Hedgehog signaling maintains resident hepatic progenitors throughout life

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Hedgehog signaling maintains resident hepatic progenitors throughout life. Am J Physiol Gastrointest Liver Physiol 290: G859–G870, 2006. First published December 1, 2005; doi:10.1152/ajpgi.00456.2005.—Hedgehog signaling through its receptor, Patched, activates transcription of genes, including Patched, that regulate the fate of various progenitors. Although Hedgehog signaling is required for endodermal commitment and hepatogenesis, the possibility that it regulates liver turnover in adults had not been considered because mature liver epithelial cells lack Hedgehog signaling. Herein, we show that this pathway is essential throughout life for maintaining hepatic progenitors. Patched-expressing cells have been identified among endodermally lineage-restricted, murine embryonic stem cells as well as in livers of fetal and adult Ptc-lacZ mice. An adult-derived, murine hepatic progenitor cell line expresses Patched, and Hedgehog-responsive cells exist in stem cell compartments of fetal and adult human livers. In both species, manipulation of Hedgehog activity influences hepatic progenitor cell survival. Therefore, Hedgehog signaling is conserved in hepatic progenitors from fetal development through adulthood and may be a new therapeutic target in patients with liver damage.

Indian hedgehog; liver; Patched; progenitor cell; Sonic hedgehog

ADULT TISSUES ROUTINELY TURNOVER, and this tissue renewal involves cells that span a wide spectrum of differentiation. Hyperplastic growth by diploid cells and hypertrophic growth by polyploid cells play important roles in this process (34, 46, 49). Ultimately, tissue renewal is fueled by cells of the stem cell compartment. Stem cell compartments have long been known to exist in adult skin and the gastrointestinal tract (10, 11) and have been assumed to exist in fetal, but not adult, livers (70). In adults, hepatic progenitors are thought to contribute to liver regeneration and turnover only when there is a significant loss of mature liver cells or when replication in mature hepatocytes is blocked (17, 69). Under these circumstances, the presence of a hepatic stem cell compartment becomes evident. This compartment is characterized by the appearance of hepatic progenitors, which are small cells (<10–12 μm in diameter) that coexpress hepatocytic and biliary antigens and accumulate in the peripoortal zone of the liver (20). The existence of a hepatic stem cell compartment in adult liver tissue has been supported further by the characterization of the antigenic and biochemical profiles of some of the hepatic progenitor populations in murine and human liver models (1, 49, 60, 65). Moreover, the Canals of Hering have been identified as the anatomic structures in which these progenitors localize in adult livers (62). In recent years, it has become apparent that subpopulations of precursors from extrahepatic sites can also acquire a hepatic fate (44, 61). However, a subsequent study (21) has questioned the contribution of such cells to liver regeneration in all but unusually severe liver injury. Thus consensus is emerging that in most settings, resident hepatic progenitors are a greater source of replacement hepatocytes than extrahepatic precursors (66). As in the skin and gut, chronic injury or oncogenic insults in the liver increase the local accumulation of progenitors (51, 69). The latter observations suggest that these cells play a greater role in tissue renewal after hepatic damage than in the normal, healthy liver.

To optimize progenitor cell participation in liver regeneration, it is necessary to understand the mechanisms that regulate their fate. One strategy to identify such mechanisms is to utilize knowledge gained from studying the hepatic specification of endodermal progenitors during embryogenesis. During gastrulation, Indian hedgehog (IHH) ligand is expressed in cells undergoing differentiation to become visceral endoderm (5, 19). Thereafter, endodermal cells that are destined to form the liver bud begin to express the DNA binding protein, GATA-4 (32). Some of these multipotent progenitors also express Patched (PTC), a receptor that engages ligands of the Hedgehog (Hh) family (57). Interaction between IHH or Sonic hedgehog (SHH) ligands and PTC activates Hh signaling and promotes the proliferation and/or differentiation of hepatic progenitor populations that ultimately form the liver bud (15).

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As the liver matures, Hh signaling abates in mature hepatocytes and cholangiocytes. Ultimately, most of the cells in adult livers do not express Ptc, a transcriptional target of the Hh pathway (6). These differentiation-dependent variations in the expression of Hh signaling components during liver development suggested to us that this pathway might identify and regulate a subset of residual, relatively primitive, intrahepatic progenitors in adult livers.

The purpose of the present study was to determine if murine and human liver precursor populations harbor such a reservoir of Ptc-expressing cells spanning from the time of endodermal differentiation to adulthood. In addition, we wanted to characterize the Ptc-expressing populations and determine if regulation of Hh signaling altered the viability of progenitors from fetal and adult livers.

Herein, we demonstrate that the Hh pathway regulates liver progenitors and that this process is conserved within the liver from embryogenesis through adulthood. Blockade of Hh activity in these cells. Because Hh signaling is required for the optimal viability of liver progenitors throughout life, this pathway is likely to modulate hepatic epithelial renewal in health and disease. Therefore, our work identifies Hh pathway components as novel therapeutic targets in chronic liver disease.

MATERIALS AND METHODS

Animal care. Adult male C57BL/6 mice were obtained from Jackson Laboratory (Bar Harbor, ME) and housed in a barrier facility. Adult male Ptc-lacZ reporter mice were obtained from Dr. P. A. Beachy (Johns Hopkins University; Baltimore, MD), and mouse embryos acquired from pregnant dams at 11.5 days postconception were obtained from Dr. E. Meyers (Duke University Medical Center; Durham, NC) (18). All animal experiments fulfilled National Institutes of Health, Johns Hopkins University, Duke University, and University of North Carolina (Chapel Hill, NC) requirements for humane animal care.

Embryonic stem cell culture and endodermal lineage restriction. Murine embryonic stem (ES) cells were cultured and endodermally lineage restricted as previously described (16). Media were changed daily, and cells were allowed to grow for 7 days before RNA isolation.

Culture of murine cell lines. A murine hepatic progenitor cell line (OV) was kindly provided by Dr. B. E. Petersen (University of Florida; Gainesville, FL) and cultured as previously described (44, 45). A well-differentiated murine hepatocytic cell line (AML-12, Hep) was obtained from the American Type Culture Collection (ATCC; Manassas, VA) and cultured according to ATCC instructions.

**Two-step real-time RT-PCR.** Total RNA was extracted from cells with the RNeasy kit followed by RNase-free DNase I treatment (Qiagen; Valencia, CA). Primers were designed using Genbank sequences or as previously described (Table 1) (6, 25, 26, 45, 54). Amplicon products were separated by electrophoresis on a 2.0% agarose gel buffered with 0.5× Tris-borate-EDTA (TBE). Products were visualized using an Alphalmager 3400 Gel Analysis System (Alpha Innotech; San Leandro, CA) with ultraviolet transillumination and were recorded using a 12-bit charge-coupled device camera that was controlled by AlphaEaseFC 4.0.1 software (Alpha Innotech). For each experiment, total RNA was reverse transcribed to cDNA templates and amplified using Ready-To-Go You-Prime First-Strand Beads (Amersham Biosciences; Piscataway, NJ) with pd(N)6 first-strand cDNA primers (Amersham Biosciences). For quantitative RT-PCR, 1.5% of the first-strand reaction was amplified using iQ-SYBR Green Supermix (Bio-Rad; Hercules, CA), an iCycler iQ Real-Time Detection System (Bio-Rad), and specific oligonucleotide primers for target sequences as well as the β-glucuronidase housekeeping gene. The PCR parameters were as follows: denaturing at 95°C for 3 min followed by 40 cycles of denaturing at 95°C for 15 s and annealing-extension at the optimal primer temperatures for 45–60 s. Threshold cycles (Ct) were automatically calculated by the iCycler iQ Real-Time Detection System. Target gene levels in the treated cells are presented as a ratio to levels detected in the corresponding control cells according to the ΔΔCt method as previously reported (35). These fold changes were determined using point and interval estimates.

### Table 1. RT-PCR primers for analysis

<table>
<thead>
<tr>
<th>Gene</th>
<th>Genbank Accession No.</th>
<th>Direction</th>
<th>Sequence</th>
<th>Amplicon Size, bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gas (25)</td>
<td>NM_000181</td>
<td>Forward</td>
<td>5’-CTCATTTCGAGATTTGCCCAGTT-3’</td>
<td>81</td>
</tr>
<tr>
<td>Ssh (54)</td>
<td>NM_000193</td>
<td>Reverse</td>
<td>5’-CCGAGAGCCAGATCGCCCTTTTT-3’</td>
<td>109</td>
</tr>
<tr>
<td>Ihh (54)</td>
<td>NM_050846</td>
<td>Reverse</td>
<td>5’-TCCAGTCTCTCCACGGACTGTT-3’</td>
<td>109</td>
</tr>
<tr>
<td>Ptc (6)</td>
<td>NM_000261</td>
<td>Forward</td>
<td>5’-CCGCTCGAGGCTGAGAAGG-3’</td>
<td>72</td>
</tr>
<tr>
<td>Shh (54)</td>
<td>NM_010368</td>
<td>Reverse</td>
<td>5’-GCTGCGCTGTTTTCGGTCTCTG-3’</td>
<td>142</td>
</tr>
<tr>
<td>Ihh (54)</td>
<td>NM_010544</td>
<td>Forward</td>
<td>5’-CCGAGAATTCCTCACTCCTGTTG-3’</td>
<td>124</td>
</tr>
<tr>
<td>Ptc (54)</td>
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<td>5’-GCAAGATGGTTGCTGTTGGA-3’</td>
<td>168</td>
</tr>
<tr>
<td>Mpk</td>
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<td>Reverse</td>
<td>5’-GCAAACTGGATGTTGACAAAGG-3’</td>
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<tr>
<td>Afp (45)</td>
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<td>Forward</td>
<td>5’-CATCTTGGGTCAGGAGG-3’</td>
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<tr>
<td>Ck-19 (26)</td>
<td>NM_008471</td>
<td>Reverse</td>
<td>5’-GTCAATCTCTCGACGAGACAG-3’</td>
<td>174</td>
</tr>
</tbody>
</table>

Patched-lacZ staining and reporter assay. We studied mice in which one allele of Ptc is replaced in-frame with the β-galactosidase gene by homologous recombination to evaluate Hh signaling in the liver. Because Ptc is a transcriptional target of Hh signaling, expression of β-galactosidase (lacZ) indicates activation of the Hh pathway (18, 58). Staining and quantification of gene expression were performed as previously described using the β-Galactosidase Detection Kit (Promega; Madison, WI) (67). Images were visualized using an Axiovert 200 microscope (Carl Zeiss) at ×10–100 with halogen illumination and were recorded using an AxioCam HRc camera (Carl Zeiss) that was controlled by Axiovision 4.4 software (Carl Zeiss).

Immunofluorescent staining of mouse livers. Sections of formalin-fixed, paraffin-embedded mouse livers (7 μm thick) were used for immunofluorescent staining. Slides were deparaffinized in xylene and serially rehydrated in sequential ethanol (100–70%). Epitope retrieval was performed by microwave-incubating slides in 10 mM sodium citrate (pH 6.0)–0.05% Tween 20 for 10 min. Slides were then rinsed twice in PBS–0.05% Tween 20 for 5 min. Sections were then blocked with 2% goat and donkey serum for 10 min followed by incubation with primary antibodies for 45 min at room temperature. The primary antibodies were mouse monoclonal anti-β-galactosidase IgG (1:100, Promega) (23, 47, 71) and rabbit polyclonal anti-human callus cytokeratin antibody (1:200, DakoCytomation; Carpinteria, CA) (48). The secondary antibodies were Alexa 568-conjugated goat anti-mouse IgG (1:75, Molecular Probes; Carlsbad, CA) and FITC-conjugated donkey anti-rabbit IgG (1:100, Jackson ImmunoResearch Laboratories; West Grove, PA). Slides were coverslipped after the completion of staining. Negative controls were performed by omitting the primary antibody from the protocol and revealed minimal background staining (data not shown). Images were visualized using an Axiovert 200 microscope (Carl Zeiss) at ×63–100 magnification with epifluorescent illumination.

Isolation of murine liver cells. The nonparenchymal cell (NPC) fraction was isolated by in situ portal vein perfusion with pronase-collagenase (Sigma Aldrich; St. Louis, MO) to destroy mature hepa-tocytes and release NPCs from the liver matrix (66). NPCs were collected by density gradient centrifugation through OptiPrep (Accurate Chemical), which separates NPCs from nonviable cells and other liver cell populations. Cell fractions were pooled from two to six mice.

Sourcing of human liver cells. Human liver tissues were obtained following approval by the Institutional Review Board for Human Studies at the University of North Carolina. Prenatal livers were obtained from fetuses at 16–20 wk gestational age (Advanced Biological Resources; San Francisco, CA). Donated postnatal livers, not suitable for orthotopic liver transplantation, were obtained from federally designated organ procurement organizations.

Processing of human livers. Human fetal livers were processed as previously described (36) while cells from postnatal human livers were isolated by enzymatic digestion as previously described (9). The freshly isolated cell suspensions were subjected to immunoselection for cells expressing epithelial cell adhesion molecule (EpCAM), which has been previously noted to be one of the surface markers identifying hepatic progenitor cells in human livers (14, 49) (U.S. Patent Appl. 20050148072 and 20020182188). Human Liver Progenitors. L. Reid et al. 2002). Immunoselection was performed using monoclonal anti-EpCAM antibody coupled to magnetic microbeads according to the manufacturer’s instructions (Miltenyi Biotec; Auburn, CA).

Cytopsin of freshly isolated, immunofluorescently stained human hepatic progenitors. After enzymatic digestion of fetal livers, 106 freshly isolated cells were centrifuged at 600 g for 5 min at 4°C. Cells were then resuspended in PBS and 4% paraformaldehyde (1:1). After fixation for 10 min at room temperature, cells were centrifuged as previously described. The supernatant was aspirated, and cells were then blocked in PBS with 2% Triton X-100 (Sigma Aldrich), 10% goat or donkey serum (depending on the secondary antibody), and 2% teleostean gelatin (Sigma Aldrich). Cells were then incubated for 1 h at room temperature and then centrifuged as described above. The primary antibodies were employed for 100 min at room temperature as follows: mouse anti-human EpCAM (1:250, Lab Vision; Fremont, CA), goat anti-SHH (1:50, Santa Cruz Biotechnology; Santa Cruz, CA), rabbit anti-IHH (1:100, Santa Cruz Biotechnology), and rabbit anti-PTC (1:100, Santa Cruz Biotechnology). Cells were then washed twice in PBS–2% Triton X-100. The appropriate secondary antibodies were employed for 60 min at room temperature with 4′,6-diamidino-2-phenylindole (DAPI) as follows: Alexa 488-conjugated goat anti-rabbit IgG (1:1,000, Molecular Probes), Alexa 568-conjugated goat anti-mouse IgG (1:1,000, Molecular Probes), Texas red-conjugated donkey anti-mouse IgG (1:100, Molecular Probes), and Alexa 488-conjugated donkey anti-goat IgG (1:1,000, Molecular Probes). Cells were then washed twice in PBS–2% Triton X-100, centrifuged, resuspended in blocking buffer, and cytospin at 800 rpm for 3 min using precoated cytoslides (Shandon Anatomical Pathology; Pittsburgh, PA). Cells were then mounted in aqueous glycerol gelatine mounting media (Sigma Aldrich) and coverslipped. Images were visualized using a Leica SP2 AOBS laser scanning confocal microscope (Leica Microsystems; Bannockburn, IL) at ×189 that was controlled by Leica SP2 TCS software (Leica Microsystems). Negative controls were performed by omitting the primary antibody from the protocol and revealed no staining (data not shown).

Human liver immunohistochemistry. Serial sections of formalin-fixed, paraffin-embedded human livers (10 μm thick) were used for immunohistochemical staining. Sections were incubated twice in PBS with 0.3% Triton X-100 (Sigma Aldrich) for 10 min and then in 0.3% hydrogen peroxide solution to block endogenous peroxidase activity. Sections were then blocked with serum and treated with an avidin-biotin blocking kit (Vector Laboratories; Burlingame, CA) to inhibit nonspecific binding in the tissue. Sections were incubated with primary antibody overnight at 4°C. Protease digestion using the Auto/Zyme Reagent Set (Biomedia; Foster City, CA) was employed for cytokeratin-19 (CK-19) staining before blockade with serum. The primary antibodies employed were as follows: mouse anti-human CK-19 (1:100, Novocastra Laboratories; Newcastle, UK), mouse anti-human EpCAM (1:200, Miltenyi Biotec), rabbit anti-IHH (1:100, Santa Cruz Biotechnology), and goat anti-SHH (1:50, Antibodies Incorporated; Davis, CA). Sections were then incubated with biotinylated secondary antibody and ABC reagents of the Vectastain Elite Universal ABC kit according to the manufacturer’s instructions (Vector Laboratories). The secondary antibody was then detected using the avidin-biotin-peroxidase method with 3,3′-diaminobenzidine as the substrate (Vector Laboratories). For each antibody, negative controls were performed by omitting the primary antibody and/or using isotype control antibodies. These controls revealed no or minimal background staining (data not shown).

Colonies of human hepatic progenitors. After immunoselection, EpCAM+ cells from human fetal livers were plated at densities of 103–104 cells/100-mm2 plate in serum-free media tailored for hepatic progenitors and referred to as “Kubota’s medium” (29, 36). These clonogenically derived colonies expand for weeks at the rate of approximately 1 division/day and coexpress EpCAM, CK-19, telomerase, and other markers of human hepatic progenitors (L. M. Reid and E. Schmelzer, unpublished observations) (49).

Immunofluorescent staining of human hepatic progenitor colonies. After 2–3 wk of culture, the medium was removed from the colonies of human hepatic progenitors. Adherent cells were rinsed three times with Hank’s balanced salt solution (HBSS). Cells were then fixed with fresh acetonitrile-methanol (1:1) for 2 h at room temperature. Fixed cells were then rinsed three times with HBSS and were then incubated with 20% goat serum in HBSS for 4–6 h at 4°C. Cells were then rinsed three times with HBSS before incubation with primary antibody for 6–12 h at 4°C. The primary antibodies employed were as follows: goat anti-SHH (1:100, Antibodies Incorporated), rabbit anti-IHH (1:100, Santa Cruz Biotechnology), and goat anti-PTC (1:100, Santa Cruz Biotechnology). Cells were then washed twice in PBS–2% Triton X-100, centrifuged, resuspended in blocking buffer, and cytospin at 800 rpm for 3 min using precoated cytoslides (Shandon Anatomical Pathology; Pittsburgh, PA). Cells were then mounted in aqueous glycerol gelatine mounting media (Sigma Aldrich) and coverslipped. Images were visualized using a Leica SP2 AOBS laser scanning confocal microscope (Leica Microsystems; Bannockburn, IL) at ×189 that was controlled by Leica SP2 TCS software (Leica Microsystems). Negative controls were performed by omitting the primary antibody from the protocol and revealed no staining (data not shown).
Cruz Biotechnology). After incubation with primary antibody, cells were rinsed three times with HBSS, and secondary antibody was added for 75 min at room temperature. The appropriate secondary antibodies were employed as follows: Alexa 488-conjugated donkey anti-goat IgG or phycoerythrin-conjugated goat anti-rabbit IgG (1:800, Molecular Probes). Cells were then rinsed three times with HBSS and preserved with 2% formaldehyde in HBSS. Images were visualized using an Olympus IX70 microscope (Olympus Imaging America; Melville, NY) at ×4–10 with epifluorescent illumination and were recorded using a Nikon BM-2 camera (Nikon Instruments; Melville, NY) that was controlled by Nikon View 6.0.1 software (Nikon Instruments). Negative controls were performed by omitting the primary antibodies from the protocol and revealed no or minimal background staining (data not shown).

Pharmacological regulation of Hh signaling. Colonies of human hepatic progenitor cells and the murine hepatic progenitor cell line were treated with regulators of Hh signaling. Human hepatic progenitor cell colonies were treated with cyclopamine (10 μM, Calbiochem; San Diego, CA) (7, 8, 22, 28). The murine hepatic progenitor cell line was treated with recombinant Shh-N ligand (10–20 nM, Stem Cell Technologies) (6).

Cell death assays. Three- to four-week-old colonies of human hepatic progenitors from three different fetal livers were cultured as previously described. Imaging of the colonies was performed before treatment with cyclopamine (Calbiochem) and every 24 h after treatment. After 48 h, control and treated colonies were stained using Vybrant Apoptosis Assay Kit No. 8 (Molecular Probes) according to the manufacturer’s instructions. Briefly, colonies were washed twice with binding buffer and incubated for 15 min at 37°C with R-phycocerythrin annexin V (1:50) and SYTOX green (0.01 μM) diluted in binding buffer. Colonies were then washed twice, and fluorescent microscopy was performed. Numbers of dead cells stained with SYTOX green (i.e., necrotic cells) or R-phycocerythrin annexin V (i.e., apoptotic cells) were assessed by morphometric analysis with the Meta Morph Imaging System (Molecular Devices; Downingtown, PA). Immunofluorescently stained cells were quantitated in four representative fields of images under ×10 magnification. For the murine hepatic progenitor cell line, apoptotic activity was measured with an Apo-ONE Homogeneous Caspase-3/7 Apoptosis Assay (Promega) according to manufacturer’s instructions (56, 59). Briefly, the murine cell line was cultured for 24 h and then treated with Shh-N-supplemented medium or control medium for 48 h. In these replicate experiments (n = 4), fluorescence measurements were then performed using a FLUOstar OPTIMA microplate reader (BMG Labtech; Durham, NC).

Statistical analysis. Comparisons between groups were made using Intercooled Stata 8.0 (Stata; College Station, TX). For replicate experiments, data are reported as means ± SD. Comparisons between groups were performed using the Student’s t-test for real-time RT-PCR analyses; changes in target mRNA levels are represented by using point and interval estimates. Significance was accepted at the 5% level.

RESULTS

Hh ligands and receptors are differentially expressed in ES cells and mature hepatocytes. ES cells are pluripotent and can be propagated indefinitely in culture (30). ES cells require Hh signaling for differentiation into ectodermal (37, 38) and endodermal (37, 35) tissues. Recently, it has been reported that mouse ES cells treated for 7 days with acidic fibroblast growth factor can be differentiated into cells that are lineage restricted to the endoderm (16). When such cells were injected into a mouse model of hemophilia B that lacked clotting factor IX, these putative endodermal precursors engrafted within the livers and acquired hepatocyte functions, including hepatocyte-specific production of clotting factor IX, and the latter corrected hemophilia in recipient mice. In the present study, we evaluated murine ES cells and ES-derived putative endodermal precursors for expression of Hh ligands and receptors. Mouse ES cells expressed Hh ligands (Shh and Ihh) and the Hh receptor Ptc both initially (ES day 0) and when cultured under conditions that promote endodermal lineage restriction (ES day 7; Fig. 1A). Interestingly, as the ES cells differentiated toward an endodermal lineage, Shh expression fell, Ihh expression increased (Fig. 1B), and Ptc expression declined slightly (Fig. 1C). This is consistent with earlier reports (5, 19) showing that Ihh is expressed in the visceral endoderm of ES cell embryoid bodies and that Ihh is involved in visceral endodermal differentiation. In contrast to undifferentiated ES cells and endodermal progenitors, the well-differentiated hepatocytic cell line AML-12 did not express Ptc but continued to produce Hh ligands (Fig. 1). Therefore, well-differentiated hepatocytic cells did not exhibit evidence of active Hh signaling, whereas undifferentiated and endodermally lineage-restricted ES cells transcribed mRNA for the Hh receptor and gene target Ptc (57).

Hh-responsive cells exist in pre- and postnatal murine livers. Ptc-lacZ mice, which carry a transgene encoding β-galactosidase driven by Hh-responsive elements in the Ptc promoter,

Fig. 1. Endodermally lineage-restricted murine embryonic stem (ES) cells express Hedgehog (Hh) ligands and receptors. A: before (day 0) and after endodermal lineage restriction (day 7), murine ES cells were evaluated for expression of Hh ligands [Sonic Hh (Shh) and Indian Hh (Ihh)], the Hh receptor [Patched (Ptc)], and the β-glucuronidase (Gus) housekeeping gene. A well-differentiated hepatocytic cell line (Hep) was also evaluated for the same transcripts. Real-time RT-PCR demonstrated effects of differentiation upon expression of Hh ligands (B) and Ptc (C). Expression in ES cells is normalized to that of Hep. †*P < 0.05; ‡*P < 0.01.
have been used to identify cellular Hh activity (18, 43). To more definitively localize Hh-responsive hepatic progenitors in fetal livers, we examined whole mounts of Ptc-lacZ mouse embryos at 11.5 days postconception, which roughly corresponds to 16 wk of gestational age in human prenatal development. At this time point, the developing neural tube, notochord, and muscularis propria of the stomach had β-galactosidase-expressing cells, as expected (data not shown). Small cells interspersed between unstained fetal liver cells also demonstrated reporter activity (Fig. 2A). Thus, in late-gestational mouse embryos, Ptc is expressed by neural tissue, cells in the muscularis propria of the gut, and cells lining the hepatic sinusoids, but not by more mature epithelial cells of the stomach and liver.

We then examined four healthy Ptc-lacZ mice (10–14 wk old) to determine if, like fetal livers, adult livers harbored cells that expressed Ptc. As expected, the gallbladder wall from these mice had numerous β-galactosidase-positive cells (data not shown). Consistent with an earlier report (6), as well as our RT-PCR analysis of the hepatocytic cell line (Fig. 1A), Hh pathway activity was not detected in mature hepatocytes or cholangiocytes at ×10 magnification (Fig. 2B) or ×100 magnification (Fig. 2B, inset). However, at ×100 magnification, we found rare β-galactosidase-positive cells in the perportal zone of adult livers (Fig. 2C). These cells were much smaller in size than adjacent mature hepatocytes. The location and size of the Ptc-expressing cells suggested to us that, like hepatic progenitors, these small cells may reside among the hepatic NPC fraction. To verify this suspicion, we perfused the livers of two additional adult Ptc-lacZ mice and isolated the hepatocyte and NPC populations. Protein extracted from the NPC fraction exhibited β-galactosidase activity more than 45,000-fold higher than the hepatocyte fraction (P < 0.001; Fig. 2D), suggesting that the Hh-responsive cells in the perportal zone (Fig. 2C) coisolate with liver progenitors that reside within the NPC fraction of adult livers.

To determine if these Ptc-expressing cells colocalized with cells expressing a hepatic progenitor marker in the adult liver,
we performed immunofluorescent staining using anti-β-galactosidase antibody to mark Ptc-positive cells and polyclonal anti-cytokeratin antibody (48) to mark committed, bipotential hepatic progenitors. β-Galactosidase-positive cells were heterogeneous in nature (i.e., some, but not all, were pancytokeratin positive by immunohistochemistry). As previously noted, β-galactosidase-positive cells were mostly located in the periportal zones of the liver. Some were “periductal null” cells (31) (i.e., ductular-like cells separate from the biliary tree) and others were within bile ductules (40, 41, 62) (Fig. 2E). Rare clusters of small hepatocyte-like cells that coexpressed β-galactosidase and cytokeratins were also observed within the hepatic parenchyma (Fig. 2F).

Fetal human hepatic progenitors secrete Hh ligands. Although Ihh is known to regulate ES cell differentiation into the endoderm (19) and Shh is known to play an essential role in the hepatic specification of embryonic endodermal progenitors (15), the specific identity, phenotype, and tissue localization of Hh-secreting cells in the fetal liver are unknown. To clarify these issues, we used immunohistochemistry to examine fetal human livers. Our results demonstrated that Ihh (Fig. 3A) but not Shh (data not shown) was expressed in the stem cell compartments of fetal livers (e.g., ductal plate and adjacent hepatoblasts) (33, 62). Furthermore, these Ihh-secreting cells colocalized with multiple markers of hepatic epithelial progenitors, namely, CK-19 (Fig. 3B) (12, 13), EpCAM (Fig. 3C) (14), and α-fetoprotein (AFP; data not shown), suggesting that progenitors identified in the stem cell compartments of human livers express Ihh but not Shh. These findings in human fetuses complement and extend our findings from fetal and adult mouse livers and together demonstrate that Hh regulation of hepatic progenitors is conserved across vertebrate species.

Hepatic progenitors from human livers express components of the Hh pathway and are Hh responsive in vitro. To further investigate the significance of our findings showing that Hh ligands and receptors are expressed in pluripotent ES cells, endodermally lineage-restricted ES cells, and resident hepatic progenitors within fetal and adult livers, the relative mRNA expression pattern of Hh pathway components was compared between freshly immunoselected human liver cells lacking EpCAM expression (i.e., mature hepatocytes and nonepithelial liver cells) and EpCAM+ cells (i.e., hepatic progenitors) (2, 14) isolated from the livers of several (n = 8) young and old individuals (age range: 2 days–58 yr). In contrast to mature liver cells (EpCAM− fraction), EpCAM+ progenitor fractions were consistently enriched with Ihh (P < 0.05) and Ptc (P < 0.05) transcripts (Fig. 4, A and B), whereas both fractions had similar expression levels of Shh. Although it appeared that the progenitor cells tended to have more Ptc expression, EpCAM− fractions also contained cells that expressed Ptc transcripts. This may be explained by the occasional failure rate of EpCAM immunoselection (L. M. Reid and E. Schmelzer, unpublished observations) as well as by recent evidence showing that hepatic stellate cells, EpCAM− liver mesenchymal cells, also express components of the Hh pathway (54). To determine if, in addition to expressing mRNA for Hh pathway components, EpCAM+ progenitor cells produce SHH, IHH, and PTC proteins, we performed immunofluorescent staining to colocalize these proteins and EpCAM. We found that freshly isolated cytopsins of EpCAM+ cells also expressed all three Hh components (Fig. 4, C–N).

To corroborate our findings showing that freshly isolated cytopsins of liver cells include cells that coexpress EpCAM and Hh pathway components, clonally derived colonies of EpCAM+ cells were established in culture for 2–3 wk to eliminate contaminating cells, and immunofluorescent staining was performed for Hh ligands and PTC (n = 2 plates of colonies/primary antibody). Staining demonstrated colony expression of SHH (Fig. 5A), IHH (Fig. 5B), and PTC (Fig. 5, C and D). SHH was most strongly expressed in cells along the edge of the colony and colocalized with areas that most strongly expressed PTC. IHH expression was fairly uniform throughout the colonies. Next, we evaluated the effect of treating EpCAM+ colonies with cyclopamine, a potent and highly specific blocker of Hh signaling (8). Upon initiation of the experiment, representative colonies from the control group (n = 3 plates of colonies; Fig. 5E) and the treatment group (n = 3 plates of colonies; Fig. 5I) appeared morphologically similar. However, after 2 days of treatment with control medium or 10 μM cyclopamine-supplemented medium (7, 28), only cyclopamine-treated colonies (Fig. 5J) developed large bare fields within their centers as compared to control colonies (Fig. 5F). Therefore, cyclopamine significantly inhibited the viability of EpCAM+ cells in vitro. To determine if this effect was due to cellular necrosis or apoptosis, fluorescent staining was performed on the same colonies. In the control colonies, there was minimal cellular necrosis (Fig. 5G) or apoptosis (Fig. 5H). In contrast, the cyclopamine-treated colonies had a slight increase in the number of necrotic cells (Fig. 5K) and substantially higher numbers of cells undergoing apoptosis (Fig. 5L). Cyclopamine-induced cell death was morphometrically quantified and demonstrated a twofold increase in cell necrosis (P < 0.05) and a threefold increase in apoptosis (P < 0.01; Fig. 5M). Together, these
findings demonstrated that Hh signaling modulated the apoptosis of human hepatic progenitors and that Hh activity was necessary for optimal viability of these cells in culture.

**Murine hepatic progenitors are regulated by Hh signaling in vitro.** To verify the possibility that Hh activity regulated hepatic progenitor cell viability, we examined a murine hepatic progenitor cell line (OV) (44, 45). This murine progenitor line strongly expressed embryonic (muscle) pyruvate kinase (Mpk), Afp, and Ck-19, all markers of hepatic progenitors (64) (Fig. 6A). These committed progenitors also expressed Ihh and Ptc but not Shh (Fig. 6B). The latter finding was consistent with our earlier data demonstrating that IHH is present in human hepatic progenitors. In addition, the expression of Ptc was >35,000 fold higher than in our well-differentiated hepatocytic cell line (P < 0.01; Fig. 6C) but only 4.5% of the level in endodermally lineage-restricted ES cells, suggesting that there is a progressive decrease in Ptc gene expression with differentiation.

In our earlier experiments using colonies of human EpCAM+ cells, we demonstrated that Hh signaling regulated the fate of hepatic progenitors and is required for optimal viability. Recently, another group (63) demonstrated that the binding of SHH ligand to PTC prevented the induction of apoptosis mediated by caspase-3, and to a lesser extent caspase-7, in neuroepithelial cells. Therefore, we investigated if treating our murine hepatic progenitor cell line with recombinant Shh-N ligand would decrease caspase-3/7 activity, an indicator of cellular apoptosis. As expected, the presence of supplemental Hh ligand significantly decreased endogenous caspase activity in a dose-dependent fashion (P < 0.05; Fig. 6D). Therefore, as in neuroepithelial cells,
viability was optimized by the addition of SHH to Ptc-expressing murine hepatic progenitors.

**DISCUSSION**

Hh signaling, long known to be important in endodermal differentiation (5, 19) and liver organogenesis (15), has been found to persist in the maintenance of hepatic progenitors throughout life. Hepatic progenitors freshly isolated from human fetal, infant, and adult livers express Hh pathway components, as do ES cells lineage-restricted toward an endodermal fate and a well-characterized murine hepatic progenitor cell line. Furthermore, both human and mouse liver progenitors depend on Hh signaling for optimal viability in culture. Our studies localized cells that secrete Hh ligands to the same stem cell compartments that harbor Hh-responsive cells in fetal and adult livers. The comparison of pluripotent ES cells, endodermally lineage-restricted ES cells, and hepatic progenitors with mature hepatocyte epithelial cells suggests that Hh activity is extinguished as progenitors differentiate toward mature liver parenchymal cells. Therefore, although mature liver epithelial cells lack Hh pathway function, resident hepatic progenitors throughout life retain Hh activity and express components of this well-known fetal morphogenetic pathway.

To our knowledge, this is the first evidence that, like the skin (10), intestine (11), and bone marrow (3), the adult liver possesses a progenitor compartment that is regulated by Hh signaling. Previous work by Zaret and colleagues (15) has established the requirement for Hh signaling during hepato-

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**Fig. 5.** Human hepatic progenitors are regulated by the Hh pathway. A: EpCAM<sup>+</sup> cells were isolated from the fetal liver and cultured for 2–3 wk. Immunohistochemistry of EpCAM<sup>+</sup> colonies demonstrated expression of SHH (A), IHH (B), and PTC (C and D). EpCAM<sup>+</sup> colonies were treated with hormonally defined, serum-free control medium (E) or medium supplemented with the potent Hh pathway inhibitor cyclopamine (I). After treatment for 2 days, the control (F) and treated (J) colonies were examined under light microscopy. Cellular necrosis (G and K) and apoptosis (H and L) were assessed in representative colonies. M: quantitative morphometric analysis of cell death after the 2-day treatment. Data are represented as numbers of positive cells (means ± SD). †P < 0.05; *P < 0.01.
genesis. The present work demonstrates simultaneous expression of Hh ligands and the Hh receptor in cells within the hepatic progenitor compartment, suggesting that liver progenitors maintain their viability via autocrine and/or paracrine Hh signaling throughout fetal and adult life. Similar localized progenitor compartments that depend on Hh signaling have been described in the brain (42) and the foregut (68). In those tissues, PTC and Hh ligands cooperate to maintain a population of transiently amplifying Hh-responsive progenitors. Because PTC is the only known receptor for Hh ligands, PTC expression marks cells that retain sensitivity to SHH. However, PTC is a double-edged sword (Fig. 7). When it is unoccupied by the ligand, the receptor spontaneously activates caspase-3 and -7, assuring a high constitutive level of apoptosis that limits the size of the progenitor compartment (63). Conversely, when the ligand availability increases, binding of SHH to PTC prevents this default induction of caspase-mediated apoptosis, activates further downstream signals that promote progenitor cell viability, and therefore expands populations of PTC-expressing cells. Thus populations of PTC-expressing progenitors amplify or shrink according to the availability of Hh ligand. This mechanism maintains a small reservoir of transiently amplifying progenitors that aid in life-long maintainence of tissue integrity while minimizing the risk for neoplasia (50).

Our studies of cultured murine hepatic progenitors suggest that they might be regulated by similar mechanisms. Like other Hh-sensitive progenitors, cultures of hepatic progenitors derived from adult mouse livers exhibited endogenous caspase-3/7 activity, and this was reduced significantly by providing SHH. In addition, cyclopamine-mediated blockade of Hh-initiated signals downstream of PTC caused cell death in colonies of human hepatic progenitor cells. Collectively, these results demonstrate that Hh signaling is necessary to maintain the optimal viability of hepatic progenitors from fetal development to adulthood.

Our findings are important because they indicate that the liver is similar to other tissues that rely upon Hh signaling for...
homeostasis (4). For example, mucosal expression of Hh ligands and their receptor, Ptc, have been demonstrated in healthy adult intestines, and Hh activity increases in several inflammatory disorders of the bowel (39). Androgen deprivation of the prostate ablates prostatic epithelium but enriches the gland with prostatic progenitors. Subsequent blockade of Hh signaling by cycloamine or Shh neutralization prevents prostatic regeneration (27). Regeneration of airway epithelium after acute lung injury is marked by the induction of Shh and Gli1, a transcriptional gene target of Hh signaling, suggesting that airway regeneration also requires Hh activation (67). The present demonstration of Hh activity in hepatic progenitors complements a recent report showing that hepatic stellate cells produce Hh ligands (54) and extends earlier findings of increased Ptc and Smo transcripts in chronically injured human livers (52). Together, these results demonstrate that cells with the capacity for Hh signaling persist and thrive in several adult tissues, including the liver, and suggest that Hh-regulated regenerative mechanisms are probably conserved among adult tissues that were derived from the endoderm.

This discovery opens novel areas for liver research that are likely to have broad implications, because transiently amplifying progenitors provide the seeds for both healthy regeneration and neoplasia (24, 40). Consistent with the latter concept, overactivation of the Hh pathway has been implicated in the formation of tumors in several other endodermally derived tissues (57), including the pancreas, gallbladder (6), prostate (27), and lung (67). A role for Hh signaling had not been considered in hepatocarcinogenesis in prior studies, because mature liver parenchymal cells lack Ptc. However, the present finding that Hh-responsive cells reside among progenitors in adult livers challenges that assumption. Furthermore, we (53) have recently shown that human hepatocellular carcinomas and liver cancer cell lines express the Hh pathway and are regulated by Hh signaling. Thus resident populations of Hh-sensitive progenitors may play a role in the genesis of liver cancer, as they do in malignancies of other endoderm-derived organs (4).

In conclusion, these findings have exciting therapeutic implications for patients with various types of chronic liver disease. For example, they suggest that targeted ablation of Hh activity in malignant liver cells might prove useful as a treatment for hepatocellular carcinoma. On the other hand, supplementing Hh activity in healthy hepatic progenitors might help to improve recovery from liver damage. Actualization of these putative therapeutic advances will require a better delineation of how the Hh pathway interfaces with other autocrine and paracrine signals that control the fate of resident hepatic progenitor populations during health as well as during states of accelerated tissue remodeling after liver injury.

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REFERENCES


HEDGEHOG SIGNALING MAINTAINS HEPATIC PROGENITORS


59. Tian YW, Smith PG, and Yeoh GC. The oval-shaped cell as a candidate for a liver stem cell in embryonic, neonatal and precancerous liver: identification based on morphology and immunohistochemical staining for...


