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The Vagal Nerve Stimulates Activation of the Hepatic Progenitor Cell Compartment via Muscarinic Acetylcholine Receptor Type 3

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In the rat the hepatic branch of the nervus vagus stimulates proliferation of hepatocytes after partial hepatectomy and growth of bile duct epithelial cells after bile duct ligation. We studied the effect of hepatic vagotomy on the activation of the hepatic progenitor cell compartment in human and rat liver. The number of hepatic progenitor cells and atypical reactive ductular cells in transplanted (denervated) human livers with hepatitis was significantly lower than in innervated matched control livers and the number of oval cells in vagotomized rat livers with galactosamine hepatitis was significantly lower than in livers of sham-operated rats with galactosamine hepatitis. The expression of muscarinic acetylcholine receptors (M1-M5 receptor) was studied by immunohistochemistry and reverse transcriptase-polymerase chain reaction. In human liver, immunoreactivity for M3 receptor was observed in hepatic progenitor cells, atypical reactive ductules, intermediate hepatocyte-like cells, and bile duct epithelial cells. mRNA for the M1-M3 and the M5 receptor, but not the M4 receptor, was detected in human liver homogenates. In conclusion, the hepatic vagus branch stimulates activation of the hepatic progenitor cell compartment in diseased liver, most likely through binding of acetylcholine to the M3 receptor expressed on these cells. These findings may be of clinical importance for patients with a transplant liver. *(Am J Pathol 2002, 161:521–530)*

Hepatic progenitor cells (HPCs) are small epithelial cells that can differentiate toward the hepatocytic and the biliary lineage.[1–6] Because HPCs have an oval nucleus, they are usually referred to as "oval cells" in the rat liver.[7] In the nondiseased liver, HPCs are located in the canals of Hering, which are the smallest ramifications of the biliary tree that connect to the hepatocytes in the periportal area.[8]

In various human liver diseases and experimental animal models that are characterized by damage and loss of hepatocytes and/or bile duct epithelial cells, the number of HPCs strongly increases and atypical reactive ductules and intermediate hepatocyte-like cells appear.[3,9–12] The former represent differentiation of the HPCs toward bile duct epithelial cells and the latter toward hepatocytes.[3,10,12] The increase in number of HPCs and atypical reactive ductules is referred to as "activation" of the HPC or oval cell compartment.[13,14] Studies in experimental models of liver injury have led to the identification of a number of autocrine and paracrine factors that are involved in the regulation of oval cell activation in rat liver. Identified factors include transforming growth factor-α, hepatocyte growth factor and its receptor c-met, and the plasminogen activator/plasmin system.[15–17] It remains to be shown whether these or other factors also have a function in the activation of the human HPC compartment.

Hepatic regeneration after partial hepatectomy (PHx) occurs through the proliferation of mature hepatocytes and bile duct epithelial cells[18] and it has previously been shown that this process is suppressed by hepatic branch vagotomy and facilitated by stimulation of the nervus vagus.[19–21] It is not clear how the nervus vagus influences proliferation of hepatocytes after PHx, but it is not via binding of acetylcholine to nicotinic acetylcholine receptors because these are not expressed in the liver.[22–25] Experiments in which bile duct-ligated rats underwent cervical vagotomy have revealed that the nervus vagus directly stimulates growth of bile duct epithelial cells via binding of acetylcholine to the muscarinic acetylcholine receptor type 3 (M3 receptor).[26] Outside the liver, *in vitro* binding of acetylcholine or an analog of acetylcholine to the M3 receptor has been shown to stimulate the proliferation of human colon cancer cells, rat astrocytes, human astrocytoma cells, human prostate cancer cells and human oligodendrocyte progenitors.[27–30] It is not known whether the nervus vagus also

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Table 1. Patient Characteristics

	K/L	Fibr	Infl	Duct	HPC	Duct+HPC	Tx	IS	P	D
I	K	4	1/1	70.0	10.7	80.7	52	M/T	PC/HCV	84
	L	4	1/1	45.3	8.7	54.0	35	T	HCV	29
II	K	0	1/1	15.3	2.7	18.0	12	Cs/C/M	HBV	120
	L	0	1/1	14.7	2.0	16.7	14	T/C/M	Alcohol	*
	L	0	1/1	18.7	7.0	25.7	12	Cs/Az	Cryptogenic	*
	L	0	1/1	12.3	0.7	13.0	12	Cs/C/Az	HCV	*
III	K	0	1/2	18.3	9.3	27.6	178	C/Az	HBV	180
	L	0	1/2	17.3	4.7	22.0	32	Cs/Az	HCV	*
IV	K	1	2/1	51.3	10.7	62.0	122	Cs/C/Az	HBV	*
	K	1	2/1	24.7	6.7	31.4	216	C/Az	HBV	156
	K	1	2/1	42.7	11.3	54.0	276	C/M	HCV	*
	L	1	2/1	23.7	2.3	26.0	112	Cs/Az	PBC	*
	L	1	2/1	13.0	1.3	14.3	19	Cs/M	Alcohol	*
	L	1	2/1	20.7	1.0	21.7	10	T/M	HCV	*
	L	1	2/1	24.0	6.3	30.3	24	Cs/M	HCV	19
	L	1	2/1	10.0	1.3	11.3	59	Cs/Az	PBC	*

I-IV, cluster of biopsies, matched for necro-inflammation and fibrosis; K, kidney transplant patient; L, liver transplant patient; Infl, inflammation score; Fibr, fibrosis score; duct, number of atypical reactive ductular cells per field; HPC, number of HPCs per field; duct+HPC, total number of cells in the HPC compartment; Tx, time elapsed (months) after transplantation (= time on immunosuppressive therapy); IS, immunosuppression scheme (M, mycophenolate mofetil; T, tacrolimus; Cs, cyclosporin; C, corticosteroids (methylprednisolon); Az, azathioprin); P, liver pathology diagnosis on biopsy for kidney transplant patients and on explant liver for liver transplant patients; D, time (months) since diagnosis of hepatitis (since diagnosis of recurrence of hepatitis, in case of liver transplantation); *, missing data.

influences the activation of oval cells, because the studied animal models (bile duct ligation and PHx) do not involve oval cell activation.[18,31]

We have previously shown that the human HPC compartment has neural/neuroendocrine features, including the expression of parathyroid hormone-related peptide, chromogranin-A, neural cell adhesion molecule (NCAM), neurotrophin 4/5 and the neurotrophin receptor tyrosine kinase B.[3,32,33] This suggests that neural/neuroendocrine factors might play a role in the activation of the human HPC compartment.

In view of these findings, we studied the expression of the muscarinic acetylcholine receptors in normal and diseased human liver and we evaluated the effect of vagotomy on the HPC compartment in both rat and human liver. This subject is of potential clinical importance because a transplanted liver is evidently denervated and reinnervation eventually only occurs in the hilum and the largest portal tracts.[34,35]

Materials and Methods

Liver Tissue Specimens

To study the effects of vagotomy on the HPC compartment in human liver, we used paraffin-embedded, B5-fixed tissue specimens of denervated and innervated livers, all affected by chronic hepatitis. The denervated liver specimens were biopsies from liver transplant patients suffering from recurrence of HCV hepatitis in the graft ($n = 5$) or from hepatitis of unknown origin ($n = 5$). They showed no signs of rejection. To control established confounding factors such as degree of necro-inflammation, degree of fibrosis, and immunosuppressive treatment,[11,12,36] the control liver biopsies (innervated) were from kidney transplant patients ($n = 6$) with chronic HBV or HCV hepatitis who were also under immunosuppressive treatment at the time of biopsy and innervated control biopsies and denervated transplant liver biopsies were matched for necro-inflammatory activity and fibrosis (see Table 1 for patient characteristics) using the Scheuer system.[37] At the time of biopsy, all 16 patients showed a bilirubin level within the normal range. In this way, all factors that are known to influence activation of the HPC compartment[11,12,36] were excluded, except presence or absence of the innervation.

To study the effects of vagotomy on oval cell activation in rat liver, we used male Wistar rats with a weight of 300 to 350 g. The rats were fed *ad libitum* and received human care in accordance with the institution's ethical guidelines. Eight rats were subjected to hepatic branch vagotomy, as previously described,[19] and eight rats were sham-operated. Immediately after the operation, all 16 rats were intoxicated. Galactosamine intoxication was induced by application of galactosamine (Sigma, Steinheim, Germany) in the abdominal cavity (galactosamine was diluted to 150 mg/ml in NaCl 0.9% and applied in a dose of 500 mg/kg). Liver biopsies, taken at 48 hours after intoxication, were snap-frozen in liquid nitrogen-cooled isopentane and stored at −70°C, until further use. The 48-hour time point is the time point of maximal hepatitis and maximal number of oval cells.[38,39]

To study the expression of the muscarinic acetylcholine receptors in normal and diseased human liver, we used 35 liver biopsies that were snap-frozen in liquid nitrogen-cooled isopentane and stored at −70°C. The 35 biopsies included 5 biopsies from normal donor livers, 2 biopsies of resected focal nodular hyperplasia, 3 biopsies from the explant liver of patients with submassive liver cell necrosis, and 25 biopsies of patients with chronic liver disease in septal or cirrhotic stage because of HCV ($n = 10$), alcoholic hepatitis ($n = 5$), primary biliary cirrhosis ($n = 8$), and primary sclerosing cholangitis ($n = 2$).

Immunohistochemistry

Immunohistochemistry was performed with mouse monoclonal antibodies against cytokeratin (CK) 7 (dilution 1/50; DAKO, Glostrup, Denmark) and NCAM (dilution 1/40; Sigma), with OV-6 (dilution 1/200; Dr. S. Sell Albany Medical College, Albany, NY) and with goat polyclonal antibodies against the muscarinic acetylcholine receptor types 1 to 5 (M1-M5 receptor) (dilutions 1/30; Santa Cruz Biotechnology, Santa Cruz, CA). The anti-muscarinic acetylcholine receptor antibodies were shown to be specific for the muscarinic acetylcholine receptor subtype against which they were raised and do not cross-react with the other subtypes. Specificity of the M3 receptor antibody was further demonstrated in a Western blotting experiment on mouse brain extract (Santa Cruz Biotechnology).

After overnight drying and fixation in acetone for 10 minutes, 5-μm-thick cryostat sections were incubated with the primary antibodies. For the goat polyclonal antibodies, the second step consisted of swine anti-goat immunoglobulins followed by goat peroxidase anti-peroxidase (both from DAKO). For the monoclonal mouse antibodies, the second and third step consisted of peroxidase-labeled rabbit anti-mouse and peroxidase-labeled swine anti-rabbit immunoglobulins (both from DAKO) for human liver and of peroxidase-labeled goat anti-mouse and peroxidase-labeled swine anti-goat immunoglobulins (both from Sigma) for OV-6 immunohistochemistry on rat liver sections. Secondary and tertiary antibodies were diluted in phosphate-buffered saline (PBS), pH 7.2, containing 10% normal human serum. All incubations were performed for 30 minutes at room temperature and followed by a wash in three changes of PBS for 5 minutes.

Four-μm-thick sections were made from the paraffin-embedded human liver biopsies. After deparaffinization, rehydration, and heating in a microwave oven for 10 minutes at 750 W, the sections were incubated with the anti-CK7 antibody and subsequently with goat anti-mouse Envision (DAKO). Both incubations were performed for 30 minutes at room temperature and followed by a wash in three changes of PBS for 5 minutes.

For all stainings, the reaction product was developed with the use of 3-amino-9-ethylcarbazole or 3,3-diaminobenzidine tetrahydrochloride and H_2O_2, 0.01%. The sections were counterstained with hematoxylin. Negative controls consisted of omission of the primary antibody and were consistently negative. Cryostat sections of normal human salivary gland tissue were used as positive control for the M3 receptor.[40] In agreement with a previous study,[41] the staining showed immunoreactivity in the acini and intercalated and striated ducts.

Double immunostaining for the M3 receptor and CK7 was performed on 5-μm-thick human liver cryostat sections using fluorescein isothiocyanate-labeled bovine anti-goat immunoglobulins (Santa Cruz Biotechnology) followed by tetramethylrhodamine isothiocyanate-labeled rabbit anti-mouse immunoglobulins (DAKO). Double immunostaining was detected using a confocal laser-scanning microscope (Zeiss LSM 410, The Netherlands). Controls consisted of omission of the antibody against the M3 receptor and/or omission of the antibody against CK7 and were consistently negative. Additional controls consisted of incubation of the polyclonal goat antibody with the rabbit anti-mouse secondary antibody and incubation of the monoclonal mouse antibody with the bovine anti-goat secondary antibody. Nonspecific labeling could not be detected.

Histopathological Evaluation

HPCs were defined as small, singular cells with an oval nucleus and scanty cytoplasm immunoreactive for CK7 and OV-6. Atypical reactive ductules were defined as anastomosing ductules with poorly defined lumina, lined by flattened cells with scanty cytoplasm immunoreactive for CK7, OV-6, and NCAM. Intermediate hepatocyte-like cells were defined as polygonal cells with morphological resemblance to hepatocytes and faint to moderate immunoreactivity for CK7 and OV-6.[3,31] Oval cells were defined as OV-6-positive small cells with an oval nucleus and little cytoplasm forming strands at the portal-parenchymal interface.[10]

Cell Counting and Statistical Evaluation

The human sections from paraffin-embedded biopsies and the cryostat rat sections that were subjected to counting were stained for CK7 and OV-6, respectively. Cell counting was performed using a counter and an eyepiece with a grid, at magnification of $\times$100. Three portal tracts with an interlobular bile duct were randomly selected and visualized with the bile duct positioned in the center of the grid. If more than one bile duct was present, the largest was selected. The number of HPCs and cells lining atypical reactive ductules (in human specimens, Table 1) or the number of oval cells (in rats) in the field defined by the grid were counted. Only cells with a clear nucleus were counted. Statistical analysis of the human data were done by calculating an exact *P* value, based on probability. For the rat data, the Mann-Whitney test was used.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Frozen human liver tissue from explant livers and a normal donor liver was used. Total RNA was extracted and reverse transcribed and PCR was performed as previously described.[33] Regions of amplification were chosen in accordance with published regions of amplification.[42,43] Primers used were: 5′-gaaagggcgtgatcgagctggc-3′ (M1 receptor forward), 5′-cttgtcccagcggcaaagcagc-3′ (M1 receptor reverse), 5′-ctaagcaaacatgcatcagaattgg-3′ (M2 receptor forward), 5′-aaggtgcacaaaaggtgttaatgag-3′ (M2 receptor reverse), 5′-accagctccgagcagatggac-3′ (M3 receptor forward), 5′-cctgcaggttgtccgatgaggg-3′ (M3 receptor reverse), 5′-tcctcaagagcccactaatgaagc-3′ (M4 receptor forward), 5′-

atctggatcttggaccatctggag-3′ (M4 receptor reverse), 5′-ggcctataagttccgattggtgg-3′ (M5 receptor forward), 5′-tgactgggacacacttgtcacag-3′ (M5 receptor reverse). Annealing temperatures were 65°C for M1 and M3, 60°C for M2, 62°C for M4, and 57°C for M5 receptor. PCR products were calculated to a length of 325 bp for M1 receptor (accession no. NM000738.1), 288 bp for M2 receptor (accession no. NM000739.1), 209 bp for M3 receptor (accession no. NM000740.1), 298 bp for M4 receptor (accession no. NM000741.1), and 293 bp for M5 receptor (accession no. NM012125.1), as was confirmed on agarose gel electrophoresis stained with ethidium bromide. Identification of all amplification products was achieved by sequencing. All PCR reactions were performed on samples that were treated with RNase-free DNase I (from Life Technologies, Merelbeke, Belgium) before reverse transcription, to exclude amplification of genomic DNA. In addition, nonreverse-transcribed samples were run in parallel, to exclude artifacts from amplification of genomic DNA (results not shown). Positive control for M1 and M2 receptor was Sk-N-SH human neuroblastoma cell line (HTB-11; ATCC, Manassas, VA).[44] Positive control for M3, M4, and M5 receptor were human peripheral blood mononuclear cells,[45] isolated by density centrifugation as previously described.[46]

Results

The Number of HPCs and Atypical Reactive Ductular Cells in Human Transplant Livers (Denervated) Is Lower than in Matched Controls (Innervated)

The probability that our testing hypothesis (the number of HPCs, atypical reactive ductular cells, and the total number of cells in the HPC compartment in denervated liver is smaller than in a comparable innervated liver) was valid and that all but one of the liver transplant cases showed a lower number of HPCs and atypical reactive ductular cells (both separately and added up) than its matched control liver, as was the case in our study (Table 1), was calculated to be 0.0078. Thus, the number of HPCs and of atypical reactive ductular cells (both separately and added up) were significantly lower in liver biopsies of liver

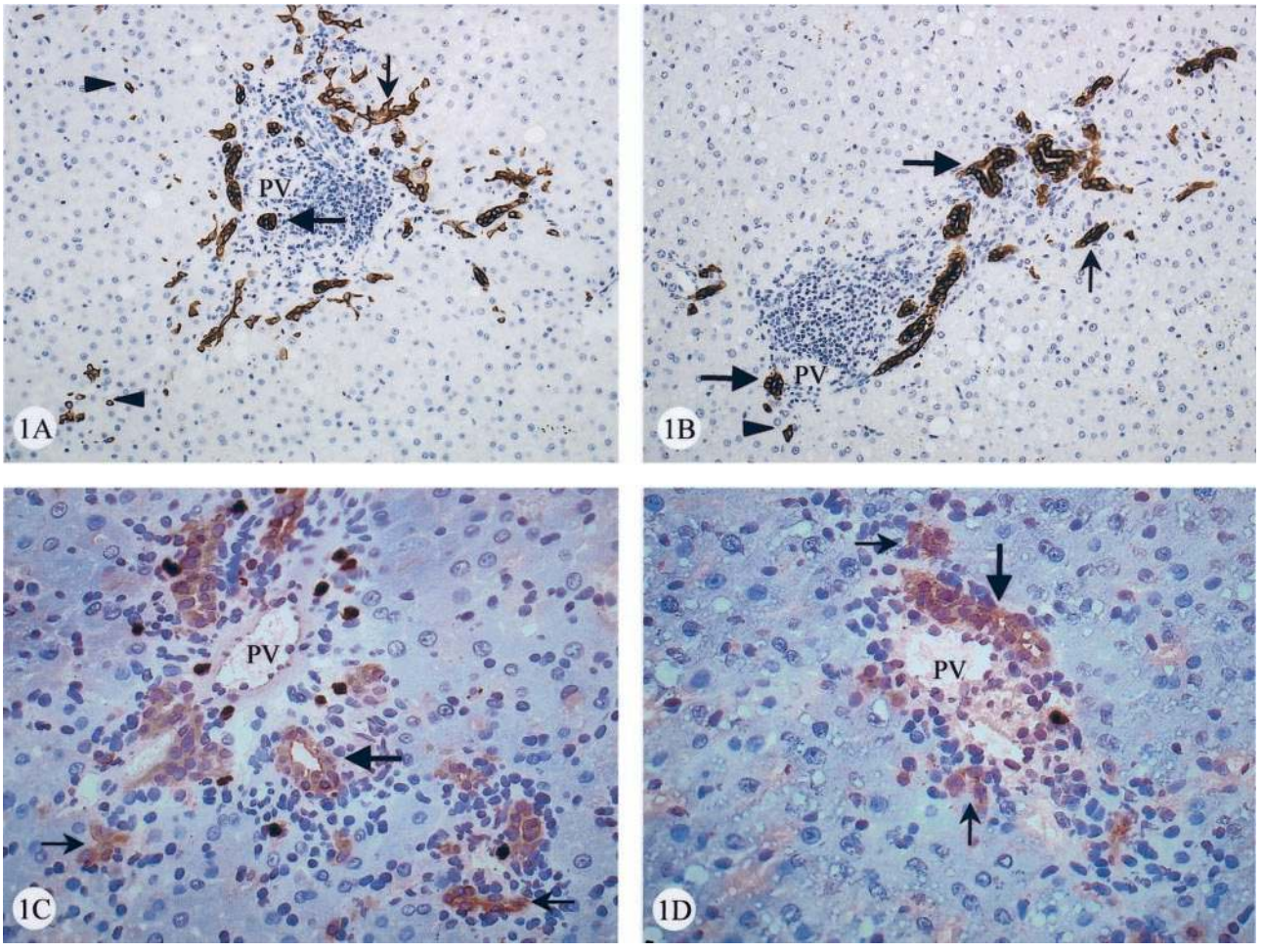

Figure 1. Immunohistochemistry for CK7 (**A** and **B**) and OV-6 (**C** and **D**). **A:** Liver biopsy from a kidney transplant patient with chronic hepatitis C (innervated). **B:** Matched biopsy from a transplant liver with recurrent chronic hepatitis C (denervated). **Small arrows**, atypical reactive ductular cells; **arrowheads**, HPCs; **large arrows**, interlobular bile duct(s). **C** and **D:** Liver biopsies of sham-operated (**C**) and vagotomized (**D**) galactosamine-intoxicated rats. **Small arrows**, oval cells; **large arrows**, interlobular bile duct; PV, portal vein. Original magnifications: ×200 (**A**, **B**); ×400 (**C**, **D**).

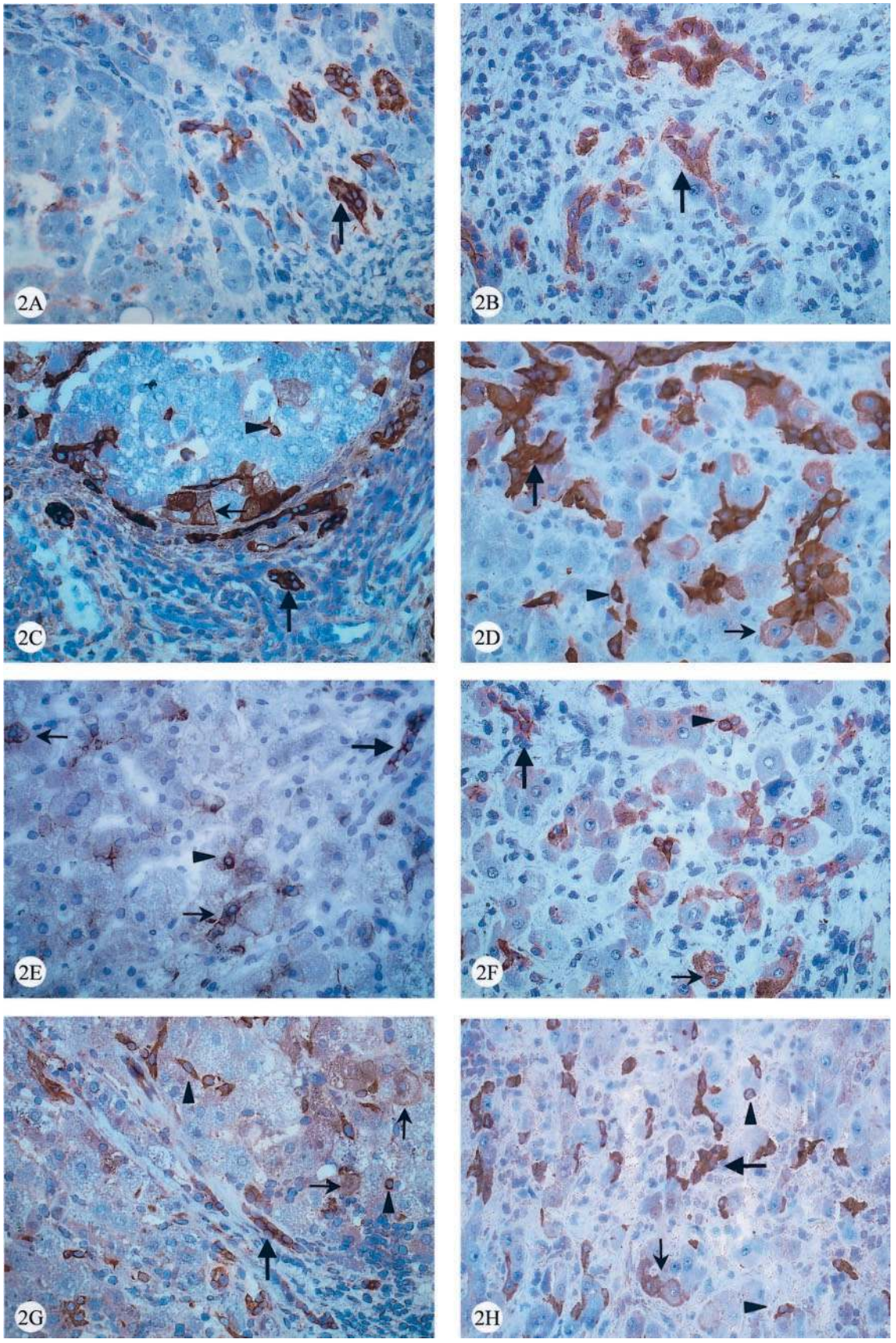

Figure 2. Immunohistochemical staining for NCAM (**A** and **B**), CK7 (**C** and **D**), OV-6 (**E** and **F**), and the M3 receptor (**G** and **H**). **A**, **C**, **E**, and **G:** Human liver, hepatitis C cirrhosis. **B**, **D**, **F**, and **H:** Human liver, toxic submassive liver cell necrosis. **Large arrows**, atypical reactive ductules; **arrowheads**, HPCs; **small arrows**, intermediate hepatocyte-like cells. Original magnifications, ×250.

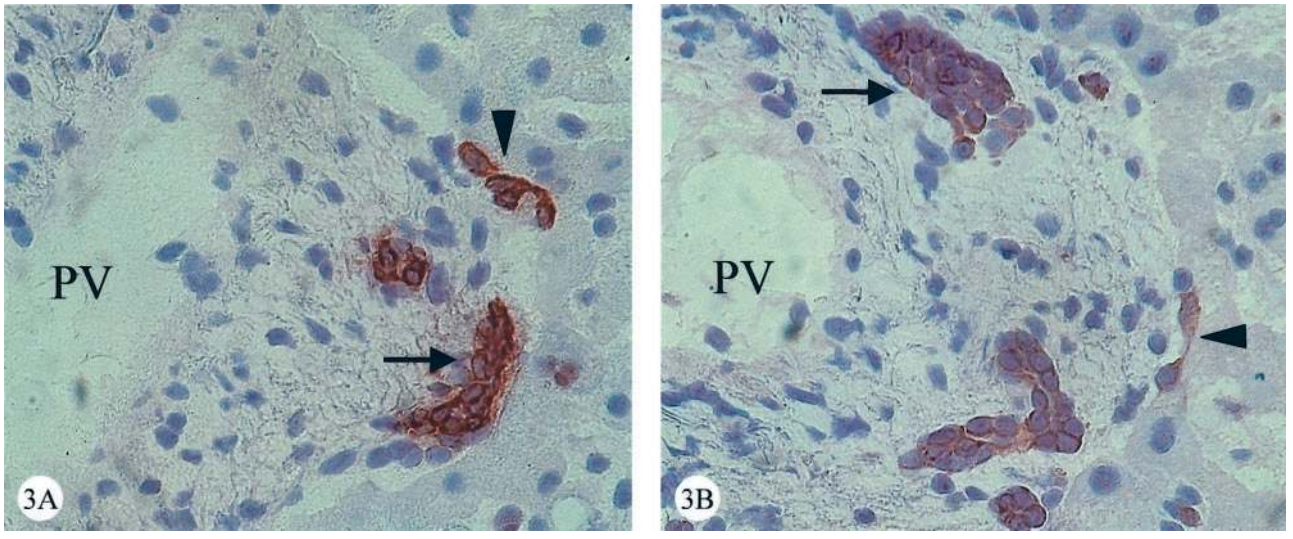

Figure 3. Normal donor liver. The interlobular bile duct (**arrow**) and the canal of Hering (**arrowhead**) are immunoreactive for CK7 (**A**) and the M3 receptor (**B**). PV, portal vein. Original magnifications, ×400.

transplant patients (denervated) than in livers of kidney transplant patients (innervated) (with $P = 0.0078$) (Figure 1, A and B).

The Number of Oval Cells after Galactosamine Intoxication Is Lower in Vagotomized Rat Livers than in Sham-Operated Rat Livers

The number of oval cells in selectively vagotomized rat livers was significantly lower than the number in sham-operated rat livers (9.17 ± 1.67 and 22.46 ± 5.07, respectively; $P = 0.0008$) at 48 hours after intoxication (Figure 1, C and D).

Human HPCs, Atypical Reactive Ductules, Intermediate Hepatocyte-Like Cells, and Cholangiocytes Express the M3 Receptor

Single immunohistochemical stainings for CK7, NCAM, and with OV-6 in specimens with primary sclerosing cholangitis, primary biliary cirrhosis, focal nodular hyperplasia and submassive liver cell necrosis were identical to what we described before (Figure 2; A to F).[3,47] Staining for the M3 receptor revealed the presence of intensely staining small cells with a similar morphology and localization as the HPCs (CK7+, OV-6+, NCAM−) in the different types of liver disease, indicating that HPCs express the M3 receptor. Atypical reactive ductules (CK7+, OV-6+, NCAM+) were always strongly positive for the M3 receptor. Furthermore, in the same areas where intermediate hepatocyte-like cells were present, cells with similar morphology and faint immunoreactivity for the M3 receptor were observed. The number of intermediate hepatocyte-like cells was lower when staining for the M3 receptor, than in sections stained for CK7 and OV-6. In all types of liver disease, cells lining the interlobular and septal bile ducts were immunoreactive for the M3 receptor. There was no immunoreactivity for the M3 receptor in hepatocytes, sinusoidal cells or any other cell type in any of the liver disorders studied (Figure 2, G and H).

In normal liver, cholangiocytes lining both large and small bile ducts were positive for the M3 receptor. Small cells located at the portal-parenchymal interface, most likely representing canals of Hering, were also positive (Figure 3). Other cell types did not show immunoreactivity.

Arterial endothelial cells were immunoreactive for the M1 receptor, both in normal and diseased liver (data not shown). Other cell types showed no immunoreactivity. Immunohistochemistry for the M2, M4 and M5 receptor revealed no immunoreactivity in normal and diseased liver. Repeated stainings with undiluted primary antibodies against the M2, M4 and M5 receptor also showed no positivity.

Immunoreactivity could not be obtained with any of the goat polyclonal antibodies against the different muscarinic acetylcholine receptor types on frozen liver sections from galactosamine-intoxicated rats. Additionally, we performed two immunohistochemical procedures (peroxidase anti-peroxidase and Envision) using rabbit polyclonal antibodies specific against the different muscarinic acetylcholine receptor subtypes (Biodesign) in different dilutions. On frozen liver sections from both human and galactosamine-intoxicated rats, immunoreactivity could not be obtained with any of these antibodies. The rabbit polyclonal antibody against the M3 receptor also yielded no immunoreactivity on frozen sections from normal human salivary gland (positive control).

In addition to the results obtained from serial sections, double immunostaining in human liver confirmed that all HPCs and atypical reactive ductules, revealed by CK7 staining, expressed the M3 receptor. Less than half of the intermediate hepatocyte-like cells staining for CK7 were also immunoreactive for the M3 receptor (Figure 4).

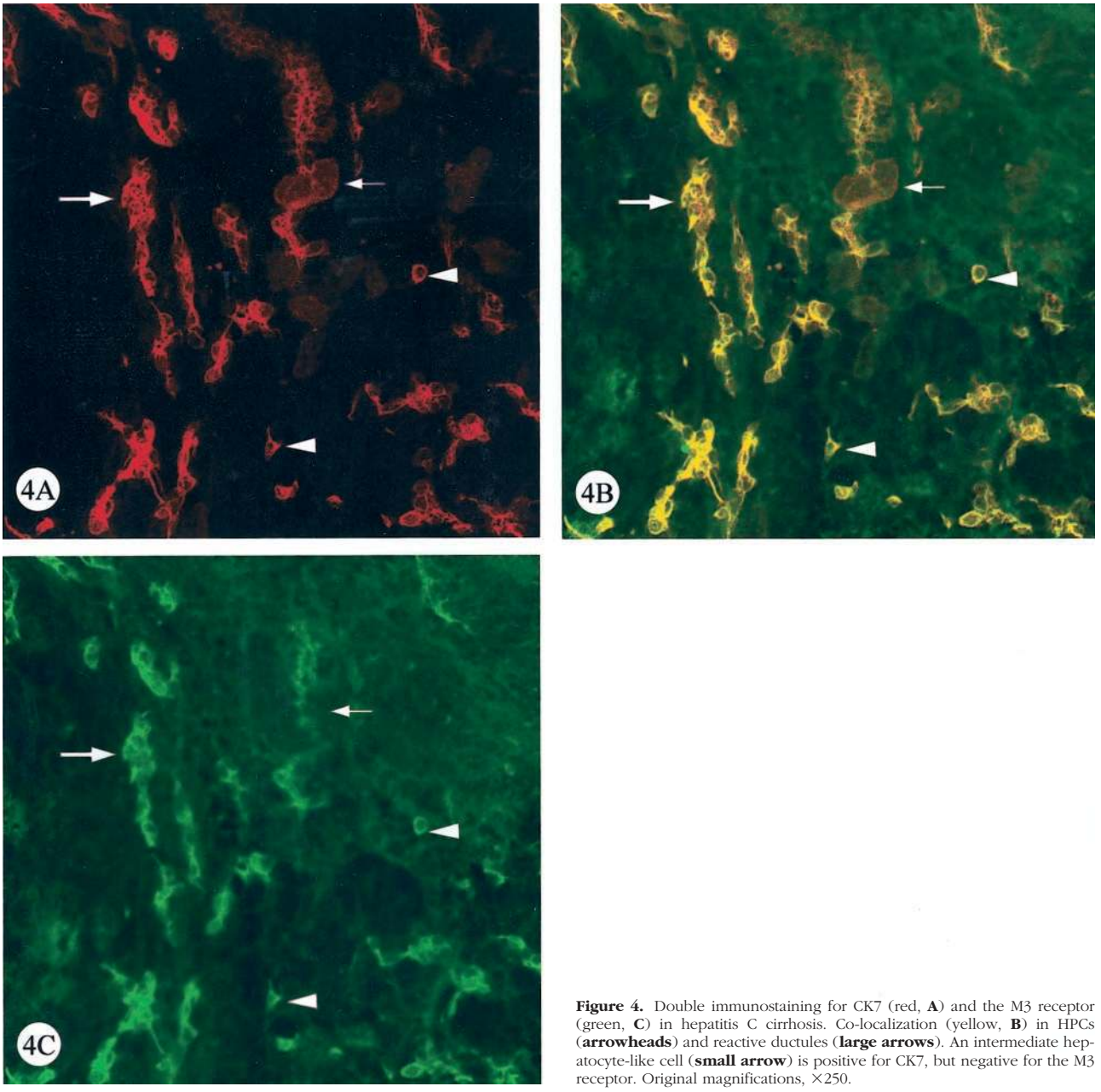

Figure 4. Double immunostaining for CK7 (red, **A**) and the M3 receptor (green, **C**) in hepatitis C cirrhosis. Co-localization (yellow, **B**) in HPCs (**arrowheads**) and reactive ductules (**large arrows**). An intermediate hepatocyte-like cell (**small arrow**) is positive for CK7, but negative for the M3 receptor. Original magnifications, ×250.

M3 Receptor mRNA Is Expressed in Human Liver

Transcription of the M1, M2, M3 and M5 receptor genes in homogenates of normal and diseased human livers was demonstrated by RT-PCR. M4 receptor mRNA could not be demonstrated in human liver, whereas the used positive control (human peripheral blood monocytes) did show M4 receptor amplification product (Figure 5).

Discussion

The results of the present study indicate that sectioning the hepatic vagus branch results in a significantly decreased activation of the HPC compartment in diseased liver. In humans, we show that transplanted (denervated) livers with hepatitis show significantly less HPCs and atypical reactive ductular cells than innervated control livers with hepatitis. We carefully matched the study and control livers for all factors that are known to influence HPC activation except presence or absence of the innervation. In addition, we only used liver tissue samples that showed no signs of rejection from patients with normal serum bilirubin levels. Nevertheless, because of the nature of a patient study, it could not be excluded that there were some unknown differences between the two groups that influenced HPC activation. To rule out this possibility and to rule out a possible influence of the adrenergic innervation, which is also lost on transplantation, we confirmed our human findings in animals by performing se-

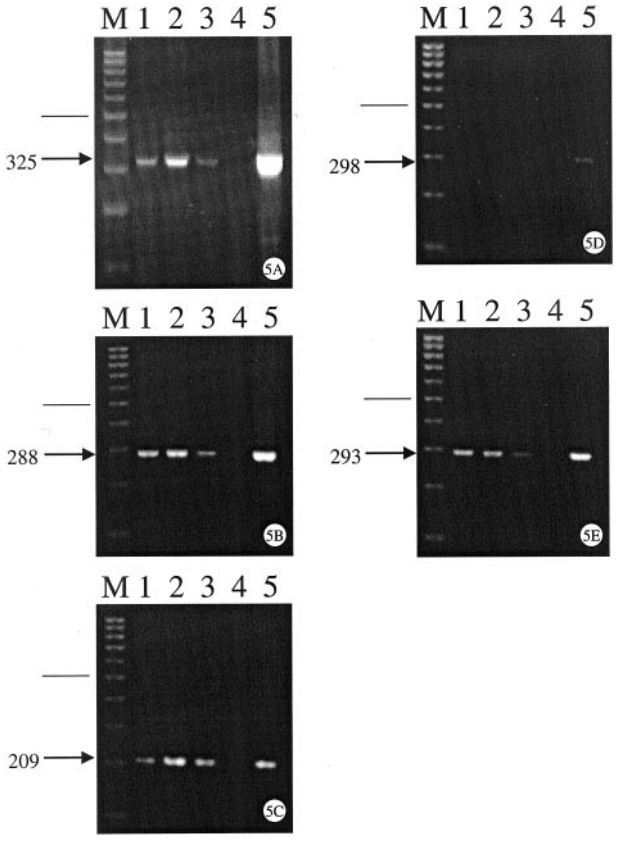

Figure 5. RT-PCR for the muscarinic acetylcholine receptor subtypes M1 to M5 (**A–E**). M: 100-bp ladder. Homogenate of a human explant liver of patients with primary biliary cirrhosis (**lane 1**) and toxic submassive liver failure (**lane 2**). **Lane 3**: Homogenate of a healthy human transplant liver, rejected on the basis of macroscopical anatomical defects. **Lane 4**: No template control. **Lane 5**: Positive control. **Line,** 500 bp; **arrow**, expected length of the amplification product.

lective hepatic vagotomy in the galactosamine-intoxicated rat. Galactosamine intoxication is a well-known animal model of liver injury that is associated with HPC activation,[13,48] in contrast to the models that were studied in combination with hepatic[19,21] or cervical[26] vagotomy. Selective hepatic vagotomy in combination with galactosamine-induced acute hepatitis in rats caused a significant decrease in the number of oval cells compared to sham operation in combination with galactosamine intoxication.

We also found that HPCs, atypical reactive ductules and intermediate hepatocyte-like cells in human liver express the M3 receptor, which strongly suggest that the stimulatory effects of the nervus vagus on the HPC compartment occurs via binding of acetylcholine to the M3 receptor present on these cell types. Although M2 and M5 mRNA transcripts were shown to be present in human liver homogenates, immunohistochemistry could not demonstrate the presence of the M2 and M5 receptor at the protein level. Posttranscriptional processes may inhibit the translation of mRNA into protein.[49]

Our data show that bile ducts in normal and diseased human liver express the M3 receptor. LeSage and colleagues[26] previously showed the presence of the M3 receptor on isolated rat cholangiocytes by Western blotting, but not by *in vivo* immunohistochemistry. Alvaro and colleagues[50] reported that isolated rat bile duct units were immunoreactive when exposed to the M35 antibody, but not when exposed to an antibody specific against the M2 receptor. Because the M35 antibody does not discriminate between muscarinic acetylcholine receptor subtypes,[51] these authors erroneously concluded that they demonstrated immunohistochemical expression of the M3 receptor on isolated rat bile duct units. Using primary antibodies from two different sources, we were not able to demonstrate immunohistochemical expression of the M3 receptor by bile ducts or other cell types in rat liver either, which indicates that there is low abundance of protein expression or that the used primary antibodies are not adequate for immunohistochemistry in rat liver.

Because hepatocytes in normal and diseased liver do not express any of the nicotinic and muscarinic acetylcholine receptors,[22–25] the stimulatory effect of the nervus vagus on proliferating hepatocytes after PHx must be indirect. It has been shown that the nervus vagus modulates sinusoidal blood flow[52,53] and the present study reveals that arterial endothelial cells express the M1 receptor. Therefore, it is very well possible that the stimulatory effect of the nervus vagus on proliferating hepatocytes after PHx is caused by the modulation of the sinusoidal blood flow.

Interestingly, hepatocytes produce and secrete cholinesterase and acetylcholinesterase is present at the cell surface membrane.[54–57] The concentration of serum cholinesterase accurately reflects the hepatocyte mass and can be used to assess the prognosis of patients with liver cirrhosis.[54,58] These observations and the findings of the present study have led us to hypothesize the following model that explains HPC behavior as observed in different conditions: in normal liver and after PHx, each HPC is surrounded by a normal number of hepatocytes that inhibits the binding of acetylcholine to the M3 receptor on the HPC by producing and secreting cholinesterase. As a consequence, the HPC compartment is not or only minimally activated in these conditions. When there is loss and impaired proliferation of hepatocytes (as is the case in almost all liver diseases), the cholinesterase activity will decrease proportionally to the topography and severity of hepatocyte loss. This allows acetylcholine to exert its trophic effects on the HPCs, until hepatocyte mass is restored again. In a transplant patient with a diseased liver, the stimulatory effect of the nervus vagus is abolished, which leads to an impaired HPC activation. Intriguingly, an atypical ductular reaction is absent in chronic liver allograft rejection,[59] despite the destruction of the bile ducts. The enigmatic paucity of atypical reactive ductules in this condition can be explained by our hypothesis. In our study, signs of rejection were absent from all included patient samples. Therefore, this factor could not have influenced the differences we observed between the numbers of HPCs and atypical reactive ductules between the study and control group.

In the hematopoietic system, which is related to the HPC compartment,[4,6,60,61] a similar mechanism seems to be present: acetylcholinesterase, which is synthesized

by mature red blood cells,[62] enhances apoptosis and reduces proliferation of mouse hematopoietic progenitor cells committed to erythroid and other lineages *in vitro*.[63] These and our findings are two examples of the nonclassical, autocrine/paracrine actions of the cholinergic system.[64,65]

In conclusion, the hepatic vagus branch stimulates activation of the HPC compartment in diseased liver, most likely through binding of acetylcholine to the M3 receptor expressed on these cells. These findings may be of clinical importance for patients with a transplant liver.

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