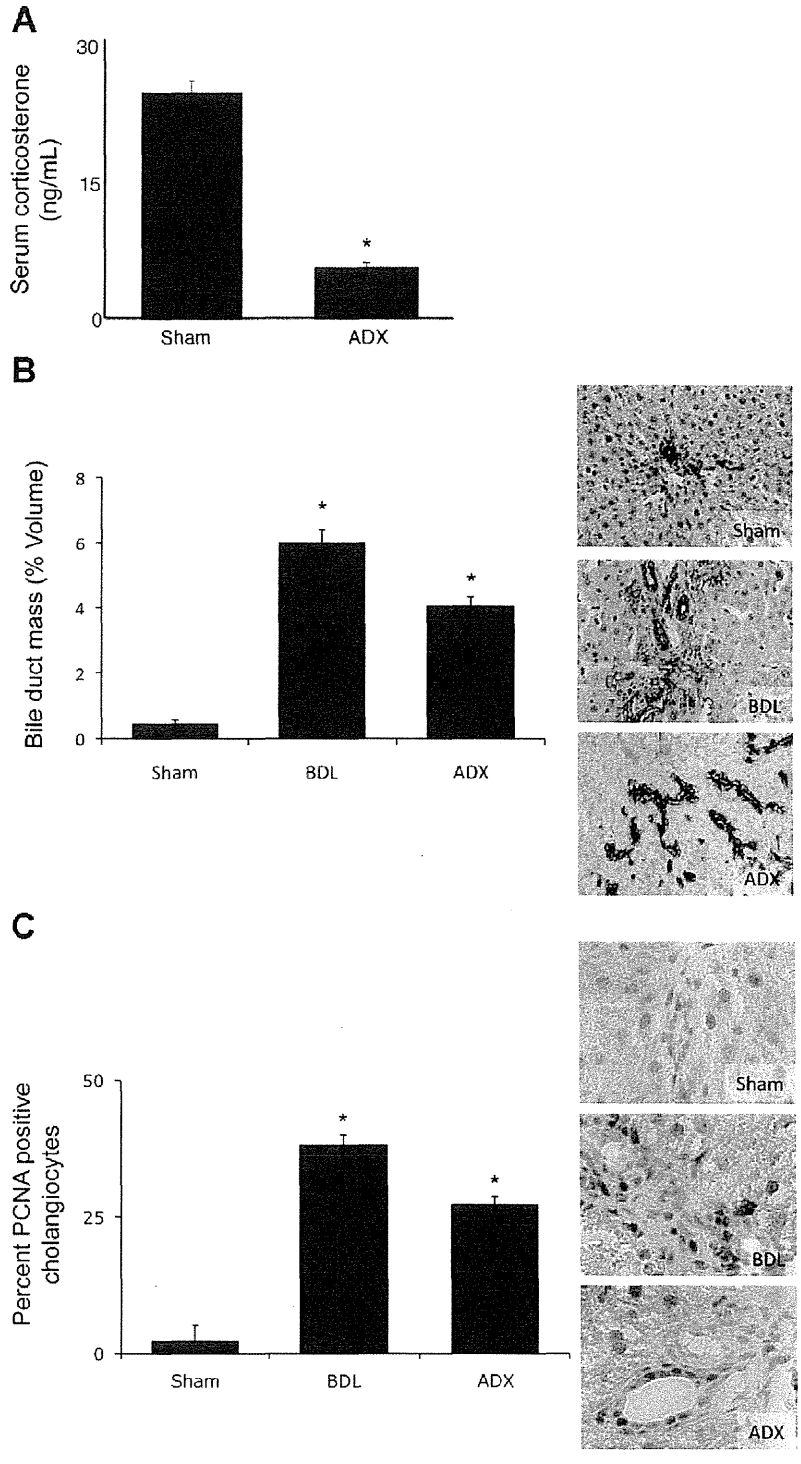


Fig. 5. Adrenalectomy (ADX) induces cholangiocyte proliferation. *A*: circulating corticosterone levels 7 days after ADX or BDL, assessed by commercially available EIA. Values are means ± SE; $n = 5$. *$P < 0.05$. *B* and *C*: cytokeratin-19 (CK-19)-positive cholangiocytes and proliferating cell nuclear antigen (PCNA)-expressing cholangiocytes in liver sections 7 days after ADX or BDL. Values are means ± SE; $n = 7$. *$P < 0.05$ vs. sham.

The second approach involved suppression of hypothalamic expression of CRH by the central administration of CRH-specific Vivo-morpholinos for 7 days. Vivo-morpholinos are antisense reagents that contain a novel transporter structure for effective use in vivo, used to block translation or interfere with RNA processing (39). Daily injection (intracerebroventricular) of CRH-specific Vivo-morpholinos decreased CRH expression by ~60% compared with naive animals. Injection of the mismatched control sequence, having 6 bp changed, had no effect on CRH expression (Fig. 6*A*). Furthermore, central administration of CRH-specific Vivo-morpholinos significantly decreased the circulating corticosterone levels (Fig. 6*B*), which suggests that the genetic knockdown of CRH expression in the hypothalamus results in an overall suppression of the HPA axis. In support of the adrenalectomy data

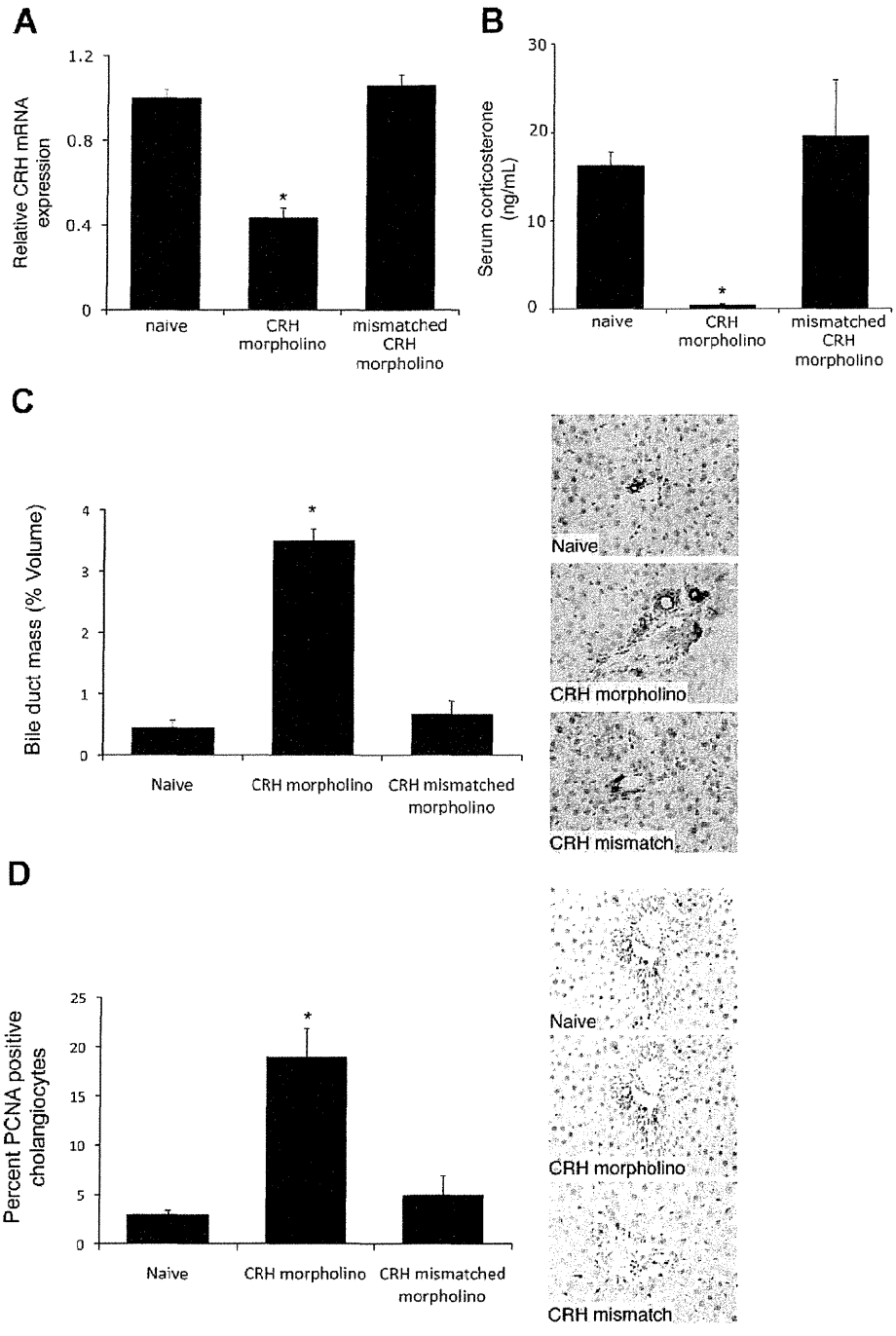


Fig. 6. Knockdown of hypothalamic CRH expression induces cholangiocyte proliferation. Rats were injected with CRH Vivo-morpholino (4 $\mu g \cdot rat^{-1} \cdot day^{-1}$ icv) or the mismatched control Vivo-morpholino for 7 days. *A*: hypothalamic CRH expression assessed by real-time PCR. Values are means ± SE; $n = 4$. $^*P < 0.05$ vs. naive. *B*: circulating corticosterone assessed by commercially available EIA. Values are means ± SE; $n = 5$. $^*P < 0.05$. *C* and *D*: CK-19-positive cholangiocytes and PCNA-expressing cholangiocytes in liver sections. Values are means ± SE; $n = 7$. $^*P < 0.05$ vs. naive.

described above, suppression of the HPA axis by knockdown of hypothalamic CRH expression also increased biliary mass, which was not observed after intracerebroventricular injections of the mismatched control Vivo-morpholino sequence (Fig. 6*C*). Furthermore, there was an increased number of PCNA-positive cholangiocytes after central administration of CRH Vivo-morpholinos compared with naive animals or after central administration of the mismatched control sequence (Fig. 6*D*). Taken together, our data suggest that suppression of the HPA axis has the capacity to induce cholangiocyte proliferation.

*Restoring HPA Axis Function Attenuates Cholangiocyte Proliferation Observed During Extrahepatic Biliary Obstruction*

We next evaluated if reactivation of the HPA axis can prevent the cholangiocyte proliferation as a result of biliary obstruction. We again took a parallel approach and administered recombinant ACTH systemically or administered CRH locally into the ventricle (intracerebroventricularly) prior to BDL or sham surgery. Both of these treatment strategies increased corticosterone to levels above those in sham control

animals (Fig. 7*A*). Reactivation of the HPA axis by systemic ACTH or local, central CRH treatment had no effect on biliary mass in sham-operated animals (Fig. 7*B*). However, both treatment regimens attenuated the increase in biliary mass after BDL surgery (Fig. 7*B*). Furthermore, there was no observable change in the number of PCNA-positive cholangiocytes after ACTH or CRH treatment in sham-operated animals, whereas there was a marked suppression of PCNA-positive cholangiocytes after CRH and ACTH treatment in BDL animals (Fig. 7*C*).

The functional output of HPA axis activation is an increase in glucocorticoid synthesis and release. To test the validity of our hypothesis that restoration of HPA axis activity inhibits cholangiocyte proliferation, we treated rats with systemic cortisol or corticosterone immediately after BDL or sham surgery. Glucocorticoid treatment had no effect on biliary mass in sham-operated animals (Fig. 8*A*), whereas glucocorticoid administration significantly reduced biliary mass 7 days after BDL surgery (Fig. 8*A*). In parallel, there was no observable change in the number of PCNA-positive cholangiocytes after treatment with cortisol or corticosterone in sham-operated animals, whereas there was a marked suppression of the number of PCNA-positive cholangiocytes in BDL animals after treatment with either glucocorticoid (Fig. 8*B*).

## DISCUSSION

The major findings of this study relate to the interrelationship between biliary obstruction and the HPA axis. Specifically, the expression and secretion of the key regulatory molecules that drive HPA axis activity and resulting glucocorticoid levels are rapidly suppressed in experimental rodent models of cholestatic liver diseases. Furthermore, our data suggest that suppression of the HPA axis, by a number of surgical and genetic techniques, stimulates cholangiocyte proliferation in a manner similar to that normally observed after BDL. Conversely, reactivation of the HPA axis after BDL attenuated hyperplastic cholangiocyte proliferation. Taken together, our data suggest that modulation of the HPA axis may be a target for the maintenance of biliary mass during cholestatic liver diseases.

In animal models of cholestasis, as well as in human cholangiopathies, cholangiocytes proliferate or are damaged (1, 2, 4). In the BDL rat model, which is widely used for evaluating the

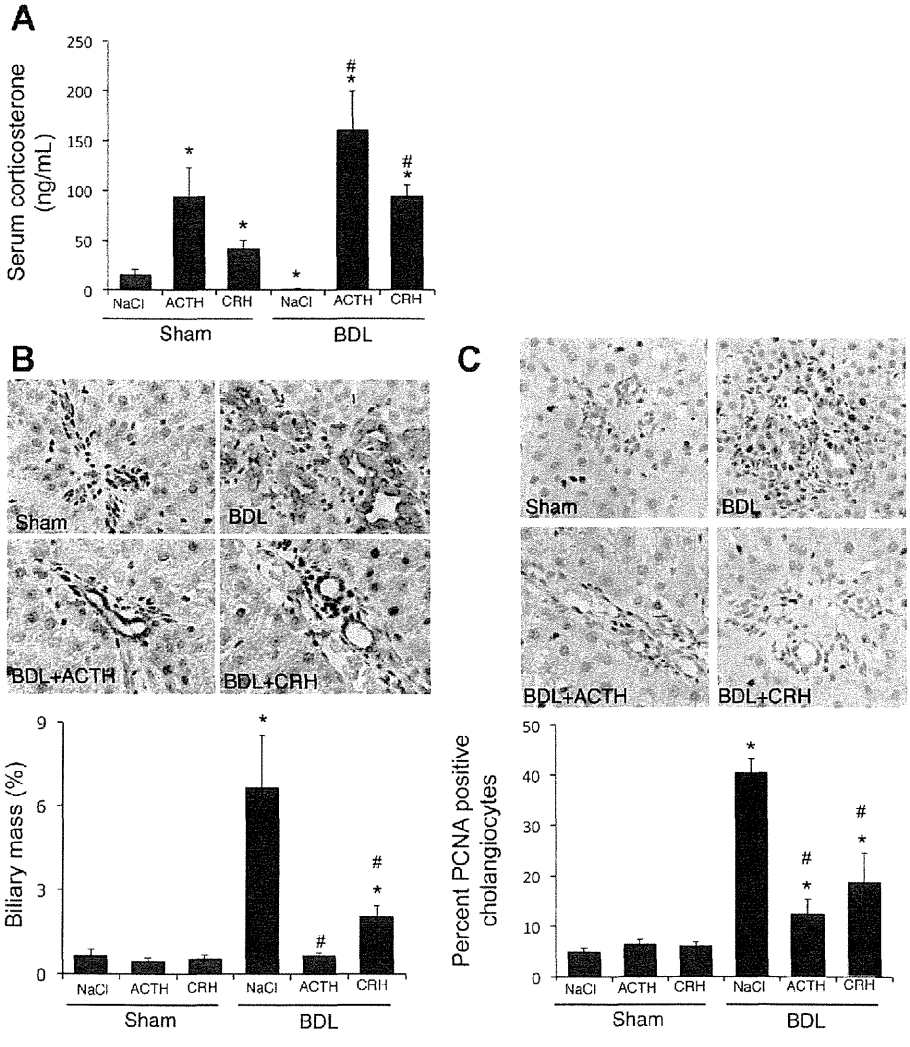


Fig. 7. Restoration of the hypothalamic-pituitary-adrenal (HPA) axis inhibits cholangiocyte proliferation after BDL. Rats underwent BDL or sham surgery and then treated systemically with ACTH (100 $\mu g \cdot kg^{-1} \cdot day^{-1}$ ip) or centrally with CRH (5 $\mu g \cdot rat^{-1} \cdot day^{-1}$ icv) for 7 days. *A*: circulating corticosterone levels assessed by commercially available EIA. Values are means ± SE; $n = 5$. *$P < 0.05$ vs. sham. #$P < 0.05$ vs. BDL. *B* and *C*: CK-19-positive cholangiocytes and PCNA-expressing cholangiocytes in liver sections. Values are means ± SE; $n = 7$. *$P < 0.05$ vs. sham. #$P < 0.05$ vs. BDL.

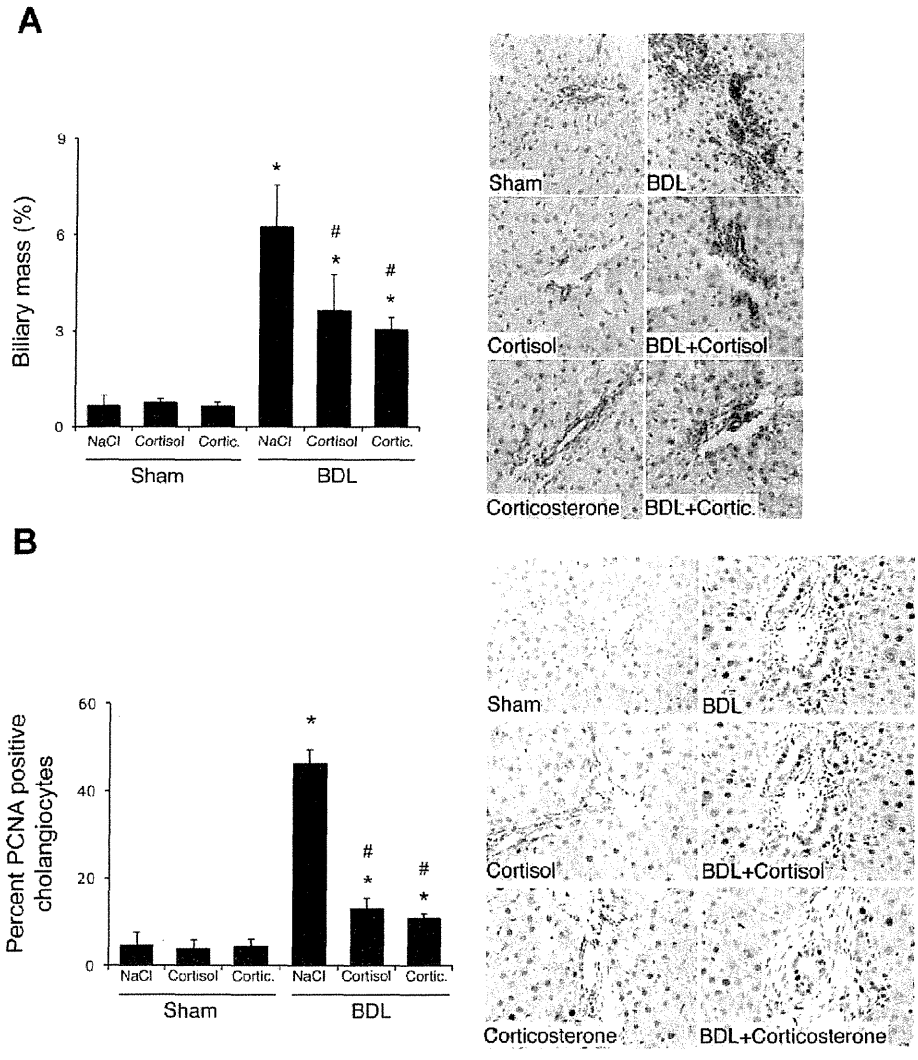


Fig. 8. Systemic treatment of rats with cortisol and corticosterone inhibited cholangiocyte proliferation after BDL. Rats underwent BDL or sham surgery and were treated with cortisol and corticosterone (1 $mg \cdot kg^{-1} \cdot day^{-1}$) for 7 days postsurgery. *A* and *B*: CK-19-positive cholangiocytes and PCNA-expressing cholangiocytes in liver sections. Values are means ± SE; $n = 7$. *$P < 0.05$ vs. sham. #$P < 0.05$ vs. BDL.

mechanisms of cholangiocyte hyperplasia (1), there is an increase in ductal mass (1, 15, 31) and secretin-stimulated cAMP levels (17, 31, 33). In humans, cholangiocyte proliferation occurs in biliary obstruction, in chronic cholestatic liver diseases, and in many forms of liver injury (2, 4, 31). Cholangiopathies share common pathological features, such as the damage of intrahepatic bile ducts and the proliferation of residual ducts (as a mechanism of compensatory repair to maintain biliary homeostasis), but they evolve toward ductopenia, which represents the terminal stage of the disease (2, 4, 31).

The present study demonstrated that the HPA axis is suppressed at every level during the course of cholestasis. CRH expression is decreased in the hypothalamus, which, in turn, has an inhibitory effect on ACTH (POMC) expression in the pituitary, leading to a decreased expression of the key steroidogenic enzymes HSD-3β and Cyp11b1 in the adrenal glands, which results in decreased circulating glucocorticoids. As mentioned above, the link between cholestasis and the HPA axis has been demonstrated clinically, with cholestatic patients often exhibiting clinical features suggestive of adrenal insufficiency, such as hypovolemia, hypotension, and renal failure (55, 57). Furthermore, patients with congenital hypopituitarism or glucocorticoid deficiency often exhibit cholestatic hepatitis (5, 20, 28). In cirrhosis, adrenal insufficiency is associated with increased mortality and hemodynamic impairment (22, 51). Treatment of cirrhotic patients with low doses of hydrocortisone resolves the hemodynamic impairment and is associated with a higher survival rate (13).

The HPA axis is also suppressed in experimental models of cholestasis (37, 46–48). Resection of the bile duct in rats leads to impaired stress-induced CRH release compared with controls, which results in decreased circulating ACTH levels (46, 48). ACTH release from the pituitary as a result of IL-1β treatment is also dampened after bile duct resection (47). Furthermore, McNeilly et al. (37) recently suggested that bile acid buildup as a result of cholestasis may have the ability to inhibit hepatic glucocorticoid clearance, thereby allowing glucocorticoid levels to increase to a level that would inhibit the HPA axis. The data presented here support the concept that the HPA axis is suppressed during cholestasis and that this suppression may have a causal link to other pathological features observed during liver damage. Furthermore, the observation that the HPA axis is not suppressed after only partial BDL in

the rat, where serum bile acids are only modestly increased (23), supports a role for perturbed bile acid and/or cholesterol homeostasis in suppression of the HPA axis after complete biliary obstruction. However, instead of affecting hepatic glucocorticoid clearance (37), we hypothesize that the bile acids may enter the brain and suppress the HPA axis directly. Others have shown that the blood-brain barrier becomes leaky in rodent models of cholestasis (12, 56), allowing access of aberrant signaling molecules to the brain. We have preliminary data to suggest that there are increased amounts of bile acids in the brain after BDL and that these bile acids have a dampening effect on the HPA axis (DeMorrow et al., unpublished observations). Whether these bile acids are working through a mechanism involving direct activation of the glucocorticoid receptor [as has been demonstrated previously (38, 49, 50)], thereby activating the negative-feedback loop to shut off the HPA axis, or via direct transcriptional suppression of CRH independent of glucocorticoid receptor activation is under investigation.

To our knowledge, this is the first study that demonstrates a causal link between the suppressed HPA axis and cholangiocyte proliferation observed after BDL. Suppression of the HPA axis by knockout of hypothalamic CRH expression or surgical removal of the adrenal glands induced cholangiocyte proliferation and increased biliary mass in vivo. Furthermore, reactivation of the HPA axis after BDL by central administration of CRH, systemic treatment with ACTH, or low levels of cortisol or corticosterone prevented the biliary outgrowth seen after BDL. Conversely, treatment of rats with high doses of the synthetic glucocorticoid dexamethasone (up to 2.7 $mg \cdot rat^{-1} \cdot day^{-1}$) increases bicarbonate secretion from cholangiocytes (3). We previously showed that the effects of a compound on ductal secretion often parallel its effects on cholangiocyte proliferation (14, 18, 19, 33, 36). The glucocorticoid treatments were ~10-fold less in the present study than in the above-mentioned study, with considerably less potency than dexamethasone, and were designed to restore the HPA axis and circulating glucocorticoid levels to physiological levels. The difference in concentrations and the potency of the glucocorticoid used in these studies may account for the difference in effects.

Treatment of cholestatic liver diseases with glucocorticoids (e.g., dexamethasone) has had varied success in alleviating symptoms (16, 42). Any beneficial effects of glucocorticoids in the management of these diseases seem to be outweighed by the increased adverse effects and complications (16, 42). Therefore, alternative strategies aimed at modulating the HPA axis without producing the nonspecific side effects are necessary. We believe that elucidation of the mechanism by which chronic cholestasis results in a suppressed HPA axis may provide a more specific target for successful management of the balance between biliary hyperplasia and ductopenia, depending on the stage of the disease.

In conclusion, we have provided further evidence for suppression of the HPA axis in an experimental model of cholestasis. However, we have extended our current knowledge by demonstrating a causal link between suppression of the HPA axis and induction of cholangiocyte proliferation after BDL. Further knowledge of the role of the HPA axis during cholestasis and the mechanism by which the HPA axis is suppressed during cholestasis may lead to development of innovative treatment paradigms for chronic liver disease.

## GRANTS

This work was supported by National Institute of Diabetes and Digestive and Kidney Diseases Grants R01-DK-082435 and K01-DK-078532 (to S. DeMorrow), Health and Labour Sciences Research Grants for Research on Measures for Intractable Diseases from the Ministry of Health, Labour, and Welfare of Japan, and Grant-in-Aid for Scientific Research C (21590822) from the Japan Society for the Promotion of Science (to Y. Ueno).

This material is the result of work supported with resources and the use of facilities at the Central Texas Veterans Health Care System.

## DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

## AUTHOR CONTRIBUTIONS

M.Q. and S.D. are responsible for conception and design of the research; M.Q., Y.U., H.Y.P., L.H., G.F., C.G., H.F., D.H., and M.M. performed the experiments; M.Q., Y.U., H.Y.P., L.H., G.F., C.G., H.F., D.H., M.M., and S.D. analyzed the data; M.Q., Y.U., and S.D. interpreted the results of the experiments; M.Q. and S.D. prepared the figures; M.Q. and S.D. drafted the manuscript; M.Q., Y.U., H.Y.P., L.H., G.F., C.G., H.F., D.H., M.M., and S.D. approved the final version of the manuscript; Y.U., H.Y.P., L.H., G.F., M.M., and S.D. edited and revised the manuscript.

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# Role of Stem Cell Factor and Granulocyte Colony-Stimulating Factor in Remodeling During Liver Regeneration

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**Functional pluripotent characteristics have been observed in specific subpopulations of hepatic cells that express some of the known cholangiocyte markers. Although evidence indicates that specific cytokines, granulocyte macrophage colony-stimulating factors (GM-CSFs), and stem cell factors (SCFs) may be candidate treatments for liver injury, the role of these cytokines in intrahepatic biliary epithelium remodeling is unknown. Thus, our aim was to characterize the specific cytokines that regulate the remodeling potentials of cholangiocytes after 70% partial hepatectomy (PH). The expression of the cytokines and their downstream signaling molecules was studied in rats after 70% PH by immunoblotting and in small and large murine cholangiocyte cultures (SMCCs and LMCCs) by immunocytochemistry and real-time polymerase chain reaction (PCR). There was a significant, stable increase in SCF and GM-CSF levels until 7 days after PH. Real-time PCR analysis revealed significant increases of key remodeling molecules, such as S100 calcium-binding protein A4 (S100A4) and miR-181b, after SCF plus GM-CSF administration in SMCCs. SMCCs produced significant amounts of soluble and bound SCFs and GM-CSFs in response to transforming growth factor-beta (TGF-β). When SMCCs were incubated with TGF-β plus anti-SCF+GM-CSF antibodies, there was a significant decrease in S100A4 expression. Furthermore, treatment of SMCCs with SCF+GM-CSF significantly increased matrix metalloproteinases (MMP-2 and MMP-9) messenger RNA as well as miR-181b expression, along with a reduction of metalloproteinase inhibitor 3. Levels of MMP-2, MMP-9, and miR-181b were also up-regulated in rat liver and isolated cholangiocytes after PH. *Conclusion*: Our data suggest that altered expression of SCF+GM-CSF after PH can contribute to biliary remodeling (e.g., post-transplantation) by functional deregulation of the activity of key signaling intermediates involved in cell expansion and multipotent differentiation.** (HEPATOLOGY 2012;55:209-221)

In addition to playing important roles in the regulation of ductal secretion, cholangiocytes are the target cells of apoptotic, proliferative, and regenerative events leading to changes in biliary damage (e.g., after carbon tetrachloride [$CCl_4$] acute administration), hyperplasia (e.g., after bile duct ligation), and regeneration (e.g., after 70% hepatic resection).[1,2] During liver development, both hepatocytes and cholangiocytes arise from common bipotential progenitors called hepatoblasts[3] or hepatic stem cells (HSCs).[4] The HSCs or hepatoblasts in the liver parenchyma differentiate into hepatocytes, whereas those adjacent to the

*Abbreviations: AP, alkaline phosphatase; BrdU, bromodeoxyuridine; $CCl_4$, carbon tetrachloride; CSFs, colony-stimulating factors; ECM, extracellular matrix; ELISA, enzyme-linked immunosorbent assay; EPO, erythropoietin; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte macrophage colony-stimulating factor; HiBECs, human intrahepatic biliary epithelial cells; HSCs, hepatic stem cells; IRCs, isolated rat cholangiocytes; LMCC, large murine cholangiocytes; MI, mitotic index; miRNA, microRNA; MMP, matrix metalloproteinase; mRNA, messenger RNA; MTS, (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium); NIRCs, normal rat intrahepatic cholangiocyte cultures; PCR, polymerase chain reaction; PH, partial hepatectomy; S100A4, S100 calcium-binding protein A4; SCF, stem cell factor; SE, standard error; SMCC, small murine cholangiocytes; TGF-β, transforming growth factor-beta; TIMP-3, tissue inhibitor of metalloproteinases-3.*

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*Received June 30, 2011; accepted August 30, 2011.*

portal mesenchyme differentiate into cholangiocytes.[4,5] During liver regeneration, the process of hepatic wound healing, a complex cascade of inflammatory signaling, recruits inflammatory cells, stimulates hepatobiliary cell proliferation, directs cell migration, and induces vascularization to restore tissue integrity.[6] Previous studies related to biliary wound healing focused mainly on the inducers of compensatory biliary proliferation after bile duct insult.[7]

Cytokines that are candidates in liver-remodeling processes include stem cell factor (SCF), erythropoietin (EPO), granulocyte colony-stimulating factor (G-CSF), and granulocyte macrophage colony-stimulating factor (GM-CSF); these regulate bone marrow production of circulating red cells, white cells, and platelets. These cytokines act on stem cells, leading to lineage specific differentiation.[8] SCF regulates the differentiation of CD34-positive stem cells, whereas other factors, such as EPO, G-CSF, and GM-CSF, modulate the synthesis of more specific cell types.[9] Colony-stimulating factors (CSFs) are involved in hepatic inflammation (via direct effects on the vascular endothelium, and/or neutrophil recruitment and activation) as well as in hepatic repair and regeneration.[10]

The potential mechanisms involved in direct actions and/or induction of additional factors that promote liver regeneration and repair are unclear. Multiple studies have suggested that there is an intricate regulatory system involved in hepatic regeneration after injury. Although many cytokines have proliferative effects on hepatobiliary cells both *in vitro* and *in vivo*, no single molecule has proven to be a key factor responsible for controlling hepatocyte or proliferation of subpopulations sharing cholangiocyte markers and *in vivo* remodeling during liver injury. Although evidence suggests that these factors may be candidate treatments for liver injury, either as potential hepatoprotectants or as hepatoreparative agents, the role of one or more of these cytokines in hepatobiliary remodeling after partial hepatectomy (PH) is undefined.

Hepatocyte proliferation may be blocked if the tissue injury is too severe.[6] During this process, cholangiocytes of the portal ductules and canals of Hering (small tubules lined by epithelium with biliary morphology, which connect the hepatocyte bile canalicular network to the portal biliary ductules) begin expressing hepatocyte-associated transcription factors.[11] It has been suggested that cholangiocytes can acquire stem cell phenotypes and, subsequently, become hepatocytes, restoring liver regeneration when hepatocytes cannot proliferate,[12] but an alternative interpretation is that liver regeneration is derived from HSCs.[4,13,14] Furthermore, HSCs are located within the canals of Hering and have markers shared with biliary epithelia,[4,13-15] and they expand in disease conditions[14] before the formation of oval cells, progenitor populations occurring in livers of hosts exposed to oncogenic insults.[16] Therefore, HSCs are one of the most important regenerative alternatives during conditions where hepatocytes fail to proliferate. The current study elucidated the possible role of cytokine-mediated remodeling during liver regeneration, especially their synergistic effects on the proliferation of cholangiocytes and their mesenchymal partners, stellate cells,[17] from human, rat, and mouse bile ducts.

## Materials and Methods

***Cell Lines and Cultures.*** Our small (SMCC) and large (LMCC) murine cholangiocytes were isolated from healthy mice (BALB/c) and immortalized by the introduction of the SV40 large-T antigen gene.[18] Healthy human intrahepatic biliary epithelial cells (HiBECs), human hepatocytes, and mediums were obtained from ScienCell Research Laboratories (San Diego, CA). All other cell-culture media and supplements were obtained from Invitrogen (Carlsbad, CA).

***Animal Protocols and 70% Hepatectomy Model.*** Male Fisher 344 rats (75-100 g) were purchased from Charles River (Wilmington, MA). The 70% partial hepatectomy (PH) surgery was performed according to the classical model of Higgins and Anderson. Tissues were collected, and intrahepatic cholangiocytes were isolated from the removed liver tissues, as previously described.[1,2,19,20]

*This study was supported by the Dr. Nicholas C. Hightower Centennial Chair of Gastroenterology from Scott & White, a Veterans Affairs Research Career Scientist Award, a Veterans Award Merit Award, and National Institutes of Health grants DK58411 and DK76898 (to G.A.).*

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*View this article online at wileyonlinelibrary.com.*

*DOI 10.1002/hep.24673*

*Potential conflict of interest: Nothing to report.*

*Additional Supporting Information may be found in the online version of this article.*

***Purified Cholangiocytes and Normal Rat Intrahepatic Cholangiocyte Cultures.*** Virtually pure cholangiocytes were isolated by immunoaffinity separation[1,2,19,20] with a monoclonal antibody (a gift of Dr. R. Faris) against an unidentified antigen expressed by all intrahepatic rat cholangiocytes.[21] The *in vitro* experiments were performed in freshly isolated rat cholangiocytes (IRCs) and our polarized normal rat intrahepatic cholangiocyte cultures (NRICs).[22]

**In Vitro *Proliferation and Migration Assay.*** Commercially available kits were used for the proliferation and migration assays in hepatobiliary cells (details in Supporting Information).

***Western Blotting.*** Protein was extracted from cultured cells or homogenized tissues, and western blotting was performed as previously described.[23] Please see the Supporting Information for more details.

***Real-Time Polymerase Chain Reaction Assays for Mature microRNAs.*** The microRNA (miRNA) was isolated as previously described,[23] and the expression of specific mature miR-181b was verified by real-time polymerase chain reaction (PCR) analysis, using a TaqMan Human MicroRNA Assay kit (Applied Biosystems, Foster City, CA).

***Enzyme-Linked Immunosorbent Assay.*** SCF (SCF enzyme-linked immunosorbent assay [ELISA] kit; BioSource International, Camarillo CA, USA) and GM-CSF (GM-CSF ELISA kit; BioSource) ELISAs were performed according to the manufacturer's instructions.

***Statistical Analysis.*** All data are expressed as mean ± standard error (SE). The differences between groups were analyzed by the Student *t* test when two groups were analyzed or analysis of variance if more than two groups were analyzed. $P < 0.05$ was used to indicate statistically significant differences.

Please see Supporting Information for more detailed information of this section.

## Results

***Altered Expression of CSF and Their Receptors After PH.*** Animals underwent 70% PH or sham and were sacrificed at 0, 1, 3, 5, and 7 days after surgery. Liver samples were obtained and analyzed for SCF, GM-CSF, G-CSF, and EPO messenger RNA (mRNA) expression by real-time PCR. Sham animals demonstrated high mRNA levels of SCF, but not GM-CSF, G-CSF, and EPO, in the liver, suggesting a large baseline hepatic reservoir of SCF (Fig. 1A). There was a significant decline in SCF expression during the first 24 hours after PH, followed by a significant, stable increase in SCF levels up to 7 days after PH (Fig. 1B). Concurrent with this increase in hepatic SCF mRNA levels, there was a significant increase in SCF receptor mRNA levels (Fig. 1C). Meanwhile, a moderate increase of GM-CSF, but not receptor mRNA, levels was observed after PH. Interestingly, the significant increases of SCF and GM-CSF were also observed in IRCs from total liver after PH (Fig. 1D). No changes in the expression of other CSFs and their receptors, such as G-CSF and EPO, were observed after PH.

***CSFs Promote the Proliferation of Primary Hepatocytes but Not Cholangiocytes.*** We evaluated *in vitro* the effects of CSF on the proliferation of human and mouse hepatocytes and cholangiocytes. Cells were exposed to media alone or 0.1, 1, 10, 20, and 50 ng/mL of CSFs, and proliferation was measured at 72 hours by (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) (MTS) assays.[24] Exposure to 10 and 20 ng/mL of SCF and GM-CSF resulted in a significant increase in the proliferation of cells of the hepatocytic lineage, but not in subpopulations sharing cholangiocyte markers (i.e., HSCs and biliary epithelia), compared with incubation with media alone (Supporting Fig. 1), suggesting different proliferative roles between cells with hepatocytic markers and those with cholangiocyte traits during regeneration after PH.

***Synergistic Role of Cytokines in Proliferation of Cells With Biliary Markers.*** The synergistic effects of SCF in combination with other cytokines have been demonstrated by many studies during the regeneration of different organ systems.[25,26] To determine whether the association between liver regeneration and the biliary epithelium is cytokine dependent, we examined the proliferation of cell subpopulations with cholangiocyte markers (e.g., HSCs and biliary epithelia) before or after stimulation with SCF+GM-CSF, G-CSF, and EPO. We observed an enhancement of cell proliferation in small murine epithelial subpopulations with biliary markers after stimulation with SCF+GM-CSF for 72 hours (Fig. 2). Addition of this cytokine combination did not change the proliferation index in other human and mouse cholangiocyte subpopulations. These results indicate a regenerative potential in either HSCs and/or committed biliary epithelial progenitors (i.e., small cholangiocytes) after stimulation with SCF+GM-CSF.

***SCF+GM-CSF Induce Subpopulations of Biliary Epithelia to Become Competent to Mitogen Signal.*** Isolated cholangiocyte subpopulations include cells that are small (7-9 μm) or large (14-15 μm).[2,20] The small ones comprise the HSCs and the committed biliary progenitors, whereas the large ones are

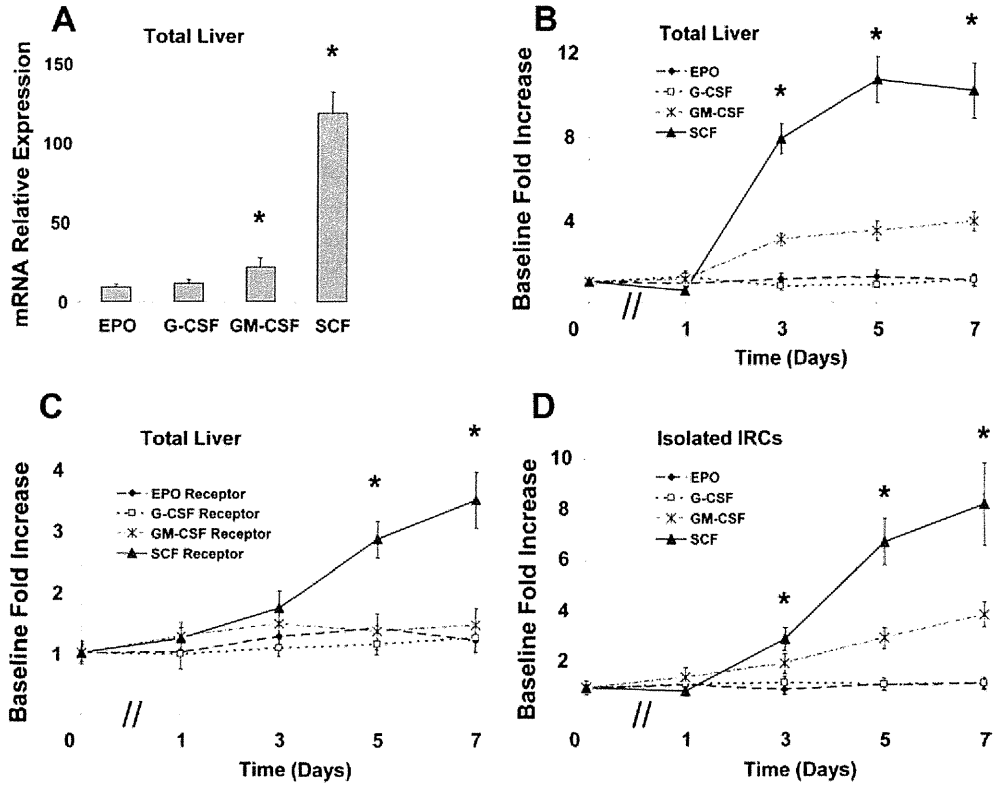


Fig. 1. The expressions of CSFs and their receptors in rat liver after 70% PH. Animals underwent 70% PH or sham control, and liver tissues as well as IRCs were obtained at various times postoperatively. RNA was isolated, and complementary DNA was generated by reverse transcription using MMLV reverse transcriptase. Basal mRNA expressions of SCF, EPO, G-CSF, and GM-CSF (A), as well as the alterations of CSFs (B) and their receptors (C) in total liver tissues, and the expressions of CSF in IRCs (D) after PH, were quantified by real-time PCR using SYBR Green as the fluorophore, and expressed relative to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA expression assessed concurrently in the same samples. (A) $*P < 0.05$, when compared with the EPO group. (B andC) $*P < 0.05$, when compared with the sham control group.

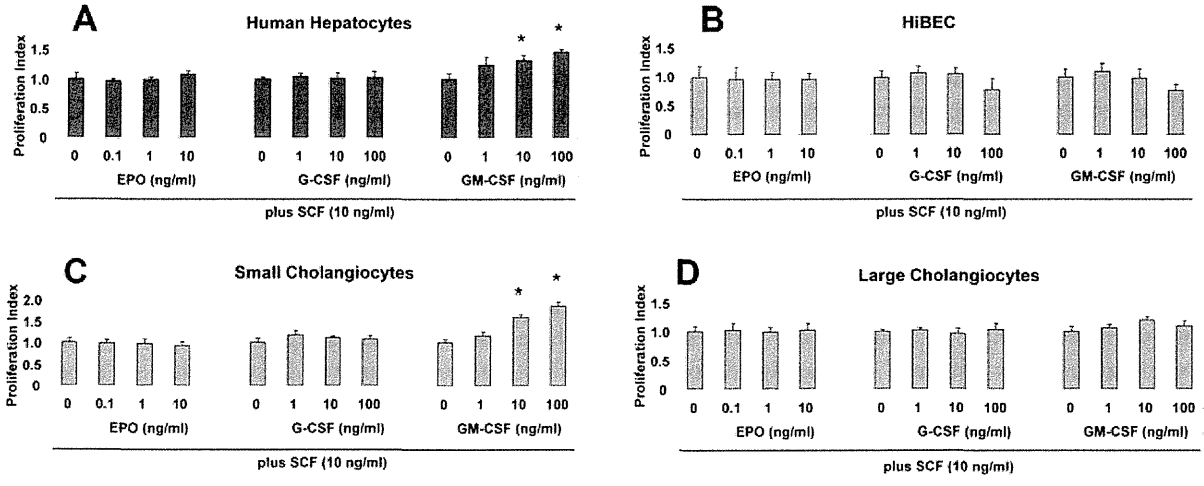


Fig. 2. SCF promotes synergistic cellular proliferation in combination with GM-CSF in small murine cells with biliary markers. Epithelial cells in 96-well plates with 10% fetal bovine serum containing culture medium and were treated with different cytokines for various concentrations; as indicated below the panels, in combination with SCF (10 ng/mL). After 72 hours, cell proliferation was assessed using the MTS assay, and the proliferation index was derived. Mean ± SE from four independent experiments, each in triplicate, at each point is illustrated. GM-CSF in combination with SCF significantly increased cellular proliferation at higher concentrations (≥10 ng/mL) in normal human hepatocytes and SMCCs. $*P < 0.05$, when compared with control SCF-only group.

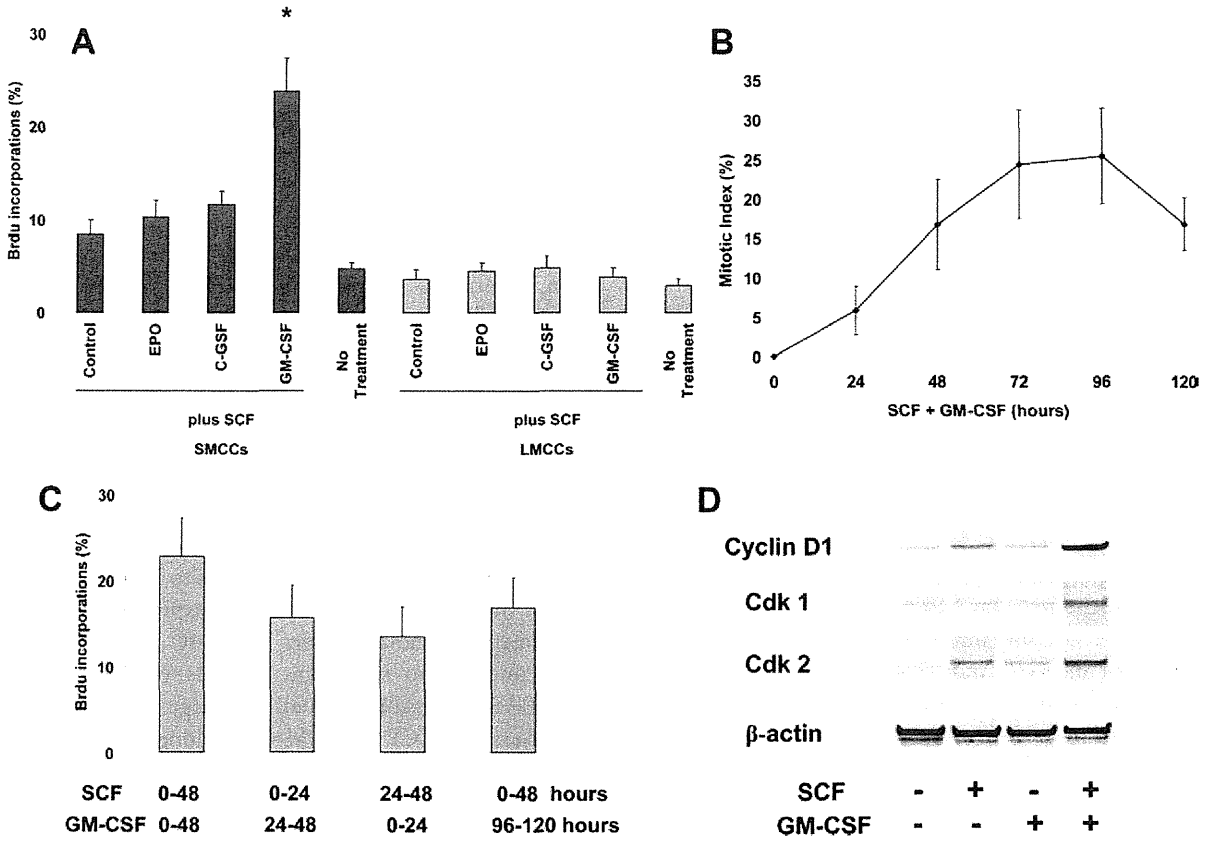


Fig. 3. SCF+GM-CSF promote cell-cycle progression in SMCC. (A) SMCCs and LMCCs were stimulated with SCF plus other CSFs (all at 10 ng/mL) for 72 hours, and percentages of BrdU-labeled cells were determined by immunocytochemistry after 24-hour BrdU incorporation at day 3. Results represent means ± SE (n = 8). (B) SMCCs were stimulated with the combination of SCF/GM-CSF (10 ng/mL) for 5 days. MI was determined every day after a 24-hour colcemid treatment. Percentages of dividing cells were determined. Values represent means ± SE (n = 6). (C) SMCCs were stimulated with (1) combination of SCF+GM-CSF for 48 hours, (2) SCF then GM-CSF for 24 hours each, (3) GM-CSF then SCF for 24 hours each, and (4) SCF for 48 hours, followed by 2 days without stimulation and then GM-CSF for 48 hours. BrdU was incorporated during the last 24 hours of treatment, and percentages of BrdU-labeled cells were determined. Results are means ± SE (n = 6). (D) Alterations of cell-cycle proteins in SCF- and/or GM-CSF-treated SMCCs. SMCCs were maintained without stimulation or treated with SCF, GM-CSF alone, or SCF+GM-CSF (10 ng/mL) for 3 days. Protein extracts from fractions enriched for cells with biliary markers were analyzed by immunoblotting using antibodies against cyclin D1, Cdk1, Cdk2, and β-actin.

cholangiocytes.[4] These usually acquire long survival potency, maintain a certain level of differentiation, and do not respond to single-cytokine stimulation. To test the capacity of cholangiocyte subpopulations to proliferate, isolated small (i.e., HSCs and committed biliary epithelia) and large cholangiocytes were exposed for 3 days to different combinations of SCF, EPO, G-CSF, and GM-CSF in a medium without fetal calf serum, and DNA synthesis was estimated in these subpopulations (Fig. 3A). In the absence of stimulation and in single CSF-treated cultures, low levels of DNA synthesis were detected in both small and large biliary subpopulations. In contrast, a combination of SCF+GM-CSF induced DNA synthesis in at least 30% of small cells with biliary markers at day 3, whereas less than 12% of large cholangiocytes incorporated bromodeoxyuridine (BrdU) when stimulated by SCF in the presence of GM-CSF. Measurement of the mitotic index (MI) in cultures stimulated with SCF+GM-CSF for 5 days showed a peak of division at day 4, reaching 25% of small cells with biliary markers (Fig. 3B). The MI matched the BrdU incorporation level.

We next tested whether the addition of GM-CSF before or after SCF would influence the replication of small cells with biliary markers. Cultures were costimulated with GM-CSF and SCF for 48 hours or stimulated for 24-hour periods with GM-CSF before or after SCF treatment (Fig. 3C). In all cases, cells replicated their DNA, and BrdU incorporation levels were similar. In addition, a pause of 2 days after SCF treatment did not change the responsiveness of cells to GM-CSF.