The Functional Role of the Secretin/Secretin Receptor Signaling During Cholestatic Liver Injury

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ABBREVIATIONS: AC= adenylyl cyclase; **AE2** = Anion Exchanger 2; **AMA**= anti mitochondrial antibody; **ARE**= adenylate uridylate-rich element; **ASBT** =apical-sodium-BAs cotransporter ; **ATP** = Adenosine Triphosphate; **cAMP** = adenosine 3',5'-cyclic monophosphate; **BA**=bile acid; **BDL**= bile duct ligated; **CCA** = cholangiocarcinoma; **CF** = Cystic Fibrosis; **CFTR** = Cystic Fibrosis Transmembrane Conductance Regulator; **Cftr$^{-/-}$** = CF transmembrane conductance regulator knockout mice; **CNS** = Central Nervous System; **dnTGF-βRII**= dominant-negative transforming growth factor-β-receptor II; **ERCP** = Endoscopic Retrograde Cholangiopancreatography; **ERK =** Extracellular Receptor Kinase; **GPCR** = G-Protein Coupled Receptor; **IFN-γ**= interferon-γ; **IP3**= inositol triphosphate; **Mdr2$^{-/-}$** = Multidrug resistance 2 (Abcb4) gene knockout mice; **MEK =** mitogen-activated protein kinase kinase; **miRNA** = microRNA; **MRCP** = Magnetic Resonance Cholangiopancreatography; **NASH=** Non Alcoholic Steato hepatitis; **NGF**= nerve growth factor; **PBC** = Primary Biliary Cholangitis; **PKA** = Protein Kinase A; **PKC** = Protein Kinase C; **PR**=purinergic receptor; **PSC** = Primary Sclerosing Cholangitis; **SR** = Secretin Receptor; **Sct**=secretin; **SSTR2** = Somatostatin Receptor 2; **TC**=Taurocholic acid; **TGF-β1**= Transforming Growth Factor-β1; **TLC**= taurolithocholic acid; **TMEM16A** = Transmembrane Member 16A; **VEGF-A** =vascular endothelial growth factor-A .

ABSTRACT

The gastrointestinal peptide, secretin (Sct) is an important homeostatic regulator of pancreatic and liver secretory function. With regard to the liver, discoveries have been made, in the last decades, indicating a key role for the secretin/secretin receptor axis during normal or cholestatic conditions. Since large cholangiocytes are the only cells to express secretin receptor in the liver, research on secretin also expanded our knowledge on biliary epithelia. In this review we examined in detail the role of the secretin/secretin receptor axis, not only on biliary secretion, but also on cholangiocyte proliferation and senescence, as well as in prompting fibrotic processes involving biliary epithelia. Relevant data on human chronic cholestatic liver diseases, such as primary biliary cholangitis or primary sclerosing cholangitis, and obtained in animal models mimicking the diseases or in correlative studies on human are also reported. The aim of this review is to provide an update on the progress regarding the interactions between secretin and the biliary epithelia in normal and pathological conditions, underlining the aspects that suggests modulation of secretin pathway as a possible therapeutic approach for chronic cholestatic human liver disease.

INTRODUCTION

Liver diseases are a major health concern and affect a large proportion of people worldwide. There are over 100 types of liver disorders, including cirrhosis, cholangiocarcinoma (CCA), hepatocellular carcinoma and hepatitis. Despite the relevant number of people that are affected by liver diseases, and the increased awareness with regard to these disorders, the number of deaths corresponding to liver injury is expected to increase in the foreseeable future. One of the possible reasons for this is that a complete comprehension of the mechanisms of hepatic damage involving specific liver anatomical districts is lacking, and, as a consequence, current treatments available are suboptimal. A major burden in the clinical setting are chronic cholestatic liver diseases (e.g., primary biliary cholangitis (PBC), primary sclerosing cholangitis (PSC) and biliary atresia), which target the biliary epithelium and are characterized by cholestasis (1, 2). Since the secretin/secretin receptor (Sct/SR) axis (expressed only by cholangiocytes in the liver) (3, 4) is the major regulator of ductal bile secretion (5, 6), it is intuitive that this axis plays a key role in the maintenance of biliary homeostasis during the progression of cholangiopathies. For instance, PBC is characterized by reduced bicarbonate secretion, a phenomenon possibly impeding the formation of an HCO_3^- canalicular film ("bicarbonate umbrella") on bile ducts, which has protective properties against highly concentrated bile acids (BAs) (1, 7, 8). In this review, we examined the molecular mechanisms by which the Sct/SR axis regulates biliary function and the homeostasis of the biliary epithelium in normal and pathophysiological conditions.

The Sct/SR Axis: The Basics

Sct, a neuroendocrine, gastrointestinal peptide of twenty-seven amino acids is mainly secreted by the intestinal S cells located in the Lieberkühn crypts. There is evidence that Sct is also secreted by other epithelia in different organs including pancreas, intestine and liver thus exerting various biological effects in many systems (9). Important roles for Sct have been demonstrated with regard to regulation of fluid homeostasis (10). Stimulation of biliary secretion is due to Sct binding to the basolateral SR expressed only by cholangiocytes (3). SR is a G protein-coupled receptor (GPCR) class B1 which contains seven membrane-spanning domains, the members of GPCR class B1 family characteristically activate their signal transduction pathway through increased adenosine 3',5'-cyclic

monophosphate (cAMP) intracellular levels (11, 12). Also, Sct stimulates ductal secretory activity by activation of the Ca^{2+}-dependent adenylyl cyclase isoforms, AC5, AC6, AC9, the Ca^{2+}/calmodulin-stimulated AC8, and the soluble sAC that are expressed in large cholangiocytes (13). Cholestatic liver diseases represent an important chapter of clinical hepatology. In this perspective, studies were undertaken during the last 50 years to identify the features of cholangiocytes and the molecular mechanisms of bile secretion by these cells in normal and cholestatic conditions.

Role of the Sct/SR Axis in the Modulation of Ductal Bile Secretion in Normal Conditions

The mechanism linking Sct to ductal secretion is the following: Sct binds to SR on large cholangiocytes increasing intracellular cAMP levels, which in turn stimulates phosphorylation of protein kinase A (PKA), opening of cystic fibrosis transmembrane conductance regulator (CFTR) and activation of the apically located Cl^-/HCO_3^- exchanger AE2, leading to enhanced secretion of bicarbonate-rich choleresis (3, 5, 14). Further research clarified that Sct stimulates water and bicarbonate secretion only in large cholangiocytes lining large bile ducts, but not small cholangiocytes lining small ducts (3, 15). This finding demonstrates that the dimensional heterogeneity of the biliary epithelium is linked to functional differences, since only large cholangiocytes express SR, CFTR and AE2 (3, 16). Moreover, these cells are reactive to other gastrointestinal peptides/neuropeptides and neurotransmitters such as somatostatin, gastrin and endothelin-1 (6). Among non-hormonal regulators of biliary secretion, bile acids (BAs) need to be underscored. A number of studies show that SR, cAMP levels and AE2 were all increased in large cholangiocytes exposed to physiologic amount of taurocholic (TC) or taurolithocholic (TLC) acids (17). The important interplay between the Sct/SR axis and BAs, in supporting hepato-biliary secretion, was further confirmed by the individuation of a specific apical-sodium-BA cotransporter (ASBT) in cholangiocytes (17). ASBT is responsible for partial reuptake of BAs from bile, allowing their recirculation in blood in a short-circuit within the liver, thus increasing hepatocyte BA-dependent bile flow (18). Interestingly, Sct was demonstrated to stimulate migration of ASBT from the cytoplasmic to the canalicular membrane of cholangiocytes and to increase BA uptake from cholangiocytes, thus enhancing cholehepatic shunting (19). Taken together, it is intuitive that BA-dependent hepatocyte bile flow and BA-independent (regulated by the Sct/SR axis) cholangiocyte secretion, are integrated in

a complex interplay. With regard to small cholangiocytes, even if these cells lack SR, CFTR and AE2, an alternative mechanism to secrete water and electrolytes has been identified involving the activation of Ca^{2+}-dependent pathways (20). Small mouse cholangiocytes have been shown to release adenosine triphosphate (ATP) and to produce purinergic (PR) receptor-mediated bile secretion (21). This suggests the presence of an alternative, compensatory pathway in small cholangiocytes to maintain biliary homeostasis when larger bile ducts are damaged (22, 23). However, when large cholangiocytes are injured, such as after carbon tetrachloride (CCl_4) treatment, small, mitotically dormant cholangiocytes proliferate, thus replacing and acquiring the phenotypic features of large cholangiocytes (23). Secretory pathways of large and small cholangiocytes are depicted in Figure 1.

Sct/SR Axis in Experimental Animal Models of Cholestasis

Cholestasis is a process exhibiting an early phase and intense "typical" cholangiocyte proliferation, possibly compensatory, followed by a second later stage represented by loss of bile ducts (ductopenia) and fibrosis, which is accompanied by an "atypical" proliferation (24). Accordingly, it seems important to examine the relationship between the Sct/SR axis and bile ducts with respect to these two different phases. In this section, the findings on the Sct/SR axis will be described respective to hyperplastic or ductopenic experimental settings.

- ***Sct/SR Axis in the Early Hyperplastic Phase of Cholestasis***

The bile duct ligated (BDL) rodent model represents an important tool to evaluate the molecular mechanisms occurring in the early hyperplastic phase of cholestasis, also reproducing "ductular reaction" (a histological finding corresponding to proliferating ductules) frequently observed in liver specimens of human cholestatic diseases (24). Pivotal studies employing this experimental system demonstrated that Sct-stimulated bile secretion is linearly related to the degree of bile duct hyperplasia, and cholangiocytes isolated from BDL rats expressed SR at a rate that was higher compared to normal cholangiocytes (4, 25). Upregulation of the Sct/SR axis corresponded to increased synthesis of intracellular cAMP that lead to increased activation of PKA, opening of CFTR channels and activation of the AE2 with subsequent enhanced biliary secretion (3, 5, 6, 14, 26). In this setting, biliary cAMP levels have been shown to play a central role in promoting choleretic signaling. In fact, down- or up-regulation of cAMP

signaling by several gastrointestinal hormones and neurotransmitters, such as somatostatin, gastrin, endothelin-1, the α2-adrenergic receptor agonists (as well as a number of BAs) proportionally changes ductal bicarbonate secretion (6). Moreover, during BDL-induced cholestasis, increased cAMP levels not only support choleresis, but also enhance the expansion of bile ducts (6, 15, 27, 28). Proliferation of bile ducts is correlated with cAMP-dependent activation of ERK 1/2 signaling; however, this mechanism is inhibited in BDL rodents lacking SR (28, 29). Further studies clarified that cAMP-mediated proliferation in this setting is sustained by both autocrine (by large cholangiocytes) and by paracrine (duodenal S cells) Sct secretion and is promoted by specific microRNAs (miR125b and let7a), that increase biliary mass and liver fibrosis by directly targeting vascular endothelial growth factor-A (VEGF-A) and nerve growth factor (NGF), respectively (27). Changes in large cholangiocyte phenotypes after BDL are depicted in Figure 2.

- ***Sct/SR Axis in Ductopenic Models of Cholestasis***

Chronic cholestasis, in both humans and rodent models, is characterized by a ductopenic stage, with reduced biliary mass and progression to liver fibrosis. A transient ductopenic cholestatic model was induced in animals using toxins, such as CCl_4 (23). CCl_4 treatment induced selective damage and loss of Sct-stimulated secretory activity in large (but not small) cholangiocytes (23). Interestingly, the consequential loss of secretive and proliferative activities in large cholangiocytes was then replaced by the expansion of small cholangiocytes, which *de novo* acquired the phenotypes of large cells (23). The relocation of Sct/SR axis from damaged (large) to non-damaged (small) cholangiocytes underscores the efforts of the biliary epithelium to maintain a pathway of paramount importance during injury. We suggest. That small cholangiocytes typically display traits of committed progenitor cells and may act as a biliary-committed progenitor niche that expands and differentiates to large cholangiocytes during cholestatic injuries in an effort to maintain secretive capacity and biliary homeostasis (24).

- ***Sct/SR Axis in Animal Models Resembling Adult Human Chronic Cholestatic Liver Diseases***

PBC is an autoimmune disease that primarily affects middle-aged women, and is characterized by anti-

mitochondrial antibodies (AMA) positivity in the blood and potentially evolves toward extended liver fibrosis and cirrhosis (30). The target of human PBC are cholangiocytes lining the interlobular bile ducts (i.e. small ducts), with consequential necrosis, apoptosis and progressive cholestasis (31). PBC, at the ultra-structural level, displays non-suppurative cholangitis with portal inflammatory infiltrates composed by autoreactive T cells surrounding bile ducts (31).

a) Sct/SR axis in PBC Animal Models

The lack of knowledge and effective medical treatments for PBC is also related to existence of adequate animal models. Lately, several systems based on genetically modified mice have been proposed. One of these models is represented by the dominant-negative transforming growth factor-β-receptor II (dnTGF-βRII) mouse, which exhibits AMA positivity, coupled with biliary inflammation and injury of small bile ducts (32). The Sct/SR axis was recently examined in an early stage PBC mouse model (dnTGF-βRII, 12 wk) to establish the role of this pathway in biliary injury, liver inflammation and fibrosis (7). This study demonstrated increased stimulation of Sct/SR signaling in this mouse model. Inhibition of SR signaling by the SR antagonist Sec5–27 decreased biliary senescence, liver inflammation and fibrosis. Interestingly, in this model, contrary to what has been canonically observed, stimulation of Sct/SR signaling did not correspond to increased bicarbonate biliary secretion. This phenomenon, in parallel with what is observed in human PBC possibly affects the formation of the protective bicarbonate umbrella (33) at the canalicular side of the biliary system, which is likely associated with miR-506-mediated CFTR/AE2 decreased expression. The importance of AE2 stimulation by the Sct/SR molecular cascade of signals has been supported by observations in Ae2a,b$^{-/-}$ mice with disruption of the AE2 gene (34). This rodent model exhibits inflammatory liver damage and biochemical signs of progressive cholestasis and cytolysis coupled with AMA positivity, thus representing another suggested animal model to study PBC for its similarity with the corresponding human disease (34). Other rodent models of PBC have been described, such as IL-2Rα$^{-/-}$ mice which is a genetically modified strain that demonstrated 100% AMA positivity, as well as mild bile duct injury, interface hepatitis and severe colitis, though the latter not typically observed in PBC patients (35). Another interesting model has been developed in mice by inducing chronic expression of interferon-γ (IFN-γ) by deletion of 3'-untranslated region adenylate uridylate-rich element (ARE) within the IFN-γ gene (36). This strain exhibits: i) 100% AMA

positivity; ii) increased levels of BAs, iii) female predominance and iv) not only portal and lobular inflammation, but also granulomas (that are a specific feature of human PBC) at histological examination; however, changes of Sct/SR axis as a function of cholestatic liver damage has not been evaluated.

a) Sct/SR Axis in PSC Animal Models

With regard to PSC, the absence of diagnostic markers for the early stages of the disease, low prevalence and difficult accessibility of the biliary tract in human patients stimulated the search for animal models to study this disease (37). Multidrug resistance 2 (Mdr2, i.e. Abcb4) gene knockout mice ($Mdr2^{-/-}$) are one system that is currently being used to study PSC. Ablation of this gene removes phospholipid secretion in bile, in turn counteracting the formation of mixed micelles in cooperation with BAs, a process that mitigates the cytopathic-detergent property of BAs . Since these mice mimic several key features of human PSC (including the development of cholangitis, irregular bile duct formation and liver fibrosis), they are generally considered a suitable tool to study this disease (38). Moreover, pictures resembling high similarities with PSC have been described in patients with defects of the MDR3 gene; the homolog of mouse Mdr2 (39). Using this model, a possible important role of the Sct/SR axis in liver fibrotic processes occurring in PSC, was proposed (40). In fact, cholangiocytes isolated from $Mdr2^{-/-}$ mice demonstrated a basal increase in Sct and SR gene expression that, in turn, was associated with the activation of transforming growth factor-β1/transforming growth factor-β1 receptor (TGF-β1/TGF-β1R) axis and SMAD2/3 phosphorylation; the latter pathway is well known for promoting liver fibrosis via hepatic stellate cell activation (41). Moreover, a direct link between SR signaling and TGF-β1 stimulation was achieved when the specific Sct inhibitor Sec 5–27 was administered to mice. In fact, suppression of the Sct/SR axis in $Mdr2^{-/-}$ mice was associated with reduced activity of TGF-β1R/SMAD2/3 pathway and a subsequent decrease of liver collagen deposition (41). These findings were then extended in the same $Mdr2^{-/-}$ mouse model that were crossed with mice containing SR gene disruption (42). The model of $Mdr2^{-/-}/SR^{-/-}$ mouse confirmed the contribution of Sct/SR axis in supporting liver fibrosis since evaluation of collagen deposit in liver tissues was markedly decreased in $Mdr2^{-/-}$ mice with disruption of SR gene. However, this model expanded these findings by demonstrating that cellular senescence (arrest of cell cycle under stress conditions) and neo-angiogenesis that are enhanced, together with fibrosis, in

Mdr2$^{-/-}$ mice were actually normalized by suppression of SR gene in the same animals. On the basis of these findings, a possible mechanism starting from Sct and involving: i) SR; ii) neo-angiogenetic factors and iii) cellular senescence to determine liver fibrosis in experimental PSC, was suggested for the first time.

SCT/SR AXIS IN ADULT HUMAN CHOLESTATIC CHRONIC LIVER DISEASES

In the following subparagraphs, the findings regarding Sct/SR axis in human PBC and PSC experimental studies are reported.

- ***Data on Sct/SR Axis in Human PBC***

The first evidences of Sct/SR pathway malfunction in PBC came from a number of studies that sequentially demonstrated reduced mRNA levels and decreased immune-reactivity for AE2 in the liver that associated with mild Sct-stimulated bicarbonate secretion in PBC patients (43). On the basis of these findings, PBC damage in humans was hypothesized to be associated with a defect in the formation of bicarbonate protective film (i.e. bicarbonate umbrella) on the canalicular side. This layer is thought to be important in the prevention of cellular damage induced by detergent BAs. In order to better clarify the mechanism of AE2 scarce response to Sct during PBC, the possible role of microRNAs (miRs) was investigated. Since miR-506 was predicted, on the basis of the available datasets, to possibly target AE2, a study focused on the influence of this miR on AE2 was undertaken (44). In fact, miR-506 was overexpressed (>3 fold increase) in PBC livers in comparison with normal controls. The possible effects of miR-506 on AE2 was then examined in isolated bile duct cells (immortalized H69 line, and primary PBC and normal cultures). These experiments showed that miR-506 directly inhibited AE2 expression and its response to Sct. A recent study specifically examined the Sct/SR axis in PBC early stage liver tissue (7). Similar to what has been observed in dnTGF-βRII mice, expression of Sct and SR were augmented in human specimens. Despite this, bicarbonate biliary secretion was reduced, supporting the view of the down-regulation of AE2 in this disease. However, this study also found that the Sct/SR axis promotes scar tissue deposition, upregulating the TGF-β1/TGF-β1R pathway. In fact, both the expression of TGF-β1 and its receptor were enhanced in the liver of early stage PBC patients. Based on data in the human samples and dnTGF-βRII mouse, it was hypothesized that liver damage during early stage PBC, was significantly related to the upregulation of Sct/SR axis (potentially compensatory) that in turn determined an increased in ductular reaction, cellular senescence and fibrosis. Decreased AE2 expression and

bicarbonate secretion may be occurring through secondary mechanisms in this setting. In this perspective, possible inhibitors of the Sct/SR axis might be considered beneficial for the treatment of human PBC.

- ***Data on Sct/SR Axis in Human PSC***

Data on the Sct/SR axis in patients with PSC are insufficient. A correlative evaluation was carried out with human samples in a study mainly involving Mdr2$^{-/-}$ mice (40). Similar to what is reported in early stage PBC, increased expression of Sct, SR, TGF-β1 and its receptor was seen in PSC liver tissue. However, these findings were extended to evaluate let-7a, which is known to be an important regulator of NGF (45). In fact, downregulation of let-7 increases secretion of NGF and neuronal tissue regeneration. In tune with this observation, increased Sct/SR expression was associated with reduce let-7a activity and subsequently increased NGF expression in human PSC. The consequential increase in NGF justifies the further onset of proliferation characterized by ductular reaction and evolution toward fibrosis. Again, as has been observed in early stage PBC, upregulation of the Sct/SR axis in PSC may exert negative effects by promoting scar tissue deposition and evolution to end-stage liver disease.

SCT/SR AXIS IN OTHER HUMAN DISEASES TARGETING THE BILIARY TREE

Few data are available with regard to Sct/SR axis and human biliary diseases other than PBC and PSC. Biliary atresia is a pediatric disorder affecting ~6 out of 100,000 newborns in the western world. Being the most important cause of severe cholestatic liver disease in children, biliary atresia is a progressive obliterative cholangiopathy, mainly involving large ducts. Therapy for biliary atresia is based on surgical approaches either with liver transplantation or Kasai procedure. The latter is based on the complete removal of the extra-hepatic portion of the biliary tract and the direct anastomosis of the gut at the base of the liver, where the smaller (less or not affected) bile ducts converge. Given the important choleretic effect of Sct, treatment with this hormone was hypothesized in the 80s (in conjunction with the surgical treatment) as a potential therapeutic for biliary atresia (46). Lately, a possible impairment of the digestive hormone system in this disease (involving Sct and cholecystokinin excretion), was suggested in a case report (46). A fetus showing a gallbladder full of sludge at 34 wk gestation (possibly

evolving toward biliary atresia) was treated, shortly after the induction of a premature birth, with Sct and a cholecystokinin analogue (46). A short course of this treatment normalized bile flow and dissolved the sludge, thus preventing the possible onset of a chronic cholestatic disease in this child. Based on this observation, the authors suggested dysregulation of choleretic hormones as a possible factor inducing biliary atresia. Several attempts, have been made to develop animal models mirroring the features of human biliary atresia; however, despite the efforts, an adequate animal system to evaluate the pathogenesis of this pediatric disease and to test therapeutic strategies is not available so far.

Finally, another important human liver disease affecting the biliary tract is CCA. Differently from other liver primary tumors, such as hepatocellular carcinoma, SR is expressed in human CCA (47). To clarify the possible role of Sct/SR axis in CCA, a study was undertaken on CCA cell lines (mainly Mz-ChA-1) and liver tissue (48). Unlike normal cholangiocytes, Sct inhibited CCA cells growth in culture, which was related to a failure in inducing an increase of the intracellular levels of cAMP as observed in normal cholangiocytes. The inhibitory effect of Sct on CCA growth was then confirmed in an *in vivo* model of BALB/c nude mice injected with Mz-ChA-1 cells. After 57 days, these mice when treated with Sct (I.P. injection 25 μg/kg/BW every three days) had CCA tumors three times smaller in volume when compared to control. The peculiar inhibitory effect of Sct on tumor growth was attributed to an erroneous coupling of SR with the G-protein $G\alpha_i$ (inhibitory) instead of the normal coupling with $G\alpha_s$. This study provided the first evidence of the possible role of Sct/SR system in modulating CCA growth. Results of main studies evaluating Sct/SR axis in specific human diseases of the biliary tract are shown in Table 1.

SUMMARY AND FUTURE. PERSPECTIVES

As reported in this review, Sct is a gastrointestinal hormone that has a multitude of effects on both healthy or injured biliary epithelia. While the attention of liver researchers was, at the beginning, captured by the important Sct effects on BA-independent ductal secretion, our knowledge has extended to recognizing a role for this hormone in regulating proliferation, cellular senescence and fibrosis of the hepatic biliary compartment. These effects are obtained by the wide interaction of the Sct/SR pathway with growth factors, miRs, BAs, cellular transporters and other biological components. Animal models of cholestasis (or cholangiocyte damage) remain of paramount

importance to further clarify molecular features of this process; however, additional efforts should be undertaken in the future to translate animal findings to human pathological conditions. Discovery and adoption of more appropriate animal models of human diseases and/or confirmation of animal data on human samples represent a promising strategy to achieve new therapeutic tools. Finally, in depth study of Sct-related biological effects would be relevant also for other important non-biliary liver diseases. In fact: i) the evidence of a cooperative role of Sct and scavenger receptor CD36 in lipid absorption and; ii) the finding of reduced fatty tissue deposition in Sct knock-out mice (49)**,** suggest manipulation of Sct/SR pathway to mitigate liver diseases characterized by increased fatty deposition.

References

1. van Niekerk J, Kersten R, Beuers U. Role of Bile Acids and the Biliary $HCO_3(^-)$ Umbrella in the Pathogenesis of Primary Biliary Cholangitis. Clin Liver Dis 2018;22:457-479.

2. Feldman AG, Sokol RJ. Neonatal cholestasis: emerging molecular diagnostics and potential novel therapeutics. Nat Rev Gastroenterol Hepatol 2019;16:346-360.

3. Alpini G, Roberts S, Kuntz SM, Ueno Y, Gubba S, Podila PV, LeSage G, et al. Morphological, molecular, and functional heterogeneity of cholangiocytes from normal rat liver. Gastroenterology 1996;110:1636-1643.

4. Alpini G, Ulrich CD, 2nd, Phillips JO, Pham LD, Miller LJ, LaRusso NF. Upregulation of secretin receptor gene expression in rat cholangiocytes after bile duct ligation. Am J Physiol Gastrointest Liver Physiol 1994;266:G922-928.

5. Alpini G, Lenzi R, Sarkozi L, Tavoloni N. Biliary physiology in rats with bile ductular cell hyperplasia. Evidence for a secretory function of proliferated bile ductules. J Clin Invest 1988;81:569-578.

6. Kanno N, LeSage G, Glaser S, Alpini G. Regulation of cholangiocyte bicarbonate secretion. Am J Physiol Gastrointest Liver Physiol 2001;281:G612-625.

7. Kennedy L, Francis H, Invernizzi P, Venter J, Wu N, Carbone M, Gershwin ME, et al. Secretin/secretin receptor signaling mediates biliary damage and liver fibrosis in early-stage primary biliary cholangitis. Faseb j 2019;33:10269-10279.

8. Sasaki M, Sato Y, Nakanuma Y. An impaired biliary bicarbonate umbrella may be involved in dysregulated autophagy in primary biliary cholangitis. Lab Invest 2018;98:745-754.

9. Hacki WH. Secretin. Clin Gastroenterol 1980;9:609-632.

10. Chu JY, Lee LT, Lai CH, Vaudry H, Chan YS, Yung WH, Chow BK. Secretin as a neurohypophysial factor regulating body water homeostasis. Proc Natl Acad Sci U S A 2009;106:15961-15966.

11. Siu FK, Lam IP, Chu JY, Chow BK. Signaling mechanisms of secretin receptor. Regul Pept 2006;137:95-104.

12. Kato A, Gores GJ, LaRusso NF. Secretin stimulates exocytosis in isolated bile duct epithelial cells by a cyclic AMP-mediated mechanism. J Biol Chem 1992;267:15523-15529.

13. Strazzabosco M, Fiorotto R, Melero S, Glaser S, Francis H, Spirli C, Alpini G. Differentially expressed adenylyl cyclase isoforms mediate secretory functions in cholangiocyte subpopulation. Hepatology 2009;50:244-252.

14. Alpini G, Ulrich C, Roberts S, Phillips JO, Ueno Y, Podila PV, Colegio O, et al. Molecular and functional heterogeneity of cholangiocytes from rat liver after bile duct ligation. Am J Physiol Gastrointest Liver Physiol 1997;272:G289-297.

15. Alpini G, Glaser S, Ueno Y, Pham L, Podila PV, Caligiuri A, LeSage G, et al. Heterogeneity of the proliferative capacity of rat cholangiocytes after bile duct ligation. Am J Physiol Gastrointest Liver Physiol 1998;274:G767-775.

16. Alpini G, Glaser S, Robertson W, Rodgers RE, Phinizy JL, Lasater J, LeSage G. Large but not small intrahepatic bile ducts are involved in secretin-regulated ductal bile secretion. Am J Physiol Gastrointest Liver Physiol 1997;272:G1064-1074.

17. Alpini G, Glaser S, Ueno Y, Rodgers R, Phinizy JL, Francis H, Baiocchi L, et al. Bile acid feeding induces cholangiocyte proliferation and secretion: evidence for bile acid-regulated ductal secretion. Gastroenterology 1999;116:179-186.

18. Xia X, Francis H, Glaser S, Alpini G, LeSage G. Bile acid interactions with cholangiocytes. World J Gastroenterol 2006;12:3553-3563.

19. Alpini G, Glaser S, Baiocchi L, Francis H, Xia X, LeSage G. Secretin activation of the apical Na+-dependent bile acid transporter is associated with cholehepatic shunting in rats. Hepatology 2005;41:1037-1045.

20. Dutta AK, Khimji AK, Kresge C, Bugde A, Dougherty M, Esser V, Ueno Y, et al. Identification and functional characterization of TMEM16A, a Ca2+-activated Cl- channel activated by extracellular nucleotides, in biliary epithelium. J Biol Chem 2011;286:766-776.

21. Woo K, Sathe M, Kresge C, Esser V, Ueno Y, Venter J, Glaser SS, et al. Adenosine triphosphate release and purinergic (P2) receptor-mediated secretion in small and large mouse cholangiocytes. Hepatology 2010;52:1819-1828.

22. Pedemonte N, Galietta LJ. Structure and function of TMEM16 proteins (anoctamins). Physiol Rev 2014;94:419-459.

23. LeSage G, Glaser S, Marucci L, Benedetti A, Phinizy JL, Rodgers R, Caligiuri A, et al. Acute carbon tetrachloride feeding induces damage of large but not small cholangiocytes from BDL rat liver. Am J Physiol Gastrointest Liver Physiol 1999;276:G1289-1301.

24. Sato K, Marzioni M, Meng F, Francis H, Glaser S, Alpini G. Ductular Reaction in Liver Diseases: Pathological Mechanisms and Translational Significances. Hepatology 2019;69:420-430.

25. Alpini G, Lenzi R, Zhai WR, Slott PA, Liu MH, Sarkozi L, Tavoloni N. Bile secretory function of intrahepatic biliary epithelium in the rat. Am J Physiol Gastrointest Liver Physiol 1989;257:G124-133.

26. Francis H, Glaser S, Ueno Y, LeSage G, Marucci L, Benedetti A, Taffetani S, et al. cAMP stimulates the secretory and proliferative capacity of the rat intrahepatic biliary epithelium through changes in the PKA/Src/MEK/ERK1/2 pathway. J Hepatol 2004;41:528-537.

27. Glaser S, Meng F, Han Y, Onori P, Chow BK, Francis H, Venter J, et al. Secretin stimulates biliary cell

proliferation by regulating expression of microRNA 125b and microRNA let7a in mice. Gastroenterology 2014;146:1795-1808.e1712.

28. Glaser S, Lam IP, Franchitto A, Gaudio E, Onori P, Chow BK, Wise C, et al. Knockout of secretin receptor reduces large cholangiocyte hyperplasia in mice with extrahepatic cholestasis induced by bile duct ligation. Hepatology 2010;52:204-214.

29. Alvaro D, Onori P, Metalli VD, Svegliati-Baroni G, Folli F, Franchitto A, Alpini G, et al. Intracellular pathways mediating estrogen-induced cholangiocyte proliferation in the rat. Hepatology 2002;36:297-304.

30. Hohenester S, Oude-Elferink RP, Beuers U. Primary biliary cirrhosis. Semin Immunopathol 2009;31:283-307.

31. Poupon R. Primary biliary cirrhosis: a 2010 update. J Hepatol 2010;52:745-758.

32. Tsuda M, Zhang W, Yang GX, Tsuneyama K, Ando Y, Kawata K, Park O, et al. Deletion of interleukin (IL)-12p35 induces liver fibrosis in dominant-negative TGFbeta receptor type II mice. Hepatology 2013;57:806-816.

33. Beuers U, Hohenester S, de Buy Wenniger LJ, Kremer AE, Jansen PL, Elferink RP. The biliary HCO(3)(-) umbrella: a unifying hypothesis on pathogenetic and therapeutic aspects of fibrosing cholangiopathies. Hepatology 2010;52:1489-1496.

34. Salas JT, Banales JM, Sarvide S, Recalde S, Ferrer A, Uriarte I, Oude Elferink RP, et al. Ae2a,b-deficient mice develop antimitochondrial antibodies and other features resembling primary biliary cirrhosis. Gastroenterology 2008;134:1482-1493.

35. Hsu W, Zhang W, Tsuneyama K, Moritoki Y, Ridgway WM, Ansari AA, Coppel RL, et al. Differential mechanisms in the pathogenesis of autoimmune cholangitis versus inflammatory bowel disease in interleukin-2Ralpha(-/-) mice. Hepatology 2009;49:133-140.

36. Bae HR, Leung PS, Tsuneyama K, Valencia JC, Hodge DL, Kim S, Back T, et al. Chronic expression of

interferon-gamma leads to murine autoimmune cholangitis with a female predominance. Hepatology 2016;64:1189-1201.

37. Fickert P, Pollheimer MJ, Beuers U, Lackner C, Hirschfield G, Housset C, Keitel V, et al. Characterization of animal models for primary sclerosing cholangitis (PSC). J Hepatol 2014;60:1290-1303.

38. Fickert P, Fuchsbichler A, Wagner M, Zollner G, Kaser A, Tilg H, Krause R, et al. Regurgitation of bile acids from leaky bile ducts causes sclerosing cholangitis in Mdr2 (Abcb4) knockout mice. Gastroenterology 2004;127:261-274.

39. Benzimra J, Derhy S, Rosmorduc O, Menu Y, Poupon R, Arrive L. Hepatobiliary anomalies associated with ABCB4/MDR3 deficiency in adults: a pictorial essay. Insights Imaging 2013;4:331-338.

40. Wu N, Meng F, Invernizzi P, Bernuzzi F, Venter J, Standeford H, Onori P, et al. The secretin/secretin receptor axis modulates liver fibrosis through changes in transforming growth factor-β1 biliary secretion in mice. Hepatology 2016;64:865-879.

41. Hu HH, Chen DQ, Wang YN, Feng YL, Cao G, Vaziri ND, Zhao YY. New insights into TGF-β/Smad signaling in tissue fibrosis. Chem Biol Interact 2018;292:76-83.

42. Zhou T, Wu N, Meng F, Venter J, Giang TK, Francis H, Kyritsi K, et al. Knockout of secretin receptor reduces biliary damage and liver fibrosis in Mdr2(-/-) mice by diminishing senescence of cholangiocytes. Lab Invest 2018;98:1449-1464.

43. Prieto J, García N, Martí-Climent JM, Peñuelas I, Richter JA, Medina JF. Assessment of biliary bicarbonate secretion in humans by positron emission tomography. Gastroenterology 1999;117:167-172.

44. Banales JM, Sáez E, Uriz M, Sarvide S, Urribarri AD, Splinter P, Tietz Bogert PS, et al. Up-regulation of microRNA 506 leads to decreased Cl^-/HCO_3^- anion exchanger 2 expression in biliary epithelium of patients with

primary biliary cirrhosis. Hepatology 2012;56:687-697.

45. Li S, Wang X, Gu Y, Chen C, Wang Y, Liu J, Hu W, et al. Let-7 microRNAs regenerate peripheral nerve regeneration by targeting nerve growth factor. Mol Ther 2015;23:423-433.

46. Isenberg JN, Schwartz MZ. Stimulation of bile output by gastrointestinal hormones following portoenterostomy for biliary atresia. J Pediatr Surg 1984;19:471-475.

47. Mavros MN, Economopoulos KP, Alexiou VG, Pawlik TM. Treatment and Prognosis for Patients With Intrahepatic Cholangiocarcinoma: Systematic Review and Meta-analysis. JAMA Surg 2014;149:565-574.

48. Onori P, Wise C, Gaudio E, Franchitto A, Francis H, Carpino G, Lee V, et al. Secretin inhibits cholangiocarcinoma growth via dysregulation of the cAMP-dependent signaling mechanisms of secretin receptor. Int J Cancer 2010;127:43-54.

49. Sekar R, Chow BK. Secretin receptor-knockout mice are resistant to high-fat diet-induced obesity and exhibit impaired intestinal lipid absorption. FASEB J 2014;28:3494-3505.

50. Michel E, Kilavuz O, Jager R, Nasir R. Biliary atresia due to delayed maturation of the gut hormones' system?--Introducing a new treatment modality. J Perinat Med 2004;32:288-292.

Figure legends

Figure 1: The differences between large and small cholangiocytes: i) in size; ii) in location within the biliary tree and iii) in the secretive pathway, are depicted. As reported in figure small cholangiocytes are able to migrate and replace the large ones when the latter are damaged. Symbols: ● = secretin. Abbreviations: **AE2** = Anion Exchanger 2; **ATP** = Adenosine Triphosphate; **cAMP** = adenosine 3',5'-cyclic monophosphate; **CFTR** = Cystic Fibrosis Transmembrane Conductance Regulator; **IP3** = Inositol trisphosphate; **PKA** = Protein Kinase A; **p-PKA** = phosphorylated-Protein Kinase A; **PR** = purine receptor; **SR**=secretin receptor; **TMEM 16A**= Transmembrane member 16A.

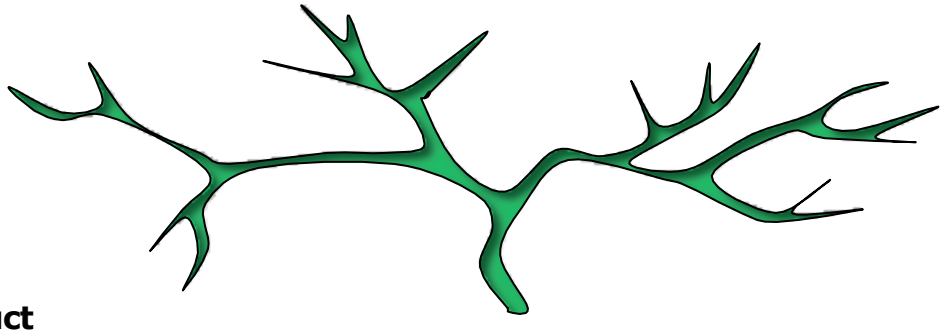
Figure 2: Changes occurring in rat large cholangiocytes by BDL-induced cholestasis. The up-regulation of secretin/SR axis enhances cAMP accumulation in the cell, stimulating the secretive and proliferative activities. Symbols: ● = secretin. Abbreviations: **AE2** = Anion Exchanger 2; **BDL =** bile duct ligated; **cAMP** = adenosine 3',5'-cyclic monophosphate; **CFTR** = Cystic Fibrosis Transmembrane Conductance Regulator; **ERK** = Extracellular Receptor Kinase; **MEK =** mitogen-activated protein kinase kinase; **PKA** = Protein Kinase A; **p-PKA** = phosphorylated-Protein Kinase A; **SR** = secretin receptor; **Src =** Proto-oncogene tyrosine-protein kinase.

Table 1 Main studies focusing on the role of Sct/SR axis in specific cholestatic human liver diseases

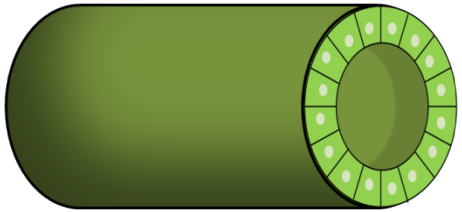
Disease	Experimental approach	Findings	Reference
	AE2a,b$^{-/-}$ mice (PBC model; AMA+)	AE2 gene disruption induces inflammatory liver damage and biochemical signs of progressive cholestasis	(34)
	Liver specimens of human PBC	miR-506 over-expression decreases the activity of AE2	(44)
	•dnTGF-βRII mice (PBC model; AMA+) •liver specimens of human PBC	•Inhibition of secretin/SR axis reduces biliary proliferation, inflammation and fibrosis. •Secretin fails to stimulate choleresis since miR-506 over-expression decreases the activity of CFTR/AE2	(7)
PSC	•Mdr2$^{-/-}$ mice (PSC model) •liver specimens of human PSC	Increased secretin and SR gene expression determines activation of TGF-β1/TGF-β1R axis and SMAD2/3 phosphorylation	(40)
	Mdr2$^{-/-}$/;SR$^{-/-}$ mice (PSC model with SR gene disruption)	Disruption of SR gene reduces fibrosis, cellular senescence and neo-angiogenesis	(42)
Biliary Atresia	Case report	Treatment with secretin and cholecystokinin analogue, shortly after birth, resolved biliary sludge possibly preventing evolution toward biliary atresia	(50)
CCA	•CCA cell lines •liver specimens of human PSC • BALB/c nude mice seeded with CCA cells	Stimulation of secretin/SR axis reduces tumor growth	(48)

Table 1:. The reference, the experimental systems employed and the main findings of each single publication are reported in columns and ordered according to the specific disease. Abbreviations**: AE2** = Anion Exchanger 2; **AMA**= anti mitochondrial antibody; **CCA**= cholangiocarcinoma; **CFTR** = Cystic Fibrosis Transmembrane Conductance Regulator; **Mdr** = Multi drug resistant; **PBC**= Primary biliary cholangitis; **PSC**=primary sclerosing cholangitis**; Sct**= secretin**; SR=** secretin receptor; **TGF**= Tumor Growth Factor.

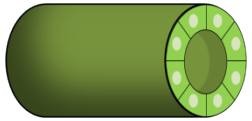
Figure 1



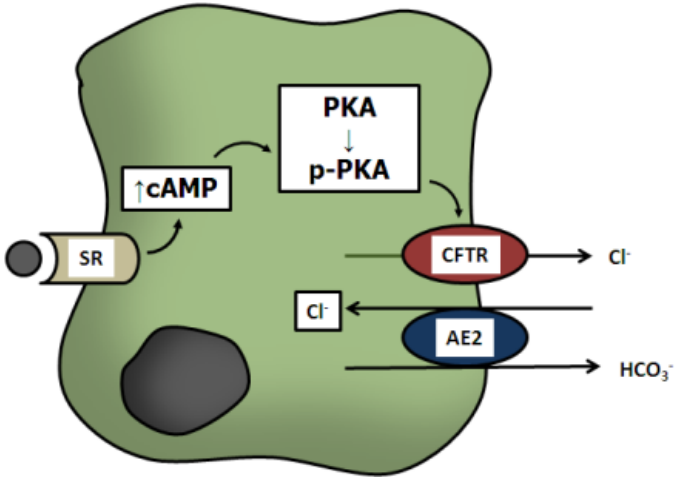
Large bile duct



Small bile duct



Large cholangiocytes (~14 µm)



Small cholangiocytes (~8 µm)

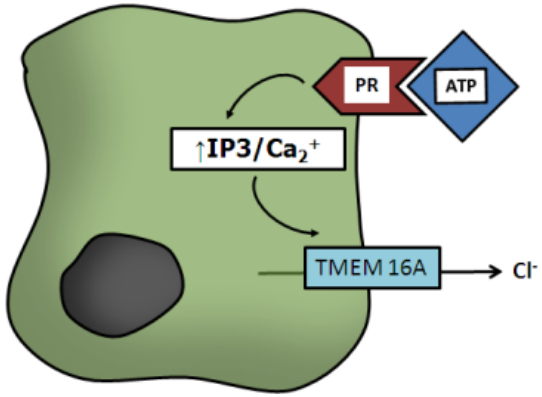
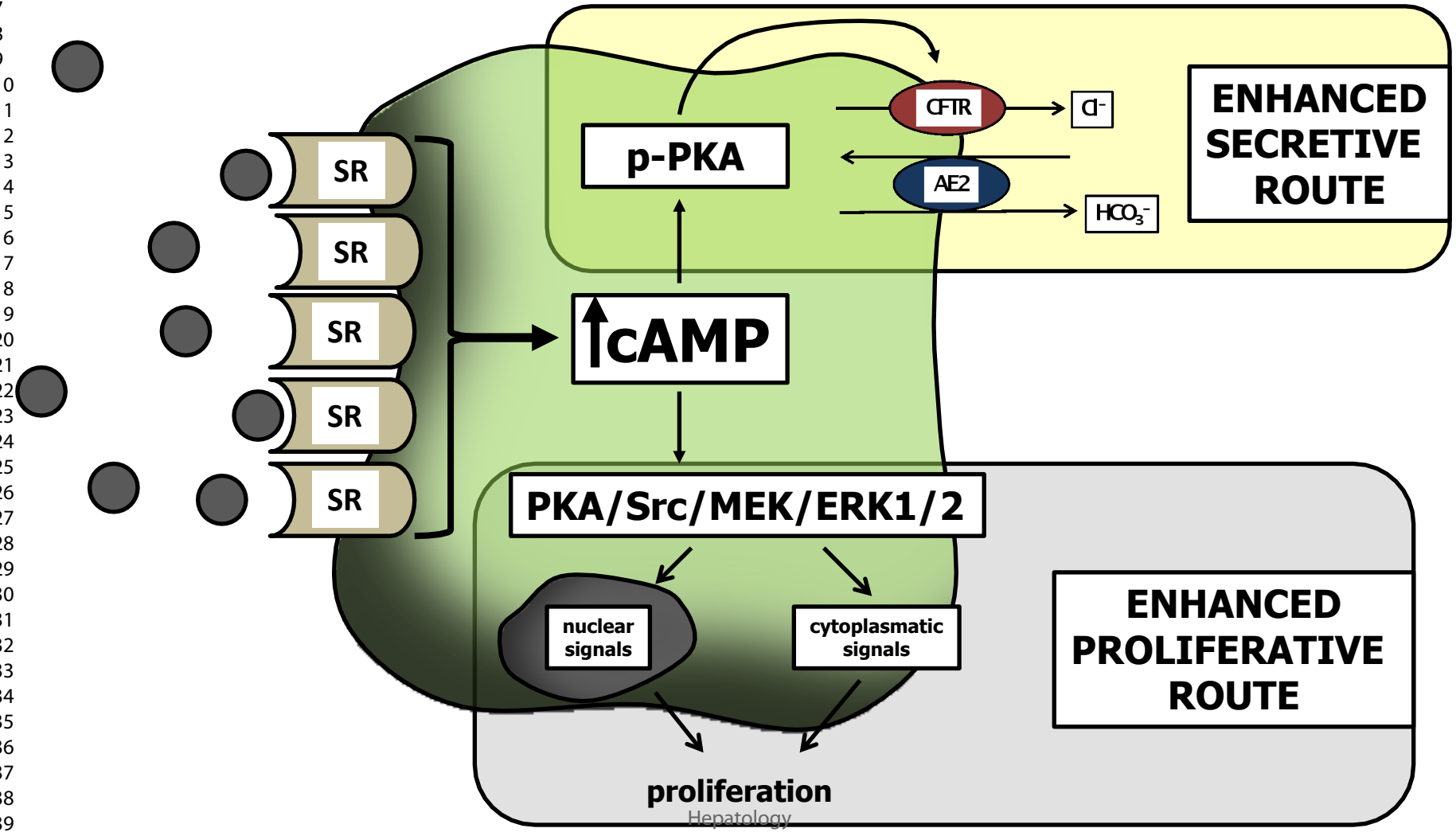


Figure 2


SR
SR
SR
SR
SR
p-PKA
CFTR
Cl⁻
AE2
HCO3⁻
ENHANCED SECRETIVE ROUTE
cAMP
PKA/Src/MEK/ERK1/2
nuclear signals
cytoplasmatic signals
ENHANCED PROLIFERATIVE ROUTE
proliferation