Scopolamine Treatment and Muscarinic Receptor Subtype-3 Gene Ablation Augment Azoxymethane-Induced Murine Liver Injury

Sandeep Khurana, Nirish Shah, Kunrong Cheng, Brian Shiu, Roxana Samimi, Angelica Belo, Jasleen Shant, Cinthia Drachenberg, Jürgen Wess, and Jean-Pierre Raufman

Division of Gastroenterology and Hepatology, VA Maryland Health Care System and University of Maryland School of Medicine, Baltimore, Maryland (S.K., N.S., K.C., B.S., R.S., A.B., J.S., J.-P.R.); Department of Pathology, University of Maryland School of Medicine, Baltimore, Maryland (C.D.); and National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland (J.W.)

Received December 21, 2009; accepted March 1, 2010

ABSTRACT

Previous work suggests that vagus nerve disruption reduces hepatocyte and oval cell expansion after liver injury. The role of postneuronal receptor activation in response to liver injury has not been ascertained. We investigated the actions of scopolamine, a nonselective muscarinic receptor antagonist, and specific genetic ablation of a key cholinergic receptor, muscarinic subtype-3 (*Chrm3*), on azoxymethane (AOM)-induced liver injury in mice. Animal weights and survival were measured as was liver injury using both gross and microscopic examination. To assess hepatocyte proliferation and apoptosis, ductular hyperplasia, and oval cell expansion, we used morphometric analysis of 5-bromo-2'-deoxyuridine-, activated caspase-3-, hematoxylin and eosin-, cytokeratin-19-, and epithelial cell adhesion molecule-stained liver sections. Sirius red staining was used as a measure of collagen deposition and its association with oval cell reaction. In AOM-treated

mice, both muscarinic receptor blockade with scopolamine and *Chrm3* ablation attenuated hepatocyte proliferation and augmented gross liver nodularity, apoptosis, and fibrosis. Compared with control, scopolamine-treated and *Chrm3*(-/-) AOM-treated mice had augmented oval cell reaction with increased ductular hyperplasia and oval cell expansion. Oval cell reaction correlated robustly with liver fibrosis. No liver injury was observed in scopolamine-treated and *Chrm3*(-/-) mice that were not treated with AOM. Only AOM-treated *Chrm3*(-/-) mice developed ascites and had reduced survival compared with AOM-treated wild-type controls. In AOM-induced liver injury, inhibiting postneuronal cholinergic muscarinic receptor activation with either scopolamine treatment or *Chrm3* gene ablation results in prominent oval cell reaction. We conclude that *Chrm3* plays a critical role in the liver injury response by modulating hepatocyte proliferation and apoptosis.

This work was supported in part by the Intramural Research Program of the National Institutes of Health National Institute of Diabetes and Digestive and Kidney Diseases; the National Institutes of Health National Cancer Institute [Grants CA107345, CA120407] (to J.-P.R.); the National Institutes of Health National Institute of Diabetes and Digestive and Kidney Diseases [Grant T32-DK067872] (to N.S.; principle investigator J.-P.R.); the National Institutes of Health Institute of Diabetes and Digestive and Kidney Diseases [Grant K08-DK081479] (to S.K.); the Office of Research and Development, Medical Research Service, Department of Veterans Affairs; and the Baltimore Research and Education Foundation.

S.K. and N.S. contributed equally to this work and share first authorship.

Parts of this work were previously published in abstract form: Shah N, Samimi R, Cheng K, Drachenberg C, Raufman J-P, and Khurana S (2008) *Gastroenterology* **134** (Suppl. 1):A-820; Shah N, Belo A, Cheng K, Shant J, Drachenberg C, Raufman J-P, and Khurana S (2009) *Hepatology* **48** (Suppl.):457A; Shah N, Shiu B, Samimi R, Drachenberg C, Shant J, Cheng K, Raufman J-P, and Khurana S (2009) *Gastroenterology* **136** (Suppl. 1):A-114; Shah N, Shiu B, Drachenberg C, Shant J, Belo A, Cheng K, Raufman J-P, and Khurana S (2009) *Gastroenterology* **136** (Suppl. 1):A-819; Shah N, Shiu B, Belo A, Drachenberg C, Shant J, Cheng K, Raufman J-P, and Khurana S (2009) *Hepatology* **50** (Suppl.):847A.

Article, publication date, and citation information can be found at <http://jpet.aspetjournals.org>.

doi:10.1124/jpet.109.165118.

Acetylcholine activates two classes of cholinergic receptors: nicotinic, which function as cation channels, and muscarinic (CHRM), which mediate G protein-coupled signaling. Five mammalian CHRM subtypes (CHRM1–5) are described; odd-numbered CHRM (CHRM1, 3, and 5) preferentially activate G_{q/11} proteins, whereas even-numbered CHRM (CHRM2 and 4) activate G_{i/o} proteins. Cell type-specific expression of CHRM subtypes mediates diverse signaling events. In the nervous system, muscarinic receptors play a major role in synaptic transmission and regulate sensory, motor, and autonomic functions. In non-neuronal tissue, muscarinic receptor activation regulates cell function (Shah et al., 2009). For example, muscarinic receptors mediate proliferation of lung and colon cancer cells (Raufman et al., 2008; Shah et al., 2009).

In the liver, stimulation of the vagus nerve induces proliferation of oval cells (designated intermediate hepatobiliary

ABBREVIATIONS: *Chrm3*, cholinergic receptor, muscarinic subtype-3; SCOP, scopolamine; AOM, azoxymethane; PBS, phosphate-buffered saline; WT, wild type; BrdU, 5-bromo-2'-deoxyuridine; H&E, hematoxylin and eosin; IHC, immunohistochemistry; EpCAM, epithelial cell adhesion molecule; HPF, high-power field; CK-19, cytokeratin-19; VIP, vasoactive intestinal peptide.

cells in humans) by release of acetylcholine and activation of muscarinic receptors (Cassiman et al., 2002). Oval cells are hepatic progenitor cells that can differentiate into either hepatocytes or cholangiocytes (Roskams et al., 2004). After severe and chronic liver injury, oval cells play a critical role in hepatic parenchymal restoration (Forbes et al., 2002; Newsome et al., 2004). Oval cell expansion and ductular hyperplasia are observed in animal models of liver injury and in human chronic liver diseases (Roskams et al., 2004; Clouston et al., 2005). Oval cells are also thought to be precursors of hepatic cancer cells (Lee et al., 2009). In animal models of liver cancer, expansion of the oval cell compartment is observed before the development of frank neoplasia (Libbrecht et al., 2000).

Human and rodent hepatic oval cells, hepatocytes, and cholangiocytes express CHRM3 subtype muscarinic receptors (Alvaro et al., 1997; Cassiman et al., 2002; Li et al., 2009). Based on several observations, it was suggested that liver injury responses to vagus nerve stimulation are mediated by activation of CHRM3 (Cassiman et al., 2002). Compared with innervated control livers, denervated livers (transplanted) have decreased numbers of bile ductules and intermediate hepatobiliary cells (Cassiman et al., 2002). In vagotomized rats, acute galactosamine-induced liver injury results in reduced oval cell proliferation compared with sham-operated controls (Cassiman et al., 2002). Modulating cholinergic input by vagal nerve disruption or stimulation in other models of liver injury alters oval cell proliferation and hepatocyte restoration. After partial hepatectomy, animals with disrupted hepatic vagus nerve branches have attenuated hepatocyte proliferation and ductular reaction (Ikeda et al., 2009). In a rat ischemia-reperfusion injury model, vagus nerve stimulation attenuates hepatic tissue necrotic factor synthesis and systemic shock (Bernik et al., 2002). In guinea pigs, bile duct ligation-induced chronic liver injury is associated with the formation and proliferation of acetylcholinesterase-positive nerve fibers that traverse bands of deposited collagen (Akiyoshi, 1989).

Collectively, these observations indicate that vagus nerve stimulation promotes ductular reaction and may regulate the liver injury response. Moreover, in animal models of liver injury and in human chronic liver disease, these findings suggest that oval cell reaction plays a key role in parenchymal restoration and possibly hepatic carcinogenesis. Nonetheless, the importance of muscarinic receptors, specifically CHRM3, in modulating oval cell and ductular reaction, and the response to liver injury is not known.

In the course of evaluating the role of muscarinic receptors and ligands in colon cancer (Raufman et al., 2008), we observed unanticipated severe liver injury in azoxymethane (AOM)-treated mice with concurrent muscarinic receptor inactivation. AOM, an ingredient in cycad palms found on the island of Guam, was first identified, and subsequently used by investigators, as a selective intestinal carcinogen (Laqueur et al., 1963; Hirono, 1981). Subsequent work in rodents revealed that low-dose AOM treatment induces hepatotoxicity with ductular hyperplasia (Ward, 1975; Shan et al., 2008). At higher doses, AOM causes severe liver damage resulting in acute hepatic failure, encephalopathy, and increased rodent mortality (Matkowskyj et al., 1999; Bélanger et al., 2006). AOM is now used by many investigators as a model of

Fig. 1. Study design. A, 129S6/SvEv $\times$ CF1 mice were treated with intraperitoneal PBS ($n = 4$) or AOM ($n = 26$; 10 mg/kg) once each week for 6 weeks. Mice were also allocated to treatment with subcutaneous PBS ($n = 13$) or SCOP (3 mg/kg/day in two divided doses; $n = 17$) 5 days/week for 20 weeks. B, *Chrm3*($-/-$) ($n = 29$) and WT mice ($n = 25$) were treated with intraperitoneal AOM (10 mg/kg once each week for 6 weeks). Additional WT ($n = 10$) and *Chrm3*($-/-$) mice ($n = 4$) treated with intraperitoneal PBS only were used as controls.

acute liver failure and hepatic encephalopathy (Chen et al., 2009).

By use of AOM to induce chronic liver injury in mice, we conducted the current study to elucidate the role of muscarinic receptors in mediating ductular hyperplasia and oval cell proliferation. To evaluate the overall role of muscarinic receptors in these processes, we used scopolamine (SCOP) butylbromide, a nonselective muscarinic receptor antagonist that does not cross the blood-brain barrier. To determine more specifically the role of CHRM3 in modulating ductular hyperplasia and oval cell proliferation, we used knockout [*Chrm3*($-/-$)] mice. Our findings show that muscarinic receptor signaling mediated by CHRM3 plays a critical role in the liver injury response by modulating oval cell expansion, ductular hyperplasia, and hepatocyte proliferation and apoptosis.

Materials and Methods

Experimental Design and Animal Procedures. All animal studies were conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* prepared by the United States National Academy of Sciences (National Institutes of Health publication 86-23, revised 1985). Mouse studies were approved by both the Institutional Animal Care and Use Committee at the University of Maryland School of Medicine and the Research and Development Committee at the VA Maryland Health Care System (Baltimore, MD). All mice [genetic background, 129S6/SvEv $\times$ CF1 (50%:50%); Taconic Farms, Germantown, NY] were housed under identical conditions in a pathogen-free environment with a 12:12-h light/dark cycle and free access to standard mouse chow and water. Mice were acclimatized for 2 weeks before any treatment. The study design is outlined in Fig. 1. In study A, 26 6-week-old male mice were treated with intraperitoneal AOM (10 mg/kg once each week for 6 weeks; Midwest Research Institute, Kansas City, MO) (Fig. 1A). In addition, these mice were allocated to treatment with subcutaneous injection of scopolamine butylbromide, a quaternary ammonium compound (<http://chem.sis.nlm.nih.gov/chemidplus>) that does not cross the blood-brain barrier (3 mg/kg/day in two divided doses, 5 days/week for 20 weeks, $n = 13$; Butler Animal Health Supply, Dublin, OH) or PBS control administered subcutaneously using the same schedule

($n = 13$). Four control mice that received intraperitoneal PBS instead of AOM were also treated with subcutaneous scopolamine. Mice were euthanized 20 weeks after the first AOM dose.

The study design using *Chrm3*-deficient [*Chrm3*($-/-$)] and muscarinic receptor wild-type (WT) control mice is shown in Fig. 1B. Generation of *Chrm3*($-/-$) mice was as described previously (Yamada et al., 2001). In study B, 54 6-week-old male WT ($n = 25$) and *Chrm3*($-/-$) mice ($n = 29$) were treated with intraperitoneal AOM (10 mg/kg once each week for 6 weeks). As additional controls, 10 WT mice were treated with intraperitoneal PBS instead of AOM and four *Chrm3*($-/-$) mice received no treatment. Mice were euthanized 20 weeks after the first intraperitoneal dose of AOM or PBS.

Mouse weight and mortality were recorded weekly. To measure cell proliferation, 2 h before euthanasia, mice were injected with 50 mg/kg 5-bromo-2'-deoxyuridine (BrdU; Sigma-Aldrich, St. Louis, MO). At euthanasia, gross liver appearance was graded by two investigators masked to study group: 0, normal; 1, mild liver surface nodularity; 2, intermediate liver surface nodularity; and 3, marked nodularity with or without ascites. Livers were harvested after visual inspection, weighed, and fixed in 4% para-formaldehyde.

Liver Histology. Five-micrometer sections obtained from formalin-fixed, paraffin-embedded liver tissue were stained with hematoxylin and eosin. The sections were analyzed by an expert pathologist blinded to study groups. H&E sections were reviewed to assess liver

fibrosis and scored using Batts-Ludwig criteria. Furthermore, liver fibrosis was quantified using morphometric analysis of Sirius red-stained sections. After dewaxing and hydration, liver sections were stained with picrosirius red solution for 1 h followed by washing in acidified water. The resultant sections were dehydrated, mounted, and examined under light microscopy using an i80 photomicroscope (Nikon, Tokyo, Japan) at 100 $\times$ magnification. To minimize variation, all sections were examined and photographed with the same microscope settings. At least five different areas were photographed from each section. Because the degree of Sirius red staining measured by the saturation of the red channel correlates well with chemically determined collagen content and morphometrically determined fibrosis, fibrosis was expressed as the percentage of summed pixels per unit area of liver section (in arbitrary units) determined using Image-Pro Plus software (version 5.0; Media Cybernetics, Silver Spring, MD).

Immunohistochemistry. Hepatocyte proliferation was assessed using primary antibodies against BrdU (BD Bioscience, San Jose, CA; dilution, 1:10). Liver sections were blocked with normal serum for nonspecific protein binding after treating with H_2O_2 for nonspecific peroxidase activity and heat-induced antigen retrieval. They were incubated overnight with biotinylated anti-BrdU antibody at 4°C. Sections were incubated with streptavidin-horseradish peroxidase for 30 min at room temperature followed by staining with

Fig. 2. Effects of SCOP on AOM-induced oval cell chronic liver injury in mice. Livers were analyzed 20 weeks after the first injection of AOM. A, animal weights were recorded over 20 weeks in AOM-treated mice cotreated with PBS or SCOP; livers from four mice treated with scopolamine only (no AOM) were also analyzed. C, representative photographs show grades of liver nodularity determined by gross inspection: 0, normal; 1, mild liver surface nodularity; 2, intermediate liver surface nodularity; and 3, marked nodularity. D, abnormal livers ($\geq$ grade 1 nodularity) were observed only in AOM-treated mice. E, liver nodularity score was greater in mice treated with AOM plus scopolamine compared with those treated with AOM plus PBS. F, H&E sections were examined to assess fibrosis using the Batts-Ludwig criteria. Compared with PBS, scopolamine cotreatment increased AOM-induced liver fibrosis. Livers from mice that were treated with scopolamine only (no AOM) appeared normal, without fibrosis. Bars represent mean values $\pm$ S.E. *, $p < 0.05$.

diaminobenzidine and counterstaining with hematoxylin. At least 1000 hepatocyte nuclei were counted at 200 $\times$ magnification and expressed as BrdU-stained nuclei/1000 cells.

IHC was performed for markers of apoptosis using anti-activated caspase-3 antibody (Cell Signaling Technology Inc., Danvers, MA; dilution 1:100) and markers of bile ducts and oval cells using anti-cytokeratin-19 (CK-19) and anti-epithelial cell adhesion molecule (EpCAM) antibodies (both from Abcam Inc., Cambridge, MA; dilution, 1:100). IHC was performed using the avidin-biotin reaction with the VECTASTAIN Elite ABC kit (Vector Laboratories, Burlingame, CA) per the manufacturer's recommendations. After deparaffinization, hydration, and endogenous peroxidase blockade (10% H₂O₂), heat-induced antigen retrieval was performed using citrate buffer, and the sections were incubated at room temperature with 5% normal goat serum (20 min), avidin blocking reagent (15 min), and biotin blocking reagent (15 min). Sections were washed three times with 0.1% Tween 20 in PBS between each step. Sections treated overnight at 4°C with primary antibody were incubated with

biotinylated goat anti-rabbit antibody (30 min) followed by incubation with streptavidin-horseradish peroxidase (30 min). They were stained with diaminobenzidine (2 min) followed by counterstaining with hematoxylin (4 min). At least 1000 hepatocytes were counted to determine activated caspase-3 activity. Bile ductular cells were defined as CK-19- or EpCAM-stained cells forming ductular structure with lumens. Oval cells were defined as CK-19- or EpCAM-stained small cells with oval nuclei and scant cytoplasm not forming ductular structures. A specific marker for oval cells has not been identified. In liver injury, antigen expression profile of proliferating nonparenchymal cells overlaps with that of oval cells; this makes identification of oval cells difficult. Hence, morphometric analysis of liver sections with two recently established oval cell markers was used not only to identify and grade but also to validate oval cell reaction (Yovchev et al., 2008; Okabe et al., 2009). At least five fields were examined at 200 $\times$ total magnification, and results expressed as the number of bile ducts/high-power field (HPF), bile duct size/HPF (number of cells forming bile ducts), and oval cells/HPF.

Fig. 3. Effects of SCOP on AOM-induced oval cell reaction. A, ductules and oval cells (stained brown) are demonstrated using IHC for CK-19 and EpCAM in liver sections from AOM-treated mice co-treated with SCOP or PBS. Liver sections from mice treated with scopolamine only (no AOM) were also stained. Arrows indicate ductules and arrowheads indicate oval cells. Treatment with scopolamine increased AOM-induced oval cell reaction with proliferation of large ductules (demonstrated by the number of cells/ductule) and oval cell expansion that was confirmed by both CK-19 (B) and EpCAM (C) staining. Ductular cells were defined as CK-19- or EpCAM-stained cells forming ductular structures with lumens. Oval cells were defined as CK-19- or EpCAM-stained small cells with oval nuclei and scant cytoplasm not forming ductular structures. Bars represent mean values $\pm$ S.E. *, $p < 0.05$ and ***, $p < 0.001$.

Statistical Analysis. All data are expressed as mean $\pm$ S.E. Data distribution was determined using the Anderson-Darling method. Based on distribution of data, Student's unpaired *t* test (normally distributed data) or the Mann-Whitney U test (nonparametric data) was used to determine significance. Nominal data were analyzed using a χ^2 test with Fisher's test. Linear correlations were determined using Pearson's r^2 . Survival was analyzed using the Kaplan-Meier method. Analysis was performed using StatView (version 5.0.1; SAS Institute, Cary, NC). Significance was defined as $p < 0.05$ and is expressed in figures as *, $p < 0.05$, **, $p < 0.01$, and ***, $p < 0.001$.

Results

Effects of Scopolamine Treatment on Gross Liver Appearance in AOM- and PBS-Treated Mice. During the 20-week treatment period (Fig. 1A), one AOM-treated mouse that received scopolamine died; there was no mortality in the PBS-treated group. Throughout the study, scopolamine- and PBS-treated mice did not differ in body weight (Fig. 2A). Likewise, liver weights were similar in all study groups (Fig. 2B). Figure 2C shows representative photographs of gross liver nodularity grades at euthanasia. Eighty-two percent of AOM-treated mice had gross liver nodularity (Fig. 2D). In mice treated with subcutaneous scopolamine alone (no intraperitoneal AOM), the liver surface was normal and there was no mortality (Fig. 2E). AOM-treated mice that received scopolamine had a higher gross liver nodularity score compared with mice treated with PBS (1.3 ± 0.2 versus 0.8 ± 0.1 ; $p < 0.05$; Fig. 2E).

Histological Studies in Scopolamine-Treated Mice. Liver fibrosis (H&E) was not observed in mice treated with scopolamine alone (no AOM; Fig. 2F). In contrast, as predicted by the nodularity scores (Fig. 2, D and E), AOM-treated mice developed fibrosis; fibrosis scores were higher in scopolamine- compared with PBS-treated mice (Batts-Ludwig fibrosis score, 2.2 ± 0.2 versus 1.2 ± 0.3 ; $p < 0.05$). These findings indicate that cotreatment with AOM plus scopolamine augments the fibrotic response to liver injury.

Previous work suggested that muscarinic receptors regulate oval cell reaction in response to galactosamine- and bile duct ligation-mediated liver injury (LeSag et al., 1999; Cassiman et al., 2002). To confirm the importance of muscarinic receptor activation in mediating oval cell reaction after AOM-induced liver injury, we examined the effects of the nonselective muscarinic receptor antagonist scopolamine (van Koppen and Kaiser, 2003) on ductular hyperplasia and oval cell expansion measured by IHC for CK-19 and EpCAM. Cells forming the ductular phenotype express these ligands. We counted oval cells and bile ducts in five randomly selected HPF (total magnification, $200\times$). As a surrogate marker for bile duct size, we counted the number of cells forming bile ducts. AOM treatment induced ductular hyperplasia and expansion of oval cells. In scopolamine- compared with PBS-treated mice, using CK-19 (Fig. 3, A and B) and EpCAM (Fig. 3, A and C) staining, ductule size and the numbers of ductules and oval cells were increased. These findings indicate that blocking muscarinic receptor activation augments oval cell reaction after AOM-induced liver injury.

In chronic hepatitis C, and alcoholic and nonalcoholic fatty liver diseases, ductular reaction correlates strongly with the stage of fibrosis (Roskams et al., 2003; Clouston et al., 2005). To determine whether hepatic collagen deposition correlated

with oval cell reaction, we used Sirius red staining to measure liver fibrosis. Mice that received scopolamine alone (no AOM) had no collagen deposition (Fig. 4A). In AOM-treated mice, Sirius red staining was increased in scopolamine-treated compared with PBS-treated mice (10.3 ± 1.0 versus $3.4 \pm 0.8\%$, SCOP versus PBS, $p < 0.001$; Fig. 4, A and B). There was a significant correlation between oval cell reaction and liver fibrosis; CK-19-stained liver sections revealed that fibrosis correlated with bile ductular size and oval cell expansion but not with duct number. Ductular and oval cell proliferation detected by EpCAM staining also correlated with liver fibrosis (Sirius red staining) (Table 1). These data indicate that in AOM-treated mice, cotreatment with scopolamine promotes oval cell reaction that correlates robustly with collagen deposition.

Hepatocyte restoration after liver injury results from a balance between hepatocyte proliferation and apoptosis. Cholinergic stimulation via the vagus nerve is reported to modulate hepatocyte proliferation (Ohtake et al., 1993; Kiba et al., 1994; Kiba, 2002). To determine the effects of scopolamine treatment on hepatocyte restoration, we measured cell

Fig. 4. Effects of SCOP on AOM-induced liver fibrosis. A, collagen deposition was evaluated by Sirius red staining. B, cotreatment with scopolamine increased liver fibrosis in AOM-treated mice. Bars represent mean values $\pm$ S.E. ***, $p < 0.001$.

TABLE 1

Correlation of fibrosis with oval cell reaction in liver sections from AOM-treated mice

See Fig. 1 and *Materials and Methods* for details.

Variable	Sirius Red-Stained Area	
	r^2 (Study A)	r^2 (Study B)
CK-19-stained		
ductules	0.31 ^a	0.74**
ductule cells	0.44*	0.64**
oval cells	0.49*	0.58*
EpCAM-stained		
ductules	0.63**	0.96***
ductule cells	0.75**	0.62*
oval cells	0.69**	0.57*

* $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

^a $p = 0.06$.

proliferation (BrdU staining) and apoptosis (activated caspase-3 staining). In AOM-treated mice that received scopolamine compared with mice that received AOM plus PBS, BrdU staining was reduced (5.7 ± 1.3 versus 10.7 ± 1.6 BrdU-stained cells/1000 hepatocytes, $p < 0.05$; Fig. 5, A and B) and apoptosis was augmented (27.8 ± 7.2 versus 7.5 ± 2.5 activated caspase-3-stained cells/1000 hepatocytes, $p < 0.05$; Fig. 5, A and C). These data indicate that scopolamine alters AOM-induced oval cell reaction by augmenting bile ductular proliferation and oval cell expansion and reducing hepatocyte proliferation.

Gross Liver Injury in AOM-Treated *Chrm3*-Deficient Mice. Previously, it was suggested in animal models of liver injury that vagus nerve stimulation augments ductular hyperplasia and oval cell proliferation by specific activation of *Chrm3* (Cassiman et al., 2002). To focus on the role of *Chrm3* in oval cell reaction and to determine whether *Chrm3* ablation mimics pharmacological blockade of muscarinic receptors, we studied *Chrm3*($-/-$) mice (Fig. 1B). Figure 6A (left) shows representative *Chrm3*($-/-$) mice treated with AOM (left mouse) or PBS (right mouse). The AOM-treated mouse had a distended abdomen and laparotomy revealed ascites (Fig. 6A, middle), with grade 3 liver nodularity (Fig. 6A, right). The liver of the PBS-treated mouse was normal. Over the course of these experiments, AOM-treated *Chrm3*($-/-$) mice gained more weight compared with AOM-treated WT mice (41.7 ± 5.4 versus $21.9 \pm 3.9\%$ initial body weight, $p < 0.05$; Fig. 6B), a finding that we attribute to fluid retention and the development of ascites (Fig. 6A). Moreover, whereas no PBS-treated mice died over the course of treatment, in the AOM treatment group, three WT and 13 *Chrm3*($-/-$) mice

died (mortality rate 12 versus 45% for WT and *Chrm3*($-/-$) mice treated with AOM, $p < 0.01$; Fig. 6C).

At laparotomy, WT and *Chrm3*($-/-$) mice treated with PBS (no AOM) had a normal liver appearance (Fig. 6D). In contrast, 30% of WT and 88% of *Chrm3*($-/-$) AOM-treated mice had liver nodularity ($p < 0.05$). Compared with WT, AOM-treated *Chrm3*($-/-$) mice had greater liver nodularity scores (Fig. 6, D and E). The highest grade, liver nodularity with ascites, was observed in approximately 25% of AOM-treated *Chrm3*($-/-$) mice and in no AOM-treated WT mice (Fig. 6E).

Histological Studies in *Chrm3*-Deficient Mice. To determine the effects of *Chrm3* ablation on AOM-induced oval cell reaction, we measured CK-19 and EpCAM staining. AOM-treated mice had marked oval cell reaction with bile ductular proliferation and oval cell expansion. In *Chrm3*($-/-$) compared with WT mice, IHC for CK-19 (Fig. 7, A and B) and EpCAM (Fig. 7, A and C) revealed increased numbers of bile ductules, ductular cells, and oval cells.

To determine the effect of *Chrm3* ablation on AOM-induced liver fibrosis, we analyzed H&E- and Sirius red-stained sections. Gross liver nodularity in AOM-treated mice correlated with the Batts-Ludwig fibrosis score (data not shown). Sirius red-stained liver sections revealed increased collagen deposition in *Chrm3*($-/-$) compared with WT mice (21.4 ± 3.2 versus $7.6 \pm 1.0\%$ Sirius red-stained area, $p < 0.001$; Fig. 8, A and B). A strong correlation was observed between liver nodularity on gross examination and the Sirius red-stained area ($r^2 = 0.82$; $p < 0.001$). As observed in scopolamine-treated animals, in AOM-treated mice oval cell and ductular hyperplasia were most prominent in areas of fibro-

Fig. 5. Effects of SCOP on hepatocyte restoration in AOM-treated mice. A, representative sections show BrdU and activated caspase-3 staining in livers from mice treated with scopolamine alone (no AOM) and from mice treated with AOM plus PBS or scopolamine. Arrows indicate BrdU- and activated caspase-3-stained hepatocytes. In AOM-treated mice, treatment with scopolamine reduced the numbers of hepatocytes that stained for BrdU (B) and increased the number of hepatocytes that stained for activated caspase-3 (C). Bars represent mean values $\pm$ S.E. *, $p < 0.05$.

Fig. 6. Effects of *Chrm3* ablation on AOM-induced chronic liver injury. A, left, *Chrm3*^{-/-} mice after treatment with AOM (left) and PBS (right) are shown. Middle and right, necropsy of the AOM-treated *Chrm3*^{-/-} mouse revealed ascites (blue arrow) and a grossly nodular liver. B, at 20 weeks, AOM-treated *Chrm3*^{-/-} mice had gained more weight than WT mice. C, as shown by the Kaplan-Meier curve, after treatment with AOM, *Chrm3*^{-/-} mice had reduced survival compared with WT mice. D, liver nodularity was not observed in WT or *Chrm3*^{-/-} mice that were not treated with AOM. Liver nodularity scores were greater in AOM-treated *Chrm3*^{-/-} compared with WT mice. E, *Chrm3*^{-/-} mice were more likely to have grossly abnormal livers than WT mice. Grade 3 liver nodularity was seen only in AOM-treated *Chrm3*^{-/-} mice. Bars represent mean values $\pm$ S.E. *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$.

sis; ductular hyperplasia and oval cell proliferation (CK-19 and EpCAM staining) correlated strongly with Sirius red staining (Table 1).

The data shown in Fig. 5 indicate that muscarinic receptor blockade modulates hepatocyte restoration by reducing hepatocyte proliferation and increasing apoptosis. To determine whether *Chrm3* ablation mimicked the actions of scopolamine, we used BrdU and activated caspase-3 staining. In PBS-treated mice (no AOM), very few BrdU-stained nuclei and little activated caspase-3 staining were observed (Fig. 9A). These findings confirm that, in the absence of injury, hepatocytes remain quiescent and do not undergo mitosis. In contrast, in liver sections from AOM-treated animals we observed increased hepatocyte proliferation. However, BrdU-stained hepatocytes were fewer in *Chrm3*^{-/-} compared with WT mice (3.1 ± 0.8 versus 9.2 ± 2.0 BrdU-stained cells/1000 hepatocytes, $p < 0.05$; Fig. 9, A and B). Moreover, in the AOM treatment group, *Chrm3*^{-/-} mice had augmented hepatocyte apoptosis compared with WT mice (36.6 ± 13.7 versus 17.3 ± 0.6 activated caspase-3-stained cells/1000 hepatocytes, $p < 0.05$; Fig. 9, A and C). These data indicate that hepatic parenchymal restoration after AOM-induced liver injury is modulated by both pharmacological inhibition of muscarinic receptor activation and by specific genetic ablation of *Chrm3*, resulting in attenuated hepatocyte proliferation, augmented apoptosis, and increased ductular hyperplasia and oval cell expansion.

Discussion

Our findings are consistent with previous observations that AOM treatment induces chronic liver injury in murine liver (Ward, 1975; Shan et al., 2008). Mice treated with AOM developed liver surface nodularity, fibrosis, and ductular hyperplasia. In addition, ductular hyperplasia and oval cell expansion after AOM treatment correlated strongly with liver fibrosis, a hallmark of chronic liver disease. Our results extend previous observations by showing that in addition to ductular hyperplasia (Shan et al., 2008), AOM treatment induces oval cell expansion. To investigate the role of muscarinic activation in mediating cellular responses to AOM-induced liver injury, we used pharmacological inhibition of muscarinic receptor activation and genetic ablation of *Chrm3*. Experiments using scopolamine-treated WT and *Chrm3*^{-/-} mice revealed reproducible augmentation of ductular hyperplasia and oval cell expansion, with increased fibrosis compared with control mice. In addition, attenuating muscarinic receptor activation with scopolamine treatment or *Chrm3* gene ablation modulated hepatocyte restoration after AOM injury by reducing hepatocyte proliferation (BrdU staining) and increasing apoptosis (activated caspase-3 staining). Collectively, these data indicate that in mice, CHRM3 activation plays a key role in protecting the liver from AOM-induced toxicity and imply that CHRM3 activa-

Fig. 7. Effects of *Chrm3* ablation on AOM-induced oval cell reaction. A, ductules and oval cells (stained brown) are demonstrated using IHC for CK-19 and EpCAM in liver sections from PBS- and AOM-treated *Chrm3*(^{-/-}) and WT mice. Arrows indicate ductules and arrowheads indicate oval cells. As demonstrated by CK-19 (B) and EpCAM (C) staining, sections from *Chrm3*(^{-/-}) mice revealed robust AOM-induced oval cell reaction with increased numbers of large ductules (demonstrated by numbers of cells/ductule) and oval cell expansion compared with those from WT mice. Ductular cells were defined as CK-19- or EpCAM-stained cells forming ductular structures with lumens. Oval cells were defined as CK-19- or EpCAM-stained small cells with oval nuclei and scant cytoplasm not forming ductular structures. Bars represent mean values $\pm$ S.E. *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$.

tion is likely to play an important role in hepatic regeneration in chronic liver disease.

Whereas scopolamine treatment increased apoptosis, oval cell reaction, and fibrosis, these effects were less robust than those observed in *Chrm3*(^{-/-}) mice. Moreover, in the AOM treatment groups, only one mouse that received scopolamine died, whereas 13 *Chrm3*(^{-/-}) mice died ($p < 0.01$) and cirrhosis with ascites after AOM treatment was seen only in *Chrm3*(^{-/-}) mice. Several factors probably account for the greater efficacy of *Chrm3* ablation compared with scopolamine treatment. To avoid systemic toxicity, we used a modest dose of scopolamine, administered the drug only twice daily, and mice had a 2-day treatment holiday each week. Hence, scopolamine treatment was not likely to achieve the same magnitude or duration of reduced muscarinic receptor activation achieved by genetic ablation. We consider the possibility that scopolamine, a nonselective muscarinic receptor antagonist, modulated liver injury by blocking other muscarinic receptor subtypes. However, a recent study revealed

that *Chrm3* is the only muscarinic receptor subtype expressed in mouse liver and other *Chrm* subtypes (1, 2, 4, and 5) are not expressed (Li et al., 2009). Hence, it is unlikely that other *Chrm* subtypes are involved in regulating AOM-induced injury in mouse liver.

All AOM-treated mice developed ductular hyperplasia and oval cell expansion that was most prominent along collagen bands. Sirius red staining correlated strongly with all components of ductular reaction. Clouston et al. (2005) demonstrated in chronic hepatitis C that fibrosis correlates strongly with periportal ductular reaction. Oval cell proliferation (intermediate hepatobiliary cells in humans) is described in a variety of chronic liver diseases with a strong association between fibrosis, ductular proliferation and oval cell numbers in nonalcoholic and alcoholic fatty liver disease (Ray et al., 1993; Roskams et al., 2003). These findings are similar to those observed in AOM-treated mice. In addition, it was shown recently that increased oval cell numbers correlates with more fibrosis (Fotiadu et al., 2004). Hence, our findings

Fig. 8. Effects of *Chrm3* ablation on AOM-induced liver fibrosis. A, collagen deposition was evaluated by Sirius red staining in *Chrm3*(^{-/-}) and WT mice treated with PBS or AOM. B, AOM-treated *Chrm3*(^{-/-}) mice had increased liver fibrosis compared with AOM-treated WT mice. Bars represent mean values $\pm$ S.E. ***, $p < 0.001$.

Fig. 9. Effects of *Chrm3* ablation on hepatocyte restoration in AOM-treated mice. A, representative sections show BrdU and activated caspase-3 staining in livers from PBS- and AOM-treated WT and *Chrm3*(^{-/-}) mice. Black arrows indicate BrdU-stained hepatocytes (top row) and activated caspase-3-stained hepatocytes (bottom row). Compared with WT mice, AOM-treated *Chrm3*(^{-/-}) mice had reduced numbers of BrdU-stained hepatocytes (B) and increased numbers of activated caspase-3-stained hepatocytes (C). Bars represent mean values $\pm$ S.E. *, $p < 0.05$.

of strong correlations between CK-19/EpCAM staining and collagen deposition are consistent with observations in animal models of liver injury and in human chronic liver disease.

In various organ systems, *Chrm3* play an important role in mediating the actions of muscarinic ligands on cell proliferation, migration, and wound healing (Shah et al., 2009). Branches of the vagus nerve provide parasympathetic inner-

vation to the liver (Kiba, 2002). Previously, vagus nerve stimulation and transection were used to investigate the cholinergic regulation of liver injury (LeSag et al., 1999; Bernik et al., 2002; Cassiman et al., 2002). The work described herein is the first to evaluate the role of postneural activation of muscarinic receptors on liver injury. Expression of *Chrm3* in bile ductules, oval cells, and hepatocytes was

confirmed by others (LeSag et al., 1999; Cassiman et al., 2002; Li et al., 2009). Their observations are consistent with our finding of increased hepatocyte apoptosis and reduced proliferation in AOM-treated *Chrm3*($-/-$) and scopolamine-cotreated mice. PBS-treated *Chrm3*($-/-$) mice and mice that received scopolamine but not AOM had no gross or histological evidence of liver injury. Moreover, our finding of diminished hepatocyte proliferation in scopolamine-treated and *Chrm3*($-/-$) mice is similar to results after partial hepatectomy, where vagotomy impairs hepatocyte proliferation and diminishes liver weight (Ohtake et al., 1993).

In contrast to our findings, two studies reported decreased ductular and oval cell proliferation after vagotomy (LeSag et al., 1999; Cassiman et al., 2002). We ascribe this discrepancy to different models of liver injury, a different method of inhibiting cholinergic signaling, and shorter time courses compared with our study. LeSag et al. (1999) used bile-duct ligation to induce liver injury and investigated cholangiocyte proliferation 1 week after surgery. Because bile duct ligation causes cholestasis, the consequent elevation in serum and liver bile acids may activate *Chrm3*, thereby stimulating cell proliferation (Raufman et al., 2002). Cassiman et al. (2002) performed studies 48 h after galactosamine treatment, an acute hepatocyte injury model that triggers oval cell reaction. In contrast to these experimental models, we studied AOM-induced injury 20 weeks after treatment. Moreover, an AOM metabolite, methyl-azoxymethanol, inhibits hepatocyte proliferation (Zedeck and Swislocki, 1975). Hence, it is possible that the predominant effect in chronic AOM-induced toxicity is hepatocyte injury and that muscarinic receptor inactivation exacerbates liver injury by attenuating hepatocyte proliferation or by augmenting hepatocyte apoptosis, thus triggering compensatory oval cell reaction (Figs. 5 and 9). Alternatively, inhibiting *Chrm3* activation may prevent differentiation of oval cells into hepatocytes, thus preventing oval cell-dependent parenchymal restoration. In addition, scopolamine treatment and *Chrm3* ablation are systemic inhibitors of muscarinic receptor activation, whereas vagotomy results in a regional effect.

Vagotomy may also modulate liver injury by preventing nicotinic receptor activation in Kupffer cells. Vagotomy promoted anti-Fas antibody-induced hepatocyte apoptosis and mortality in mice; that effect was blocked by nicotine and an $\alpha 7$ nicotinic receptor agonist (Hiramoto et al., 2008). Studies using vagotomy to investigate cholinergic regulation of liver injury may also create an imbalance between sympathetic and parasympathetic regulation of liver regeneration and oval cell proliferation. A growing body of evidence suggests that the sympathetic nervous system plays a major role in regulating liver injury. Specifically, sympathetic neural input inhibition reduces hepatocyte injury and promotes oval cell proliferation (Oben and Diehl, 2004). Finally, oval cells were shown to express vasoactive intestinal peptide (VIP) receptors and receive input from VIP-containing neurons (Miyazawa et al., 1988; Akiyoshi et al., 1998; Cassiman et al., 2007). Hence, in addition to acetylcholine, the vagus nerve stores and releases other neurotransmitters, including VIP that may modulate liver injury responses.

In conclusion, our findings demonstrate convincingly that in mice, pharmacological inhibition or genetic ablation of *Chrm3* augments AOM-induced chronic liver injury. In particular, we observed attenuated hepatocyte restoration and

augmented ductular hyperplasia and oval cell expansion. Potential therapeutic implications of these findings include pharmacological or other approaches to modulating muscarinic receptor activity as a means of preventing liver injury or hastening repair. Future work will be directed at defining the role of muscarinic receptors in mediating responses to liver injury caused by other hepatotoxins (e.g., ethanol and acetaminophen) and determining whether stimulating muscarinic receptor activation attenuates hepatic injury.

References

- Akiyoshi H (1989) Ultrastructure of cholinergic innervation in the cirrhotic liver in guinea pigs. Neurohistochemical and ultrastructural study. *Virchows Arch B Cell Pathol Incl Mol Pathol* **57**:81–90.
- Akiyoshi H, Gonda T, and Terada T (1998) A comparative histochemical and immunohistochemical study of aminergic, cholinergic and peptidergic innervation in rat, hamster, guinea pig, dog and human livers. *Liver* **18**:352–359.
- Alvaro D, Alpini G, Jezequel AM, Bassotti C, Francia C, Fraioli F, Romeo R, Marucci L, Le Sage G, Glaser SS, et al. (1997) Role and mechanisms of action of acetylcholine in the regulation of rat cholangiocyte secretory functions. *J Clin Invest* **100**:1349–1362.
- Bélanger M, Côté J, and Butterworth RF (2006) Neurobiological characterization of an azoxymethane mouse model of acute liver failure. *Neurochem Int* **48**:434–440.
- Bernik TR, Friedman SG, Ochani M, DiRaimo R, Susarla S, Czura CJ, and Tracey KJ (2002) Cholinergic antiinflammatory pathway inhibition of tumor necrosis factor during ischemia reperfusion. *J Vasc Surg* **36**:1231–1236.
- Cassiman D, Libbrecht L, Sinelli N, Desmet V, Denef C, and Roskams T (2002) The vagal nerve stimulates activation of the hepatic progenitor cell compartment via muscarinic acetylcholine receptor type 3. *Am J Pathol* **161**:521–530.
- Cassiman D, Sinelli N, Bockx I, Vander Borgh S, Petersen B, De Vos R, van Pelt J, Nevens F, Libbrecht L, and Roskams T (2007) Human hepatic progenitor cells express vasoactive intestinal peptide receptor type 2 and receive nerve endings. *Liver Int* **27**:323–328.
- Chen F, Ohashi N, Li W, Eckman C, and Nguyen JH (2009) Disruptions of occludin and claudin-5 in brain endothelial cells in vitro and in brains of mice with acute liver failure. *Hepatology* **50**:1914–1923.
- Clouston AD, Powell EE, Walsh MJ, Richardson MM, Demetris AJ, and Jonsson JR (2005) Fibrosis correlates with a ductular reaction in hepatitis C: roles of impaired replication, progenitor cells and steatosis. *Hepatology* **41**:809–818.
- Forbes S, Vig P, Poulsom R, Thomas H, and Alison M (2002) Hepatic stem cells. *J Pathol* **197**:510–518.
- Fotiadu A, Tzioufa V, Vrettou E, Koufogiannis D, Papadimitriou CS, and Hytioglou P (2004) Progenitor cell activation in chronic viral hepatitis. *Liver Int* **24**:268–274.
- Hiramoto T, Chida Y, Sonoda J, Yoshihara K, Sudo N, and Kubo C (2008) The hepatic vagus nerve attenuates Fas-induced apoptosis in the mouse liver via $\alpha 7$ nicotinic acetylcholine receptor. *Gastroenterology* **134**:2122–2131.
- Hirono I (1981) Natural carcinogenic products of plant origin. *Crit Rev Toxicol* **8**:235–277.
- Ikedo A, Ozaki M, Murata S, Matsuo R, Nakano Y, Watanabe M, Hisakura K, Myronovych A, Kawasaki T, Kohno K, et al. (2009) Autonomic regulation of liver regeneration after partial hepatectomy in mice. *J Surg Res* **152**:218–223.
- Kiba T (2002) The role of the autonomic nervous system in liver regeneration and apoptosis—recent developments. *Digestion* **66**:79–88.
- Kiba T, Tanaka K, Numata K, Hoshino M, and Inoue S (1994) Facilitation of liver regeneration after partial hepatectomy by ventromedial hypothalamic lesions in rats. *Pflügers Arch* **428**:26–29.
- Laqueur GL, Mickelsen O, Whiting MG, and Kurland LT (1963) Carcinogenic properties of nuts from *Cycas circinalis* L. indigenous to Guam. *J Natl Cancer Inst* **31**:919–951.
- Lee TK, Castilho A, Ma S, and Ng IO (2009) Liver cancer stem cells: implications for a new therapeutic target. *Liver Int* **29**:955–965.
- LeSag E, Alvaro D, Benedetti A, Glaser S, Marucci L, Baiocchi L, Eisel W, Caligiuri A, Phinzy JL, Rodgers R, et al. (1999) Cholinergic system modulates growth, apoptosis, and secretion of cholangiocytes from bile duct-ligated rats. *Gastroenterology* **117**:191–199.
- Li JH, Gautam D, Han SJ, Guettier JM, Cui Y, Lu H, Deng C, O'Hare J, Jou W, Gavrilova O, et al. (2009) Hepatic muscarinic acetylcholine receptors are not critically involved in maintaining glucose homeostasis in mice. *Diabetes* **58**:2776–2787.
- Libbrecht L, Desmet V, Van Damme B, and Roskams T (2000) The immunohistochemical phenotype of dysplastic foci in human liver: correlation with putative progenitor cells. *J Hepatol* **33**:76–84.
- Matkowskyj KA, Marrero JA, Carroll RE, Danilovich AV, Green RM, and Benya RV (1999) Azoxymethane-induced fulminant hepatic failure in C57BL/6J mice: characterization of a new animal model. *Am J Physiol* **277**:G455–G462.
- Miyazawa Y, Fukuda Y, Imoto M, Koyama Y, and Nagura H (1988) Immunohistochemical studies on the distribution of nerve fibers in chronic liver diseases. *Am J Gastroenterol* **83**:1108–1114.
- Newsome PN, Hussain MA, and Theise ND (2004) Hepatic oval cells: helping redefine a paradigm in stem cell biology. *Curr Top Dev Biol* **61**:1–28.
- Oben JA and Diehl AM (2004) Sympathetic nervous system regulation of liver repair. *Anat Rec A Discov Mol Cell Evol Biol* **280**:874–883.
- Ohtake M, Sakaguchi T, Yoshida K, and Muto T (1993) Hepatic branch vagotomy can suppress liver regeneration in partially hepatectomized rats. *HPB Surg* **6**:277–286.
- Okabe M, Tsukahara Y, Tanaka M, Suzuki K, Saito S, Kamiya Y, Tsujimura T, Nakamura K, and Miyajima A (2009) Potential hepatic stem cells reside in EpCAM+ cells of normal and injured mouse liver. *Development* **136**:1951–1960.

- Raufman JP, Chen Y, Cheng K, Compadre C, Compadre L, and Zimniak P (2002) Selective interaction of bile acids with muscarinic receptors: a case of molecular mimicry. *Eur J Pharmacol* **457**:77–84.
- Raufman JP, Samimi R, Shah N, Khurana S, Shant J, Drachenberg C, Xie G, Wess J, and Cheng K (2008) Genetic ablation of M3 muscarinic receptors attenuates murine colon epithelial cell proliferation and neoplasia. *Cancer Res* **68**:3573–3578.
- Ray MB, Mendenhall CL, French SW, and Gartside PS (1993) Bile duct changes in alcoholic liver disease. The Veterans Administration Cooperative Study Group. *Liver* **13**:36–45.
- Roskams T, Yang SQ, Koteish A, Durnez A, DeVos R, Huang X, Achten R, Verslype C, and Diehl AM (2003) Oxidative stress and oval cell accumulation in mice and humans with alcoholic and nonalcoholic fatty liver disease. *Am J Pathol* **163**:1301–1311.
- Roskams TA, Theise ND, Balabaud C, Bhagat G, Bhathal PS, Bioulac-Sage P, Brunt EM, Crawford JM, Crosby HA, Desmet V, et al. (2004) Nomenclature of the finer branches of the biliary tree: canals, ductules, and ductular reactions in human livers. *Hepatology* **39**:1739–1745.
- Shah N, Khurana S, Cheng K, and Raufman JP (2009) Muscarinic receptors and ligands in cancer. *Am J Physiol Cell Physiol* **296**:C221–C232.
- Shan W, Nicol CJ, Ito S, Bility MT, Kennett MJ, Ward JM, Gonzalez FJ, and Peters JM (2008) Peroxisome proliferator-activated receptor-beta/delta protects against chemically induced liver toxicity in mice. *Hepatology* **47**:225–235.
- van Koppen CJ and Kaiser B (2003) Regulation of muscarinic acetylcholine receptor signaling. *Pharmacol Ther* **98**:197–220.
- Ward JM (1975) Dose response to a single injection of azoxymethane in rats. Induction of tumors in the gastrointestinal tract, auditory sebaceous glands, kidney, liver and preputial gland. *Vet Pathol* **12**:165–177.
- Yamada M, Miyakawa T, Duttaroy A, Yamanaka A, Moriguchi T, Makita R, Ogawa M, Chou CJ, Xia B, Crawley JN, et al. (2001) Mice lacking the M3 muscarinic acetylcholine receptor are hypophagic and lean. *Nature* **410**:207–212.
- Yovchev MI, Grozdanov PN, Zhou H, Racherla H, Guha C, and Dabeva MD (2008) Identification of adult hepatic progenitor cells capable of repopulating injured rat liver. *Hepatology* **47**:636–647.
- Zedeck MS and Swislocki NI (1975) Effect of methylazoxymethanol acetate on adenylate cyclase and 5'-nucleotidase in rat liver plasma membranes. *Cancer Lett* **1**:109–112.

Address correspondence to: Dr. Sandeep Khurana, Division of Gastroenterology and Hepatology, University of Maryland School of Medicine, 22 South Greene St., N3W50, Baltimore, MD 21201. E-mail: skhurana@medicine.umaryland.edu