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Pleiotrophin/Heparin-Binding Growth-Associated Molecule as a Mitogen of Rat Hepatocytes and Its Role in Regeneration and Development of Liver

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Previously pleiotrophin (PTN) was identified among proteins secreted by Swiss 3T3 cells as a mitogen for cultured adult rat hepatocytes. The present study showed that the growth of rat hepatocytes was enhanced when cultured with rat hepatic stellate cells (HSCs). HSCs expressed PTN mRNA and secreted its protein in the co-cultures. Recombinant PTN enhanced the growth of hepatocytes in culture, suggesting that HSCs stimulate the growth of hepatocytes through the action of PTN. To know the biological role of PTN in the growth of hepatocytes *in vivo*, we examined the expression of PTN in four regeneration models of adult liver and embryonic liver of rat. The expression of PTN mRNA in the liver was markedly up-regulated by the treatment with D-galactosamine (GalN) or with acetylaminofluorene followed by partial hepatectomy. HSCs expressed PTN mRNA in response to GalN treatment and its protein was found on hepatocytes. The mRNA expression of *N*-syndecan, a PTN receptor, was up-regulated in GalN-treated hepatocytes. The mesenchymal cells in the septum transversum enclosing the embryonic liver, but not embryonic HSCs, expressed PTN mRNA. We suggest that PTN is secreted from activated adult HSCs and embryonic mesenchymal cells as a mitogen of parenchymal cells in adult and embryonic liver, respectively. *(Am J Pathol 2002, 160:2191–2205)*

Adult hepatocytes have been shown to replicate repeatedly *in vivo*,[1] whereas they hardly replicate *in vitro*.[2] Attempts have been made to induce the growth of functional hepatocytes *in vitro* by incorporating into culture media growth factors such as epidermal growth factor, hepatocyte growth factor (HGF), and transforming growth factor-α.[3] Among these growth factors, HGF is thought to be the most potent mitogen for hepatocytes.[3,4]

We established a culture method by which primary rat hepatocytes can be maintained for more than 1 month without losing their differentiated phenotypes and growth potential.[5–7] The incorporation of liver nonparenchymal cells (NPCs) is one of the important features of this method. Hepatic stellate cells (HSCs) effectively supported the growth of hepatocytes, especially, small-sized hepatocytes,[7] suggesting that the NPCs secrete growth factors for hepatocytes as a result of the hepatocyte to NPC interactions. Furthermore, we found that Swiss 3T3 cells also stimulate the growth of cultured rat hepatocytes.[8] Such a stimulatory effect was replaceable with the conditioned medium (CM) of Swiss 3T3 cells. The activity in the CM was purified and identified as pleiotrophin (PTN).[8] Actually, our previous data indicated that purified PTN or recombinant PTN enhanced the DNA synthesis of hepatocytes.[8] However, the physiological significance of PTN in the growth of hepatocytes remains to be clarified.

PTN, also termed heparin-binding growth-associated molecule, is an 18-kd heparin-binding protein and shows 50% identity to midkine (MK).[9,10] This protein is a basic secreted protein and contains five disulfide bonds.[11] PTN and MK form a family that is distinguished from fibroblast growth factors in their amino acid sequences. Although developing rat fetuses express PTN mRNA in a variety of tissues, its expression in the adult is restricted to brain, bones, and genital organs.[9,12,13] PTN mRNA was mainly found in neuroectoderm and mesoderm of rat fetus, whereas its protein was found in endoderm and nerve tissues,[12,13] suggesting that PTN acts as a mesenchymal factor on epithelial tissues. It is known that PTN plays a role as a mesenchymal factor regulating branching mor-

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phogenesis of the ureteric bud during kidney development.[14] PTN shows an angiogenic activity toward endothelial cells, and mitogenic activity toward epithelial lens cells.[15,16] *N*-syndecan (syndecan-3), protein tyrosine phosphatase ζ (PTPζ), and anaplastic lymphoma kinase (ALK) have been known as functional PTN receptors.[17–19]

The present study firstly asked whether PTN is a liver NPC-derived mitogen for hepatocytes. Previously, we suggested that HSCs among the NPCs most contribute to inducing the growth of hepatocytes *in vitro*.[7] To test this suggestion, rat hepatocytes were co-cultured with rat HSCs in this study. The result showed that the HSCs actually stimulate the growth of hepatocytes. HSCs were shown to express PTN mRNA and secrete its protein. In addition, PTN significantly enhanced the growth of hepatocytes in culture. The second aim of this study was to gain insights into the biological function of PTN in liver regeneration and development. We examined the expression of PTN in four different rat models of regeneration: regeneration induced by the treatment with D-galactosamine (GalN),[20] with acetylaminofluorene (AAF) followed by a partial hepatectomy (PHx) (AAF/PHx),[21] with carbon tetrachloride (CCl_4)[22] and with PHx.[23] These models are known to regenerate the liver through different mechanisms. Oval cells, a specific population of liver progenitor-like cells, emerge and proliferate in the regenerating liver of GalN and AAF/PHx models,[20,21] whereas parenchymal hepatocytes (PHs) around the central vein start to proliferate in response to the damage caused by CCl_4.[22] The present study showed that HSCs began to express PTN mRNA in the GalN-treated liver before the onset of DNA synthesis. Immunohistochemistry in the GalN and AAF/PHx models detected PTN protein on the hepatocytes. Furthermore, hepatocytes in the GalN model and cultured hepatocytes expressed *N*-syndecan gene. CCl_4-treated liver tissues moderately up-regulated PTN gene expression, its time of expression being well correlated with the peak of induced DNA synthesis of hepatocytes reported previously.[24] PHx did not induce such a marked stimulation of the PTN mRNA expression before the onset of DNA synthesis. The embryonic liver bud is formed as the hepatic diverticulum that is induced from the endoderm of the ventral foregut by the cardiac mesoderm.[25] We showed that mesenchymal cells enclosing the hepatic diverticulum intensely expressed PTN mRNA and its protein was found on the hepatoblasts in the developing liver. These results suggest that pathologically active HSCs in the adult liver and biologically active mesenchymal cells in the embryonic liver promote the growth of hepatocytes by secreting PTN.

Materials and Methods

Isolation and Culture of Hepatocytes

Ten-week-old male Fischer 344 rats were obtained from Charles River Japan, Inc. (Yokohama, Japan). The liver was perfused with collagenase to disaggregate its cells.[26] The liver cells were isolated and washed three times with Dulbecco's modified Eagle's medium (Life Technologies Inc., Rockville, MD) containing 10% fetal bovine serum (HyClone Laboratories, Inc., Logan, UT). The cells were precipitated by centrifugation at 50 $\times$ *g* for 1 minute at each time of washing. The washed cells were centrifuged through 45% Percoll at 50 $\times$ *g* for 10 minutes and the pellets obtained were used as a fraction of PHs.[27] The supernatants obtained at the centrifugation of the collagenase perfusate were further centrifuged at 150 $\times$ *g* for 5 minutes and the pellets were similarly centrifuged twice and used as a fraction containing small hepatocytes (SHs) and NPCs.[7] This fraction was suspended and centrifuged at 50 $\times$ *g* for 2 minutes to separate it into the SH-enriched fraction as a pellet and the NPC-enriched fraction as a supernatant. SHs were purified from the SH-enriched fraction by the Percoll isodensity centrifugation at 50 $\times$ *g* for 24 minutes. The NPC-enriched fraction was plated on hydrophobic plastic dishes coated with *N*-*p*-vinylbenzyl-*O*-β-D-galactopyranosyl-(1,4)-D-gluconamide.[28] A fraction of NPCs was obtained as cells that had not adhered to the dishes at 4 hours after plating. The fraction of NPCs was plated on the plastic dishes and the cells attached to the dishes were used as NPCs. The purity of the fractions of SHs and PHs was determined as follows. Cells of these two fractions were placed on Celldesks (Sumitomo Bakelite Co., Tokyo, Japan) for 1 day and were immunocytochemically identified. The antibodies against the following proteins were used for the immunohistochemical study: rat albumin (Cappel, Durham, NC) for the identification of hepatocytes, chicken desmin (Monosan, Uden, The Netherlands) for HSCs, SE-1 (IBL, Gunma, Japan) for endothelial cells, and ED-2 (Sigma Chemical Co., St. Louis, MO) for Kupffer cells. The purity of hepatocytes in the fractions of PHs and SHs in normal liver was 98.2% and 97.6% (the average of two determinations), respectively, and those in GalN-treated liver, 97.4% and 95.9%, respectively. Hepatocytes were suspended in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 20 mmol/L *N*-2-hydroxyethylpiperazine-*N*′−2-ethane sulfonic acid (Life Technologies, Inc.), 15 μg/ml L-proline (Life Technologies, Inc.), 0.25 μg/ml insulin (Sigma), 10^{-7} mol/L dexamethasone (Sigma), 44 mmol/L $NaHCO_3$, 10 mmol/L nicotinamide, 10 ng/ml epidermal growth factor, 0.1 mmol/L L-ascorbic acid 2-phosphate, and antibiotics of 100 IU/ml penicillin G (Life Technologies, Inc.) and 100 μg/ml streptomycin (Life Technologies, Inc.) (HCGM).[5,6]

Co-Culture of Hepatocytes

Swiss 3T3 cells were obtained from the American Type Culture Collection (Rockville, MD). They were serially cultured according to the standard procedure.[29] Rat HSCs were isolated according to Kawada and colleagues[30] and were serially subcultured. Rat skin fibroblasts (RSFs) of Fischer 344 rats were serially cultured as previously described.[31,32] Hepatocytes were co-cultured with one of the HSCs, 3T3 cells, and RSFs as follows. PHs were

Table 1. Oligonucleotide Primers and TaqMan Probes Used in the Real-Time RT-PCR Experiments

Gene	Forward primers (5′–3′)	Reverse primers (5′–3′)	TaqMan probes (5′–3′)*
PTN rat	GGAAGAAGCAGTTTGGAGCTG	GGCGGTATTGAGGTCACATTC	TGCAAATACCAGTTCCAGGCTTGGG
PTN mouse	GGAAGAAGCAGTTTGGAGCTG	GGCGGTATTGAGGTCACATTC	TGCAAGTACCAGTTCCAGGCTTGGG
HGF rat	AAAACAAGGTCTGGACTCACATGT	TAGCGTCTGGCTCCCAGAA	TGGGACAAGAATATGGAGGATTTACACCGT
HGF mouse	AAAACAAGGTCTGGACTTACATGT	TAGCATCTGGCTCCCAGAA	TGGGACAAGAATATGGAGGATTTACACCGT
MK rat	GCATCGAGCCGGCC	GAGGGCAACAAGGGCTAGAA	TGAGCGAGATGCAGCACCGAAGTT
MK mouse	TGATGGGAGCACTGGCAC	CATTGTACCGCGCCTTCTT	AAAGCCCGCCAAGGGACCCT
N-syndecan rat	AAGAGGAAGGAAGCCCTGG	GGCCATCTTAACCCTGGC	TGCTGGTTGAAACATGGGCGG
PTPζ rat	GTGCAAACAGAGGAGCAATACGT	TTTTGTCTTGCCTGATGGTCC	CCATACTCAGCAAAGAGACAGAGGTGCCTG
ALK rat	GGAGGTGTATGAAGGCCAGGTAT	CAGCTCATCTTGCTCTGAACACA	AATGCCCAATGACCCAAGCCCTCT
GAPDH rat	TGCCATCACTGCCACTCAG	TGCCCCACGGCCAT	ACTGTGGATGGCCCCTCTGGAAAG
GAPDH mouse	GGGAAGCCCATCACCATCT	GCCTCACCCCATTTGATGT	CAGGAGCGAGACCCCACT

*FAM and TAMURA were conjugated with the 5′- and 3′-end of the TaqMan probes, respectively.

inoculated at 4×10^3 cells/cm^2 on Celldesks. HSCs, 3T3 cells, and RSFs were lethally treated with 10 μg/ml of mitomycin C (Sigma) and inoculated at three different densities (6×10^2, 2×10^3, and 6×10^3 cells/cm^2) on the hepatocytes at 1 day after the plating of hepatocytes. They were cultured at 37°C for 10 days in an atmosphere of 5% CO_2 and 95% air. The cells were fixed in cold ethanol on days 1 and 10, and stained with hematoxylin and eosin (H&E). The number of hepatocytes was counted in 10 randomly selected different microscopic fields and the ratio of hepatocyte number at 10 days to that at 1 day was calculated as a measure of growth rate of hepatocytes.[7]

Quantification of mRNA

ALK cDNA was cloned from RNA of rat small intestine by reverse transcriptase-polymerase chain reaction (RT-PCR) using PowerScript reverse transcriptase (Clontech), Oligo(dT)12-18 primer (Life Technologies, Inc.), and a set of primers; 5′-GACCCCGAGAGCCACAAAGTC and 5′-CCATCCGTCCTCCGCTGGTGA according to the manufacturer's instruction. A 1054-bp PCR product of ALK was subcloned into pCRII vectors (Invitrogen, Carlsbad, CA). Rat ALK cDNA (accession no. AB073169) had 94% and 91% similarity with mouse and human ALK, respectively.

Total RNAs of cultured cells were extracted by a RNeasy total RNA system (Qiagen, Tokyo, Japan). The RNA samples were treated with RNase-free DNase I (Takara, Tokyo, Japan) at 37°C for 10 minutes and were electrophoresed on a 1% formaldehyde agarose gel to evaluate the quality of the samples. cDNAs were synthesized using 1 μg of total RNA, PowerScript reverse transcriptase, and Oligo(dT)12-18 primer according to the manufacturer's instruction. The expression of mRNAs was measured by real-time RT-PCR, a method to precisely quantify mRNA. This method has been proved to be as valid as Northern blot analysis.[33] The diluted cDNA was amplified with a set of gene-specific primers, a gene-specific *Taq*Man probe, and Universal PCR master mix (Applied Biosystems, Tokyo, Japan) according to the manufacturer's protocol in a PRISM 7700 Sequence Detector (Applied Biosystems). Primers and *Taq*Man probes used in the real-time RT-PCR experiments are summarized in Table 1. FAM (6-carboxyfluorescein) and TAMURA (6-carboxytetramethyl-rhodamine) were conjugated with the 5′- and 3′-end of the *Taq*Man probes, respectively. Amplified PCR products can be monitored directly by measuring the increase of the dye intensity of the FAM that is removed from the probes by the 5′- to 3′-exonuclease activity of *Taq* DNA polymerase. A series of diluted plasmid cDNAs containing each gene was used for making the standard amplification curves. Copy numbers of mRNA in the cDNA samples were calculated using the standard amplification curves. Real-time RT-PCR was performed in triplicate for each sample.

Quantification of Proteins of PTN and HGF in CM

HSCs, 3T3 cells, and RSFs were inoculated at 1 to 2.5×10^5 cells in 100-mm plastic tissue culture dishes containing 10 ml of Dulbecco's modified Eagle's medium and 10% fetal bovine serum and cultured for 4 days. The culture medium (CM) was collected and incubated for 1 hour at 4°C with gels of Affi-Gel Heparin Gel (1 ml; Bio-Rad Laboratories, Hercules, CA) suspended in solution of 0.15 mol/L NaCl and 10 mmol/L phosphate buffer, pH 7.3. The gels were precipitated by centrifuging the reaction mixture at 12 krpm for 10 minutes and were resuspended in 1.5 mol/L NaCl and 10 mmol/L phosphate buffer, pH 7.3, which were centrifuged as above. The supernatant was desalted and concentrated with an Ultrafree ultrafiltration system (Millipore, Bedford, MA), and was subjected to polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The gel was transferred to a nitrocellulose membrane. The membrane was blocked for 1 hour at room temperature with 5% skim milk dissolved in 0.15 mol/L NaCl and 20 mmol/L Tris-HCl, pH 7.4 (Tris-buffered saline), and incubated overnight at 4°C with 40-fold diluted polyclonal antibodies against human recombinant (hr) PTN (R&D Systems, Minneapolis, MN) in Tris-buffered saline. After washing with Tris-buffered saline in 0.1% Tween 20, the membrane was incubated for 1 hour at room temperature with a 200-fold diluted horseradish peroxidase-labeled anti-goat IgG (Vector Laboratories, Burlingame, CA). The signals were visualized with Konica

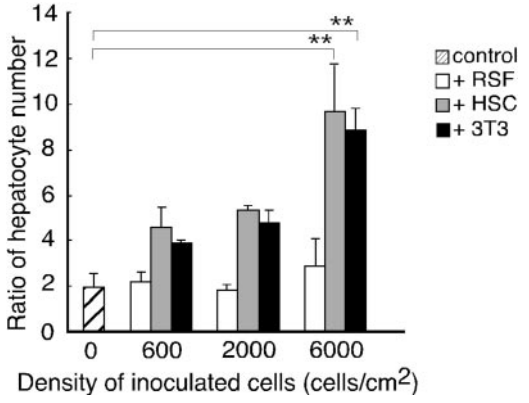


Figure 1. Growth of hepatocytes in the presence of RSFs, HSCs, or 3T3 cells. Hepatocytes were inoculated at 4×10^3 cells/cm^2 together with or without mitomycin C-treated RSFs, HSCs, or 3T3 cells at the indicated inoculation density. The hepatocytes were allowed to grow for 10 days and the ratio of number of hepatocytes at day 10 to that at day 1 was determined as a measure of growth rate. Each value represents the mean ± SD of triplicate experiments. The difference between the values indicated by **brackets** was statistically significant at a level of ** for $P < 0.01$.

Immunostain HRP-1000 (Konica, Tokyo, Japan). Concentrations of PTN were estimated by Western blot using a series of diluted hrPTN proteins as a standard. Concentrations of HGF in the CM were measured with a rat HGF EIA kit (Institute of Immunology, Tokyo, Japan).

Effect of Growth Factors and CM on Growth of Hepatocytes

3T3 cell CM was prepared as previously described.[8] Hepatocytes were cultured at 4×10^3 cells/cm^2 on the Celldesks in HCGM with or without 100 ng/ml of hrPTN (R&D Systems), 5 ng/ml of hrHGF (Becton Dickinson, Bedford, MA), and 50% 3T3 cell CM. The cultures were fixed at 10 days of culture in ethanol and stained with H&E. The ratio of number of hepatocytes was calculated as described above.

Induction of Liver Regeneration

Four rat models of liver regeneration were produced as follows, using male Fischer 344 rats at 9 to 10 weeks of age: models of GalN, AAF/PHx, CCl_4, and PHx. GalN model was obtained by intraperitoneally injecting rats with GalN dissolved in saline at a dose of 70 mg/100 g body weight.[20] Rats of AAF/PHx model were given first AAF by gavage for 4 days at a dose of 1.5 mg/day and then a two-thirds PHx. One day later, AAF treatment was resumed for additional 5 days.[21] Those of CCl_4 model were intramuscularly injected with CCl_4 dissolved in the equal volume of olive oil at a dose of 0.4 ml/100 g body weight. PHx model was produced by subjecting rats to a two-thirds PHx.[23] Rats of the sham-operated group were only treated with laparotomy. At appropriate periods after these treatments or the operations, rats were sacrificed to excise the liver. Total RNAs of liver tissues were extracted by Isogen (Nippon Gene, Tokyo, Japan). The RNA samples were treated with RNase-free DNase I and their quantity was evaluated by RNA electrophoresis as

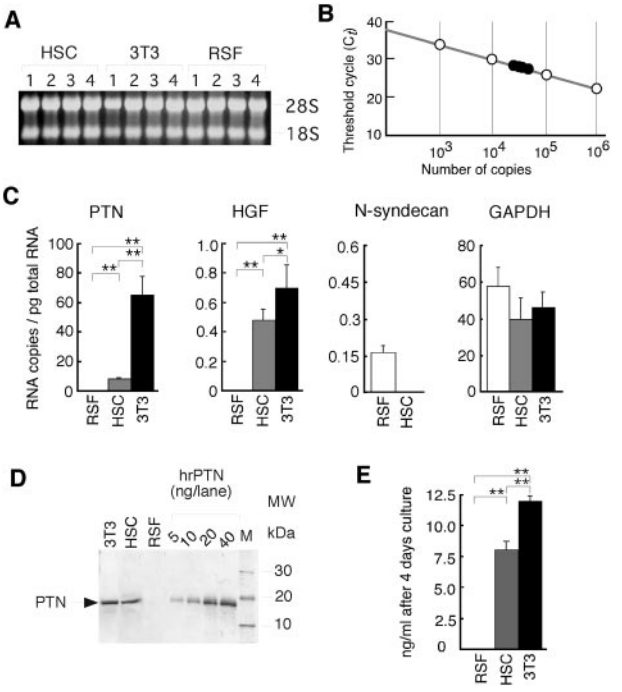


Figure 2. Expression of mRNAs and proteins of PTN and HGF by HSCs, 3T3 cells, and RSFs. **A:** Electrophoresis of RNA purified from HSCs (HSC), 3T3 cells (3T3), and RSFs (RSF). **B:** The quantification of PTN mRNA expressed by HSCs. A standard curve was made by a series of diluted plasmids containing PTN cDNA (**open circles**) *versus* threshold cycle (Ct). Copy numbers of PTN mRNA (**closed circles**) in RNA of HSCs were calculated using the standard curve. **C:** RSFs, HSCs, and 3T3 cells were cultured for 4 days and were used for determining the expression of mRNAs of PTN, HGF, *N*-syndecan, and GAPDH by real-time RT-PCR. Both HSCs and 3T3 cells, but not RSFs expressed mRNAs of both PTN and HGF. RSFs, but not HSCs, expressed *N*-syndecan mRNA. **D:** Western blot of PTN. The media wherein 3T3 cells, HSCs, or RSFs had been cultured as in **C** were collected and used for determining the concentration of PTN by Western blot using hrPTN protein as a standard (**right**, hrPTN). The PTN antibody reacted with the protein whose size (18 K) matched to the size of the hrPTN protein. The concentration of PTN in the media was 18 ng/ml for 3T3 cells and 12 ng/ml for HSCs, whereas RSFs did not secrete measurable amounts of PTN. MW represents the molecular weight determined by the migration of marker proteins. **E:** HGF secreted in the media. The media collected as in **C** were used for determining HGF by EIA. Both HSCs and 3T3 cells, but not RSF, secreted HGF at a concentration of 8 ng/ml and 12 ng/ml, respectively. Each value represents the mean ± SD. The difference between the values indicated by **brackets** was statistically significant at a level of * for $P < 0.05$ and ** for $P < 0.01$($n = 4$ for **C** and $n = 3$ for **E**).

above. cDNAs were synthesized using 1 μg of total RNA and used as templates of real-time RT-PCR.

In Situ *Hybridization and Immunohistochemistry*

Rat PTN cDNA was cloned from GalN-treated rat liver RNA by RT-PCR using a ThermoScript RT-PCR system (Life Technologies, Inc.) with a set of primers of 5′-GACAGAACGGAATTGAGTGAAGGC and 5′-CCTCAATCCTACCATCTATCATG. The PCR product was subcloned into pGEM-T vectors (Promega, Tokyo, Japan). Digoxigenin-labeled cRNA probes were synthesized with a DIG RNA labeling kit (Roche Molecular Biochemicals, Tokyo, Japan). Liver tissues of fetuses and adults of rats were fixed overnight at 4°C with 4% paraformaldehyde in phosphate-buffered saline (PBS) (137 mmol/L NaCl, 2.7 mmol/L KCl, 10 mmol/L Na_2HPO_4, and 2 mmol/L

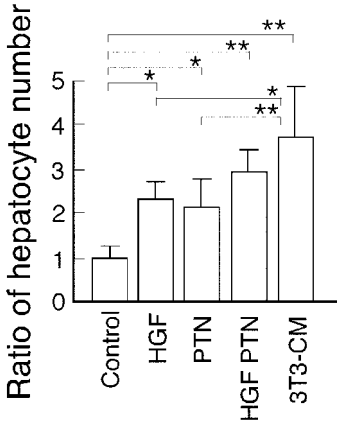


Figure 3. Effect of PTN and HGF on the growth of hepatocytes. Hepatocytes were cultured at 4×10^3 cells/cm^2 in the medium containing either of hrHGF (5 ng/ml) and hrPTN (100 ng/ml), or both of them. The cells were also cultured in 3T3-CMs. Growth of hepatocytes is shown as the ratio of hepatocyte number at 10 days to that at 1 day. Each value represents the mean ± SD of triplicate measurements. The difference between the values indicated by **brackets** is significant at a level of * for $P < 0.05$ and ** for $P < 0.01$ ($n = 4$).

KH_2PO_4) and were embedded in paraffin. The sections were cut at 5 μm, mounted onto glass slides, rehydrated, digested with 1 μg/ml of proteinase K in PBS at 37°C for 7 minutes, and postfixed in 4% paraformaldehyde in PBS for 20 minutes. mRNA *in situ* hybridization was performed using cRNA probes as described elsewhere.[34] Hybridized cRNA probes were detected by nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl-phosphate (Roche Molecular Biochemicals). Some sections were subsequently subjected to immunohistochemistry with anti-desmin antibodies. The sections were incubated in Target Retrieval Solution (DAKO Japan, Kyoto, Japan) to revive their immunoreactivity. After blocked with 10% normal goat sera, the sections were incubated with 100-fold diluted rabbit anti-chicken desmin antibodies (Monosan), washed with PBS, and were incubated with 200-fold diluted fluorescein-labeled anti-rabbit IgG (Vector Laboratories). The photographs were taken in the bright field for *in situ* hybridization and then in the fluorescence field for immunohistochemistry.

Immunohistochemistry was performed using goat anti-hrPTN or rabbit anti-human α1-fetoprotein (AFP) (DAKO). Paraffin sections were rehydrated and were incubated in Target Retrieval Solution. Endogenous peroxidase activity was inactivated by 0.3% H_2O_2 in methanol for 30 minutes, and endogenous biotin was blocked by a biotin-blocking kit (Vector Laboratories). The sections were blocked with 10% horse sera in the former or goat sera in the latter, incubated with 100-fold diluted anti-PTN antibodies in the former or anti-AFP antibodies in the latter, and were washed with PBS. The sections were then incubated with 200-fold diluted horseradish peroxidase-labeled anti-goat antibodies in the former or anti-rabbit IgG antibodies in the latter and were washed with PBS. The second antibodies were colored using a Vectastain ABC kit (Vector Laboratories). Goat IgG (Biogenesis, Poole, England) was used as negative staining instead of the anti-PTN antibody. The sections were counterstained with hematoxylin. To assess the validity of immunohistochemistry, the anti-PTN antibody was absorbed by hrPTN as follows. The hrPTN was incubated overnight with the antibody at 4°C and the reaction mixture was incubated with Affi-Gel Heparin at 4°C for 2 hours. The supernatant of the reaction mixture did not show any immunoreactivity when used for immunohistochemistry on liver tissues, indicating reliability and validity of the antibody for the immunohistochemical analysis performed in the present study.

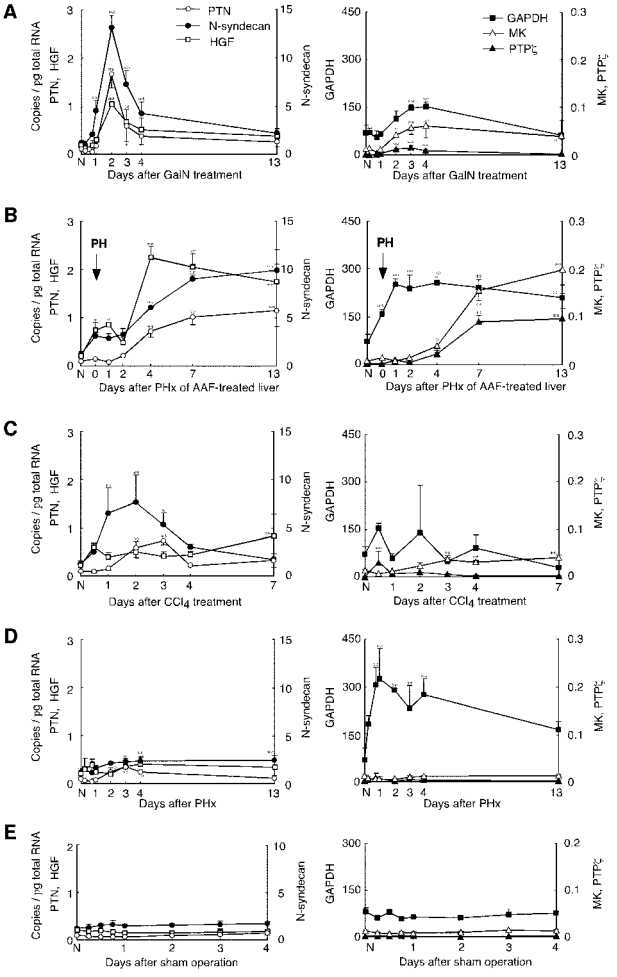
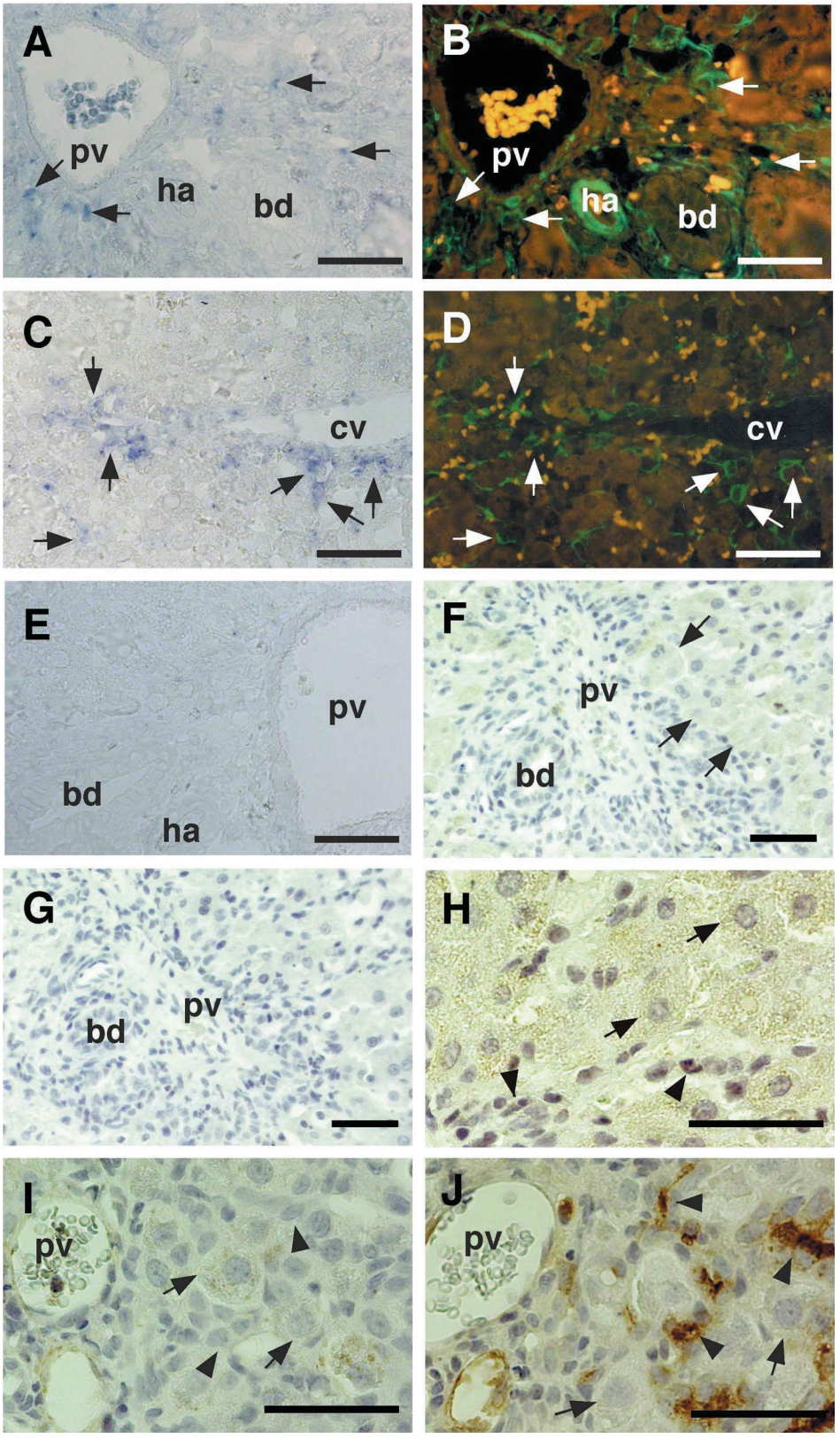


Figure 4. Gene expression of PTN and its receptors during regeneration. Rats were treated with GalN (**A**), AAF/PHx (**B**), CCl_4 (**C**), and PHx (**D**), or subjected to sham operation (**E**). The liver specimens were obtained at indicated days of the treatment or after the operation, and were used to measure by real-time RT-PCR mRNAs of PTN (**open circles**), *N*-syndecan (**closed circles**), HGF (**open squares**), GAPDH (**closed squares**), MK (**open triangles**), and PTPζ (**closed triangles**). Normal rats were used as controls and are shown as N in the horizontal axis of each graph. The vertical axis in each graph shows mRNA copies per pg total RNA for PTN and HGF (**left**, left vertical axis), *N*-syndecan (**left**, right vertical axis), GAPDH (**right**, left vertical axis), and MK and PTPζ (**right**, right vertical axis). Each value represents the mean ± SD of triplicate measurements. Statistical differences were tested between normal and treated groups. The level of statistical significance is shown by * for $P < 0.05$ and ** for $P < 0.01$.

Quantification of mRNAs in Hepatocytes

Hepatocytes in the PH and SH fraction of normal rats were cultured at 4×10^3 cells/cm^2 in 35-mm plastic tissue culture dishes containing HCGM. Total RNAs were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isolated from the cultured hepatocytes at 1, 2, 5, and 10 days of culture ($n = 3$) by a RNeasy total RNA system. cDNAs were synthesized using 200 ng of total RNA. The purity of hepatocytes in the fraction of PHs and SHs at 10 days of culture was 96.4% and 98.1% ($n = 2$), respectively.

Liver cells and their fractions (PHs, SHs, and NPCs) were prepared from normal ($n = 4$) and experimental livers treated with GalN 2 days ($n = 4$). Total RNAs were isolated from these cells by a RNeasy total RNA system. A yield of PHs from GalN-treated liver was significantly lower than that from normal liver. Therefore, cDNAs were synthesized using 50 ng of total RNAs from the GalN-treated and normal samples. mRNA was determined by real-time RT-PCR using the cDNAs as templates as described above.

Statistical Tests

Statistical tests for the significance of differences between control and treated groups were made by one-way analysis of variance followed by Dunnet *post hoc* test. Multiple- and two-sample comparison were made by one-way analysis of variance followed by Fisher protected least significant difference (PLSD) *post hoc* test and by Welch's *t*-test, respectively. A level of $P < 0.05$ was considered statistically significant.

Results

Culture of Hepatocytes with HSCs

HSCs are located in the space of Disse and thought to play important roles in the maintenance of physiological and pathological activities of hepatocytes.[35] Mitomycin C-treated HSCs were placed as feeder cells in cultures of hepatocytes to directly examine the effect of HSCs on the growth of hepatocytes. Co-cultures of hepatocytes with mitomycin C-treated Swiss 3T3 cells or RSFs served as control experiments. We previously showed that 3T3 cells enhance the DNA synthesis of cultured hepatocytes.[8] HSCs and 3T3 cells stimulated the replication of hepatocytes, the rate of stimulation depending on the number of HSCs introduced, whereas RSFs did not show such a stimulatory effect (Figure 1). This result strongly suggested that HSCs secrete PTN as a mitogen of hepatocytes as in the case of 3T3 cells.[8] This possibility was tested by measuring mRNA of PTN in cultured HSCs. RNA was extracted from these three types of feeder cells that had been cultured for 4 days. mRNAs of PTN in the RNA was quantified by real-time RT-PCR together with those of HGF. The integrity of RNA was verified by electrophoresis (Figure 2A). Copy numbers of mRNA in the samples were calculated using the standard amplification curves made by a series of diluted plasmids of each of the two genes. Such a calculation is shown for PTN mRNA as an example in Figure 2B. mRNAs of both PTN and HGF were measurable for both HSCs and 3T3 cells, but not for RSFs (Figure 2C). The expression level of the two genes was significantly higher in 3T3 cells than in HSCs. *N*-syndecan, PTPζ, and ALK are known as PTN receptors.[17–19] RSFs but not HSCs expressed *N*-syndecan. Both RSFs and HSCs did not express both PTPζ and ALK (data not shown). Expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in RSFs was higher than that in HSCs and 3T3 cells, although the difference was not statistically significant. MK was not measurable in these cells (data not shown). These three types of feeder cells were cultured for 4 days and the media were collected to determine the concentration of PTN by Western blot. The media of 3T3 cells and HSCs, but not RSFs gave a band whose molecular weight (18 kd) was identical to hrPTN protein (Figure 2D). The band densities of PTN was used to determine its concentration using hrPTN as a standard, which gave 18 ng/ml for 3T3 cells and 12 ng/ml for HSCs (Figure 2D). We also measured the concentration of HGF in the media with EIA and obtained the value of 8 ng/ml for HSCs and 12 ng/ml for 3T3 cells (Figure 2E). RSFs did not express proteins of both PTN and HGF. These results suggested that both 3T3 cells and HSCs stimulate the growth of hepatocytes through the action of either HGF or PTN, or through their cooperative actions. These measurements also supported the fact that RSFs did not serve as feeder cells for the growth of hepatocytes shown in Figure 1.

Effect of PTN and HGF on the Growth of Hepatocytes

We previously reported that the DNA synthesis of hepatocytes was significantly stimulated when they were cultured in CMs of 3T3 cells.[8] The present study showed that the 3T3-CM enhanced the replication of hepatocytes approximately three times as compared to normal media (Figure 3). We tested if this effect of 3T3 cell CM can be explained by the action of either of HGF and PTN or both factors. When either 5 ng/ml of hrHGF or 100 ng/ml of hrPTN was added to the cultures of hepatocytes, their growth rate was stimulated approximately two times. The simultaneous presence of the two factors

Figure 5. Identification of PTN mRNA and its protein-expressing cells in the liver treated with GalN and AAF/PHx. Rats were treated with GalN for 2 days (**A–D**, **F–H**) or AAF/PHx for 13 days (**I**, **J**). *In situ* hybridization of PTN mRNA was performed in GalN-treated (**A**, **C**) and normal (**E**) liver. Immunohistochemistry was performed with antibodies against desmin (**B**, **D**), PTN (**F**, **H**, **I**), normal goat IgG (**G**), and AFP (**J**). **A** and **C:** PTN mRNA *in situ* hybridization. Sections around the portal (**A**) and central vein (**C**) are presented. PTN mRNA (blue) is seen in nonparenchymal tissues (**arrows**). **B** and **D:** Sections of **A** and **C** were used for the desmin immunostaining that produced sections of **B** and **D**, respectively. PTN mRNA-expressing cells indicated by **arrows** in **A** and **C** are simultaneously positive for the desmin staining (green fluorescent cells indicated by **arrow** in **B** and **D**). Parenchymal cells and blood cells are stained yellow-red and yellow, respectively, because of the autofluorescence of these cells. **E:** *In situ* hybridization of PTN mRNA in normal liver. A section around the portal vein is presented. No positive cells are seen. **F:** Hepatocytes are positive for the PTN staining (brown, **arrows**). **G:** Normal goat IgG was used as the first antibody of immunostaining that served as a negative control. There is no signal in the liver. **H:** A high magnification of **F**. Hepatocytes (**arrows**), but not NPCs (**arrowheads**) are positive in the staining. **I:** Hepatocytes (**arrows**), but not oval cells (**arrowheads**), are positive for PTN. **J:** Oval cells (**arrowheads**) but not hepatocytes (**arrows**) are positive for AFP. bd, bile duct; cv, central vein; ha, hepatic artery; pv, portal vein. Scale bar, 50 μm.

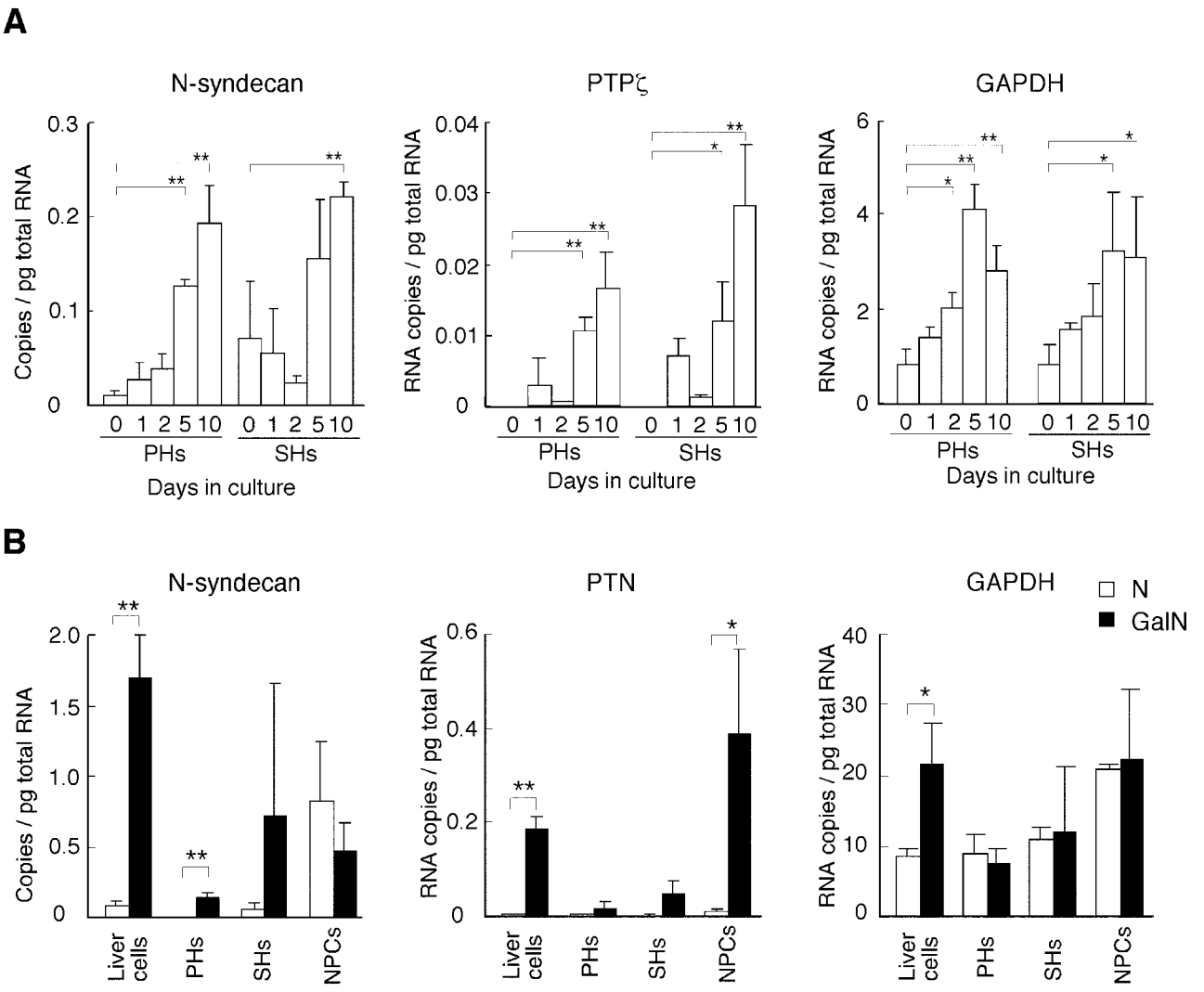


Figure 6. Expression profiles of PTN receptor genes in liver cells. **A:** Expression level of mRNA of *N*-syndecan, PTPζ, ALK, and GAPDH was determined by real-time RT-PCR. PHs and SHs were isolated from normal liver and cultured in HCGM for the indicated days. mRNAs of *N*-syndecan and PTPζ were up-regulated in their expression during the culture. ALK mRNA was not measurable in these cells. **B:** Expression of mRNA of *N*-syndecan and PTN in liver cells of the GalN model. PHs, SHs, and NPCs were isolated from livers of normal rats (**open bars**) and those treated with GalN for 2 days (**closed bars**). RNAs were isolated from these cells to measure the expression level of mRNAs of *N*-syndecan, PTN, PTPζ, ALK, and GAPDH. Expressions of *N*-syndecan and PTN mRNAs were much higher in GalN-treated liver cells than in those of normal ones. GalN treatment induced NPCs to up-regulate mRNA of PTN, but not *N*-syndecan. The GalN treatment induced both PHs and SHs to up-regulate *N*-syndecan mRNA. PHs and SHs weakly expressed PTN mRNA. mRNAs of PTPζ and ALK were not measurable in these cells. Each value represents the mean ± SD. The level of statistical significance is shown by * for $P < 0.05$ and ** for $P < 0.01$ ($n = 3$ for **A**, $n = 4$ for **B**).

stimulated the replication more than the single presence of either of the two. However, it cannot be said that PTN and HGF show the synergistic action on the growth of hepatocytes, because the effect of PTN and HGF did not significantly differ from that of each of the two factors. The result shown in Figure 3 showed that the growth promoting effect of the CM was almost compensated by the presence of both factors. Thus, it was concluded that PTN acts on hepatocytes as a mitogen by itself.

Expression of PTN mRNA in Regenerating Liver

To have an insight into the role of PTN *in vivo*, we examined the expression of PTN mRNA by real-time RT-PCR in four different models of liver regeneration: models of GalN, AAF/PHx, CCl_4, and PHx. As reported previously,[36] we noticed in the present study that expression of GAPDH, a widely used internal control marker gene, was significantly increased during the liver regeneration of these four models (the right panel in Figure 4; A–D). Furthermore, β-actin, another widely used control marker gene, was drastically increased in these models (data not shown). Thus, here we show the level of mRNA expression of each gene as copy numbers per pg of total RNA present in each sample. Expression of PTN was transiently but markedly increased during the initial phase of GalN treatment, reaching to the peak of 1.65 ± 0.26 copies at 2 days of the treatment. This level was as high as 16-fold more than the normal value (Figure 4A, left) and then the level drastically decreased at 3 days. The AAF/PHx model started to increase the expression of PTN at 4 days after the PHx, the increase continuing by 13

days when the expression was 1.15 ± 0.33 copies (11-fold level more than the normal value) (Figure 4B, left). The CCl_4-treated liver moderately elevated its expression with the peak of 0.73 ± 0.1 copies at 3 days, stimulation being sevenfold as compared with the control level (Figure 4C, left). In the PHx model, the expression was transiently elevated with the peak of 0.36 ± 0.09 copies at 3 days, a 3.5-fold stimulation more than the control level (Figure 4D, left). The stimulation in the models of CCl_4 and PHx was significantly lower than that obtained in the models of GalN and AAF/PHx. HGF was transiently increased at 2 days in the GalN model and highly increased in the AAF/PHx model (Figure 4, A and B) as previously reported.[21,37] These expression profiles of HGF were similar to those of PTN in the GalN and AAF/PHx models. We confirmed the previous reports[38] on expression profiles of HGF in PHx model (Figure 4D, left). HGF rose its expression level more early than PTN. The expression of MK was stimulated in the models of GalN, AAF/PHx, and CCl_4 (Figure 4; A, B, and C, right). The level peaked at 4 days (fivefold more than the control level) in the first model, at 13 days (13-fold) in the second model, and at 7 days (threefold) in the third model. These expression levels were fivefold to eightfold lower than those of PTN. The sham operation did not change the expression of these genes (Figure 4E).

N-syndecan, PTPζ, and ALK have been known as PTN receptors.[17–19] In the present study we asked whether their expression is correlated with that of PTN. *N*-syndecan transiently but markedly increased its expression during the initial phase of GalN treatment with the peak of 13.07 ± 1.33 copies at 2 days of the treatment, which was 11-fold higher than the control value (Figure 4A, left). This stimulation pattern was correlated well with that of PTN mRNA. Similarly, the stimulation patterns of *N*-syndecan and PTN were well correlated in the AAF/PHx model (Figure 4B, left). *N*-syndecan started to raise its expression at 4 days after the PHx in this model and continued to increase through 13 days when the expression reached to 9.94 ± 2.13 copies (eightfold more than the normal value). The CCl_4-treated liver elevated the *N*-syndecan gene expression with the peak of 7.68 ± 2.86 copies at 2 days (sixfold stimulation) (Figure 4C, left), which preceded that of the ligand. The liver of the PHx model showed a weak elevation of *N*-syndecan (Figure 4D, left). Models of both GalN and AAF/PHx also up-regulated PTPζ (Figure 4, A and B, right). However, it should be noticed that the expression level of PTPζ was much lower than that of *N*-syndecan in the liver. For example, the maximum expression of *N*-syndecan in the GalN and AAF/PHx model was 13.07 copies at 2 days and 9.94 copies at 13 days, respectively. By contrast the value for PTPζ in the same models was 0.015 copies at 3 days and 0.096 copies at 13 days, respectively. Thus, the ratio of the maximum expression level of *N*-syndecan to that of PTPζ was calculated as 871 for the GalN model and 104 for the AAF/PHx model. ALK mRNA was not measurable in all these liver regeneration models by the presently adopted method (data not shown).

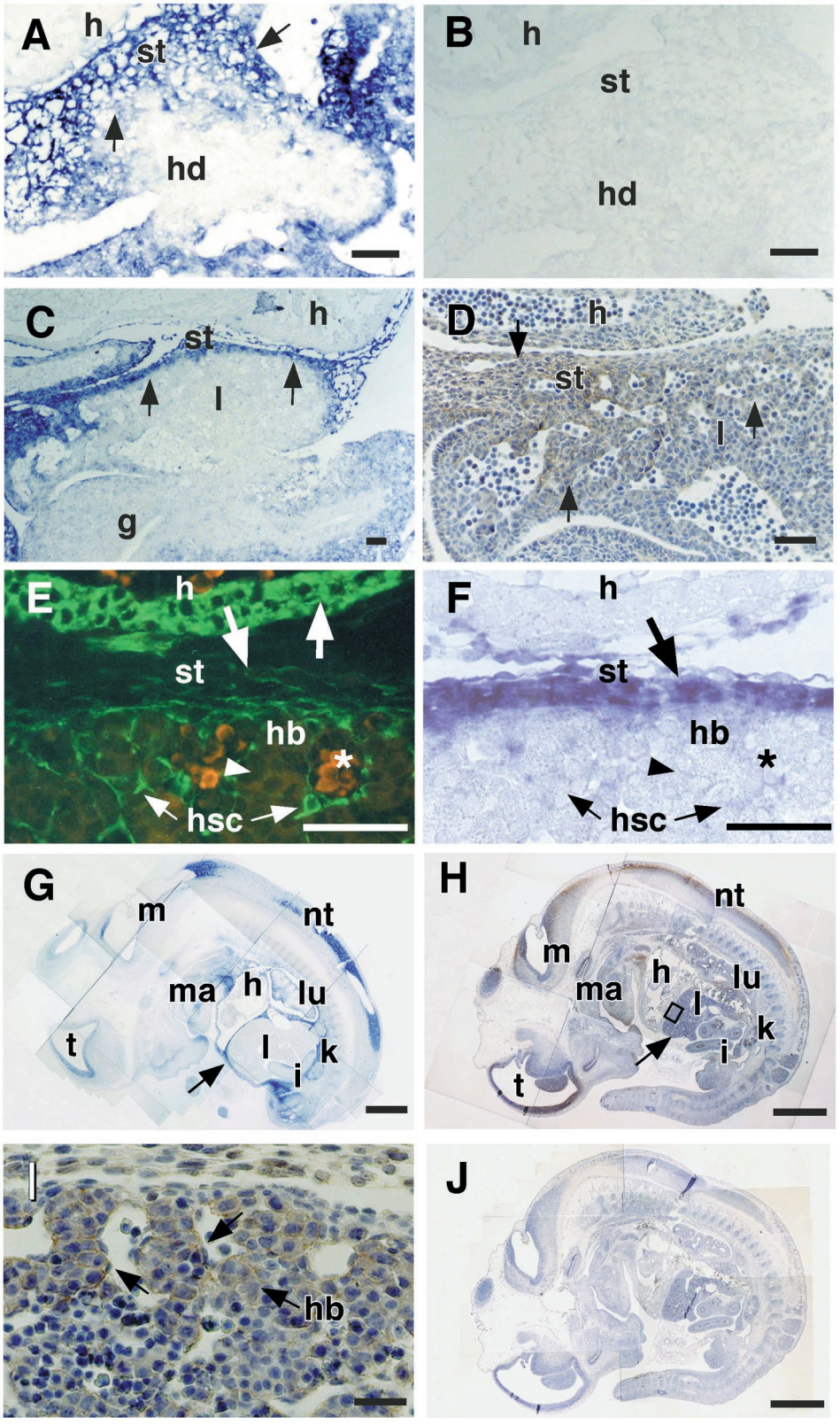
Localization of PTN mRNA and Its Protein in GalN- and AAF/PHx-Treated Liver

The *PTN* gene showed a higher and more sensitive response in its expression to the regeneration-inducing treatment and its expression kinetics were better correlated with those of the gene of its possible receptor, *N*-syndecan, in the model of GalN and AAF/PHx than the other two models. Therefore, the expression of PTN was further characterized using the model of GalN and AAF/PHx. *In situ* hybridization experiments located PTN mRNAs in the nonparenchymal regions around the portal (Figure 5A) and central veins (Figure 5C) in the livers at 2 days after the GalN-treatment. To identify these positive cells, the sections shown in Figure 5, A and C, were subsequently subjected to immunohistochemistry of desmin (Figure 5, B and D). Desmin is a marker protein of HSCs and also expressed in smooth muscle cells and mesenchymal cells in Glisson's capsules.[39] Most of PTN mRNA-expressing cells (Figure 5, A and C) were simultaneously desmin-positive (Figure 5, B and D). These desmin-positive cells appeared to be HSCs in their morphology and location in the liver tissues. No signals were seen in the normal liver (Figure 5E).

Hepatocytes were weakly PTN-positive in immunohistochemistry at 2 days of GalN-treatment (Figure 5, F and H). These weak signals were meaningful because we could see reproducibly the signals and negative immunostaining with goat normal IgG as the primary antibody produced no signals (Figure 5G). No PTN-positive cells were present in normal liver (data not shown). These results suggest that HSCs express PTN mRNA in response to the GalN treatment and its protein distributes on hepatocytes. Hepatocytes were also positive for PTN antibodies at 13 days of AAF/PHx treatment (Figure 5I). Oval cells were identified as AFP-positive cells at 13 days of AAF/PHx treatment (Figure 5J). These oval cells were negative for PTN staining (Figure 5I).

Expression of PTN Receptors in Liver Cells

Each of *N*-syndecan, PTPζ, and ALK has been reported to be a PTN receptor.[17–19] Both HSCs and Kupffer cells, but not hepatocytes express *N*-syndecan in the normal rat liver.[40] PTPζ is expressed in the normal rat brain.[18] To further study the possible role of PTN in the liver regeneration, we examined expression profiles of these reported PTN receptors in liver cells (Figure 6). PHs and SHs were isolated from normal livers and were cultured for 10 days (Figure 6A). Both PHs and SHs just after the isolation contained a measurable amount of *N*-syndecan mRNA in their RNA and increased its amounts during the culture period. Freshly isolated PHs and SHs did not express PTPζ mRNA, but started to express it when cultured, increasing the expression level as the culture period increased. The expression level of PTPζ mRNA was much lower (approximately one tenth) than that of *N*-syndecan mRNA. Liver cells, PHs, SHs, and NPCs were isolated from normal and GalN-treated livers, and mRNAs of PTN and its receptors were measured in these cells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(Figure 6B). The expression of *N*-syndecan and PTN was quite low or not measurable in PHs as reported previously[40] and in SHs isolated from the normal liver. The expression of PTN in normal NPCs was also quite low. By contrast normal NPCs expressed *N*-syndecan at a relatively high level. *N*-syndecan mRNA was increased in liver cells by GalN treatment. PHs and especially SHs in the GalN-treated liver much increased its expression, whereas NPCs rather decreased it. Thus, it can be said that the observed increase of syndecan mRNA in the GalN-treated liver cells was because of the increase in hepatocytes. The treatment of GalN up-regulated the expression of PTN mRNA in liver cells, PHs, SHs, and NPCs (Figure 6B). The expression of mRNA in PHs and SHs was very weak as compared with that in NPCs, indicating that the up-regulation of PTN mRNA in GalN-treated NPCs most contributed to its observed up-regulation in the GalN liver cells. We could not reliably determine PTPζ mRNA expression in these cells because of its low expression. No ALK expression was measurable in all these cells.

Localization of PTN mRNA and Its Protein in Embryonic Liver Cells

The above-described study suggests that PTN is a hepatocyte-mitogen in the HSC-associated liver regeneration. We examined the expression of PTN mRNA and its protein during embryonic liver development, another biological process in which hepatocytes actively proliferate, to obtain a further support for the notion that PTN is a mitogen of hepatocytes. The primordial hepatic diverticulum emerged at 11 days postcoitus (dpc) of rat fetuses. mRNA *in situ* hybridization experiments showed that the cells in the diverticulum were albumin-positive (data not shown). The mesenchymal cells in the septum transversum around the hepatic diverticulum strongly expressed PTN mRNA, whereas the hepatic diverticulum itself did not (Figure 7A). As the liver parenchyme developed from 11 to 12 dpc, the expression of PTN mRNA became much stronger in these cells (Figure 7C). PTN protein was located on both the hepatic diverticulum and the septum transversum at 12 dpc (Figure 7D). The immuno-signals were stronger in the hepatoblasts that located near the septum transversum than those located apart from it. Desmin-positive cells appeared in the mesenchymal cells of septum transversum and liver at 13 dpc, the latter being morphologically identified as HSCs (Figure 7E, small arrows). The mesenchymal cells in the septum transversum strongly expressed PTN mRNA, whereas the desmin-positive HSCs did not (Figure 7F). As previously reported,[12] nerve tissues, mesenchymal tissues of intestine, lung, and kidney were major tissues that contained PTN mRNA-expressing cells at 14 dpc (Figure 7G). In addition, the mesenchymal cells in the septum transversum strongly expressed PTN mRNA (Figure 7G, arrow). Immunohistochemistry identified the neural tube, epithelial tissues of intestine, lung, and kidney, and a part of brain as the PTN-positive tissues at 14 dpc (Figure 7H) as previously reported.[13] Hepatoblasts at 14 dpc were also positive in PTN immunostaining (Figure 7, H and I). The validity of the immunohistochemistry with the anti-PTN antibody used in the present study was shown in the experiment shown in Figure 7J, wherein the PTN antibody was first absorbed with hrPTN protein and was used as the first antibody. This absorption completely abolished the staining shown in Figure 7H.

Discussion

Adult hepatocytes hardly replicate repeatedly *in vitro* regardless of their remarkable growth potential *in vivo*.[1,2] Culture of hepatocytes is a useful experimental model to study the growth potential and the function of hepatocytes in well-defined conditions. We have been seeking an appropriate culture method of adult rat hepatocytes by which these cells can replicate repeatedly.[5,6] In the course of the study, we found that 3T3 cells markedly enhanced the growth of hepatocytes when incorporated in cultures of hepatocytes as feeder cells and the CM from 3T3 cells also stimulated the DNA synthesis of hepatocytes.[8] We identified PTN from the CM of 3T3 cells as an active principle that stimulates the DNA synthesis of hepatocytes, suggesting that PTN is a new member of hepatocyte mitogens. However, the biological significance of PTN in the regulation of the growth of hepatocytes has remained to be clarified.

The present study was performed to obtain data that support the assertion that PTN plays a role(s) in stimulating the growth of hepatocytes in biological and pathological processes taking place *in vivo*. First, we showed that HSCs as well as 3T3 cells promote the growth of rat hepatocytes in cultures. Both HSCs and 3T3 cells synthesized mRNAs of PTN and HGF, and secreted these proteins into the culture medium. RSFs showed no such

Figure 7. Profiles of expression of PTN mRNA and its protein in developing fetuses. Sagittal sections were prepared from rat fetuses at 11 (**A**, **B**), 12 (**C**, **D**), 13 (**E**, **F**), and 14 dpc (**G–J**). Sections were subjected to *in situ* hybridization using PTN anti-sense (**A**, **C**, **F**, **G**) and sense probes (**B**) or immunohistochemistry using antibodies against PTN (**D**, **H**, **I**) and desmin (**E**). **A:** PTN mRNA is seen in the septum transversum (st; **arrows**, blue), but not in the hepatic diverticulum (hd); h; heart. **B:** No signals with the PTN sense probe. **C:** Strong signals of PTN mRNA are seen in the septum transversum (**arrows**), whereas no signals are seen in the developing liver (l). g; gut. **D:** Hepatoblasts and the septum transversum are positive for anti-PTN antibodies (brown, **arrows**). **E:** HSCs (hsc, green-fluorescence, **small arrows**) are positive for desmin immunostaining as well as the mesenchymal cells of the septum transversum and heart (**thick arrows**). The hepatoblasts (**arrowhead**) and blood cells (**asterisk**) are seen yellow-red and orange because of the autofluorescence of these cells, respectively. **F:** PTN mRNA *in situ* hybridization. The section shown in **E** was used for this experiment. Specific and high expression of PTN mRNA is seen in the septum transversum (**thick arrow**), whereas no expression is seen in HSCs (**small arrows**) and hepatoblasts (**arrowhead**). **G:** PTN mRNA is expressed in nerve tissues and the mesenchymal tissues. The septum transversum strongly expresses PTN mRNA (**arrow**). i; intestine, k; kidney, lu; lung, m; metencephalon, ma; mandibular arch, nt; neural tube, t; telencephalon. **H:** Immunoreactivity to anti-PTN antibodies is seen in the nerve tissues and the epithelial tissues. The liver is weakly positive (**arrow**). **I:** The region enclosed by a **small square** in **H** is enlarged. Hepatoblasts are positive in PTN immunostaining (**arrows**). **J:** The same immunostaining was performed as in **H** using anti-PTN antibodies that had been absorbed by hrPTN protein. No immunoreactivity is seen. Scale bars, 50 μm (**A–F** and **I**); 1 mm (**G**, **H**, and **J**).

growth-promoting activity on hepatocytes and did not synthesize and secrete PTN and HGF. Thus, PTN and HGF would be causative factors for the growth promotion of hepatocytes manifested by HSCs and 3T3 cells. HGF is known as a strong mitogen for hepatocytes.[3,4] Actually, the present study showed that the presence of HGF in the culture media enhanced the growth of hepatocytes.

Although the mitogenic activity of PTN for hepatocytes is approximately one twentieth of that of HGF on the basis of the effective dose required for a similar growth promotion, hrPTN significantly enhanced the growth of cultured hepatocytes. We previously demonstrated that purified PTN from 3T3-CM had a higher activity in stimulating the synthesis of DNA of cultured hepatocytes than hrPTN and chemically synthesized PTN.[8] The present study used hrPTN produced by baculovirus-transfected insect cells. Such a recombinant PTN produced by insect cells may have a weaker biological activity on hepatocytes than the native PTN, because it is known that recombinant PTN produced by either prokaryotic cells or insect cells has neurite growth activity on rat neurons, but not mitogenic activity on several cell lines.[41,42] Therefore, we assume that the native PTN shows a much higher potency to promote the growth of hepatocytes than hrPTN used in the present study. We found that significant amounts of PTN were bound to the surface of 3T3 cells, ~50% of the secreted PTN being present there (data not shown). This fact indicates that the actual capacity of 3T3 cells to synthesize PTN should be higher than that estimated by its concentration in the CM.

The physiological role of PTN in regulating the growth of hepatocytes was studied by examining the expression of PTN in four different liver regeneration models. We also examined the expression of HGF and MK in these models. PHx induces the replication of hepatocytes,[23] with the peak of their DNA synthesis at 24 hours after PHx.[38] The peak of the level of HGF mRNA expression in the liver is seen at 12 hours after PHx.[38] On the other hand, the present study showed that the peak of PTN mRNA expression was seen at 3 days after the PHx, suggesting that PTN does not play a role for the onset of DNA synthesis in the PHx-induced regeneration. A moderate up-regulation of the PTN gene was seen in the CCl_4 model. It is known that CCl_4 causes hepatocellular damage around the central vein and induces DNA synthesis of hepatocytes at 48 hours with the peak at 60 hours after the CCl_4 treatment.[22,24] PTN mRNA started to be up-regulated at 48 hours and peaked at 72 hours after the CCl_4 treatment, suggesting that PTN plays a role(s) for the onset of DNA synthesis of hepatocytes in the regeneration of CCl_4-damaged liver.

GalN specifically inhibits the protein synthesis of hepatocytes and caused their necrosis.[20] GalN accelerates the consumption of uridine nucleotides and UDP-glucose, and causes the accumulation of slowly metabolizing UDP-hexosamines and UDP-*N*-acetylhexosamines (uridylate trapping) in the hepatocytes. As a result, the synthesis of RNA and then protein of the hepatocytes is blocked, which leads to the liver damage.[20] In the AAF/PHx model, AAF first inhibits the growth of hepatocytes, then PHx induces the regeneration.[43] In both the GalN and AAF/PHx models, the damage to hepatocytes commonly results in the emergence of oval cells, a specific population of liver progenitor-like cells.[20,21,43–45] It is thought that bile duct cells dedifferentiate to oval cells in this situation.[44,45] Oval cells express specific markers of both hepatocytes and bile duct cells, and are thought to have the potential to differentiate into these two types of cells.[20,44,45] Thus, the proliferation and differentiation of oval cells seem to be crucial events taking place in liver regeneration of these models. In addition, the level of induced PTN mRNA expression was much higher in the GalN and AAF/PHx model than that of PHx and CCl_4 model, suggesting that PTN has a unique role in the former two models. Oval cells are known to emerge commonly in the liver of the two models. Oval cells begin to proliferate at 1 day in the GalN model[20] and at 1 to 3 days in the AAF/PHx model.[43,45] The present study showed that expression of PTN mRNA sharply and transiently rose its amounts at 2 days in the GalN model. On the other hand, PTN mRNA gradually increased during 4 to 13 days in the AAF/PHx model. Thus, it is unlikely that PTN directly affects the onset of oval cell proliferation. The target of action of PTN in these models should be hepatocytes, because immunohistochemistry localized PTN on these cells. The DNA synthesis of hepatocytes in the GalN model peaks at 60 hours,[24] which supports this interpretation. Hepatocytes began to proliferate on day 2 in the GalN model and reached their maximum level on day 5.[20] Bile duct cells generate both oval cells and SHs in GalN-treated liver.[44] The SHs increase their number during 2 to 4 days of GalN-induced regeneration.[44] Oval cells were demonstrated to differentiate into basophilic hepatocytes in the AAF/PHx model,[43] and these hepatocytes start to actively proliferate at 9 days. Based on these previous and our present studies we speculate that bile duct cells dedifferentiate into oval cells in the regeneration model of GalN and AAF/PHx, and these oval cells proliferate and differentiate into hepatocytes to replace the damaged hepatocytes. In addition, other investigators suggested that injured hepatocytes can recover from the injury and proliferate again.[20,44] We consider that activated HSCs in the GalN and AAF/PHx model secrete PTN that might act as a mitogen on the two types of hepatocytes, one derived from oval cells and the other recovered from the damaged hepatocytes.

As discussed above, *in situ* hybridization and immunohistochemistry of the GalN-treated liver localized PTN mRNA in HSCs and its protein on hepatocytes, respectively. Such a different localization of PTN mRNA in HSCs and its protein on hepatocytes was also reported previously by other investigators in embryonic tissues. The mesenchymal tissues of gut express PTN mRNA, whereas its protein is found in the epithelial tissues.[12,13] The differential expression of PTN mRNA and its protein suggests that HSCs are activated in response to GalN intoxication and begin to express the PTN gene, and then PTN acts as a mitogen on hepatocytes. This notion is also supported by the fact that the presence of HSCs enhances the growth of hepatocytes in culture, wherein cultured HSCs were proven to synthesize and secrete PTN protein in the present study.

Each of *N*-syndecan, PTPζ, and ALK has been reported to function as a receptor of PTN.[17–19] *N*-syndecan is a cell surface heparan sulfate proteoglycan.[17] This proteoglycan as the receptor of PTN mediates the neurite outgrowth activating the *Src* kinase-cortactin pathway.[17] PTPζ is involved in the neuronal migration in the brain.[18] In the present study we showed that the hepatocytes in the liver exposed to GalN increased the expression of *N*-syndecan mRNA, the time of its expression well correlating with that of PTN mRNA. This correlation supports that *N*-syndecan acts as PTN receptors in hepatocytes in this model. As we showed previously[8] and in the present study, PTN stimulates the growth of hepatocytes in culture. On the other hand, the present study demonstrated that hepatocytes in culture spontaneously much increase the expression of *N*-syndecan mRNA or start to express PTPζ mRNA. Thus, it is suggested that PTN/*N*-syndecan or/and PTN/PTPζ signaling works in the cultured hepatocytes and stimulates their growth. However, it should be noted that the expression of PTPζ was extremely low as compared with that of *N*-syndecan. Thus, the biological significance of PTPζ in the liver regeneration and in the growth of cultured hepatocytes is unclear at present. ALK is expressed in neuronal tissues and is an orphan receptor of an insulin receptor subfamily of tyrosine kinase.[46] The intracellular domain of the ALK is fused to the 5′ half of nucleophosmin in the t(2,5) (p23;q35) translocation and the chimeric protein is known to be associated with the non-Hodgkin lymphoma. More recently, ALK was identified as a receptor of PTN.[19] We did not detect any expression of ALK in cultured hepatocytes and livers of the four liver regeneration models. Therefore, we assume that ALK does not act as the PTN receptor at least in the liver. Considering these results together, we suggest that PTN is secreted from activated HSCs and acts as a growth factor via *N*-syndecan on hepatocytes during liver regeneration induced by GalN treatment or AAF/PHx.

The embryonic heart mesoderm induces the gut endoderm to differentiate into the primordial liver.[25] Growth factors such as fibroblast growth factors and still-unidentified factors participate in this induction process.[47] The present study showed that the mesenchymal cells in the septum transversum surrounding the embryonic liver strongly expressed PTN mRNA. Its protein was found in both the septum transversum and the hepatic diverticulum composed of hepatoblasts. The expression of PTN in fetus liver gradually decreased as the liver bud developed. These results suggest that PTN plays a role in the early epithelial-mesenchymal interaction taking place in the embryonic liver development.

We examined the expression of MK mRNA in the liver regeneration models. Its expression was significantly increased in GalN, AAF/PHx, and CCl_4 models, although the expression level was quite low. MK may play some roles in liver regeneration. We previously observed that synthetic human MK also stimulates the DNA synthesis of cultured rat hepatocytes.[8] MK mRNA was not detectable in cultured HSCs and 3T3 cells, which differed from the situation seen in PTN mRNA. Although MK is expressed in developing liver,[13] MK-deficient mice did not show any gross anatomical abnormalities.[48] PTN- or *N*-syndecan-deficient mice also showed no gross anatomical abnormalities.[49,50] These gene-deletion experiments do not necessarily indicate that PTN or MK is not required for the normal development of embryonic liver, because it is likely that PTN compensates MK and vice versa.

The type of cells that express PTN in the liver is different between the regeneration process in adults and the organogenesis process in embryos. HSCs in the regenerating adult liver expressed PTN mRNA, whereas embryonic HSCs in the developing primordial liver did not. Instead, mesenchymal cells of the septum transversum expressed it in the latter case. It has been postulated from a morphological study that HSCs stem from the mesenchymal cells of the embryonic septum transversum.[51] We assume that each of biologically active mesenchymal cells in embryos and pathologically active HSCs in the damaged adult liver promotes the growth of hepatocytes by secreting PTN as a hepatocyte mitogen. In this context we consider that HSCs in embryonic and normal adult liver are in a biologically quiescent state because they do not express PTN as shown in the present study. The embryonic origin of HSCs is controversial. Some of HSCs in the adult liver express neural cell markers such as glial fibrillary acidic protein and nestin, which led investigators to claim that HSCs are heterogeneous in their origin.[52,53] We showed that most of desmin-positive HSCs express PTN mRNA. At present, it is not clear whether this expression profile was caused by the heterogeneity of HSCs or the different sensitivity of *in situ* hybridization of PTN mRNA among HSCs. In either case, it should be emphasized that there exist HSCs that express PTN in response to the intoxication with GalN and possibly stimulate the growth of hepatocytes. From the results obtained in the present study we propose that PTN plays a role as a growth factor for hepatocytes in the developmental and pathological processes taking place in the liver.

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