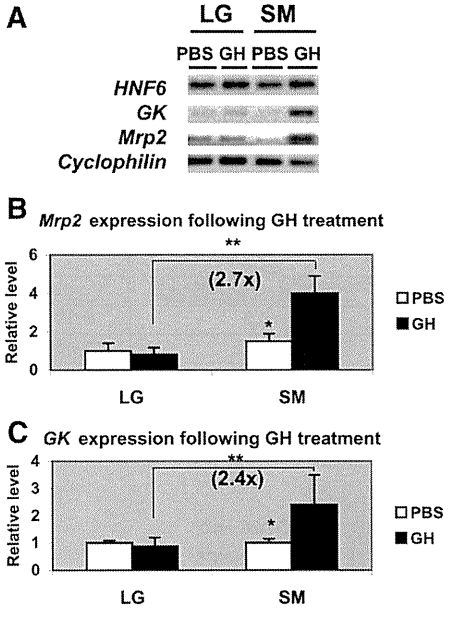

HNF6	PBS	GH	p-value
LG	1 +/- 0.2	1.5 +/- 0.3*	0.05
SM	1.2 +/- 0.3	2 +/- 0.4*	0.04

Mrp2	PBS	GH	p-value
LG	1 +/- 0.4	1.5 +/- 0.4	0.07
SM	0.8 +/- 0.37	4 +/- 0.9 *	<0.001

GK	PBS	GH	p-value
LG	1 +/- 0.09	1 +/- 0.15	0.99
SM	0.87 +/- 0.33	2.4 +/- 1.1 *	0.05

Fig. 6. Effects of growth hormone (GH)-mediated *HNF6* increases on BEC gene expression. LG and SM BEC were isolated from PBS- or GH-treated mice (n = 3). Total RNA was extracted for real-time PCR analyses of *HNF6*, *mrp2*, *GK*, and *cyclophilin*. *A*: micrograph shows representative real-time PCR gel results, and table shows *HNF6* gene levels with the corresponding *P* values. *B* and *C*: bar graphs show gene levels for *mrp2* (*B*) and *GK* (*C*) in PBS- and GH-treated BEC. *Significant differences in levels between PBS- vs. GH-treated BEC. **Significantly higher *mrp2* and *GK* levels (2.7- and 2.4-fold, respectively) in GH-treated SM relative to LG cholangiocytes. Tables show the gene levels with the corresponding *P* values.

target gene response are pending to shed light on the true physiological significance of these in vitro findings. The relevance of the suppressive transcriptional response of HNF4a to HNF6 overexpression in SV40BEC is unclear, but does not diminish the remarkable findings that Foxa2, HNF1a, and HNF4a remain dominant transcription factors in the AdHNF6-infected small BEC, with commensurate higher expression levels of their target genes, *ntcp*, *oatp1*, and *mrp2*.

Following AdHNF6 infection, despite unchanged *Foxa2* and *HNF1a*, yet suppressed *HNF4a* expression, *HNF1a-*, *HNF4a-*, and/or *Foxa2* target gene *ntcp*, *oatp1*, and *mrp2* levels were significantly enhanced in both large and small SV40 cells, suggesting that the response pattern of these bile transport genes is more complex than just straightforward transcriptional regulation by *HNF1a*, *HNF4*, *Foxa2*, or *HNF6*, since HNFs commonly function in a cross-regulatory fashion. With respect to *ntcp*, transient transfection of CMV-HNF6 expression vectors in HepG2 cell lines did not activate *ntcp* reporter gene constructs (data not shown). Furthermore, prior AdHNF6 liver infection by tail vein injection did not enhance whole liver *ntcp* or *mrp2* expression (40), suggesting that *ntcp* and *mrp2* gene response cannot be directly attributed to simple HNF6 transcriptional effects on their promoters. Since HNF commonly participate in a transcriptional network with the proper complement of HNF and cross-interaction among HNFs controlling the target gene profile, BEC upregulation of *ntcp* and *mrp2* expression following AdHNF6 infection could be due to potential molecular synergistic interactions between HNF6 and HNF1a/HNF4a/Foxa2, perhaps through the recruitment of coactivators to orchestrate target gene activation. A precedence for this mechanism has been described with HNF6-HNF4a activation of *glucose-6-phosphatase* promoter by joint engagement of the peroxisome proliferator-activated receptor-γ coactivator-1α (5); or with HNF6-C/EBPa recruitment of the CREB binding protein CBP coactivator to enhance *Foxa2* promoter activities (33, 42). Of note, *oatp1* hepatic gene expression and *oatp1* promoter occupancy by HNF6 nuclear proteins are severely diminished in HNF6 liver conditional null mice (manuscript submitted), suggesting that o*atp1* is an authentic HNF6 target gene. Upregulation of *oatp1* expression in AdHNF6 treated cells is consistent with direct transcriptional stimulation of *oatp1* by HNF6 or HNF1a-HNF6 interaction.

Gene profiling (12, 38) and functional studies (14) have shown that large BEC participate in choleresis, immune, and hormone regulation. Our gene expression data suggest that small BEC may carry a more substantial role in the transport of bile acid and bile acid constituents than large BEC. Further analyses for the respective contribution of these cell populations to the liver adaptive response to injury in experimental models of cholestasis are pending to assess the physiological significance of these observations.

Overall, the results lend support to our hypothesis that the large and small cholangiocytes have different transcriptional characteristics, thus providing a potential mechanistic basis for their functional heterogeneity. The data also imply that the well-described intricate interactions among hepatocyte transcription factors in coordinating the transcriptional profile of end genes in hepatocytes may also exist in BECs. Further characterization of the complexities of promoter regulation of biliary-enriched genes in the large and small BECs will enhance our understanding of their differential susceptibility to disease processes in an effort toward modulating their individual pathological responses.

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DISCLOSURES

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REFERENCES

1. **Alpini G, Glaser S, Robertson W, Phinizy JL, Rodgers RE, Caligiuri A, LeSage G.** Bile acids stimulate proliferative and secretory events in large but not small cholangiocytes. *Am J Physiol Gastrointest Liver Physiol* 273: G518–G529, 1997.
2. **Alpini G, Prall RT, LaRusso NF.** The pathobiology of biliary epithelia. In: *The Liver; Biology & Pathobiology,* edited by Arias IM, Boyer JL, Chisari FV, Fausto N, Jakoby W, Schachter D, and Shafritz DA. Philadelphia, PA: Lippincott Williams & Wilkins, 2001, p. 421–435.
3. **Alpini G, Roberts S, Kuntz SM, Ueno Y, Gubba S, Podila PV, LeSage G, LaRusso NF.** Morphological, molecular, and functional heterogeneity of cholangiocytes from normal rat liver. *Gastroenterology* 110: 1636–1643, 1996.
4. **Andrejko KM, Raj NR, Kim PK, Cereda M, Deutschman CS.** IL-6 modulates sepsis-induced decreases in transcription of hepatic organic anion and bile acid transporters. *Shock* 29: 490–496, 2008.
5. **Beaudry JB, Pierreux CE, Hayhurst GP, Plumb-Rudewiez N, Weiss MC, Rousseau GG, Lemaigre FP.** Threshold levels of hepatocyte nuclear factor 6 (HNF-6) acting in synergy with HNF-4 and PGC-1alpha are required for time-specific gene expression during liver development. *Mol Cell Biol* 26: 6037–6046, 2006.
6. **Benedetti A, Bassotti C, Rapino K, Marucci L, Jezequel AM.** A morphometric study of the epithelium lining the rat intrahepatic biliary tree. *J Hepatol* 24: 335–342, 1996.
7. **Bochkis IM, Rubins NE, White P, Furth EE, Friedman JR, Kaestner KH.** Hepatocyte-specific ablation of Foxa2 alters bile acid homeostasis and results in endoplasmic reticulum stress. *Nat Med* 14: 828–836, 2008.
8. **Clotman F, Jacquemin P, Plumb-Rudewiez N, Pierreux CE, Van der Smissen P, Dietz HC, Courtoy PJ, Rousseau GG, Lemaigre FP.** Control of liver cell fate decision by a gradient of TGF beta signaling modulated by Onecut transcription factors. *Genes Dev* 19: 1849–1854, 2005.
9. **Clotman F, Lannoy VJ, Reber M, Cereghini S, Cassiman D, Jacquemin P, Roskams T, Rousseau GG, Lemaigre FP.** The onecut transcription factor HNF6 is required for normal development of the biliary tract. *Development* 129: 1819–1828, 2002.
10. **Costa RH, Kalinichenko VV, Holterman AX, Wang X.** Transcription factors in liver development, differentiation, and regeneration. *Hepatology* 38: 1331–1347, 2003.
11. **Dietrich CG, Martin IV, Porn AC, Voigt S, Gartung C, Trautwein C, Geier A.** Fasting induces basolateral uptake transporters of the SLC family in the liver via HNF4alpha and PGC1alpha. *Am J Physiol Gastrointest Liver Physiol* 293: G585–G590, 2007.
12. **Fukushima K, Ueno Y.** Bioinformatic approach for understanding the heterogeneity of cholangiocytes. *World J Gastroenterol* 12: 3481–3486, 2006.
13. **Geier A, Martin IV, Dietrich CG, Balasubramaniyan N, Strauch S, Suchy FJ, Gartung C, Trautwein C, Ananthanarayanan M.** Hepatocyte nuclear factor-4alpha is a central transactivator of the mouse Ntcp gene. *Am J Physiol Gastrointest Liver Physiol* 295: G226–G233, 2008.
14. **Glaser S, Francis H, Demorrow S, Lesage G, Fava G, Marzioni M, Venter J, Alpini G.** Heterogeneity of the intrahepatic biliary epithelium. *World J Gastroenterol* 12: 3523–3536, 2006.
15. **Glaser SS, Gaudio E, Rao A, Pierce LM, Onori P, Franchitto A, Francis HL, Dostal DE, Venter JK, DeMorrow S, Mancinelli R, Carpino G, Alvaro D, Kopriva SE, Savage JM, Alpini GD.** Morphological and functional heterogeneity of the mouse intrahepatic biliary epithelium. *Lab Invest* 89: 456–469, 2009.
16. **Hayhurst GP, Lee YH, Lambert G, Ward JM, Gonzalez FJ.** Hepatocyte nuclear factor 4alpha (nuclear receptor 2A1) is essential for maintenance of hepatic gene expression and lipid homeostasis. *Mol Cell Biol* 21: 1393–1403, 2001.
17. **Holterman AX, Tan Y, Kim W, Yoo KW, Costa RH.** Diminished hepatic expression of the HNF-6 transcription factor during bile duct obstruction. *Hepatology* 35: 1392–1399, 2002.
18. **Jacquemin P, Lannoy VJ, Rousseau GG, Lemaigre FP.** OC-2, a novel mammalian member of the ONECUT class of homeodomain transcription factors whose function in liver partially overlaps with that of hepatocyte nuclear factor-6. *J Biol Chem* 274: 2665–2671, 1999.
19. **Kanno N, LeSage G, Glaser S, Alvaro D, Alpini G.** Functional heterogeneity of the intrahepatic biliary epithelium. *Hepatology* 31: 555–561, 2000.
20. **Kitanaka S, Sato U, Igarashi T.** Regulation of human insulin, IGF-I, and multidrug resistance protein 2 promoter activity by hepatocyte nuclear factor (HNF)-1beta and HNF-1alpha and the abnormality of HNF-1beta mutants. *J Endocrinol* 192: 141–147, 2007.
21. **Lahuna O, Rastegar M, Maiter D, Thissen JP, Lemaigre FP, Rousseau GG.** Involvement of STAT5 (signal transducer and activator of transcription 5) and HNF-4 (hepatocyte nuclear factor 4) in the transcriptional control of the hnf6 gene by growth hormone. *Mol Endocrinol* 14: 285–294, 2000.
22. **Lannoy VJ, Decaux JF, Pierreux CE, Lemaigre FP, Rousseau GG.** Liver glucokinase gene expression is controlled by the onecut transcription factor hepatocyte nuclear factor-6. *Diabetologia* 45: 1136–1141, 2002.
23. **Lazaridis KN, Strazzabosco M, Larusso NF.** The cholangiopathies: disorders of biliary epithelia. *Gastroenterology* 127: 1565–1577, 2004.
24. **Lee CS, Friedman JR, Fulmer JT, Kaestner KH.** The initiation of liver development is dependent on Foxa transcription factors. *Nature* 435: 944–947, 2005.
25. **Lee YH, Magnuson MA, Muppala V, Chen SS.** Liver-specific reactivation of the inactivated Hnf-1alpha gene: elimination of liver dysfunction to establish a mouse MODY3 model. *Mol Cell Biol* 23: 923–932, 2003.
26. **Liu JJ, Glickman JN, Masyuk AI, Larusso NF.** Cholangiocyte bile salt transporters in cholesterol gallstone-susceptible and resistant inbred mouse strains. *J Gastroenterol Hepatol* 23: 1596–1602, 2008.
27. **Maher JM, Slitt AL, Callaghan TN, Cheng X, Cheung C, Gonzalez FJ, Klaassen CD.** Alterations in transporter expression in liver, kidney, and duodenum after targeted disruption of the transcription factor HNF1alpha. *Biochem Pharmacol* 72: 512–522, 2006.
28. **Marzioni M, Glaser SS, Francis H, Phinizy JL, LeSage G, Alpini G.** Functional heterogeneity of cholangiocytes. *Semin Liver Dis* 22: 227–240, 2002.
29. **Odom DT, Zizlsperger N, Gordon DB, Bell GW, Rinaldi NJ, Murray HL, Volkert TL, Schreiber J, Rolfe PA, Gifford DK, Fraenkel E, Bell GI, Young RA.** Control of pancreas and liver gene expression by HNF transcription factors. *Science* 303: 1378–1381, 2004.
30. **Parviz F, Matullo C, Garrison WD, Savatski L, Adamson JW, Ning G, Kaestner KH, Rossi JM, Zaret KS, Duncan SA.** Hepatocyte nuclear factor 4alpha controls the development of a hepatic epithelium and liver morphogenesis. *Nat Genet* 34: 292–296, 2003.
31. **Plumb-Rudewiez N, Clotman F, Strick-Marchand H, Pierreux CE, Weiss MC, Rousseau GG, Lemaigre FP.** Transcription factor HNF-6/OC-1 inhibits the stimulation of the HNF-3alpha/Foxa1 gene by TGF-beta in mouse liver. *Hepatology* 40: 1266–1274, 2004.
32. **Qadri I, Hu LJ, Iwahashi M, Al-Zuabi S, Quattrochi LC, Simon FR.** Interaction of hepatocyte nuclear factors in transcriptional regulation of tissue specific hormonal expression of human multidrug resistance-associated protein 2 (abcc2). *Toxicol Appl Pharmacol* 234: 281–292, 2009.
33. **Rausa FM 3rd, Hughes DE, Costa RH.** Stability of the hepatocyte nuclear factor 6 transcription factor requires acetylation by the CREB-binding protein coactivator. *J Biol Chem* 279: 43070–43076, 2004.
34. **Raynaud P, Carpentier R, Antoniou A, Lemaigre FP.** Biliary differentiation and bile duct morphogenesis in development and disease. *Int J Biochem Cell Biol.* In press.
35. **Samadani U, Costa RH.** The transcriptional activator hepatocyte nuclear factor 6 regulates liver gene expression. *Mol Cell Biol* 16: 6273–6284, 1996.
36. **Schrem H, Klempnauer J, Borlak J.** Liver-enriched transcription factors in liver function and development. I. The hepatocyte nuclear factor network and liver-specific gene expression. *Pharmacol Rev* 54: 129–158, 2002.
37. **Strazzabosco M, Fabris L, Spirli C.** Pathophysiology of cholangiopathies. *J Clin Gastroenterol* 39: S90–S102, 2005.
38. **Ueno Y, Alpini G, Yahagi K, Kanno N, Moritoki Y, Fukushima K, Glaser S, LeSage G, Shimosegawa T.** Evaluation of differential gene expression by microarray analysis in small and large cholangiocytes isolated from normal mice. *Liver Int* 23: 449–459, 2003.
39. **Velho G, Froguel P.** Maturity-onset diabetes of the young (MODY), MODY genes and non-insulin-dependent diabetes mellitus. *Diabetes Met* 23, *Suppl* 2: 34–37, 1997.

40. **Wang M, Chen M, Zheng G, Dillard B, Tallarico M, Ortiz Z, Holterman AX.** Transcriptional activation by growth hormone of HNF-6-regulated hepatic genes, a potential mechanism for improved liver repair during biliary injury in mice. *Am J Physiol Gastrointest Liver Physiol* 295: G357–G366, 2008.
41. **Wang M, Tan Y, Costa RH, Holterman AX.** In vivo regulation of murine CYP7A1 by HNF-6: a novel mechanism for diminished CYP7A1 expression in biliary obstruction. *Hepatology* 40: 600–608, 2004.
42. **Yoshida Y, Hughes DE, Rausa FM 3rd, Kim IM, Tan Y, Darlington GJ, Costa RH.** C/EBPalpha and HNF6 protein complex formation stimulates HNF6-dependent transcription by CBP coactivator recruitment in HepG2 cells. *Hepatology* 43: 276–286, 2006.

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Review Article

Treatment of Primary Biliary Cirrhosis: A new challenge?

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Primary biliary cirrhosis (PBC) is characterized by unknown etiologies, anti-mitochondrial antibodies, injury of the biliary duct and the lack of a definite remedy. The etiologies of PBC have been well-discussed, including microorganisms and xenobiotics as the triggers for initiating the disease, and an abnormality of immune-tolerance. Recently, several animal models of PBC have been developed that may lead to the development of new therapies. Here, we reviewed the articles that address the etiology of PBC and the therapy for this disease for the confirmation of our current positions and future directions.

Key words: corticoid, MMTV, primary biliary cirrhosis, PBA-AIH overlap, retrovirus, therapy

PATHOPHYSIOLOGY AND ETIOLOGY OF PRIMARY BILIARY CIRRHOSIS (PBC)

Molecular mimicry and immunological tolerance

THE PATHO-PHYSIOLOGY of PBC is based mainly on damage of the biliary ducts, especially of the interlobular bile ducts, and the resulting cholestasis. Although bile duct injury is essential pathological findings, the mechanism of these cholangiopathies has not been clarified yet.[1–3] The evidence of an epitope of T-cell receptors derived from the E2 component of pyruvate d ehydrogenase complex (*PDC-E2*)[4] and disease manifestation with other auto-immunities suggests an immunological disorder in this disease. The relevant genetic factors including *HLA* class II,[5] tumor necrosis factor (TNF) locus[6] and *CTLA4* as an immune-suppressor have been investigated.[7] These genetic factors may be responsible for the vulnerability of the immune system that might be considered to be broken by additional factors such as molecular mimicry.[3] Bacteria,[8] xenobiotics[9] and viruses[10] are candidate environmental factors that could trigger the breakdown of immune-tolerance. A fascinating hypothesis concerning environmental factors that mimic *PDC-E2* has been raised and tested by immunologists. Interestingly, certain xenobiotics can cause cholangitis that mimics PBC in rodents.[11] There still are several issues to be considered regarding the pathogenesis of anti-mitochondrial antibodies (AMA). AMA are even transiently detectable in the serum of acute liver failure at the frequency of 40.6%.[12] The positivity of AMA is also recognized in serum samples of patients with biliary damage after bone marrow transplantation.[13] AMA-autoimmune hepatitis (AIH) with positivity of AMA is subcategorized under AIH without any evidence of cholangiopathy. Therefore, AMA production would also be regarded as the result of damage of the biliary ducts without any relevance to the pathogenesis. The mechanism of specific AMA production along with biliary damage is explained by the lack of the cellular protective mechanism of the inner lipoyl domain of *PDC-E2* during the apoptotic process by glutathiolation from the chemical modification by certain xenobiotics.[14] The modified *PDC-E2* would be recognized as a target by an immune system with genetic vulnerability.[15] The recent development of animal models of PBC may help clarify the mechanism of the immunological basis of the pathogenesis. Interleukin2 receptor (IL2R: CD25)alfa(−/−),[16] dominant negative transforming growth factor-beta (TGFβ) RII under the regulation of CD4 promoter,[17] and NODc3c4 mice[18] show properties similar to those of human PBC in terms of AMA positivity and cholangiopathy. The underlying feature shared by these rodents is the impairment of the function of CD4+CD25+FOXP3+ regulatory T-cells due to the lack of the alfa-subunit of IL2R, suppression of

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the TGFβ signal pathway, TGFβ-related cell cycle regulator, or *cdkn2b* locus that exists in the *abd* locus which is altered in NODc3.c4. An abnormality of regulatory T-cells in human PBC has also been described.[19] Moreover, Scurfy mice with the feature of FOXP3 (−/−) show the features of PBC.[20] Therefore, dysregulation of regulatory T-cells might be a clue to the etiology of PBC. In fact, the result of recent our association study for identifying genetic backgrounds for patients with PBC demonstrated the strong diseases susceptibilities nearby HLA class II locus.[6] Thus, although not determined the ultimate responsible genes, there seems strong genetic backgrounds influencing the disease susceptibility for the pathogenesis of PBC.

Emerging retroviral theory

In terms of the etiology of PBC, potential genetic or epigenetic abnormalities with regard to the immunological tolerance may not explain fully the histological recurrence rate of PBC after liver transplantation that reaches up to 10% of the recipients.[21] Retroviruses (Table 1) are likely to have a close association with human diseases such as oncogenesis and autoimmunity, probably because of their genomic integration or specific viral-encoding proteins. Mason *et al.* demonstrated that a substantial proportion of the serum samples from biliary disorders are affected by retroviruses by immunoblot in 1998.[22] In the succeeding report, the existence of the virion and the β-retroviral specific sequence in the abdominal lymph nodes of PBC patients was described.[23] The viral sequence was cloned as a human homologue of mammary tumor virus (MMTV).[10] However, the reproducibility of the results was doubted by Selmi *et al.*[24] The envelope sequence of MMTV was detected subsequently in diseased livers, and was not seen in normal livers.[25] MMTV is orally transmitted via milk and can be a cause of mouse mammary tumor. The MMTV sequence is also transmissible to offspring when it is integrated into the genome of germ cells. The contagiousness of MMTV to human was partially proved by *in vitro* experimental infection to lymphocytes and mammary epithelial cells.[26] Of note, the receptor for the initiation of infection of MMTV was identified as transferrin receptor I[27] in murine cells, but this is not the case in human cells.[28] Actually, we have found that transferring receptor I was expressed in mouse cholangiocytes.[29] It is also speculated that MMTV plays a part in the pathogenesis of human breast cancer.[30,31] Nevertheless, as for the relationship between MMTV and PBC, no association between PBC and breast cancer in terms of the incidence has been shown.[32] MMTV uses its superantigen for spreading out in the host through proliferation of T-cells that express TCR with each superantigen-specific Vβ together with its gp52 envelope.[33] Therefore, superantigen potentially causes a partly specific T-cell expansion, which would lead to the disequilibrium of the immune system of the host. *Gag* of MMTV is essential for the oncogenesis of the epithelium of mouse mammary gland.[34] *Env* of MMTV has been detected in PBC liver while *gag* is equivocal, which is consistent with the rare incidence of biliary tumors in PBC livers. All of IL2R (CD25) alfa(−/−) mice, dominant negative TGFβ RII under the regulation of CD4 promoter and NODc3c4 mice have high copy numbers of MMTV provirus integrated in the genome. Some of those are even likely to be transcriptionally active. These findings may support hypothetically the retroviral interference theory with regard to the etiology

Table 1 Classification of retrovirus

Genus	Viruses
Alpharetrovirus	Avian leukosis virus (ALV), Rous sarcoma virus (RSV), Fujinami sarcoma virus (FuSV)
Betaretrovirus	*Mouse mammary tumor virus (MMTV)
Gammaretrovirus	*Xenotropic endogenous murine leukemia virus (XMRV), Murine leukemia virus (MLV), Feline leukemia virus (FeLV), Guinea pig typeC oncovirus (GPCOV), Porcine typeC oncovirus (PCOV)
Deltaretrovirus	Bovine leukemia virus (BLV), Human T-lymphotropic virus-1 (HTLV-1), *Human T-lymphotropic virus-2 (HT:LV-2)
Epsilonretrovirus	Walleye dermal sarcoma virus (WDSV)
Lentivirus	*Human immunodeficiency virus 1 (HIV-1), Feline immunodeficiency virus (FIV), Simmian immunodeficiency virus (SIV), Equine immunodeficiency virus (EIV), Bovine imuunodeficiency virus (BIV)
Others	Chimpanzee foamy virus (CFV), Porcine Endogenous Retrovirus (PERV)

*The viruses with relevance or susceptibility to human diseases.

of the PBC-like status in these animal models. However, the viremia of MMTV was confirmed in neither NODc3c4 mice nor PBC cases by us (unpublished data), which may indicate that the open reading frame of MMTV would not be fully conserved at least in the same locus. Therefore, if MMTV can cause PBC, there are several possibilities that could affect host systems, including the possibility that transient infection of MMTV breaks immune-tolerance, fragmented proviral genome of MMTV integrated into the host genome after initial exposure is activated transcriptionally to interfere with the host system, or the possibility that MMTV fragments directly influence the expression or the function of host molecules. Another question is how the biliary epithelial cells become victims. Vulnerability due to the impairment of regulatory T-cells may not be a sufficient explanation. Infectivity of MMTV to the biliary epithelia and succeeding active transcription of a part of MMTV might explain the heterogeneous manner of biliary injury in PBC liver and the localization of biliary damage to the interlobular bile ducts. Infection of MMTV to the biliary epithelial cells may induce re-localization of *PDC-E2* or immunologically similar molecules to the cell surface,[23] or present peptides derived from viral specific products which may evoke the immunogenicity of biliary epithelial cells. Another feature of MMTV that would support its pathogenicity for PBC is that transcriptional activation of the proviral genome of MMTV is induced by glucocorticoids,[35] progesterone and dihydrotestosterone,[36] and maturation of MMTV would be affected by estrogen.[37] Thus, sex and the sexual cycle may influence the expression of MMTV transcripts, which could be immunogenic.

Human endogenous retrovirus (HERV), which was integrated into the human germ line in ancient times and occupies up to 8% of the whole human genome, has also been suspected of being pathogenic for human diseases. HERV-W, which has fusogenic envelope protein, is transcriptionally activated in schizophrenia[38] and multiple sclerosis.[39] HERV-K has specific rec sequence[40] which may cause germ cell tumor. HERV-K113, a provirus integrated in the genome of a limited population with the full length of original sequence, has been implicated as a cause of Sjögren syndrome.[41] HERV-K18 provirus contains superantigen as a transcript as well as np9 that interacts with rec sequence becoming oncogenic,[42] which has been suggested to be involved in the pathogenesis of juvenile rheumatoid arthritis[43] or multiple sclerosis.[44] The transcriptional activation of each HERV has been explored mainly. A recent advance has made with interference of HERV transcript, env, in investigations of the induction of env specific-CTL from the lymphocytes of breast cancer patients.[45]

CURRENT THERAPEUTIC CONCEPTS FOR PBC

Classification of PBC and corticosteroid therapy

THERE HAVE BEEN several types of PBC described based on the clinical course or manifestation. Though there has not built a consensus for the terminology of those types of PBC, the concepts of sub-categorization would be useful for the clinical management of PBC. Auto-antibodies to the self-antigens such as gp210, p62, sp100 and centromere[46] have been investigated for the classification of PBC. The serum positivity of antibody against gp210 that forms nuclear pore complex is shown to be related to the lobular inflammation and the progression of the disease stage, which should be addressed by a larger clinical study to explain the inconsistency with other studies.[47] Anti-centromere antibody is likely to be related to a better prognosis, histological ductular reaction and prominent portal hypertension.[48] PBC-AIH overlap is characterized by both of the features of PBC, such as AMA positivity and chronic non-suppurative destructive cholangitis, and those of AIH. The similar term "PBC hepatitic form"[49] reflects a higher level of serum concentration of transaminases, which would overlap with the same features of PBC-AIH. These sub-categories need to be reevaluated for consistency in clinical practice. Additionally, the disease status tends to vary during the course, which should be taken into account for the definition. Another sub-category is AMA-AIH that lacks cholangiopathy, which is regarded as a subtype of AIH.[50] As for the therapy, immune-suppressants are effective in AMA-AIH, which is in contrast to classical PBC. In terms of the definition of PBC-AIH overlap, Poupon *et al.*[51] defined PBC as meeting 2 of the following criteria (i) alkaline phosphatase ≥ twice or gamma glutamyltranspeptidase ≥5 times of the upper limit (ii) AMA positivity (iii) florid bile duct lesion. And AIH was defined as meet 2 items of the following criteria (i) alanine aminotransferase ≥5 times of the upper limit (ii) serum IgG ≥ twice of the upper limit or positivity of anti-smooth muscle antibody (iii) moderate or severe periportal or peri-septal lymphocytic piecemeal necrosis. PBC-AIH overlap was defined as the group meeting both of the criteria. The combination of ursodeoxycholic acid (UDCA) and corticosteroid was shown to be

effective for Poupon's overlap. Another definition was proposed by Lindor *et al.*[52] in Mayo Clinic who employed the revised international autoimmune hepatitis group (IAHG) score[53] for the definition of the AIH feature of PBC patients. They demonstrated a poor prognosis in the patients with over 10 points of the Revised IAHG score in terms of the incidence of liver transplantation, death, portal hypertension, formation of esophageal varices, upper gastro-intestinal bleeding and ascites. Though Poupon's overlap contained 17 cases whose liver histology was unrevealed, the IAHG score was from 10 to 17 (13 on average), which would meet Lindor's criteria. As for corticosteroid therapy, though a consensus has not been achieved yet because of the lack of benefit for the overall prognosis of the participants as well as decrease of bone mineral density, an improvement of histological staging and inflammatory markers has been demonstrated by meta-analysis.[54] Therefore, the therapeutic option of including corticosteroid in this small subcategory group should be investigated by a large clinical study. The histological featuring of the livers would be the challenging theme with regard to the sub-classification of PBCs. For the interface hepatitis is characterized as AIH feature that is, however, commonly seen in advanced stage PBC. Bile duct lesions are the diagnostic basis for the PBC, which is observed even in the viral hepatitis.[55] The heterogeneity of the bile duct lesions leads to the under-diagnosis for PBC or to the misdiagnosis for the overlap. Laparoscopic findings of the PBC or AIH including reddish patch, mesh-like white marking, gentle undulation, reddish marking and surface depression might shed light on these problems if its invasiveness is minimized.

Re-evaluation of the complemental therapies and clinical trials

Biochemical improvement and retardation of the progression of fibrosis of Stage I & II PBC were achieved by UDCA by meta-analysis.[56] As for the mortality and incidence of liver transplantation, the advantage of the using UDCA is under discussion.[57] However, the group that achieved a biochemical response was shown to have a better prognosis regardless of the histological stages.[58] UDCA exerts a protective effect on the liver through well-documented mechanisms.[59,60] After the administration of UDCA for 3 months, the effectiveness of UDCA reaches a plateau. A succeeding or alternative option is not available yet when a full biochemical response is not achieved by UDCA monotherapy. However, several trials are being conducted as listed in Table 2. Fibrate, one of the promising medicines, increases the excretion of phospholipid into the bile via peroxisome proliferator-activated receptor α, leading to the formation of micelle that protects cells from the toxicity of hydrophobic bile acid. Preliminary results[61] and a succeeding trial demonstrated the efficacy of fibrate in terms of the biochemical response and improvement of fibrotic markers.[62] Meta-analysis could not demonstrate the efficacy of colchicine,[63] methotrexate,[64] penicillamine,[65] azathioprine,[66] or cyclosporine[67] so far, which should be re-checked by the subgroup analysis as described above. Another immune-suppressant, Rituximab, a chimeric monoclonal antibody against CD20, is under trial, which should be carefully observed for the possible involvement of microorganisms or of viremia in the triggering of PBC and the suppressive effect on liver damage by B cell populations in an animal model of PBC.[68] Other candidates include etanercept, tumor necrosis factor receptor IgG chimera,[69,70] and S-adenosylmethionine.[71] However, efficacy has only been suggested by a non-controlled trial or case reports. Therefore, in addition to 13–15 mg of daily administration of UDCA as the consensus therapy based on the recommendation of American Association for the Study of Liver Diseases, subgrouping of patients according to the features of the disease and large scale clinical trials should be encouraged.

Potency of reverse-transcriptase inhibitors

Reverse-transcriptase activity was identified in PBC and other auto-immune disorders.[72] The human homologue of MMTV, retro-transposons including HERV fragments, or unknown retroviruses might be responsible for the enzyme activity. Such reverse-transcriptase activity would be interesting in terms of the interaction with human genomic systems and diseases like PBC. However, it might be premature to intervene in clinical studies with certain reverse-transcriptase inhibitors.[73] Mason *et al.* performed a trial with a combination of Lamivudine and Zidovudine for PBC.[74] 59 entries showing plasma alkaline phosphatase activity over 1.5 times the cut off limit were randomized to the lamivudine/zidovudine group or the placebo group. The lamivudine/zidovudine group showed significant improvement of plasma activity of alkaline phosphatase, aspartate amino-transferase and alanine amino-transferase compared to the placebo control group. Thus, reverse-transcriptase inhibitors are expected to suppress retroviral proliferation, which would reduce the expression of the target peptide on the surface of HLA molecules. We once independently tried 90 day's

Table 2 Recent trials for primary biliary cirrhosis (PBC)

Trial	Design	Estimated enrollment	Primary outcome	Start date	Status	Investigator
Study of INT 747 in combination with ursodeoxycholic acid (UDCA) in patients with primary biliary cirrhosis (PBC)	Randomized Controlled Trial	140	1. Alkaline phosphatase (AP) levels 2. Safety	Nov-07	Complete	Erin Castelloe
A pilot study of Combivir therapy for patients with primary biliary cirrhosis	Randomized Controlled Trial	60		Oct-01	Complete	JM Neuberger
Randomized Controlled Pilot Study of Combivir for Patients With Primary Biliary Cirrhosis	Randomized Controlled Trial	59	The percentage of patients with either (i) normalized alkaline phosphatase, (ii) normalized AST and ALT or (iii) normal alkaline phosphatase, AST and ALT will be recorded. [Time Frame: During the 6 months of therapy]	Jan-04	Complete	Andrew L Mason Bruce Bacon Keith Lindor James Neuberger Catherine Vincent
Initial Study of Rituximab to Treat Primary Biliary Cirrhosis	Non-Randomized, Open Label, Uncontrolled	10	Safety	Jul-07	Recruiting	M. Eric Gershwin
Use of Fenofibrate for Primary Biliary Cirrhosis	Non-Randomized, Open Label, Uncontrolled	20	Serum level of alkaline phosphatase, serum immunoglobulin M level and Mayo risk score	Aug-07	Recruiting	Cynthia Levy
Stem Cell Transplantation in Patients With Primary Biliary Cirrhosis	Non-Randomized, Open Label, Active Control	10	• No liver-related death or LTx over the 2-year (extended to 5 years) follow-up; Normalization of serum alkaline phosphatase over 6 months; Amelioration of PBC histological stage with reduction of both inflammation and fibrosis scores 42	Jan-06	Recruiting	Richard Burt
Oral Budesonide in the Treatment of Patients With Primary Biliary Cirrhosis and Overlap Features of Autoimmune Hepatitis (PBC)	Open Label, Active Control, Single Group	30	The main endpoint will be the percentage of patients with improvement in alkaline phosphatase to less than 1.5 times normal over one year and the percentage of patients with a reduction in their Mayo Risk Score over one year	Dec-07	Recruiting	Keith D Lindor, MD
Low-Dose Oral Methotrexate Versus Colchicine for Primary Biliary Cirrhosis	Randomized, Double-Blind, Placebo Control	90	No description	Nov-89	Complete	Marshall M. Kaplan
SAMe to Treat Biliary Cirrhosis Symptoms	Randomized, Double-Blind, Crossover	50		Jul-05	Complete	No description

Search term: primary bilary cirrhosis (across multiple registers including the NHS and US ClinicalTrials.gov at http://www.controlled-trials.com/).

administration of lamivudine to 20 PBC patients with insufficient biochemical response to UDCA as a randomized double blind control trial. As a result, the safety of lamivudine for PBC patients was proved. However, there no advantages were seen in the lamivudine group compared with the placebo group. Of interest, one case showed a decrease of AMA titers and biochemical response. The results were very similar to those of the pilot trial by Mason *et al.*[75] However, we did not confirm MMTV transcripts from the serum samples of the participants. We think it is necessary to establish a sensitive & quantifiable assay for serum MMTV for future clinical studies. MMTV is still a controversial topic, but further clarification could promote the development of fundamental therapies and enable the avoidance of potentially contraindicated immunosuppressants.

CONCLUSION

LIVER TRANSPLANTATION IS currently the mainstay therapy for end-stage PBC. On the other hand, the development of animal models, further clinical efforts to sub-categorize PBC and research on its etiology are expected to lead to improvements in terms of the management of this disease.

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REFERENCES

1 Hiramatsu K, Aoyama H, Zen Y, Aishima S, Kitagawa S, Nakanuma Y. Proposal of a new staging and grading system of the liver for primary biliary cirrhosis. *Histopathology* 2006; 49: 466–78.
2 Nakanuma Y, Harada K. Primary cholangiohepatitis as an alternative name for primary biliary cirrhosis. *Pathol Int* 2003; **53**: 412–14.
3 Ueno Y, Moritoki Y, Shimosegawa T, Gershwin ME. Primary biliary cirrhosis: what we know and what we want to know about human PBC and spontaneous PBC mouse models. *J Gastroenterol* 2007; **42**: 189–95.
4 Kita H, Lian ZX, Van de Water J *et al.* Identification of HLA-A2-restricted CD8(+) cytotoxic T-cell responses in primary biliary cirrhosis: T-cell activation is augmented by immune complexes cross-presented by dendritic cells. *J Exp Med* 2002; **195**: 113–23.
5 Onishi S, Sakamaki T, Maeda T *et al.* DNA typing of HLA class II genes; DRB1*0803 increases the susceptibility of Japanese to primary biliary cirrhosis. *J Hepatol* 1994; **21**: 1053–60.
6 Yahagi K, Ueno Y, Nomura E *et al.* Mapping of a disease susceptibility locus in the HLA region for Primary Biliary Cirrhosis in Japan. *Hepatol Res* 2007; **37**: 270–5.
7 Juran BD, Atkinson EJ, Schlicht EM, Fridley BL, Petersen GM, Lazaridis KN. Interacting alleles of the coinhibitory immunoreceptor genes cytotoxic T-lymphocyte antigen 4 and programmed cell-death 1 influence risk and features of primary biliary cirrhosis. *Hepatology* 2008; **47**: 563–70.
8 Olafsson S, Gudjonsson H, Selmi C *et al.* Antimitochondrial antibodies and reactivity to N. aromaticivorans proteins in Icelandic patients with primary biliary cirrhosis and their relatives. *Am J Gastroenterol* 2004; **99**: 2143–6.
9 Selmi C. Environmental factors in primary biliary cirrhosis. *Hepatol Res* 2007; **37** (Suppl 3): S370–6.
10 Xu L, Sakalian M, Shen Z, Loss G, Neuberger J, Mason A. Cloning the human betaretrovirus proviral genome from patients with primary biliary cirrhosis. *Hepatology* 2004; **39**: 151–6.
11 Wakabayashi K, Lian ZX, Leung PS *et al.* Loss of tolerance in C57BL/6 mice to the autoantigen E2 subunit of pyruvate dehydrogenase by a xenobiotic with ensuing biliary ductular disease. *Hepatology* 2008; **48**: 531–40.
12 Leung PS, Rossaro L, Davis PA *et al.* Antimitochondrial antibodies in acute liver failure: Implications for primary biliary cirrhosis. *Hepatology* 2007; **46**:1436–42.
13 Siegert W, Stemerowicz R, Hopf U. Antimitochondrial antibodies in patients with chronic graft-versus-host disease. *Bone Marrow Transplant* 1992; **10**: 221–7.
14 Odin JA, Huebert RC, Casciola-Rosen L, LaRusso NF, Rosen A. Bcl-2-dependent oxidation of pyruvate dehydrogenase-E2, a primary biliary cirrhosis autoantigen, during apoptosis. *J Clin Invest* 2001; **108**: 223–32.
15 Mao TK, Davis PA, Odin JA, Coppel RL, Gershwin ME. Sidechain biology and the immunogenicity of *PDC-E2*, the major autoantigen of primary biliary cirrhosis. *Hepatology* 2004; **40**: 1241–8.
16 Wakabayashi K, Lian ZX, Moritoki Y *et al.* IL-2 receptor alpha(−/−) mice and the development of primary biliary cirrhosis. *Hepatology* 2006; **44**: 1240–9.
17 Oertelt S, Lian ZX, Cheng CM *et al.* Anti-mitochondrial antibodies and primary biliary cirrhosis in TGF-beta receptor II dominant-negative mice. *J Immunol* 2006; **177**: 1655–60.
18 Nakagome Y, Ueno Y, Kogure T *et al.* Autoimmune cholangitis in NOD.c3c4 mice is associated with cholangiocyte-specific Fas antigen deficiency. *J Autoimmun* 2007; **29**: 20–9.

19 Lan RY, Cheng C, Lian ZX *et al.* Liver-targeted and peripheral blood alterations of regulatory T-cells in primary biliary cirrhosis. *Hepatology* 2006; **43**: 729–37.
20 Zhang W, Sharma R, Ju ST *et al.* Deficiency in regulatory T-cells results in development of antimitochondrial antibodies and autoimmune cholangitis. *Hepatology* 2009; **49**: 545–52.
21 Yamagiwa S, Ichida T. Recurrence of primary biliary cirrhosis and primary sclerosing cholangitis after liver transplantation in Japan. *Hepatol Res* 2007; **37** (Suppl 3): S449–54.
22 Mason AL, Xu L, Guo L *et al.* Detection of retroviral antibodies in primary biliary cirrhosis and other idiopathic biliary disorders. *Lancet* 1998; **351** (9116): 1620–4.
23 Xu L, Shen Z, Guo L *et al.* Does a betaretrovirus infection trigger primary biliary cirrhosis? *Proc Natl Acad Sci USA* 2003; **100**: 8454–9.
24 Selmi C, Ross SR, Ansari AA *et al.* Lack of immunological or molecular evidence for a role of mouse mammary tumor retrovirus in primary biliary cirrhosis. *Gastroenterology* 2004; **127**: 493–501.
25 Johal H, Scott GM, Jones R, Camaris C, Riordan S, Rawlinson WD. Mouse mammary tumour virus-like virus (MMTV-LV) is present within the liver in a wide range of hepatic disorders and unrelated to nuclear p53 expression or hepatocarcinogenesis. *J Hepatol* 2009; **50**: 548–54.
26 Indik S, Gunzburg WH, Salmons B, Rouault F. Mouse mammary tumor virus infects human cells. *Cancer Res* 2005; **65**: 6651–9.
27 Wang F, Lothrop AP, James NG *et al.* A novel murine protein with no effect on iron homoeostasis is homologous with transferrin and is the putative inhibitor of carbonic anhydrase. *Biochem J* 2007; **406**: 85–95.
28 Wang E, Obeng-Adjei N, Ying Q *et al.* Mouse mammary tumor virus uses mouse but not human transferrin receptor 1 to reach a low pH compartment and infecT-cells. *Virology* 2008; **381**: 230–40.
29 Ueno Y, Alpini G, Yahagi K *et al.* Evaluation of differential gene expression by microarray analysis in small and large cholangiocytes isolated from normal mice. *Liver Int* 2003; **23**: 449–59.
30 Amarante MK, Watanabe MA. The possible involvement of virus in breast cancer. *J Cancer Res Clin Oncol* 2009; **135**: 329–37.
31 Lawson JS. Do viruses cause breast cancer? *Methods Mol Biol* 2009; **471**: 421–38.
32 Piscaglia F, Sagrini E. Malignancies in primary biliary cirrhosis. *Eur J Gastroenterol Hepatol* 2008; **20**: 1–4.
33 Golovkina TV, Chervonsky A, Prescott JA, Janeway CA Jr, Ross SR. The mouse mammary tumor virus envelope gene product is required for superantigen presentation to T-cells. *J Exp Med* 1994; **179**: 439–46.
34 Swanson I, Jude BA, Zhang AR, Pucker A, Smith ZE, Golovkina TV. Sequences within the gag gene of mouse mammary tumor virus needed for mammary gland cell transformation. *J Virol* 2006; **80**: 3215–24.
35 Bonovich MT, List HJ, Zhang S, Danielsen M, Riegel AT. Identification of glucocorticoid receptor domains necessary for transcriptional activation of the mouse mammary tumor virus promoter integrated in the genome. *Exp Cell Res* 1998; **239**: 454–62.
36 Otten AD, Sanders MM, McKnight GS. The MMTV LTR promoter is induced by progesterone and dihydrotestosterone but not by estrogen. *Mol Endocrinol* 1988; **2**: 143–7.
37 Peralta Soler A, Aoki A. Estrogen influence on maturational pathway of murine mammary tumor virus: an immunoelectron microscopy study. *Exp Mol Pathol* 1989; **50**: 16–25.
38 Karlsson H, Schroder J, Bachmann S, Bottmer C, Yolken, RH. HERV-W-related RNA detected in plasma from individuals with recent-onset schizophrenia or schizoaffective disorder. *Mol Psychiatry* 2004; **9**: 12–13.
39 Jolivet-Reynaud C, Perron H, Ferrante P, Becquart L, Dalbon P, Mandrand B. Specificities of multiple sclerosis cerebrospinal fluid and serum antibodies against mimotopes. *Clin Immunol* 1999; **93**: 283–93.
40 Ehlhardt S, Seifert M, Schneider J, Ojak A, Zang KD, Mehraein Y. Human endogenous retrovirus HERV-K(HML-2) Rec expression and transcriptional activities in normal and rheumatoid arthritis synovia. *J Rheumatol* 2006; **33**: 16–23.
41 Moyes DL, Martin A, Sawcer S *et al.* The distribution of the endogenous retroviruses HERV-K113 and HERV-K115 in health and disease. *Genomics* 2005; **86**: 337–41.
42 Buscher K, Hahn S, Hofmann M *et al.* Expression of the human endogenous retrovirus-K transmembrane envelope, Rec and Np9 proteins in melanomas and melanoma cell lines. *Melanoma Res* 2006; **16**: 223–34.
43 Sicat J, Sutkowski N, Huber BT. Expression of human endogenous retrovirus HERV-K18 superantigen is elevated in juvenile rheumatoid arthritis. *J Rheumatol* 2005; **32**: 1821–31.
44 Tai AK, O'Reilly EJ, Alroy KA *et al.* Human endogenous retrovirus-K18 Env as a risk factor in multiple sclerosis. *Mult Scler* 2008; **14**: 1175–80.
45 Wang-Johanning F, Radvanyi L, Rycaj K *et al.* Human endogenous retrovirus K triggers an antigen-specific immune response in breast cancer patients. *Cancer Res* 2008; **68**: 5869–77.
46 Invernizzi P, Selmi C, Ranftler C, Podda M, Wesierska-Gadek J. Antinuclear antibodies in primary biliary cirrhosis. *Semin Liver Dis* 2005; **25**: 298–310.
47 Bogdanos DP, Liaskos C, Pares A *et al.* Anti-gp210 antibody mirrors disease severity in primary biliary cirrhosis. *Hepatology* 2007; **45**: 1583; author reply –4.
48 Nakamura M, Kondo H, Mori T *et al.* Anti-gp210 and anti-centromere antibodies are different risk factors for the progression of primary biliary cirrhosis. *Hepatology* 2007; **45**: 118–27.
49 Lohse AW, zum Buschenfelde KH, Franz B, Kanzler S, Gerken G, Dienes HP. Characterization of the overlap syn-

drome of primary biliary cirrhosis (PBC) and autoimmune hepatitis: evidence for it being a hepatitic form of PBC in genetically susceptible individuals. *Hepatology* 1999; **29**: 1078–84.
50 Farias AQ, Goncalves LL, Bittencourt PL *et al.* Applicability of the IAIHG scoring system to the diagnosis of antimitochondrial/anti-M2 seropositive variant form of autoimmune hepatitis. *J Gastroenterol Hepatol* 2006; **21**: 887–93.
51 Chazouilleres O, Wendum D, Serfaty L, Rosmorduc O, Poupon R. Long term outcome and response to therapy of primary biliary cirrhosis-autoimmune hepatitis overlap syndrome. *J Hepatol* 2006; **44**: 400–6.
52 Silveira MG, Talwalkar JA, Angulo P, Lindor KD. Overlap of autoimmune hepatitis and primary biliary cirrhosis: long-term outcomes. *Am J Gastroenterol* 2007; **102**: 1244–50.
53 Alvarez F, Berg PA, Bianchi FB *et al.* International Autoimmune Hepatitis Group Report: review of criteria for diagnosis of autoimmune hepatitis. *J Hepatol* 1999; **31**: 929–38.
54 Prince M, Christensen E, Gluud C. Glucocorticosteroids for primary biliary cirrhosis. *Cochrane Database Syst Rev* 2005; **2**: CD003778.
55 Mihm S, Fayyazi A, Hartmann H, Ramadori G. Analysis of histopathological manifestations of chronic hepatitis C virus infection with respect to virus genotype. *Hepatology* 1997; **25**: 735–9.
56 Lindor K. Ursodeoxycholic acid for the treatment of primary biliary cirrhosis. *N Engl J Med* 2007; **357**: 1524–9.
57 Gong Y, Huang ZB, Christensen E, Gluud C. Ursodeoxycholic acid for primary biliary cirrhosis. *Cochrane Database Syst Rev* 2008; **3**: CD000551.
58 Kuiper EM, Hansen BE, de Vries RA *et al.* Improved prognosis of patients with primary biliary cirrhosis that have a biochemical response to ursodeoxycholic acid. *Gastroenterology* 2009; **136**: 1281–7.
59 Marzioni M, Francis H, Benedetti A *et al.* Ca2+-dependent cytoprotective effects of ursodeoxycholic and tauroursodeoxycholic acid on the biliary epithelium in a rat model of cholestasis and loss of bile ducts. *Am J Pathol* 2006; **168**: 398–409.
60 D'Aldebert E, Biyeyeme Bi Mve MJ, Mergey M *et al.* Bile salts control the antimicrobial peptide cathelicidin through nuclear receptors in the human biliary epithelium. *Gastroenterology* 2009; **136**: 1435–43.
61 Nakai S, Masaki T, Kurokohchi K, Deguchi A, Nishioka M. Combination therapy of bezafibrate and ursodeoxycholic acid in primary biliary cirrhosis: a preliminary study. *Am J Gastroenterol* 2000; **95**: 326–7.
62 Ohmoto K, Yoshioka N, Yamamoto S. Long-term effect of bezafibrate on parameters of hepatic fibrosis in primary biliary cirrhosis. *J Gastroenterol* 2006; **41**: 502–3.
63 Gong Y, Gluud C. Colchicine for primary biliary cirrhosis. *Cochrane Database Syst Rev* 2004; **2**: CD004481.
64 Gong Y, Gluud C. Methotrexate for primary biliary cirrhosis. *Cochrane Database Syst Rev* 2005; **3**: CD004385.
65 Gong Y, Frederiksen SL, Gluud C. D-penicillamine for primary biliary cirrhosis. *Cochrane Database Syst Rev* 2004; **4**: CD004789.
66 Gong Y, Christensen E, Gluud C. Azathioprine for primary biliary cirrhosis. *Cochrane Database Syst Rev* 2007; **3**: CD006000.
67 Gong Y, Christensen E, Gluud C. Cyclosporin A for primary biliary cirrhosis. *Cochrane Database Syst Rev* 2007; **3**: CD005526.
68 Moritoki Y, Zhang W, Tsuneyama K *et al.* B cells suppress the inflammatory response in a mouse model of primary biliary cirrhosis. *Gastroenterology* 2009; **136**: 1037–47.
69 Ogata A, Terabe F, Nakanishi K *et al.* Etanercept improved primary biliary cirrhosis associated with rheumatoid arthritis. *Joint Bone Spine* 2009; **76**: 105–7.
70 Spadaro A, Scrivo R, Riccieri V, Valesini G. Effect of tumor necrosis factor alpha antagonists in a patient with rheumatoid arthritis and primary biliary cirrhosis. *Joint Bone Spine* 2008; **75**: 87–9.
71 Avezov SA, Mansurova F. [Efficacy of combined use of ursodeoxycholic acid and heptral in the treatment of primary biliary cirrhosis]. *Klin Med (Mosk)* 2004; **82**: 46–9.
72 McDermid J, Chen M, Li Y *et al.* Reverse transcriptase activity in patients with primary biliary cirrhosis and other autoimmune liver disorders. *Aliment Pharmacol Ther* 2007; **26**: 587–95.
73 Gershwin ME, Selmi C. Apocalypsal versus apocryphal: the role of retroviruses in primary biliary cirrhosis. *Am J Gastroenterol* 2004; **99**: 2356–8.
74 Mason AL, Lindor KD, Bacon BR *et al.* Clinical trial: randomized controlled trial of zidovudine and lamivudine for patients with primary biliary cirrhosis stabilized on ursodiol. *Aliment Pharmacol Ther* 2008; July 9.
75 Mason AL, Farr GH, Xu L, Hubscher SG, Neuberger JM. Pilot studies of single and combination antiretroviral therapy in patients with primary biliary cirrhosis. *Am J Gastroenterol* 2004; **99**: 2348–55.

Knockout of Secretin Receptor Reduces Large Cholangiocyte Hyperplasia in Mice With Extrahepatic Cholestasis Induced by Bile Duct Ligation

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During bile duct ligation (BDL), the growth of large cholangiocytes is regulated by the cyclic adenosine monophosphate (cAMP)/extracellular signal-regulated kinase 1/2 (ERK1/2) pathway and is closely associated with increased secretin receptor (SR) expression. Although it has been suggested that SR modulates cholangiocyte growth, direct evidence for secretin-dependent proliferation is lacking. SR wild-type (WT) ($SR^{+/+}$) or SR knockout ($SR^{-/-}$) mice underwent sham surgery or BDL for 3 or 7 days. We evaluated SR expression, cholangiocyte proliferation, and apoptosis in liver sections and proliferating cell nuclear antigen (PCNA) protein expression and ERK1/2 phosphorylation in purified large cholangiocytes from WT and $SR^{-/-}$ BDL mice. Normal WT mice were treated with secretin (2.5 nmoles/kg/day by way of osmotic minipumps for 1 week), and biliary mass was evaluated. Small and large cholangiocytes were used to evaluate the *in vitro* effect of secretin (100 nM) on proliferation, protein kinase A (PKA) activity, and ERK1/2 phosphorylation. SR expression was also stably knocked down by short hairpin RNA, and basal and secretin-stimulated cAMP levels (a functional index of biliary growth) and proliferation were determined. SR was expressed by large cholangiocytes. Knockout of SR significantly decreased large cholangiocyte growth induced by BDL, which was associated with enhanced apoptosis. PCNA expression and ERK1/2 phosphorylation were decreased in large cholangiocytes from $SR^{-/-}$ BDL compared with WT BDL mice. *In vivo* administration of secretin to normal WT mice increased ductal mass. *In vitro*, secretin increased proliferation, PKA activity, and ERK1/2 phosphorylation of large cholangiocytes that was blocked by PKA and mitogen-activated protein kinase kinase inhibitors. Stable knockdown of SR expression reduced basal cholangiocyte proliferation. SR is an important trophic regulator sustaining biliary growth. *Conclusion:* The current study provides strong support for the potential use of secretin as a therapy for ductopenic liver diseases. (HEPATOLOGY **2010;52:204-214**)

Cholangiocytes line the intrahepatic biliary system, which modifies the bile of canalicular origin into its final composition before reaching the small intestine.[1,2] Several gastrointestinal peptides/hormones, including bombesin, gastrin, and secretin, regulate cholangiocyte secretory activity.[1-3] Among these factors, secretin plays a key role in the biliary secretion of water and bicarbonate, because secretin receptor (SR) is expressed in rodent and human liver by larger bile ducts.[1,4-6] In large cholangiocytes, secretin

Abbreviations: BDL, bile duct ligation; BSA, bovine serum albumin; cAMP, cyclic adenosine monophosphate; CCl_4, carbon tetrachloride; ERK1/2, extracellular signal-regulated kinase; FACS, fluorescence-activated cell sorting; IBDM, intrahepatic bile duct mass; MEK, mitogen-activated protein kinase kinase; PCNA, proliferating cell nuclear antigen; PCR, polymerase chain reation; PKA, protein kinase A; SEM, standard error of the mean; SR, secretin receptor; WT, wild-type.

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increases cyclic adenosine monophosphate (cAMP) levels[1,4,5,7,8] and induces the opening of the Cl^- channel (cystic fibrosis transmembrane conductance regulator, CFTR)[9] leading to the activation of the Cl^-/HCO_3^- anion exchanger 2[10] and secretion of bicarbonate in bile.[2,3]

Human cholangiocytes are the target cells in several cholangiopathies, including primary biliary cirrhosis and primary sclerosing cholangitis, diseases associated with dysregulation of the balance between cholangiocyte proliferation/apoptosis.[11] Rodent cholangiocytes, which are normally mitotically quiescent,[12,13] markedly proliferate in animal models of cholestasis including extrahepatic bile duct ligation (BDL) or acute carbon tetrachloride (CCl_4) administration.[12,14] The proliferative response of the intrahepatic biliary epithelium to BDL is heterogeneous, because large (but not small) cholangiocytes proliferate through the activation of cAMP-dependent ERK1/2 signaling[12,15] leading to enhanced ductal mass.[5,12,14]

Because SR is only expressed by large cholangiocytes in the liver,[1,4,5,9,12,14] changes in the functional expression of this receptor have been suggested as a pathophysiological tool for evaluating changes in the degree of cholangiocyte growth/loss.[5,12,14] Indeed, we have shown that (1) cholangiocyte hyperplasia (after BDL or 70% hepatectomy) is associated with enhanced SR expression and secretin-stimulated cAMP levels and bicarbonate secretion[12,13,16-18] and (2) cholangiocyte damage (after CCl_4) decreases the functional expression of SR in large cholangiocytes.[14] In pathological conditions—such as the CCl_4 model, which is characterized by lack or damage of the hormonally responsive large cholangiocytes—small cholangiocytes proliferate and express SR *de novo*.[14]

The hormonal actions of secretin through SR have been studied in the pancreas, stomach, and biliary epithelium.[19] Although it has been suggested that SR modulates cholangiocyte growth,[2,12-14] the direct link between SR expression and its possible role in the regulation of biliary proliferation has not been elucidated. The aim of our study was to determine the role that SR plays in sustaining large cholangiocyte growth during cholestasis induced by BDL.

Materials and Methods

Materials. Reagents were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise stated. The nuclear dye 4′,6-diamidino-2-phenylindole was obtained from Molecular Probes, Inc. (Eugene, OR). Porcine secretin was purchased from Peninsula Laboratories (Belmont, CA). The polyclonal SR antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was raised against a peptide mapping at the C terminus of SR of human origin and cross-reacts with mouse.[20] The antibody against proliferating cell nuclear antigen (PCNA) was purchased from Santa Cruz Biotechnology. The mouse anti–cytokeratin-19 antibody was purchased from Caltag Laboratories Inc. (Burlingame, CA). Goat phosphorylated ERK1/2 and total ERK1/2 (44-42 kDa) polyclonal affinity purified antibodies were purchased from Santa Cruz Biotechnology. The RIA kits for the determination of intracellular cAMP levels in cholangiocytes were purchased from Perkin Elmer (Shelton, CT).

Animal Models. All animal experiments (Table 1) were performed in accordance with a protocol approved by the Scott & White and Texas A&M Health Science Center Institutional Animal Care and Use Committee and conformed to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (Publication No. 85-23, revised 1996). Our $SR^{+/+}$ (wild-type [WT]) or SR knockout ($SR^{-/-}$)[21] mice were maintained in a temperature-controlled environment (20-22°C) with a 12:12-hour light/dark cycle. We used adult male WT and $SR^{-/-}$ mice (approximately 25-30 g) of the N5 generation: (1) as normal treated with saline (0.9% NaCl) or secretin (2.5 nmol/kg/day, a dose similar to that used by us for another gastrointestinal hormone, gastrin, in rodents)[18] by way of intraperitoneally implanted Alzet osmotic minipumps (Alzet, CA) for 7

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Potential conflict of interest: Nothing to report.

Table 1. Evaluation of Body Weight, Biliary Expression of SR, Lobular Necrosis, Percentage of PCNA- or TUNEL-Positive Large Cholangiocytes, and Large IBDM in Liver Sections

Groups	Body Weight (g)	Percentage of Large Cholangiocytes Positive for SR	Lobular Necrosis	Percentage of Large Cholangiocytes Positive for PCNA	Large IBDM	Percentage of Large Cholangiocytes Positive by TUNEL
WT normal + NaCl, 1 week	27.8 ± 0.8	19.83 ± 1.96	(−)	6.20 ± 0.97	0.17 ± 0.03	ND
WT normal + secretin, 1 week	25.6 ± 0.5	30.60 ± 2.04*	(−)	40.80 ± 2.29*	0.35 ± 0.02*	ND
Normal $SR^{-/-}$ + NaCl, 1 week	28.6 ± 0.7	ND	(−)	4.20 ± 0.66	0.18 ± 0.02	ND
Normal $SR^{-/-}$ + secretin, 1 week	29.0 ± 1.8	ND	(−)	5.33 ± 1.08	0.18 ± 0.03	ND
WT BDL, 3 days	23.2 ± 0.7	39.0 ± 2.07*	(+)	60.62 ± 2.30	1.26 ± 0.06	ND
$SR^{-/-}$ BDL, 3 days	22.0 ± 0.5	ND	(++)	39.67 ± 2.16†	0.57 ± 0.06†	10.50 ± 1.08
WT BDL, 7 days	23.2 ± 0.7	41.33 ± 2.35*	(+)	47.67 ± 1.50	2.51 ± 0.12	ND
$SR^{-/-}$ BDL, 7 days	26.2 ± 0.6	ND	(++)	30.83 ± 2.07†	1.40 ± 0.11†	13.33 ± 0.88

Body weight values are derived from 10-20 animals per each group. Evaluations were performed in liver sections (4- to 5-μm-thick). IBDM was measured as the area occupied by cytokeratin-19-positive bile duct/total area x 100. Lobular necrosis was scored as follows: −, 0 foci; +/−, <2 foci; +, 2-4 foci; ++, >4 foci. Data are expressed as the mean ± SEM.

Abbreviations: ND, not detected; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling.

*$P < 0.05$ versus the corresponding value of WT normal mice treated with NaCl for 1 week.

†$P < 0.05$ versus the corresponding value of WT mice with BDL for 3 and 7 days, respectively.

days; or (2) for sham operation or BDL (for 3 and 7 days).[5,20,22] Because our previous studies[21] showed that $SR^{-/-}$ mice have a renal defect in water reabsorption and associated polyuria and polydipsia, experiments were performed to determine whether the response of $SR^{-/-}$ mice to BDL was due to the lack of SR rather than severe dehydration. Thus, we evaluated changes in body weight and mortality rate in the experimental groups of Table 1. In addition, both WT and $SR^{-/-}$ mice (after BDL or administration of secretin) received oral hydration therapy, consisting of up to 1 ml of normal saline subcutaneously up to twice daily along with water in gel form on the ground and food supplements. Because there were no differences in cholangiocyte proliferation between normal WT and normal $SR^{-/-}$ mice and their corresponding sham mice, we did not show the results from the sham animals.

Immortalized and Freshly Isolated Cholangiocytes. The *in vitro* studies were performed in freshly isolated or immortalized[5,8] large cholangiocytes. The rationale for performing these studies only in large cholangiocytes is based on the fact that secretin stimulated *in vivo* the proliferation of only large bile ducts and that following BDL, large but not small cholangiocytes proliferate.[5] Freshly isolated large cholangiocytes (≈99% by cytokeratin-19 immunohistochemistry)[5,20] were purified by centrifugal elutriation[4,9,14] followed by immunoaffinity separation by a monoclonal antibody, rat IgG_{2a} (provided by Dr. R. Faris, Brown University, Providence, RI), against an antigen expressed by all mouse cholangiocytes.[5] Our large mouse cholangiocyte lines, which display morphological, phenotypic, and functional features similar to that of freshly isolated large cholangiocytes were cultured as described.[5,8,9]

Evaluation of Secretin Receptor Expression. We evaluated the expression of SR by immunohistochemistry in paraffin-embedded liver sections from the experimental groups of Table 1. Because immunohistochemistry shows that only large bile ducts from WT (but not knockout) animals express SR, we evaluated the expression of SR by way of immunofluorescence and real-time polymerase chain reaction (PCR) in freshly isolated large cholangiocytes from normal and 3- and 7-day BDL WT mice. Semiquantitative immunohistochemical analysis of SR expression in sections was performed as described.[5] Light microscopy photographs of liver sections were taken by Leica Microsystems DM 4500 B Light Microscopy (Weltzlar, Germany) with a Jenoptik Prog Res C10 Plus Videocam (Jena, Germany). Immunofluorescence for SR was also performed in large cholangiocytes from normal and 3- and 7-day BDL WT mice.[5,20] Images were visualized using an Olympus IX-71 confocal microscope. For all immunoreactions, negative controls (with normal serum from the same species substituted for the primary antibody) were included.

In freshly isolated large cholangiocytes from normal and BDL WT mice, messenger RNA and protein expression of SR were evaluated by way of real-time PCR[23] and western blot analysis, respectively.[20] For real-time PCR, RNA was extracted from cholangiocytes using the RNeasy Mini Kit (Qiagen Inc, Valencia, CA) and reverse-transcribed using the Reaction Ready First Strand cDNA synthesis kit (SuperArray, Frederick, MD). These reactions were used as templates for the PCR assays using an SYBR Green PCR

master mix and specific primers designed against the mouse secretin receptor gene NM_001012322,[24] and glyceraldehyde 3-phosphate dehydrogenase, the housekeeping gene (SuperArray, Frederick, MD) in the real-time thermal cycler (ABI Prism 7900HT sequence detection system). A ΔΔCt analysis was performed using normal large cholangiocytes as the control sample. Data are expressed as fold-change of relative messenger RNA levels ± standard error of the mean (SEM) (n = 6).

Evaluation of Liver Histology, Cholangiocyte Apoptosis, and Proliferation. All liver sections were scored by two board-certified pathologists who were blinded to the identity of the samples. Lobular necrosis was evaluated in liver sections stained with hematoxylin-eosin.[25] Lobular necrosis was scored as follows: −, 0 foci; +/−, <2 foci; +, 2-4 foci; ++, >4 foci.[25] Sections were examined in a coded fashion by BX-51 light microscopy (Olympus, Tokyo, Japan) equipped with a camera. We measured (1) the percentage of cholangiocyte apoptosis by semiquantitative terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling kit (Apoptag; Chemicon International, Inc.); (2) cholangiocyte proliferation by evaluation of the percentage of small and large cholangiocytes positive for PCNA[5]; and (3) intrahepatic bile duct mass (IBDM)[5] of small (<15 μm)[1] and large (>15 μm)[1] bile ducts. IBDM was measured as the area occupied by cytokeratin-19–positive bile duct/total area × 100. Proliferation was evaluated by immunoblots[20] for PCNA in protein (10 μg) from lysate from spleen (positive control) and large cholangiocytes from WT and $SR^{-/-}$ BDL mice. Blots were normalized by β-actin.[5] The intensity of the bands was determined by way of scanning video densitometry using the Storm 860 and the ImageQuant TL software version 2003.02 (GE Healthcare, Little Chalfont, Buckinghamshire, England).

Measurement of cAMP Levels and Phosphorylation of ERK1/2. These experiments were performed in large cholangiocytes from WT and knockout 7-day BDL mice, a period where a marked ductal hyperplasia is observed.[2,12] We evaluated basal and secretin-stimulated cAMP levels (a functional parameter of cholangiocyte growth)[13,18] by commercially available RIA kits[20]; and phosphorylation of ERK1/2 by immunoblots in protein (10 μg) from cholangiocyte lysate. The intensities of the bands were determined by scanning video densitometry using a phospho-imager.

***In Vitro* Effect of Secretin on the Proliferation, Protein Kinase A Activity, and ERK1/2 Phosphorylation of Large Cholangiocytes.** Our small (negative control) and large cholangiocytes[8] were treated at 37°C with 0.2% bovine serum albumin (BSA) (basal) or secretin (100 nM) for 48 hours in the absence or presence of preincubation (1 hour) with H89 (protein kinase A [PKA] inhibitor, 30 μM) or PD98059 (mitogen-activated protein kinase kinase [MEK] inhibitor, 10 nM) before evaluating proliferation by CellTiter 96 Cell Proliferation Assay[20] (Promega Corp., Madison, WI). Absorbance was measured at 490 nm on a microplate spectrophotometer (Molecular Devices, Sunnyvale, CA). Data were expressed as the fold change of treated cells compared with vehicle-treated controls. In separate experiments, large cholangiocytes were treated with 0.2% BSA (basal) or secretin (100 nM) for 6 hours in the absence or presence of H89 (30 μM) or PD98059 (10 nM) before evaluating PCNA expression by way of immunoblotting,[5] PKA activity,[20] and phosphorylation of ERK1/2 by way of immunoblotting.[5] The intensity of the bands was determined as described above.

Stable Transfected Knockdown of Secretin Receptor in Large Cholangiocytes. To provide conclusive evidence that SR is a key proproliferative regulator sustaining large cholangiocyte growth, we stably knocked down the expression of this receptor in large cholangiocyte lines.[8] The mouse cell line lacking SR was established using SureSilencing short hairpin RNA (SuperArray, Frederick, MD) plasmid for mouse SR containing a marker for neomycin resistance for the selection of stably transfected cells, according to the instructions provided by the vendor as described.[23] A total of four clones were assessed for the relative knockdown of the SR gene using real-time PCR and a single clone with the greatest degree of knockdown was selected for subsequent experiments. In selected and mock-transfected clones, the degree of SR knockdown was also evaluated by way of fluorescence-activated cell sorting (FACS) analysis and western blot analysis as described.[26]

The two cell lines—mock-transfected clone (transfected with control vector) and the SR knockdown clone (80% knockdown efficiency of the message by real-time PCR [data not shown] and 50% knockdown of protein expression by FACS)—were then treated with 0.2% BSA (basal) or secretin (100 nM for 5 minutes) before evaluation of cAMP levels by way of RIA[4,7,9,18] or 0.2% BSA (basal) or secretin (100 nM) before measuring proliferation by way of MTS assay (48-hour incubation). The mock-transfected and SR knockdown clones in large cholangiocytes were incubated in culture medium before evaluating basal proliferative activity by MTS proliferation assay (after incubation for 6, 24, 48, and 72 hours).

Statistical Analysis. All data are expressed as the mean ± SEM. Differences between groups were analyzed using the Student unpaired *t* test when two

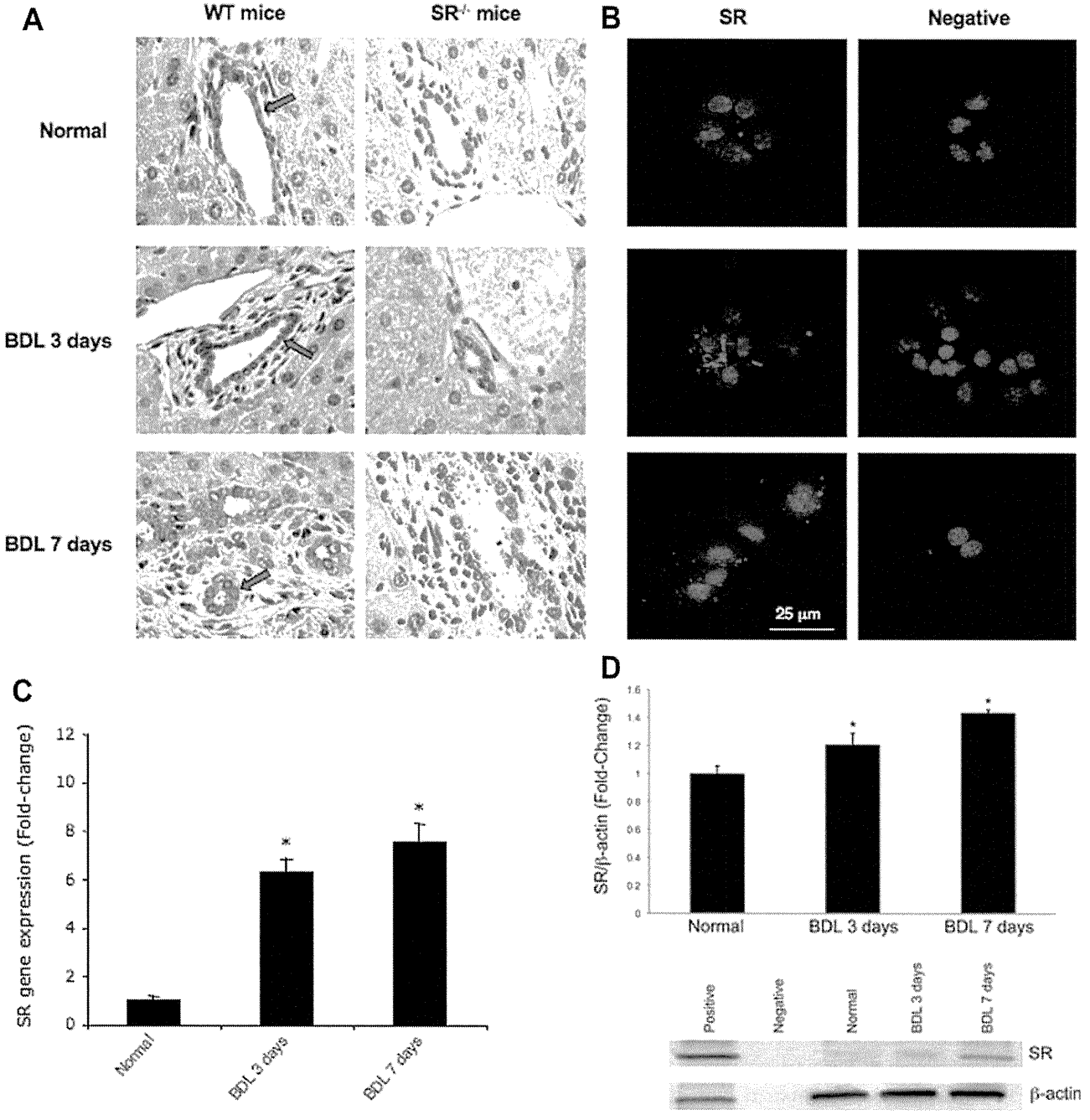

Fig. 1. Evaluation of SR expression by (A) immunohistochemistry in liver sections from WT and $SR^{-/-}$ normal mice, and mice with BDL for 3 and 7 days, (B) immunofluorescence, (C) real-time PCR, and (D) immunoblots in freshly isolated large cholangiocytes from normal and 3- and 7-day BDL WT mice. (A) Large bile ducts from normal and BDL WT mice express SR (red arrows). Original magnification ×40. (B) Specific immunoreactivity for SR in representative fields is shown in red; cell nuclei were stained with 4′,6-diamidino-2-phenylindole (blue). Scale bar = 25 μm. (C,D) Data are expressed as the mean ± SEM of six experiments. *$P < 0.05$ versus normal.

groups were analyzed, and by way of analysis of variance when more than two groups were analyzed, followed by an appropriate *post hoc* test.

Results

Evaluation of Secretin Receptor Expression. In liver sections, we demonstrated that large but not small bile ducts from normal and BDL WT mice express SR (Fig. 1A and Table 1). The expression of SR in large bile ducts was higher in: normal WT mice treated with secretin compared to saline-treated mice (Table 1) and WT BDL compared with normal WT mice (Table 1). There was no positive staining for SR in bile ducts from normal and BDL $SR^{-/-}$ mice (Fig. 1A). The expression of SR was confirmed by way of

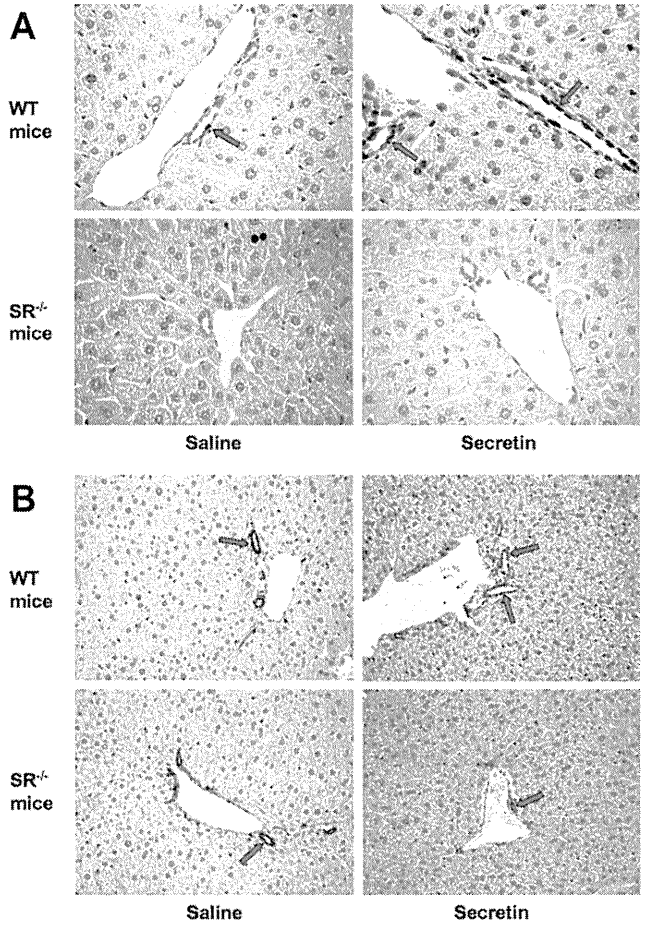

Fig. 2. Evaluation of the number of (A) large PCNA-positive cholangiocytes and (B) large IBDM in normal mice treated with saline or secretin for 1 week. In WT mice treated with secretin, there was an increase in the number of (A) large PCNA-positive cholangiocytes (red arrows) and (B) large IBDM (red arrows) compared with normal WT mice treated with saline. Original magnification ×40 (A) and ×20 (B).

immunofluorescence in large cholangiocytes purified from normal and BDL WT mice (Fig. 1B). Real-time PCR and immunoblot assay revealed that the expression of SR messenger RNA and protein was higher in large BDL cholangiocytes compared with normal large cholangiocytes (Fig. 1C,D).

Evaluation of Liver Weight, Lobular Necrosis, Cholangiocyte Apoptosis, and Proliferation. No significant differences in body weight and mortality rates were observed among the experimental groups of Table 1. No difference in lobular necrosis was observed in normal WT and $SR^{-/-}$ mice, whereas the typical necrosis present in the BDL model showed only a smaller increase (not significant) in $SR^{-/-}$ BDL mice compared with WT BDL mice. The chronic administration of secretin to normal WT mice increased the percentage of large PCNA-positive cholangiocytes and large IBDM compared with normal WT mice treated with saline (Fig. 2A,B and Table 1); secretin did not increase the proliferation of small ducts that do not express SR (not shown).[5] In normal $SR^{-/-}$ mice, secretin did not induce changes in cholangiocyte proliferation or apoptosis (Fig. 2A,B and Table 1). Following BDL, there was an increase in the percentage of PCNA expressing cholangiocytes and IBDM in large bile ducts compared with normal mice (Fig. 3A,B and Table 1). Similar to previous studies,[16] large IBDM was enhanced in parallel with the increased duration of BDL (Fig. 3B and Table 1). Knockout of SR reduces large cholangiocyte proliferation and large IBDM induced by BDL[5,20] compared with WT BDL mice (Fig. 3A,B and Table 1).

Evaluation of Proliferation, cAMP Levels, and Phosphorylation of ERK1/2 in Isolated Large Cholangiocytes. In large cholangiocytes from 7-day $SR^{-/-}$ BDL mice, there was decreased PCNA expression compared with cholangiocytes from WT BDL mice (Fig. 4A). Basal cAMP levels of large cholangiocytes from $SR^{-/-}$ BDL mice were significantly lower than

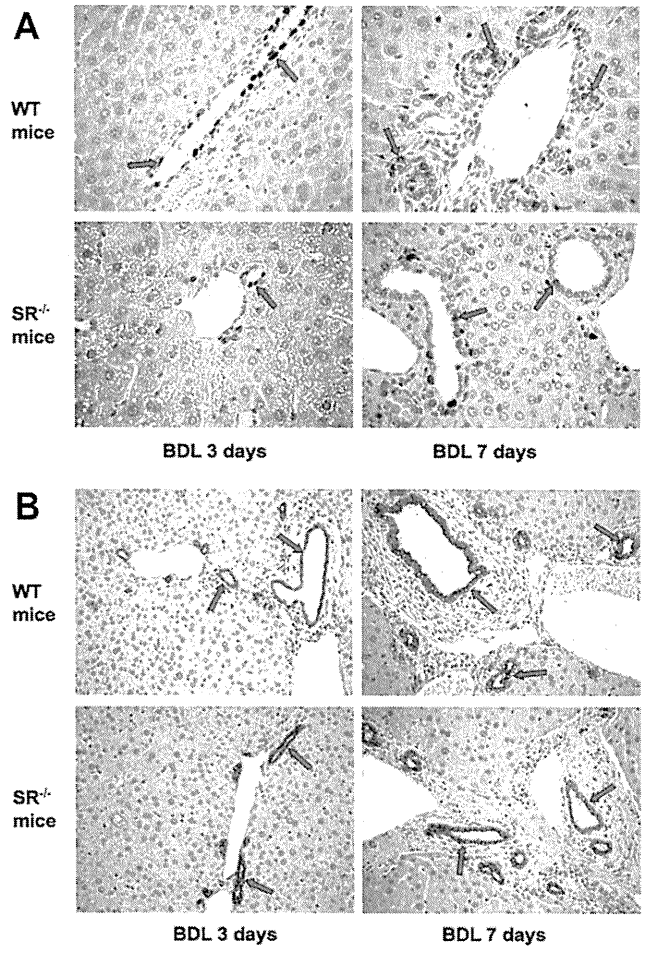

Fig. 3. Evaluation of the number of (A) large PCNA-positive cholangiocytes and (B) large IBDM in WT and $SR^{-/-}$ mice with BDL for 3 and 7 days. Original magnification ×40 (A) and ×20 (B).

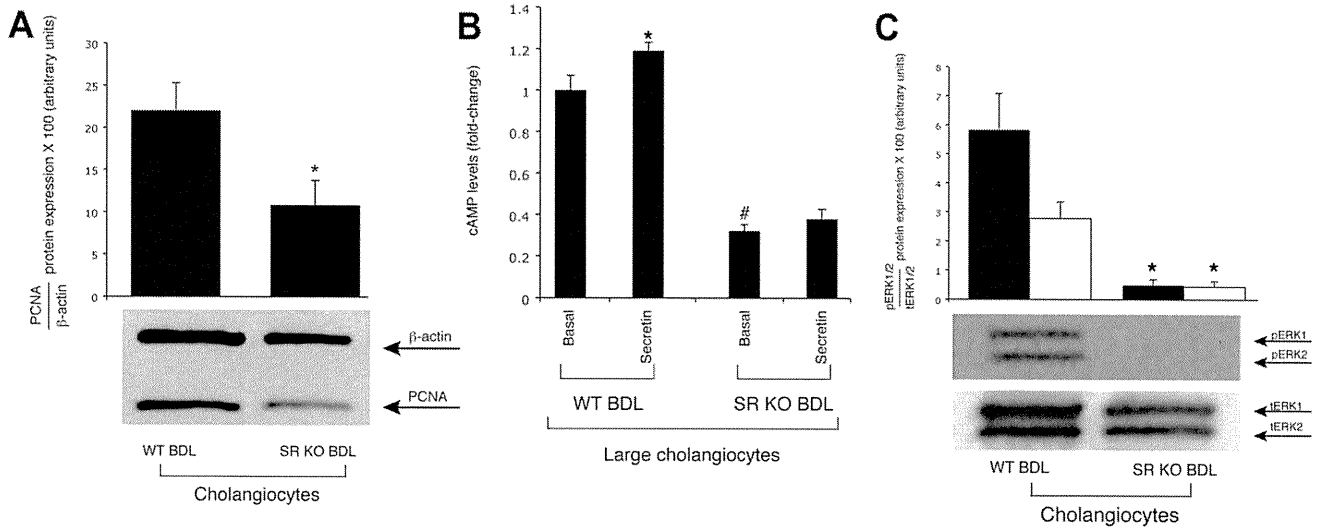

Fig. 4. Evaluation of (A) PCNA protein expression, (B) basal and secretin-stimulated cAMP levels, and (C) ERK1/2 phosphorylation in large cholangiocytes from WT and $SR^{-/-}$ 7-day BDL mice. (A) Data are expressed as the mean ± SEM of seven experiments. *$P < 0.05$ versus PCNA protein of large cholangiocytes from WT 7-day BDL mice. (B) Data are expressed as the mean ± SEM of seven experiments. *$P < 0.05$ versus 0.05 versus basal cAMP levels of large cholangiocytes from WT 7-day BDL mice. (C) Data are expressed as the mean ± SEM of seven experiments. *$P < 0.05$ versus 0.05 versus ERK1/2 phosphorylation of large cholangiocytes from WT 7-day BDL mice.

the corresponding levels of cholangiocytes from WT BDL mice (Fig. 4B). Secretin increased cAMP levels of large cholangiocytes from WT (but not $SR^{-/-}$) BDL mice (Fig. 4B). In large cholangiocytes from $SR^{-/-}$ BDL mice, there was a decreased ERK1/2 phosphorylation compared with large cholangiocytes from WT BDL mice (Fig. 4C).

Secretin Stimulates In Vitro Large Cholangiocyte Proliferation. Large (but not small) cholangiocytes proliferate after the administration of secretin (Fig. 5A). Secretin-stimulation of large cholangiocyte proliferation was blocked by H89 and partially by the MEK inhibitor, PD98059 (Fig. 5A). Secretin increased PCNA expression of large cholangiocytes, an increase that was blocked by H89 and PD98059 (Fig. 5B). There was increased PKA activity (Fig. 5C) and ERK1/2 phosphorylation (Fig. 5D) in large cholangiocytes treated with secretin compared to BSA-treated cells.

Silencing of the Secretin Receptor Gene Decreases the Proliferative Capacity of Large Cholangiocytes. The knockdown of SR protein expression by 50%, as demonstrated by FACS (Fig. 6B), was confirmed by way of western blot analysis (Fig. 6A). When we knocked down the gene for SR in large cholangiocytes, secretin did not increase cAMP levels (Fig. 6C) and proliferation (Fig. 6D, 48 hours of incubation) in these cells compared with the increase shown in large mock-transfected cholangiocytes. In support of the hypothesis that SR is a key trophic regulator in the regulation of biliary growth, there was a decrease in the basal proliferative capacity (Fig. 7) of SR-silenced large cholangiocytes compared with large mock-transfected cholangiocytes.

Discussion

In our study, we show that SR is an important trophic regulator sustaining large cholangiocyte proliferation during extrahepatic cholestasis. In the $SR^{-/-}$ mouse model, we show that proliferation of large cholangiocytes[12,14] is reduced (≈50%) during BDL compared with BDL WT mice, concomitant with elevation of biliary apoptosis. The reduction of cholangiocyte hyperplasia was associated with a decrease in both basal and secretin-stimulated cAMP levels and phosphorylation of ERK1/2 in large cholangiocytes compared with BDL cholangiocytes. *In vitro*, secretin increased the proliferation of large cholangiocytes by activation of cAMP→PKA→ERK1/2 signaling. Silencing of the SR gene induces a decrease in the basal proliferative capacity of large cholangiocytes compared with large mock-transfected cholangiocytes.

In our evaluation of SR expression, we found a time-dependent increase in the expression of SR in large cholangiocytes during BDL compared with normal large cholangiocytes. This finding was consistent with previous studies showing that: (1) in the rodent liver SR is only expressed by large cholangiocytes,[1,4,5,9,12] (2) SR expression is up-regulated following BDL ligation in large cholangiocytes,[14,17] and (3) the extent of secretin effects on cholangiocyte

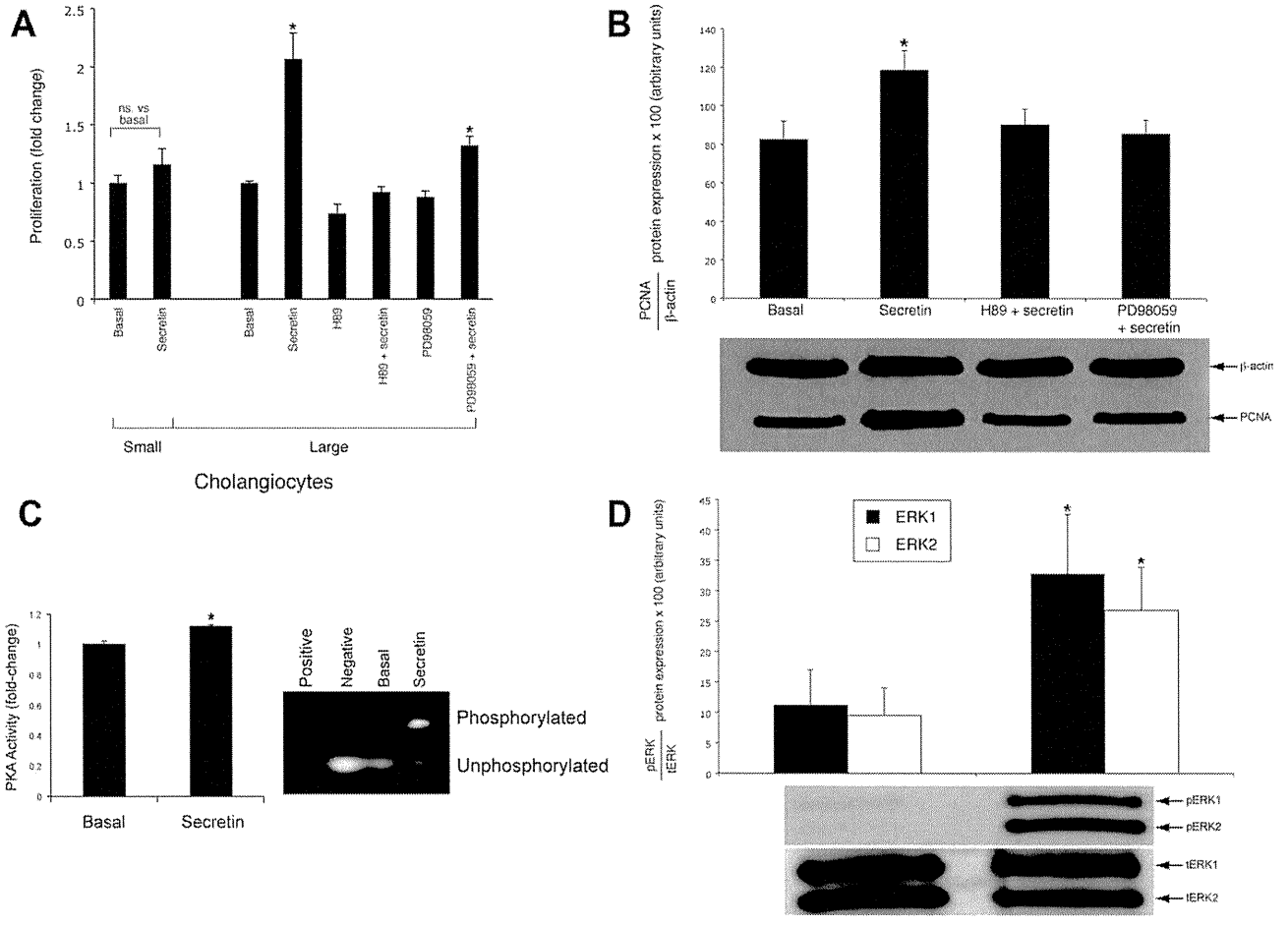

Fig. 5. (A) Effect of 0.2% BSA (basal) or secretin (100 nM) for 48 hours at 37°C on the proliferation of small and large cholangiocytes (MTS assay). Data are expressed as the mean ± SEM of 14 experiments. *$P < 0.05$ versus its corresponding basal value. (B) Data are expressed as the mean ± SEM of 14 experiments. *$P < 0.05$ versus its corresponding basal value. Secretin increased (C) PKA activity (n = 4) and (D) ERK1/2 phosphorylation (n =7) in large cholangiocytes compared with large cholangiocytes treated with BSA. *$P < 0.05$ versus its corresponding basal value.

functions parallel with the duration of BDL.[16] This finding parallels recent findings that mouse cholangiocytes share a similar heterogeneous profile as rat cholangiocytes[5] and freshly isolated and immortalized large mouse cholangiocytes are the only cell types to express the SR.[5,8,14] In human, SR expression is present in the biliary tract in normal bile ducts and ductules and the majority of cholangiocarcinomas, but is not present in hepatocytes or hepatocellular carcinoma.[26,27] Consistent with animal models of cholestasis, SR expression was up-regulated in ductular reactions in liver cirrhosis.[27]

In our *in vivo* model, the level of the reduction of cholangiocyte proliferation is consistent with the paradigm that cholangiocyte proliferation is regulated in autocrine and paracrine mechanisms by a number of stimulatory neurohormonal factors.[18,20,28] In a knockout mouse model for α-calcitonin gene-related peptide, the lack of circulating α-calcitonin gene-related peptide also reduces biliary proliferation during BDL to a similar degree as the lack of SR,[20] which indicates that the regulation of biliary proliferation during extrahepatic cholestasis is multifactorial and a complex regulatory system.[18,20,28]

The trophic effects of secretin were dependent upon the activation of the cAMP/PKA/ERK1/2 signaling. The second messenger system, cAMP, is a key factor for the function of large cholangiocytes.[1,4,7,9,13] Secretin stimulates bicarbonate secretion of large bile ducts through activation of cAMP-dependent CFTR→Cl^-/HCO_3^- anion exchanger 2.[1,4,7,9,13] Also, the activation of the cAMP/PKA/ERK1/2 pathway modulates cholangiocyte proliferation.[12,15,18,29] In fact, the direct stimulation of adenylyl cyclase activity by the chronic administration of forskolin stimulates normal cholangiocyte proliferation both *in vivo* and *in vitro*, which is associated with activation of the PKA/Src/MEK/ERK1/2 pathway.[29] Maintenance of cAMP levels by

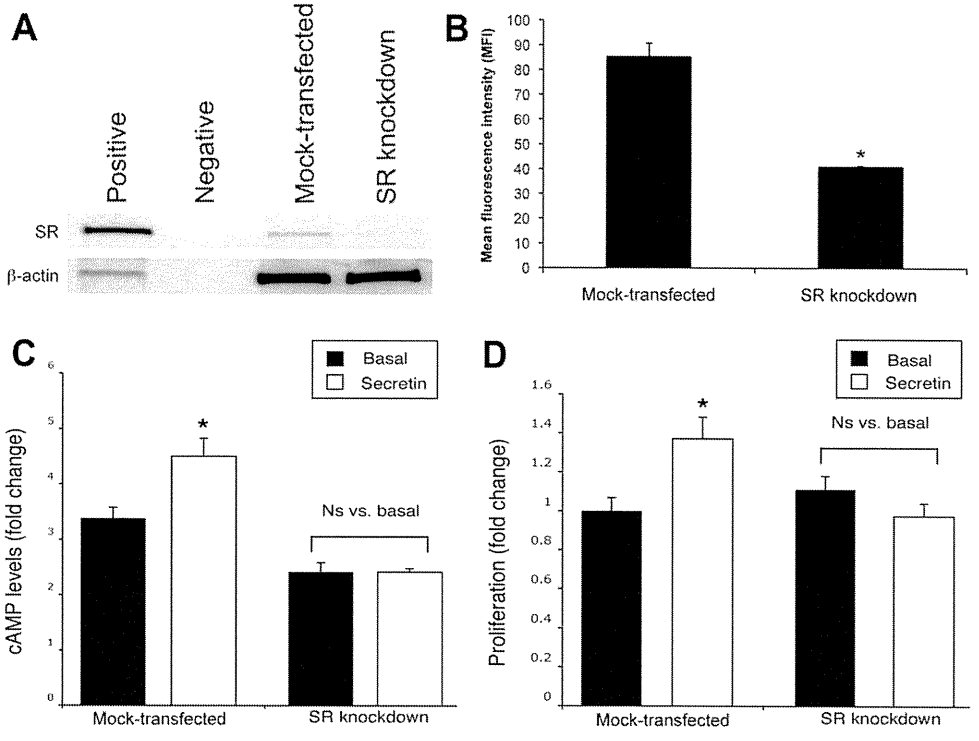

Fig. 6. Knockdown of secretin receptor protein expression in large cholangiocytes was evaluated by (A) western blotting and (B) FACS. Effect secretin receptor gene silencing on the effects of secretion on (C) cAMP levels, and (D) proliferation (by MTS assays) of large cholangiocytes. Data are expressed as the mean ± SEM of six experiments. *$P < 0.05$ versus its corresponding value of mock-transfected large cholangiocytes.

forskolin administration prevents the impairment of cholangiocyte proliferation and enhancement of biliary apoptosis induced by vagotomy.[30] Furthermore, Banales et al. have shown[31] that cAMP stimulates cholangiocyte proliferation through two downstream effectors (i.e., PKA and Epacs) in an animal model of autosomal recessive polycystic kidney disease. Down-regulation of cAMP levels and cAMP-dependent signaling reduces biliary growth and increases cholangiocyte damage by apoptosis.[12,14,20,30] The involvement of the cAMP-dependent ERK1/2 pathway in secretin-dependent biliary proliferation during cholestasis was

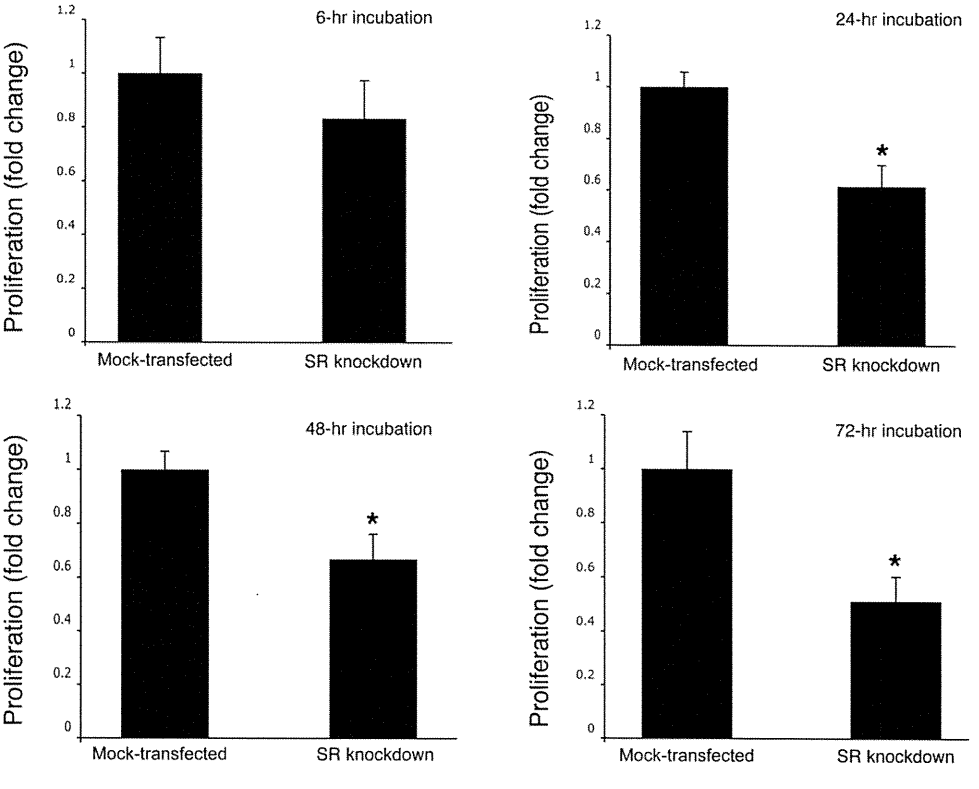

Fig. 7. Effect of secretin receptor gene silencing on the basal proliferative activity of large cholangiocytes following incubation for 6, 24, 48, and 72 hours with 0.2% BSA (MTS assay). Data are expressed as the mean ± SEM of four experiments. *$P < 0.05$ versus its corresponding value of mock-transfected large cholangiocytes.