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Expression of Hepatocyte Growth Factor and c-*met* Genes during Hepatic Differentiation and Liver Development in the Rat

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Hepatocyte growth factor (HGF) is a potent mitogen for mature hepatocytes* in vitro. *The receptor for HGF has recently been characterized as the product of the proto-oncogene c-*met. *We have examined the possible involvement of HGF in hepatic growth and differentiation in the rat. The experimental systems used were acetylaminofluorene treatment combined with partial hepatectomy to induce proliferation and differentiation of oval cells in adult liver and the pre- and postnatal liver. In the acetylaminofluorene model, Northern blot analysis showed that level of HGF transcripts increased one day after partial hepatectomy, reached a peak by day 6, were maintained at that level until day 13, and then declined, reaching normal level at 20 days. The expression of c-*met *also increased gradually, reached a peak around 9 to 13 days after partial hepatectomy, at which time oval cell proliferation was most prominent. In the developing liver, an elevated level of HGF transcripts was found between 4 and 21 days after birth. The expression of c-*met *also slightly increased at the same time.* In situ *hybridization showed that the transcripts for HGF were localized in desmin-positive Ito cells, whereas the transcripts for c-*met *were strongly expressed by oval cells. We have shown earlier that Ito cells and oval cells proliferate simultaneously and exist in close proximity in the acetylaminofluorene model and that Ito cells are a primary source of growth factors such as transforming growth factor-α and acidic fibroblast growth factors. The data presented here suggest that HGF is, in combination with other growth factors, involved in the proliferation and differentiation of oval cells via a paracrine mechanism. (Am J Pathol 1993, 142:1823–1830)

The capacity of the liver to regenerate after a reduction in liver mass induced by chemical intoxication or surgical removal is quite remarkable. The most widely studied example of this phenomenon is regeneration after partial hepatectomy in rats, in which normal liver mass is restored within ten days. This model provides a unique *in vivo* system to characterize the regulatory factors that control normal cell proliferation. The molecular mechanisms of liver regeneration and differentiation still remain the central topic in the field of liver biology.

Over the past decade, it has been demonstrated that growth factors play a pivotal role in liver regeneration. They act as either potent mitogenic or mitoinhibitory regulators of hepatocyte proliferation.[1,2] Several polypeptide growth factors such as epidermal growth factor, transforming growth factor-α (TGF-α), TGF-β1, acidic fibroblast growth factor (aFGF), and hepatocyte growth factor (HGF) are involved in liver regeneration.[1–6] Although some of the growth factors important for liver regeneration may also be involved in the development and differentiation of liver,[7–9] much less is known about this aspect of growth factor action in liver study.

Recently, HGF has aroused increasing interest due to its powerful mitogenic effect and multiple functions on various cell types.[10,11] HGF was originally identified in the serum of partially hepatectomized rats as a potent mitogen for normal adult rat hepatocytes in culture[12,13] and was subsequently cloned from both human and rat.[14,15] HGF is synthesized as a single polypeptide precursor chain of 728 amino acids, which is processed to form a mature heterodimer composed of an α-unit (69 kd) and a β-unit (34 kd).

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Because elevated levels of HGF in the plasma and HGF messenger (m)RNA in the rat liver were observed after both partial hepatectomy and necrosis induced by hepatotoxins, such as carbon tetrachloride and D-galactosamine, at a time that precedes the initiation of DNA synthesis, HGF was presumed to act as a trigger for liver regeneration.[5,16] HGF can also stimulate proliferation of a wide variety of other epithelial cells, including renal tubular epithelial cells, epidermal keratinocytes, and melanocytes.[17–20] The scatter factor that can induce dissociation of epithelial cells and increase cell motility in culture has been shown to be identical to HGF.[21–23] Montesano et al[24] have shown that treatment of Madin–Darby canine kidney epithelial cells grown in collagen gels with HGF induces the formation of branching tubules. These results suggest that HGF can act both as a motogen and a morphogen in addition to its mitogenic effect. The receptor for HGF has recently been identified as the product of the proto-oncogene c-*met*[25,26] and is present on numerous epithelial cell types.

It has been proposed by this laboratory, as well as by others, that a stem cell compartment exists in the adult rat liver.[27,28] This hepatic stem cell compartment is usually dormant except under conditions in which the adult differentiated hepatocytes are unable to regenerate the liver following loss of mass.[27] The so-called oval cells are thought to be a progeny from the putative stem cells, and these cells can expand significantly and differentiate into hepatocytes.[29,30] At the same time, mesenchymal cells (Ito cells) also proliferate vigorously in close association to the oval cells, suggesting an important interaction between these two cell groups during oval cell expansion and differentiation. We have shown earlier that Ito cells are a rich source of growth factors, including TGF-α and aFGF, that are associated with hepatic growth and differentiation.[7,9] The fact that HGF is produced by mesenchymal cells and is localized in the nonparenchymal cells in the liver[10] led us to hypothesize that HGF may participate as a paracrine effector in the proliferation and differentiation of the liver stem cell compartment. In the present paper, we provide data supporting the notion that HGF is involved in hepatic growth and differentiation via a paracrine mechanism.

Materials and Methods

Treatment of Animals

Male Fischer rats weighing 150 g were used. Rats were fed standard Purina chow pellet (National Institutes of Health supply) and allowed to drink water *ad libitum*. The administration of 2-acetylaminoflurorene (AAF) combined with partial hepatectomy (PH) was used to induce the proliferation and differentiation of oval cells. This included administration of AAF by gavage (1.5 mg/day) for 4 days before and 5 days after PH. The animals were killed between 1 and 20 days after PH. Two animals were included in each time point. In another group, fetal and postnatal animals were killed at various time points as indicated in the figures. Livers were pooled together from five to 10 animals at each time point for the isolation of RNA.

Histology and Immunocytochemistry

Immediately after removal, two small pieces of liver were cut by a razor blade. One piece was frozen at −50 C for *in situ* hybridization and immunocytochemistry. Another piece was fixed in Bouin's fixative and embedded in paraffin. Sections were stained with hematoxylin and eosin. The rest of the liver was instantly frozen in liquid nitrogen and kept at −70 C until use.

A monoclonal antibody, OV6 (kindly provided by Dr. Harold Dunsford, Galveston, TX) that recognizes both bile duct epithelial cells and oval cells was used to identify oval cells. A monoclonal antibody against porcine desmin (Dako, Carpinteria, CA) was used to localize Ito (perisinusoidal stellate) cells in the liver. The ABC immunostaining method was carried out by utilizing the Vectastain Elite kit (Vector Laboratories, Burlingame, CA).

Probes

A 600-bp complementary (c)DNA fragment encoding the 3′ end of rat HGF (kindly provided by Dr. Brian Carr, Univ. of Pittsburgh School of Medicine, Pittsburgh, PA) was subcloned into the pBluescript SK vector. A 570-bp *Eco*RI fragment of mouse c-*met* cDNA (kindly provided by M. Oskarson, NCI, Frederick Cancer Research and Development Center) was subcloned into the plasmid of pGEM7Z. Anti-sense and sense riboprobes of both HGF and c-*met* were labeled with ^{32}P and ^{35}S for Northern blot and *in situ* hybridization, respectively. The cDNA probe coding for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was labeled by nick translation and used for Northern blot analyses as an internal standard.

Northern Blot Analyses

Total RNA was isolated from the livers by guanidine isothiocyanate and centrifugation in cesium chloride. Poly(A+) RNA was selected by oligo(dT)cellulose chromatography. Ten μg of poly(A+) selected

RNA from each sample were electrophoresed in 0.8% agarose gel and then transblotted to nylon membrane. Blots were hybridized with ^{32}P-labeled riboprobes at 60 C and cDNA probe at 42 C overnight. After washing off the nonspecific binding, filters were exposed to Kodak XAR film.

In Situ *Hybridization*

In situ hybridization was performed on the frozen liver sections as previously described.[31] A combination of *in situ* hybridization and immunocytochemistry with anti-desmin and OV6 antibodies was employed to localize the specific signals produced by Ito cells and oval cells.

Results

The histological changes were consistent with our previous reports.[8,29,30] In our AAF model of hepatic differentiation, the proliferation of both oval cells and desmin-positive mesenchymal cells (Ito cells) is evident within a few hours after PH (unpublished data) in the periportal areas. However, a significant penetration of oval and Ito cells from portal areas into liver parenchyma was observed around day 4 after the operation and reached the peak around day 9. The differentiation of oval cells into hepatocytes was evident 11 to 15 days after the operation and by day 20, only a few oval cells were still present.

Northern Blot Analyses

A low level of HGF was observed in the normal rat liver. In contrast, a significant increase of the HGF gene expression was observed in the AAF-treated rats after PH. The level of HGF transcripts increased as early as 1 day after the operation, gradually reached a peak by day 6, was maintained at a high level until day 13, and then gradually declined to a normal level at day 20 (Figure 1a). At the same time, the expression of the c-*met* gene also increased gradually (Figure 1b). A significant increase in c-*met* expression was detected by day 1 after PH, and the highest level was observed around 9 to 13 days; by day 15 it started to decline. Similar results were also obtained from other independent experimental series.

As controls, we checked the expression of both HGF and c-*met* in animals with AAF administration or PH only. No increase of HGF and c-*met* transcripts was found up to 20 days after 9 doses of AAF administration (data not shown). In the PH animals, we got a transient increase of HGF expression which peaked at 12 hours as reported by other investigators.[5,6]

During the perinatal development of the rat liver, the first significant increase of HGF expression was noticed 4 days after birth. The hepatic HGF expression reached a peak level in animals about 1 to 2 weeks old (Figure 2a) and then started to decrease at 3 weeks. The level of c-*met* transcripts also showed a slight increase over that seen in normal adult rat liver during the same postnatal period (Figure 2b).

In Situ *Hybridization*

The distribution of HGF mRNA was similar in both the adult and postnatal livers. They were localized in the nonparenchymal cells as reported by other investigators.[32,33] During the period of oval cell and Ito cell proliferation, HGF-positive cells are also frequently found in close proximity to oval cells (Figure 3a), and there are seemingly more positive cells in oval cell areas than in the other areas of the liver

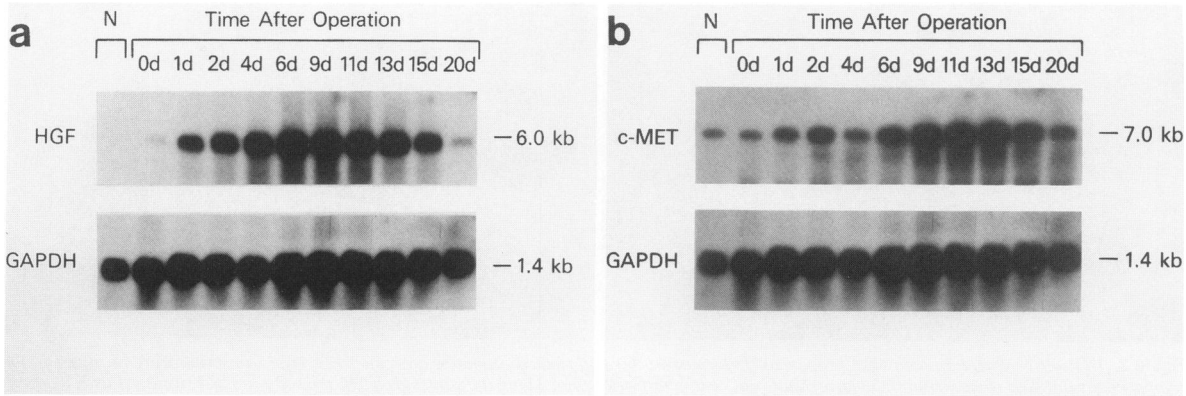


Figure 1. *Northern blot hybridization of poly(A)-selected mRNA from normal rat liver and livers of AAF-treated animals at the time of partial hepatectomy (0 day) and 1, 2, 4, 6, 9, 11, 13, 15, and 20 days after the operation. ^{32}P-labeled riboprobes for HGF* (a) *and* c-met (b) *were used. Glyceraldehyde-3-phosphate dehydrogenase was used as an internal standard.*

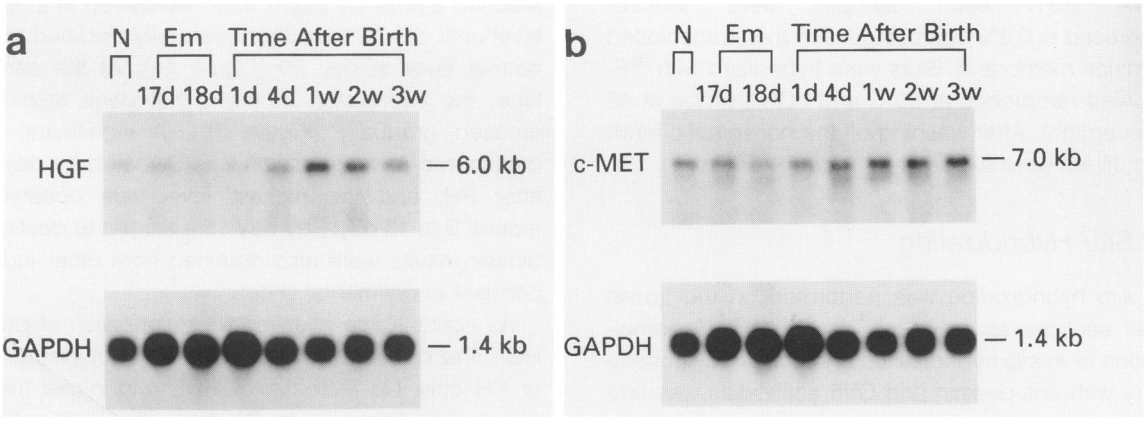


Figure 2. *Northern blot hybridization of poly(A)-selected mRNA from livers of normal adult rat (N), embryonic rats (Em) 17 and 18 days after gestation, and postnatal rats 1, 4, 7, 14, and 21 days after birth. ^{32}P-labeled riboprobes for HGF* (a) *and c-*met (b) *were used. Glyceraldehyde-3-phosphate dehydrogenase was used as an internal standard.*

lobule (Figure 3a). In a serial section hybridized with the sense probe, very few grains could be seen (Figure 3b). To identify the cell type that expresses HGF, we have utilized a combination of *in situ* hybridization with immunocytochemistry. This has allowed us to demonstrate clearly that HGF transcripts are specifically localized in desmin-positive Ito cells (Figure 4). In the oval cell areas, HGF signals were also found in the desmin-positive Ito cells surrounding the ductal structures of oval cells (Figure 4a) or interspersed with the oval cells (Figure 4b). Interestingly, obvious heterogeneity was observed in HGF expression among the desmin-positive Ito cells.

In the normal adult and perinatal rat livers, a few silver grains representing c-*met* transcripts were found on the hepatocytes. During hepatic differentiation in the AAF model, the c-*met* transcripts were

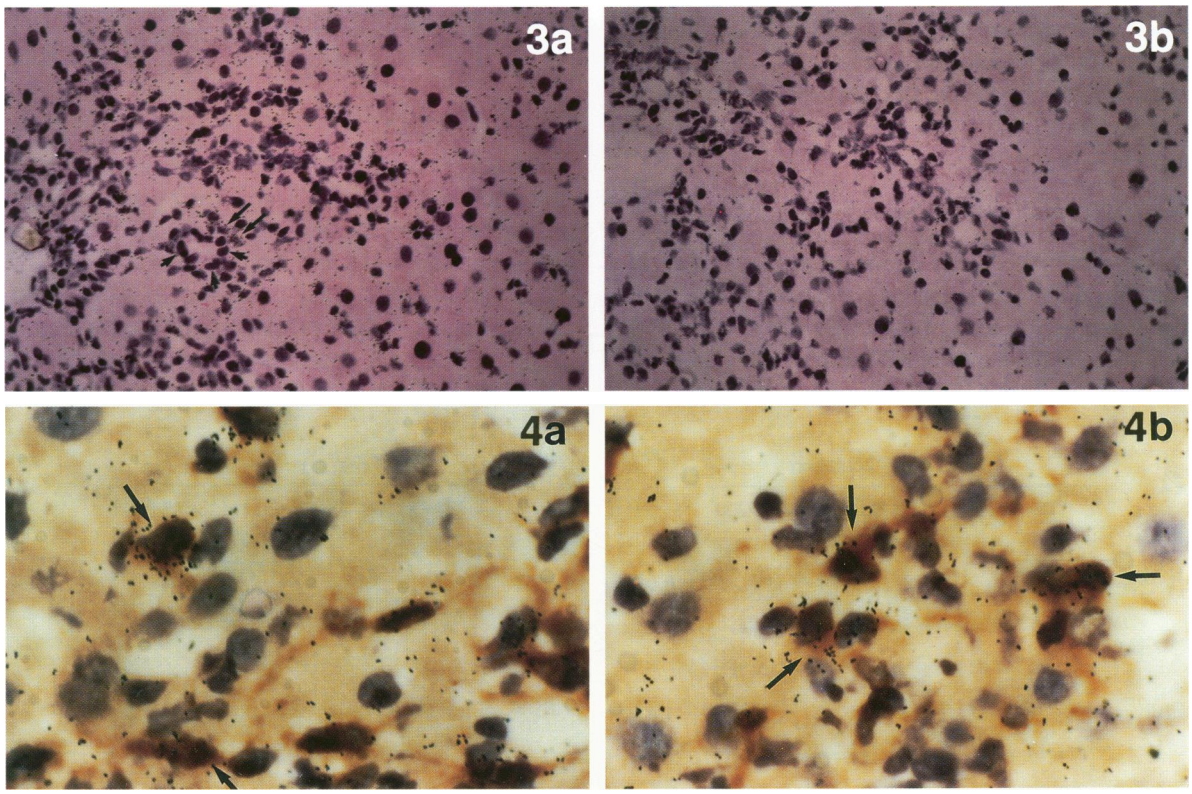


Figure 3. In situ *hybridization with HGF probe was performed in the AAF-treated animal 6 days after PH. HGF transcripts were localized in the nonparenchymal cells (long arrows) either around oval cells (short arrows) or along the sinusoids when the section was hybridized with the antisense probe* (a). *Few grains can be seen on a serial section hybridized with the HGF sense probe* (b). *Original magnification ×40.*

Figure 4. *The combination of* in situ *hybridization with desmin immunostaining of the liver from the AAF-treated animal 6 days after PH. In the oval cell areas, the transcripts for HGF are localized in the desmin-positive Ito cells (arrows) around the ductal structure of oval cells* (a) *or interspersed with oval cells* (b). *Original magnification ×100.*

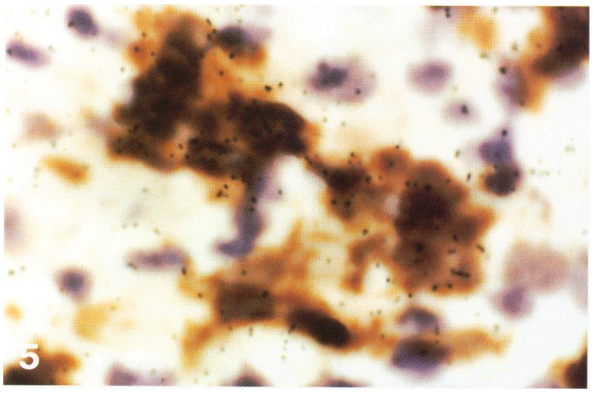
clearly localized in oval cells (Figures 5 and 6), whereas the desmin-positive Ito cells were entirely negative (Figures 6 and 7). The transcripts for c-*met* were also detected in the transitional type of oval cells (Figure 7) when oval cells were undergoing differentiation between 11 to 15 days. No increase of the c-*met* transcripts was found in the quiescent adult hepatocytes.

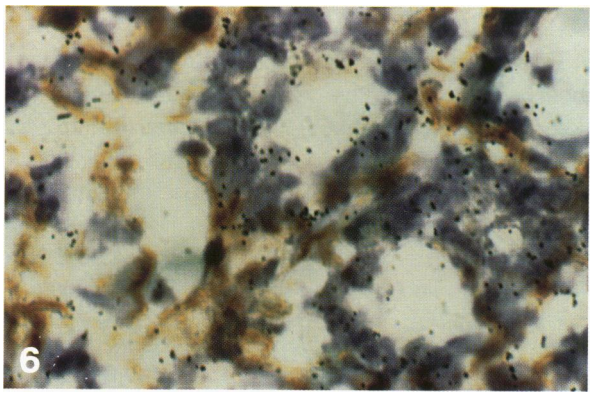
Discussion

The HGF receptor c-*met* is similar to other growth factor receptors with tyrosine kinase activity, including TGF-α/EGFR and aFGF/FGFRs, and constitutes a part of the cytokine/receptor systems that are activated in the early hepatic proliferation after PH.[1,4,34] These cytokine/receptor systems are thought to function in a coordinated fashion resulting in the proliferation of fully differentiated hepatocytes and regeneration of the liver after PH. It is not known whether the HGF/c-*met* system becomes activated when the liver is regenerating via the oval cell/stem cell compartment. In the present study, we provide evidence suggesting the involvement of the HGF/c-*met* system in both regeneration via the oval cell compartment in adult liver and in liver development during the perinatal period.

The HGF transcripts in the liver have previously been localized in the nonparenchymal cells by *in situ* hybridization.[33] This was also confirmed by immunocytochemical staining with HGF antibody.[34] Recently, two groups showed by cell fractionation and Northern blot analysis that HGF mRNA is mainly produced by Ito cells.[35,36] To identify the cells that express the HGF transcripts, we have utilized the combination of *in situ* hybridization with immunocytochemistry. The use of cell type-specific antibodies allows us to identify the cell types that are expressing the genes of interest. Our results show that the HGF transcripts were uniquely distributed in the desmin-positive Ito cells. Therefore, our combination study further confirmed the Northern blot analyses reported by Schirmacher et al[35] and Ramadori et al.[36] The failure in finding the HGF transcripts in all the Ito cells may be due to the fact that only the Ito cells in certain stages of differentiation can express HGF transcripts. This notion is supported by the observation that Ito cells lose the ability to produce HGF when they transit into myofibroblast and fibroblast.[35]

The recent characterization of the c-*met* protein as the receptor for HGF[25,26] has further defined both the cell targets and mechanism of HGF effects. Our *in situ* hybridization demonstrates that c-*met* transcripts are expressed in both oval and transitional cells (Figures 5 and 7). These data indicate that oval cells are the target for HGF in our hepatic differentiation model system. In contrast,





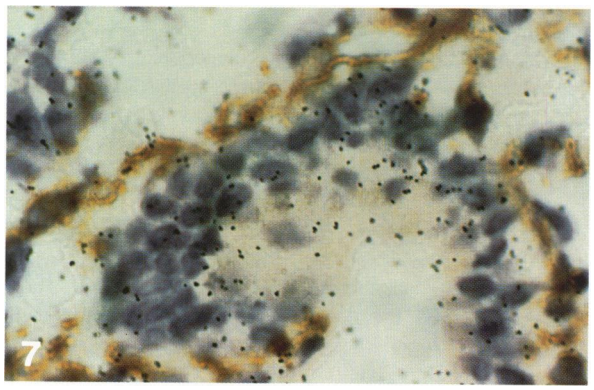


Figure 5. *The combination of* in situ *hybridization with OV6 immunostaining of the liver from an AAF-treated rat 6 days after the operation shows that the transcripts for* c-met *are expressed in the OV6-positive oval cells. Original magnification* × *100.*
Figure 6. *The combination of* c-met in situ *hybridization with desmin immunostaining in the animal 9 days after PH showed that the* c-met *transcripts were preferentially expressed in oval cells, whereas the desmin-positive Ito cells around the oval cells were negative. Original magnification* × *100.*
Figure 7. In situ *hybridization of* c-met *combined with desmin immunostaining in an AAF-treated animal 13 days after PH shows that* c-met *transcripts are present in the transitional type of oval cells. The surrounding Ito cells are negative. Original magnification* × *100.*

c-*met* transcripts were not detected in Ito cells and no HGF transcripts were found in oval cells. Thus the mechanism of action for this cytokine receptor system in our model seems to be paracrine, where Ito cells are the source of the growth factor and oval cells are the target. Furthermore, the high density of c-*met* transcripts in the oval cells may indicate a higher level of the receptor that would make them more sensitive to HGF.

The interaction between stem cells and mesenchymal cells has been proposed in various systems.[37,38] The mesenchymal cells may have a supporting role for the stem cells by providing an appropriate microenvironment. In the liver, Ito cells can produce the components of extracellular matrices such as type IV collagen and laminin which are known to be important in cell growth and differentiation.[37,39–41] Our present data demonstrate that the mesenchymal cells may also be involved in the activation of the stem cell compartment by secreting growth factor such as HGF, in addition to their indirect role of providing a supporting extracellular matrix.

The mitogenic role of HGF has been established in various types of epithelial cells *in vitro*.[11] An increased expression of HGF is also found to be associated with cell proliferation *in vivo*.[5,6] In the present model, increased levels of HGF transcripts are observed as early as one day after PH, at which time oval cell proliferation is also detectable (unpublished data). Furthermore, the expression of HGF reaches the peak when oval cell proliferation is most prominent and then declines when differentiation of oval cells into hepatocytes occurs. The coincidence of HGF expression and oval cell expansion strongly indicates that HGF can act as a mitogen for oval cells. In addition, the HGF/c-*met* system seems to be activated earlier than the other cytokines that we have studied previously.[7,9] This may indicate that the HGF/c-*met* system, possibly together with some yet unknown factors, is involved in triggering the proliferation of liver stem cells.

Scatter factor, which is now identified as the same factor as HGF,[21–23] can enhance the motility of several epithelial and endothelial cell lines by causing cell dissociation and migration, the so-called scattering effects.[42] At the same concentration, HGF has both mitogenic and scattering effects on cultured hepatocytes. The addition of HGF to hepatocyte cultures induces the formation of long processes and cell migration.[10] *In vivo*, both cell proliferation and migration are important in the process of hepatic differentiation. Thus, the scattering effects of HGF may be responsible for the migration of proliferating oval cells from portal areas into the hepatic lobules.

Active proliferation and differentiation of hepatocytes also occur during liver development. An increased level of HGF transcripts was found at the postnatal stage between 4 days and 3 weeks, with a peak at 1 week. The level of c-*met* gene expression is also slightly increased at the same time. Because this is the time when both vigorous hepatocyte proliferation and hepatic morphogenesis, which lead to the formation of acinar configuration with a single cell hepatic plate, are taking place,[43] the results may indicate that HGF exerts both mitogenic and morphogenic effects during liver development.

In our model system of hepatic differentiation and liver development, the expression of several cytokines including HGF, TGF-α, and aFGF is overlapping. Among these growth factors, HGF acts as a paracrine effector, whereas TGF-α and aFGF function via both autocrine and paracrine mechanisms.[7,9] Because all these growth factors can induce proliferation, differentiation, motility, and morphogenesis of various cells, they may work synergistically or each play a major role in a particular aspect in this complex system.

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