

# Hepatocyte Growth Factor Induces Proliferation and Differentiation of Multipotent and Erythroid Hemopoietic Progenitors

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**Abstract.** Hepatocyte growth factor (HGF) is a mesenchymal derived growth factor known to induce proliferation and "scattering" of epithelial and endothelial cells. Its receptor is the tyrosine kinase encoded by the *c-MET* protooncogene. Here we show that highly purified recombinant HGF stimulates hemopoietic progenitors to form colonies in vitro. In the presence of erythropoietin, picomolar concentrations of HGF induced the formation of erythroid burst-forming unit colonies from CD34-positive cells purified from human bone marrow, peripheral blood, or umbilical cord blood. The growth stimulatory activity was restricted to the erythroid lineage. HGF also stimulated the formation of multipotent CFU-GEMM colonies. This effect is synergized by stem cell

factor, the ligand of the tyrosine kinase receptor encoded by the *c-KIT* protooncogene, which is active on early hemopoietic progenitors. By flow cytometry analysis, the receptor for HGF was found to be expressed on the cell surface in a fraction of CD34<sup>+</sup> progenitors. Moreover, in situ hybridization experiments showed that HGF receptor mRNA is highly expressed in embryonic erythroid cells (megaloblasts). HGF mRNA was also found to be produced in the embryonal liver. These data show that HGF plays a direct role in the control of proliferation and differentiation of erythroid progenitors, and they suggest that it may be one of the long-sought mediators of paracrine interactions between stromal and hemopoietic cells within the hemopoietic microenvironment.

**H**EPATOCYTE growth factor (HGF)<sup>1</sup> (Nakamura et al., 1989; Miyazawa et al., 1989), also known as scatter factor (Naldini et al., 1991a; Wiedner et al., 1991), has the unique feature of combining mitogenic and motogenic activities on its target cells. HGF is mitogenic for hepatocytes (Michalopoulos, 1990) and other epithelial cells, such as kidney tubular epithelium, melanocytes, and keratinocytes (Kan et al., 1991; Rubin et al., 1991; Halaban et al., 1992; Matsumoto et al., 1991). In these cells, HGF also promotes "scattering" (Stoker et al., 1987; Gherardi et al., 1989; Weidner et al., 1990, 1991; Naldini et al., 1991a) and matrix invasion (Weidner et al., 1990; Naldini et al., 1991a), and it has chemotactic properties (Morimoto et al., 1991; Giordano et al., 1993). The factor stimulates extracel-

lular matrix degradation by enhancing the synthesis of enzymes involved in extracellular matrix proteolysis (Pepper et al., 1992; Boccaccio et al., 1994). HGF acts as a morphogen during neuroectodermal development in vivo (Stern et al., 1990), and it induces three-dimensional organization of epithelial cells in vitro (Montesano et al., 1991). The factor also promotes the progression of carcinoma cells toward malignant invasive phenotypes (Weidner et al., 1990).

The HGF molecule shares several structural similarities with plasminogen and other proteins of the blood coagulation cascade. These similarities include four characteristic "kringle" domains at the amino terminus, a zymogen activation site, and a carboxy-terminal serine protease-like domain (Miyazawa et al., 1989; Nakamura et al., 1989; Sottrup-Jensen et al., 1978). HGF is secreted by cells of mesodermal origin as an inactive single chain precursor (pro-HGF), which is converted to the active form in the extracellular environment by urokinase and/or a thrombin-activated serum protease (Naldini et al., 1992; Miyazawa et al., 1993; Shimomura et al., 1993). Mature HGF is a disulfide-linked heterodimer of a 60-kD  $\alpha$  chain and a 32-kD  $\beta$  chain (Nakamura et al., 1989; Gohda et al., 1988).

The receptor for HGF is the tyrosine kinase encoded by the *MET* protooncogene (Naldini et al., 1991a, 1991b; Bot-

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1. *Abbreviations used in this paper:* BFU-E, burst-forming unit-erythroid; CFU-GEMM, colony-forming unit-granulocyte, erythroid, monocyte, megakaryocyte; CFU-GM, colony-forming unit-granulocyte, monocyte; CFU-meg, colony-forming unit-megakaryocyte; dpc, days postcoitum; GM-CSF, granulocyte-macrophage colony-stimulating factor; HGF, hepatocyte growth factor; IMDM, Iscove modified Dulbecco's medium; MNAC, mononuclear nonadherent cells; SCF, stem cell factor.

taro et al., 1991), a 190-kD heterodimer of an extracellular 50-kD  $\alpha$  subunit, disulfide-linked to a transmembrane 145-kD  $\beta$  subunit (Park et al., 1987; Giordano et al., 1989a). Both subunits derive from glycosylation and proteolytic cleavage of a 170-kD single chain precursor (Giordano et al., 1989b).

HGF binding triggers autophosphorylation of two tyrosine residues (Y<sup>1234</sup>, Y<sup>1235</sup>) located in the catalytic domain of the receptor, upregulating its kinase activity (Naldini et al., 1991a; Ferracini et al., 1991; Longati et al., 1994). Negative regulation occurs through phosphorylation of a unique serine residue (S<sup>985</sup>) in the juxtamembrane domain of the  $\beta$  chain (Gandino et al., 1990, 1991, 1994).

Signaling by the HGF receptor involves multiple pathways, as expected from the pleiotropic response that its ligand elicits in target cells (Bardelli et al., 1992; Graziani et al., 1991, 1993; Ponzetto et al., 1993). As in other tyrosine kinase receptors, signal transduction is mediated by the interaction ("docking") between specific phosphotyrosines and cytoplasmic effectors containing SH2 domains (Koch et al., 1991; Pawson and Schlessinger, 1993; Cantley et al., 1991). We recently showed that the pleiotropic action of HGF is caused by ligand-induced autophosphorylation of two tyrosines in the receptor tail (Y<sup>1349</sup>, Y<sup>1356</sup>) generating a nonconventional docking site capable of activating multiple cytoplasmic signal transducers (Ponzetto et al., 1994).

The HGF receptor is expressed in adult epithelial tissues, including liver, intestine and kidney (Prat et al., 1991a; Di Renzo et al., 1991). It has been reported to be expressed in early stages of development of epithelial organs (Sonnenberg et al., 1993), and it is often overexpressed in epithelial cancer (Prat et al., 1991a; Di Renzo et al., 1991). We have shown that the receptor is also expressed in endothelial cells, and that HGF is a potent angiogenic factor both in vitro and in vivo (Bussolino et al., 1992; Grant et al., 1993). We recently cloned the promoter region of the HGF receptor gene (*c-MET*). Interestingly, a GATA-1 consensus sequence was found within 300 bp upstream from the transcription start site (Gambarella et al., 1994). The GATA motif is known to identify genes preferentially expressed during erythroid differentiation (Martin et al., 1989). This finding prompted us to investigate the expression of the HGF receptor in hemopoietic cell lineages and the possible role of HGF in human hemopoiesis.

Hemopoietic cell lineages derive from pluripotent progenitors through committed precursors, characterized by the expression of receptors for specific factors. Recently, it has been suggested that some growth factors of nonhemopoietic origin may be involved in controlling proliferation and self-renewal of early and committed hemopoietic precursors (Correa and Axelrad, 1991). In this work, we show that HGF selectively stimulates growth and differentiation of multipotent and erythroid progenitors.

## Materials and Methods

### Recombinant HGF

Full-length HGF cDNA was cloned from human liver mRNA (Naldini et al., 1991a), and it was inserted as a BamHI-EcoRI fragment into the baculovirus transfer vector PVL1393 (Invitrogen, San Diego, CA). The recombinant vector was cotransfected with the BsuI-digested BacPak6 viral DNA (Clontech Laboratories, Palo Alto, CA) into *Spodoptera frugiperda*

insect cells (Sf9) by the lipofectin procedure. Positive viral clones isolated by dot-blot hybridization, and plaque assays were used for large-scale infection. HGF was obtained from culture supernatant of Sf9-infected cells 72 h after infection, by affinity chromatography on a heparin-Sepharose FPLC column (Bio Rad Laboratories, Hercules, CA) eluted with a linear 0.5–1.8 M NaCl gradient. The unprocessed recombinant factor (pro-HGF) was detected by Coomassie blue staining as a band of 90 kD in SDS-PAGE. Protein concentration was estimated by Coomassie blue staining and comparison with a standard curve obtained with increasing amounts of bovine serum albumin.

### HGF Labeling and Immunoprecipitation

Pure HGF was radiolabeled as previously described (Naldini et al., 1992). The radiolabeled factor was immunoprecipitated, as described elsewhere (Naldini et al., 1992), with anti-HGF monoclonal antibodies.

### Scatter Assay

The dissociation assay on MDCK cells was performed as previously described (Stoker et al., 1987; Weidner et al., 1990). Cells were seeded at low density in six-well plates and exposed to HGF in fresh medium overnight. The scattering effect was monitored by light microscopy. 1 U of activity was defined as the lowest amount of factor per milliliter that clearly dissociated MDCK cells.

### Thymidine Incorporation

Primary cultures of rat hepatocytes were prepared as described (Zarnegar et al., 1991) and seeded on collagen (Boehringer Mannheim Biochemicals, Indianapolis, IN)-coated six-well cell culture cluster (Costar Corp., Cambridge, MA) in M199 medium (Sigma Immunochemicals, St. Louis, MO) supplemented with 100 ng/ml insulin. Medium was changed after 4 h, and the cultures were used within 48 h. The factors were added at the indicated concentrations for 24 h, then [<sup>3</sup>H]thymidine (28 Ci/mmol, 1  $\mu$ Ci/ml) was added in fresh medium, and the cultures were further incubated for 18 h. Cell growth was evaluated by [<sup>3</sup>H]thymidine incorporation into TCA-precipitable material.

### Recombinant Growth Factors

Human recombinant multipotent GM-CSF and human recombinant IL-3 were kindly supplied by Sandoz (Wien, Austria) and stored at  $-70^{\circ}\text{C}$  for no more than 3 mo before use. Human recombinant stem cell factor was provided by Amgen Biologicals (Thousand Oaks, California).

### CD34<sup>+</sup> Cell Separation

Bone marrow, fetal umbilical cord blood, and adult peripheral blood samples were obtained from volunteers after informed consent. Heparinized samples of bone marrow, cord blood, or peripheral blood were diluted with an equal volume of PBS, and were separated by Ficoll-Hypaque 1077 SD (Pharmacia Fine Chemicals, Piscataway, NJ) density gradient centrifugation at 550 g for 30 min. Light-density mononuclear cells were collected, washed twice in PBS, and resuspended in Iscove modified Dulbecco's medium (IMDM) (Gibco BRL, Gaithersburg, MD) supplemented with 5% FCS (Biological Industries, Kibbutz Beit Haemek, Israel). Mononuclear adherent cells were then removed by a two-step incubation of 30 min each in plastic flasks at 37°C.

Mononuclear nonadherent cells (MNAC) were incubated with neuraminidase-treated sheep erythrocytes for 15 min at 37°C, centrifuged, and incubated for 45 min at 4°C. T lymphocyte-depleted MNAC were separated by Ficoll-Hypaque 1077 SD (Pharmacia) density gradient centrifugation.

T lymphocyte-depleted MNAC were then incubated for 45 min at 4°C with the following antibodies: anti-CD3, anti-CD4, anti-CD8, anti-CD11c, anti-CD19, anti-CD57; most of the remaining B and T lymphocytes, monocytes, and granulocytes were thus removed by incubation for 45 min at 4°C with immunomagnetic beads coated with anti-mouse IgG (M-450 Dynabeads; Dynal, Inc., Great Neck, NY), subsequently collected by a magnet (MPC-1 Dynabeads; Dynal).

A positive selection of the CD34<sup>+</sup> cells was then performed: cells were incubated with an antibody anti-CD34 (My-10; Becton-Dickinson Immunocytometry Systems, Mountain View, CA) for 45 min at 4°C, then for 45 min at 4°C with immunomagnetic beads coated with anti-mouse IgG; a 4:1 bead/cell ratio was found to provide the best recovery. CD34<sup>+</sup> cells bound to the beads were then collected by a magnet (MPC-1 Dynabeads;

Dynal), and were resuspended in IMDM supplemented with 10% FCS. An overnight incubation at 37°C was then performed; to allow CD34<sup>+</sup> cell detachment, the beads were subjected to shearing forces by repeated flushing through a Pasteur pipette. Further details about the negative/positive double selection procedure used have been published previously (Bagnara et al., 1991).

### Cell Cultures

The colony assay for erythroid burst-forming units (BFU-E) and for multipotent granulocyte-erythroid-monocyte-megakaryocyte colony-forming units (CFU-GEMM) was performed according to Iscove et al. (1974). Cord blood, bone marrow, or peripheral blood CD34<sup>+</sup> cells were plated in 24-well cell culture clusters (Costar) at a density of  $2.5 \times 10^3$  cells/well, in a medium containing IMDM, 30% FCS,  $2 \times 10^{-4}$  M hemin,  $5 \times 10^{-5}$   $\beta$ -mercaptoethanol and 0.9% methylcellulose. The cells were stimulated with the following growth factors alone or in combination: Epo, 2 ng/ml; IL-3, 2 ng/ml; granulocyte-macrophage colony-stimulating factor (GM-CSF), 50 ng/ml; stem cell factor (SCF), 20 ng/ml; pro-HGF, 2, 10, or 40 ng/ml. Colonies scored positive only when they appeared dark red and contained more than four aggregates.

The assay for the 14-d granulocyte-monocyte colony-forming units was performed as previously described (Iscove et al., 1971). Cord blood, bone marrow, or peripheral blood CD34<sup>+</sup> cells were plated in 24-well cell culture clusters (Costar) at a density of  $2.5 \times 10^3$  cells/well in a medium containing IMDM, 20% FCS, 0.3% Noble agar (Difco Laboratories Inc., Detroit, MI), and the following growth factors alone or in combination: IL-3, 2 ng/ml; GM-CSF, 50 ng/ml; SCF, 20 ng/ml; pro-HGF, 2, 10, or 40 ng/ml.

For the megakaryocyte colony-forming unit assay, plasma clot assay was performed according to Vainchenker et al. (1979). Cord blood, bone marrow, or peripheral blood CD34<sup>+</sup> cells were plated in 24-well cell culture clusters (Costar) at a density of  $2.5 \times 10^3$  cells/well in a medium containing IMDM, 20 mg/ml L-asparagine (Sigma), 3.4  $\mu$ g/ml CaCl<sub>2</sub>, 10% bovine plasma citrated (Gibco BRL), 1% detoxified BSA (fraction V Chon; Sigma), 10% of a preselected batch of heat-inactivated, pooled human AB serum, and the following growth factors alone or in combination: IL-3, 2 ng/ml; GM-CSF, 50 ng/ml; SCF, 20 ng/ml; pro-HGF, 2, 10, or 40 ng/ml. After 12 d of incubation, the plasma clot was fixed in situ with methanol-acetone 1:3 for 20 min, washed with PBS, and air dried. Fixed plates were stored at -20°C until immunofluorescence staining was performed; megakaryocyte colony-forming unit colonies were scored as aggregates of 3-100 cells intensively fluorescent to monoclonal antibody CD41 (Immunotech, Marseille, France) directed against the IIb/IIIa glycoprotein complex. Binding was shown by fluorescein-conjugated goat anti-mouse Ig (Becton-Dickinson Immunocytometry Systems, Mountain View, CA).

### Flow Cytometry Analysis

Cells were stained for flow cytometry analysis using phycoerythrin-conjugated anti-CD34 (Becton-Dickinson), unconjugated monoclonal antibodies DO-24 (anti-Mer; Prat et al., 1991b), and SR-1 (anti-Kir; Bridgel et al., 1992), kindly provided by V. Broudy (University of Washington Medical School, Seattle, WA). Detection of unconjugated antibodies was facilitated using fluorescein-tagged goat anti-mouse Ig (Becton-Dickinson). Each analysis included 10,000 events collected in list mode on a FACScan<sup>®</sup> flow cytometer (Becton-Dickinson) and analyzed using FACstar<sup>®</sup> Consort 30 software. The instrument was gated on the basis of forward and 90° angle scatter to eliminate debris and aggregates. For two-color analysis, 10,000 events were acquired gating on red fluorescence. Controls included cells stained with the second antibody alone or with a monoclonal antibody directed against the intracellular domain of the HGF receptor (DQ13; Prat et al., 1991b).

### Mice

C57/B16 mice were mated between 9 and 10 p.m. Day 0.5 postcoitum was assumed to begin on the middle of the day of vaginal plugging. Pregnant female mice were killed by cervical dislocation, and embryos were staged according to Theiler et al. (1989), collected in ice-cold PBS under a dissection microscope (SV11; Carl Zeiss, Inc., Thornwood, NY), and fixed in 4% paraformaldehyde overnight.

### In Situ Hybridization

In situ hybridization was carried out as described (Wilkinson and Green, 1990; Wilkinson, 1992), with minor modifications. Both paraffin-embedded

and cryostat sections were analyzed. Dissected embryos were prefixed in 0.1 M sodium phosphate buffer (pH 7.3), 4% paraformaldehyde at 4°C overnight, and embedded in Tissue-Tek<sup>™</sup> (Miles Laboratories Inc., Elkhart, IN). 8- $\mu$ m thick cryosections were transferred onto gelatin/chromium III potassium sulphate-subbed slides and dried at room temperature. Before hybridization, slides were postfixed. The probes used were antisense strands corresponding to nucleotides 2199-2718 for murine *c-Met*, as well as the entire coding sequence for HGF. Transcription reactions with T7 or Sp6 polymerase (Riboprobe Kit; Promega Biotec, Madison, WI) were carried out in the presence of [<sup>35</sup>S]CTP (Amersham Corp., Arlington Heights, IL). The template was then degraded with RNase-free DNase (Pharmacia), and the labeled RNA was purified through a Sephadex G-50 column and progressively degraded by random alkaline hydrolysis to improve access to RNA in situ. The probes were dissolved at a working concentration of  $1 \times 10^5$  cpm/ml in the hybridization mix; 30 ml of the appropriate probe was added to each slide. Hybridization was carried out overnight at 55°C. The slides were then washed under stringent conditions (65°C, 2 $\times$  SSC, 50% formamide) and treated with RNase. Autoradiography was performed with Kodak NT/B2 emulsion. Exposure times were between 15 and 25 d.

Negative controls included the *c-MET* and the HGF sense strands, and probes for the homeotic genes *Otx1*, *Otx2*, *Emx1*, and *Emx2* (Simeone et al., 1992), which were tested on sections adjacent to those hybridized with the specific antisense strands to determine the basal background of the hemopoietic cells. These probes never gave a detectable signal.

## Results

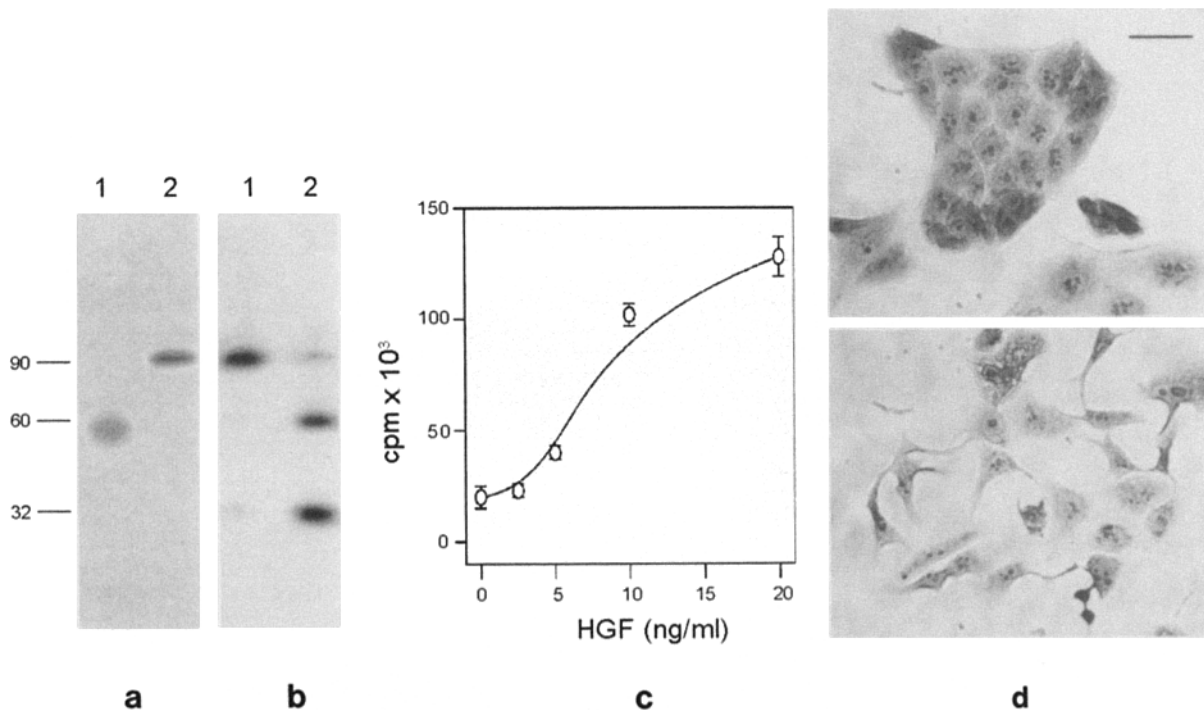
### Production of Human Recombinant HGF in Insect Cells

Since purity is mandatory to assess the properties of a growth factor in biological assays, we produced human HGF in insect cells by means of a recombinant baculovirus. Insect cells can be grown in serum-free medium, thus the recombinant protein can be recovered devoid of serum-derived, biologically active contaminants. Moreover, because of the phylogenetic distance between insects and humans, the possible interference of other contaminating molecules produced by insect cells is negligible.

The full-length human HGF cDNA (Naldini et al., 1991a) was inserted into the baculovirus genome by a transfer vector, as described in Materials and Methods. Sf9 insect cells infected with the recombinant virus released the human protein in the culture medium as single-chain unprocessed pro-HGF. The protein was purified from spent medium by heparin affinity chromatography. No contaminants could be detected in SDS-PAGE by Coomassie blue staining. The baculovirus pro-HGF migrated with the apparent molecular masses of 90 or 65 kD, under reducing or nonreducing conditions, respectively (Fig. 1a). The observed shift is caused by the reduction of intrachain disulfide bonds (Gohda et al., 1988).

We have previously shown that also mammalian cells secrete HGF in the extracellular environment as an inactive single-chain precursor. Pro-HGF is converted into the mature two-chain biologically active form upon proteolytic cleavage by urokinase or a Factor XII-like serum protease (Naldini et al., 1992; Miyazawa et al., 1993; Shimomura et al., 1993). Since the biological assays used in this study were performed in the presence of serum (see above), we tested whether the baculovirus pro-HGF could be processed after incubation for 2 h with 10% FCS. Under these conditions, the <sup>125</sup>I-labeled precursor was fully processed, generating the two-chain mature form (Fig. 1b).

The biological activity of the recombinant baculovirus HGF was assessed in two different biological assays, and



**Figure 1.** Biochemical and biological properties of human recombinant HGF, produced in the baculovirus expression system. (a) Under nonreducing conditions (lane 1), pro-HGF shows an apparent molecular mass of  $\sim 60$  kD. Under reducing conditions (lane 2), the band shifts to a molecular mass of 90 kD. No contaminants can be detected in the preparation by SDS-PAGE and Coomassie blue staining. (b) Maturation of pro-HGF. Immunoprecipitation and SDS-PAGE analysis of  $^{125}\text{I}$ -labeled unprocessed pro-HGF (lane 1). In vitro processing of pro-HGF was obtained by a 2-h incubation with fetal calf serum (10%); under these conditions, pro-HGF is cleaved, and it generates the 60-kD  $\alpha$  chain and the 32-kD  $\beta$  chain (lane 2). (c) Mitogenic activity of HGF measured by  $^3\text{H}$ thymidine incorporation assay on primary rat hepatocytes. (d) "Scatter" of MDCK cells cultured in standard medium (top) or after an 18-h incubation with pro-HGF (10 ng/ml).

titrated by comparison with HGF obtained from mammalian cells. The mitogenic activity was measured in a  $^3\text{H}$ thymidine incorporation assay, using primary rat hepatocytes. The cell "scatter" activity (Gherardi et al., 1989; Weidner et al., 1990) was evaluated on Madin-Darby canine kidney cells (Fig. 1, c and d). The recombinant molecule was active in both assays. It should be noted that the "scatter" assay was performed in the presence of 10% serum, while the  $^3\text{H}$ -thymidine incorporation assay was performed in the absence of serum. However, it is known that hepatocytes can process pro-HGF as they express the appropriate convertase(s) (Naka et al., 1992; Mizuno et al., 1992, 1994).

### **HGF Stimulates Erythroid and Multipotent Hemopoietic Progenitors In Vitro**

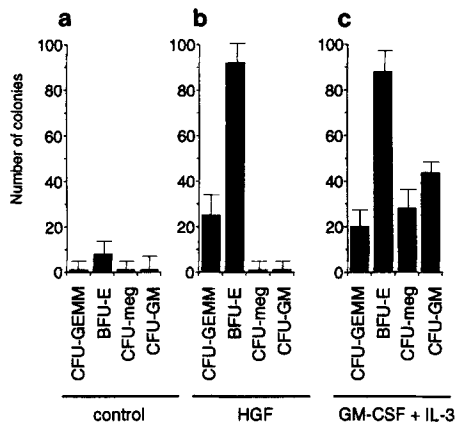
The effect of HGF on the growth and differentiation of hemopoietic progenitors was studied in colony formation assays. CD34<sup>+</sup> hemopoietic progenitors (Civin et al., 1984) were purified from adult bone marrow, fetal umbilical cord blood, or adult peripheral blood, as described in Materials and Methods. The recovered cells were morphologically unidentifiable blast elements on May-Grunwald-Giemsa staining, slightly contaminated by promyelocytes. Flow cytometry analysis indicated that the percentage of CD34<sup>+</sup> cells in the selected cell preparations varied between a minimum of 30% (when the starting material was bone marrow) and a maximum of 50% (when the starting material was umbilical

cord blood). Contamination by CD4<sup>+</sup>, CD2<sup>+</sup>, CD16<sup>+</sup>, or CD19<sup>+</sup> cells was constantly <1%.

CD34<sup>+</sup> cells were cultured in the presence of HGF alone, or in combination with other growth factors. No colonies were observed when the factor was added alone. However, in the presence of standard concentrations of erythropoietin (2 ng/ml), HGF dramatically increased the number of colonies derived from the BFU-E precursors (Fig. 2). HGF also stimulated the growth of colonies derived from multipotent CFU-GEMM progenitors. The number of colonies was comparable to that obtained by combining known hemopoietic factors such as GM-CSF and IL-3 (Bot et al., 1988; Gasson, 1991; McNiece et al., 1991; Miyajima et al., 1993). It should be noted, however, that the HGF effect was restricted to the stimulation of CFU-GEMM and BFU-E. Neither granulo-monocytic nor megakaryocytic colonies were ever observed in response to HGF.

The response to HGF was dose-dependent and could be observed at concentrations of HGF as low as 5 pM both in erythroid and multipotent colonies (Fig. 3).

The HGF action was also studied on CD34<sup>+</sup> fetal hemopoietic progenitors enriched from human umbilical cords blood. It is known that this population contains a percentage of primitive stem cells higher than the population purified from adult bone marrow or peripheral blood (Broxmeyer et al., 1992; Lu et al., 1993). As observed in the case of adult hemopoietic progenitors, HGF stimulated both BFU-E- and CFU-GEMM-derived colonies (data not shown).



**Figure 2.** Colony formation from CD34<sup>+</sup> human bone marrow progenitors grown in the presence of standard concentration of erythropoietin (2 ng/ml) and various combinations of human recombinant growth factors. (a) Erythropoietin alone. (b) 5 ng/ml HGF. (c) 50 ng/ml GM-CSF and 2 ng/ml IL-3. The data reported are the total colonies counted in four distinct wells (2,500 CD34<sup>+</sup> cells were seeded per well). Error bars indicate standard deviation. BFU-E colonies were scored positive only if containing four or more cell aggregates.

### HGF Synergizes with SCF in Stimulating Multipotent Hemopoietic Progenitors

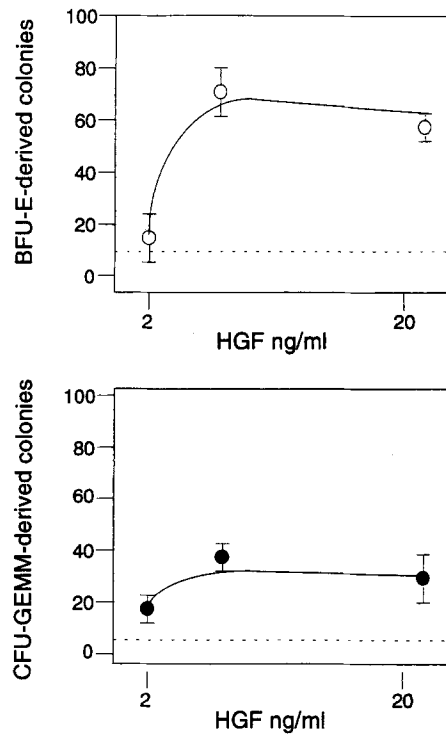
In the presence of both HGF and SCF, a significant increase in the number of CFU-GEMM-derived colonies was observed (Fig. 4). Interestingly, fewer erythroid colonies could be seen compared to those developed in the cultures stimulated by HGF alone. This suggests that the combination of HGF and SCF preferentially affects proliferation of multipotent progenitors. The erythroid colonies grown in the presence of both growth factors were extremely large, and they showed a high hemoglobin content. The size of CFU-GEMM-derived colonies grown in these conditions was also increased and, within each colony, the erythroid lineage was predominant (Fig. 5).

In these assays, HGF did not synergize with GM-CSF and IL-3, either tested individually or in combination (data not shown).

### The HGF Receptor Is Expressed in a Subpopulation of Adult Hemopoietic Progenitors (CD34<sup>+</sup>)

The presence of HGF receptor at the surface of hemopoietic progenitors was studied by flow cytometry analysis of bone marrow and peripheral blood mononuclear cells. Monoclonal antibodies directed against extracellular epitopes of the HGF receptor  $\beta$  chain were used. A small but clearly identifiable subpopulation of bone marrow cells stained positive for the HGF receptor (Table I). About half of the cells expressing the HGF receptor also coexpressed the CD34 marker and could, thus, be identified as hemopoietic progenitors (Civin et al., 1984).

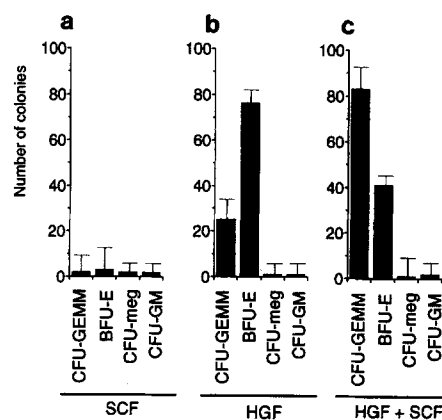
As described above, HGF synergized with the SCF in stimulating the growth and differentiation of CFU-GEMM-derived colonies. In line with this observation, a subpopulation of cells coexpressing the HGF and the SCF receptors was identified using a monoclonal antibody against extracellular epitopes of the SCF-R (*c-Kit*) protein.



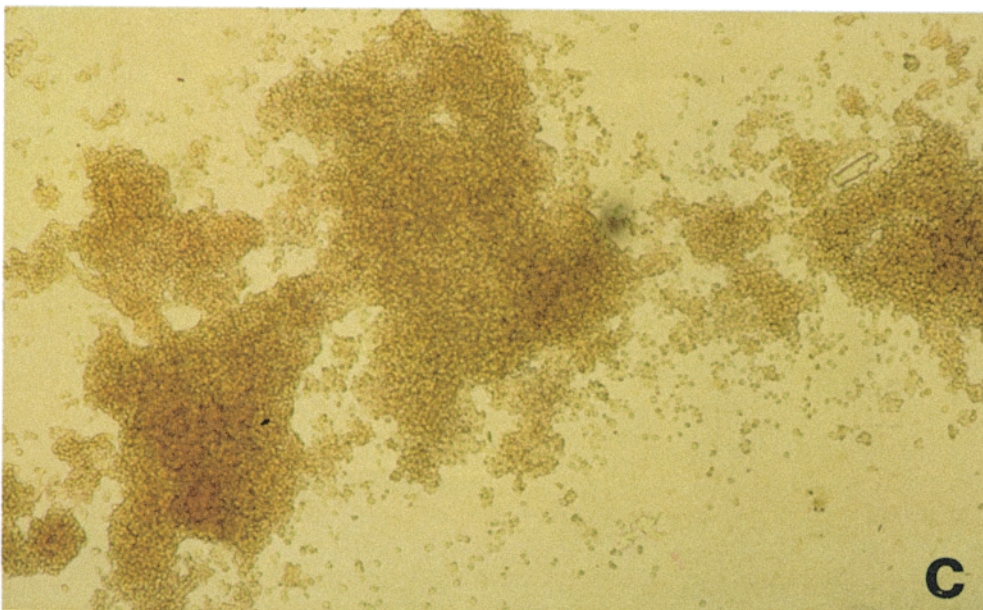
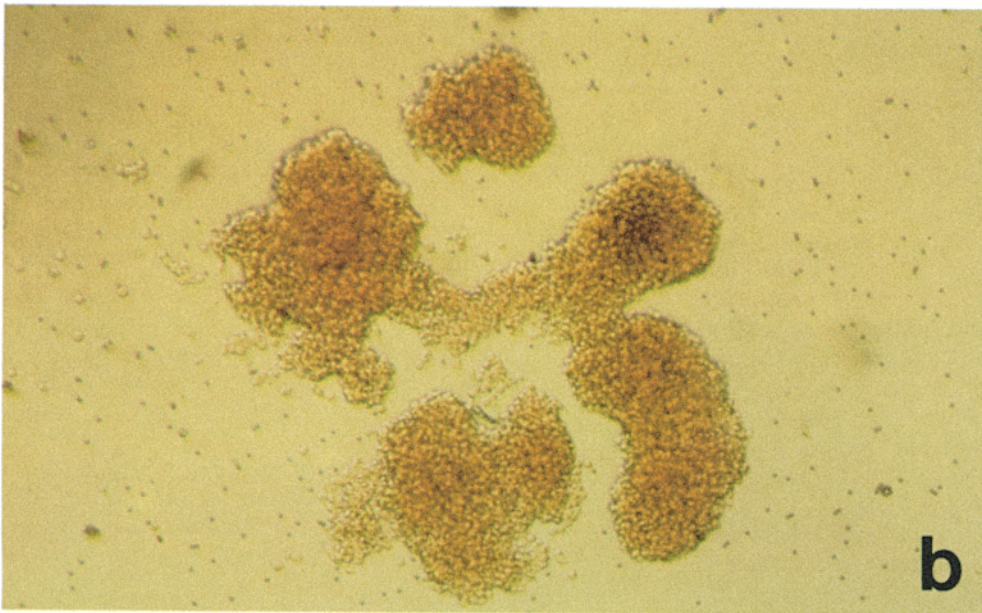
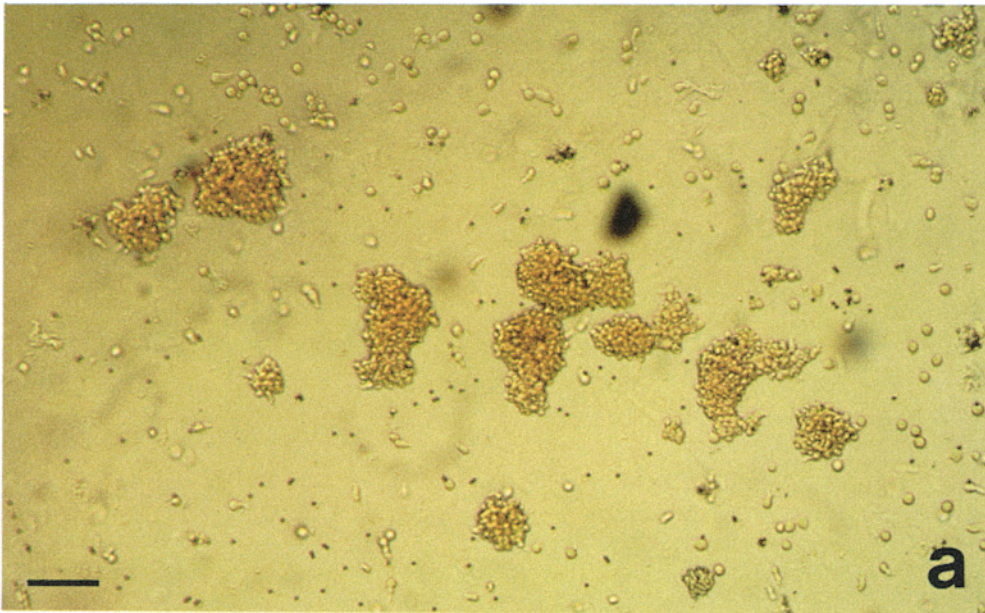
**Figure 3.** HGF-induced colony formation from CD34<sup>+</sup> human bone marrow progenitors. The action of HGF on the formation of both BFU-E and CFU-GEMM colonies is dose dependent with a peak at the concentration of  $\sim 5$  ng/ml. All tests were performed in the presence of 2 ng/ml erythropoietin. The dashed line indicates the number of colonies obtained in the presence of erythropoietin alone.

Similar results were obtained by flow cytometry analysis of CD34<sup>+</sup> progenitors circulating in the peripheral blood (data not shown).

Flow cytometry analysis with anti-HGF-receptor antibodies was also performed on cells harvested from the colonies developed in vitro in response to HGF. Table I shows that HGF receptor-positive cells were present.



**Figure 4.** Synergistic effect of HGF and SCF on colony formation from CD34<sup>+</sup> human bone marrow progenitors grown in the presence of standard concentration of erythropoietin. (a) 20 ng/ml SCF alone. (b) 5 ng/ml HGF. (c) 20 ng/ml SCF + 5 ng/ml HGF.



**Figure 5.** Synergistic effect of HGF and SCF in promoting erythroid differentiation under the conditions described in the legends of Figs. 2 and 4. (a) BFU-E-derived colony developed in the presence of HGF (5 ng/ml); (b) BFU-E-derived colony grown in the presence of HGF (same concentration) and SCF (20 ng/ml). (c) CFU-GEMM-derived colony in the presence of HGF and SCF, as in b. Among the mixed lineages, the erythroid elements are predominant.

**Table I. Expression of HGF Receptor (HGF-R) in Human Hemopoietic Progenitors**

	Phenotype	Percent of Positive Cells
Unfractionated bone marrow	HGF-R+	0.6 ± 0.1
	HGF-R+/CD34+	0.3 ± 0.05
	HGF-R+/CD34-	0.3 ± 0.1
	HGF-R+/SCF-R+	0.2 ± 0.1
	HGF-R+/SCF-R-	0.4 ± 0.1
CFU-GEMM-derived colonies	HGF-R+	15.3 ± 1.5
BFU-E-derived colonies	HGF-R+	9.7 ± 1.2

### **HGF and its Receptor Are Expressed during the Embryonal Development of Hemopoietic Cells**

The expression of the HGF receptor was studied in embryonal hemopoietic cells by in situ hybridization of histological sections of mouse embryos. Using an antisense *MET* probe, the HGF receptor mRNA could be clearly detected in megaloblastic cells located within the cavity of the developing heart and aorta from 10 to 10.5 d postcoitum (dpc) (Fig. 6, *a'* and *c*). Specific mRNA could also be detected in the hepato/biliary primordium, which at this stage contains hemopoietic precursors (Fig. 6 *b'*). In this developing organ, both epithelial cells and erythroid islands stained positive. Hemopoietic cells showed a higher level of HGF receptor mRNA, compared to the level of expression observed in the surrounding hepatocytes. From 11 dpc, the hemopoietic embryonal liver also expresses HGF mRNA (Fig. 7). The resolution of in situ hybridization with <sup>35</sup>S-labeled probes does not allow positive assignment of hybridization signals to single cells (Sonnenberg et al., 1993). However, although we were not able to identify the subpopulation of cells that express the HGF mRNA in the hepato/biliary primordium, the presence of these elements in close proximity and within the islands of erythroid progenitors suggests juxtacrine interactions.

### **Discussion**

Hemopoietic cell growth and differentiation is under the control of a complex network of cytokines, which act on their target cells via specific receptors (Metcalf, 1984; Clark and Kamen, 1987). Among these receptors, some are transmembrane tyrosine kinases expressed by different hemopoietic lineages. The macrophage colony-stimulating factor receptor kinase, encoded by the *c-FMS* protooncogene, is involved in monocyte differentiation (Stanley et al., 1983; Sherr, 1990). The receptor for SCF, encoded by the *c-kit* gene, is critical for early hemopoiesis and mast cell differentiation (Besmer et al., 1991; Chabot et al., 1988; Broxmeyer et al., 1992). Some new members of the receptor tyrosine kinase superfamily (*FLT-1*, *FLT-3*, *FLT-4*, and *FLK-1*) have recently been identified in hemopoietic cells (Rosnet et al., 1991; Shibuya et al., 1990; Matthews et al., 1991; Aprelikova et al., 1992).

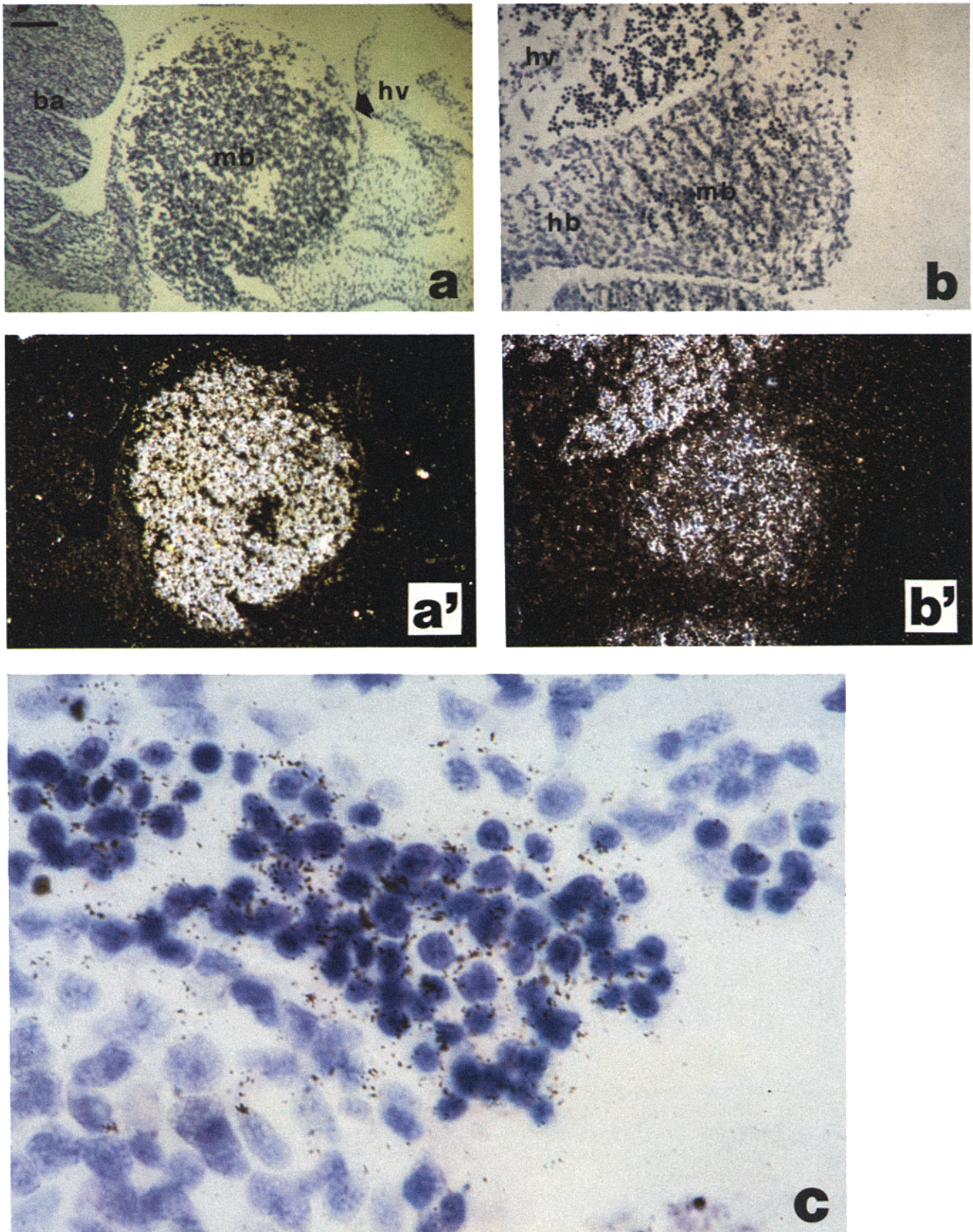
Two transmembrane tyrosine kinases encoded by genes related to the *c-MET* protooncogene are known to be involved in hemopoiesis. (*a*) The *v-sea* oncogene, originally isolated from a strain of avian erythroblastosis virus, causes erythroblastosis and anemias in chickens (Smith et al., 1989). (*b*) The recently cloned *MRK* (*MET*-related kinase)

gene is expressed in early erythroid progenitors and in differentiated hemopoietic cell lineages (Yee et al., 1993; Tamagnone et al., 1993; Stacker et al., 1993).

The *c-MET* protooncogene encodes the tyrosine kinase receptor for HGF (Naldini et al., 1991*a*, 1991*b*; Bottaro et al., 1991). The expression of this gene has been considered, so far, mainly restricted to epithelial tissues (Prat et al., 1991*a*; Di Renzo et al., 1991). We recently identified in the *c-MET* promoter, among other consensus sequences for transcription factors, a GATA consensus motif (Gambartotta et al., 1994). The GATA-1 transcription factor is the first characterized member of a multigene family considered to be of major importance in the establishment and maintenance of the erythroid phenotype (Martin et al., 1989). In this work, we demonstrate that the HGF receptor is indeed expressed in the adult by hemopoietic progenitors, as well as in the embryo by erythroblast ancestors (megaloblasts). We also show that HGF, traditionally regarded as a non-hemopoietic growth factor, is a potent stimulator of the proliferation and differentiation of the erythroid lineage. This action is lineage specific because it does not affect either granulo-monocytic or megakaryocytic lineage precursors. Several lines of evidence suggest that HGF action on hemopoietic cells is direct. (*a*) In the adult, HGF receptor is expressed by a fraction of CD34<sup>+</sup> hemopoietic progenitors. (*b*) In the embryo, HGF receptor mRNA is expressed by primordial erythrocytes (megaloblasts), both in peripheral blood and in hemopoietic liver. (*c*) The HGF and the SCF receptors are coexpressed in a subpopulation of progenitors, in agreement with the synergistic effect of the two ligands. (*d*) HGF stimulates only erythroid and multipotent progenitors. Granulo-monocytic colony formation would be expected in the case of an HGF-induced secretion of cytokines (e.g., IL-3 or GM-CSF; Gasson, 1991; Miyajima et al., 1993). However, the direct action of HGF on hemopoietic progenitors does not exclude, in principle, a possible additional indirect effect in vivo mediated by cells of the bone marrow stroma responsive to the growth factor. Notably, endothelial cells express the HGF receptor and respond to HGF (Bussolino et al., 1992; Grant et al., 1993).

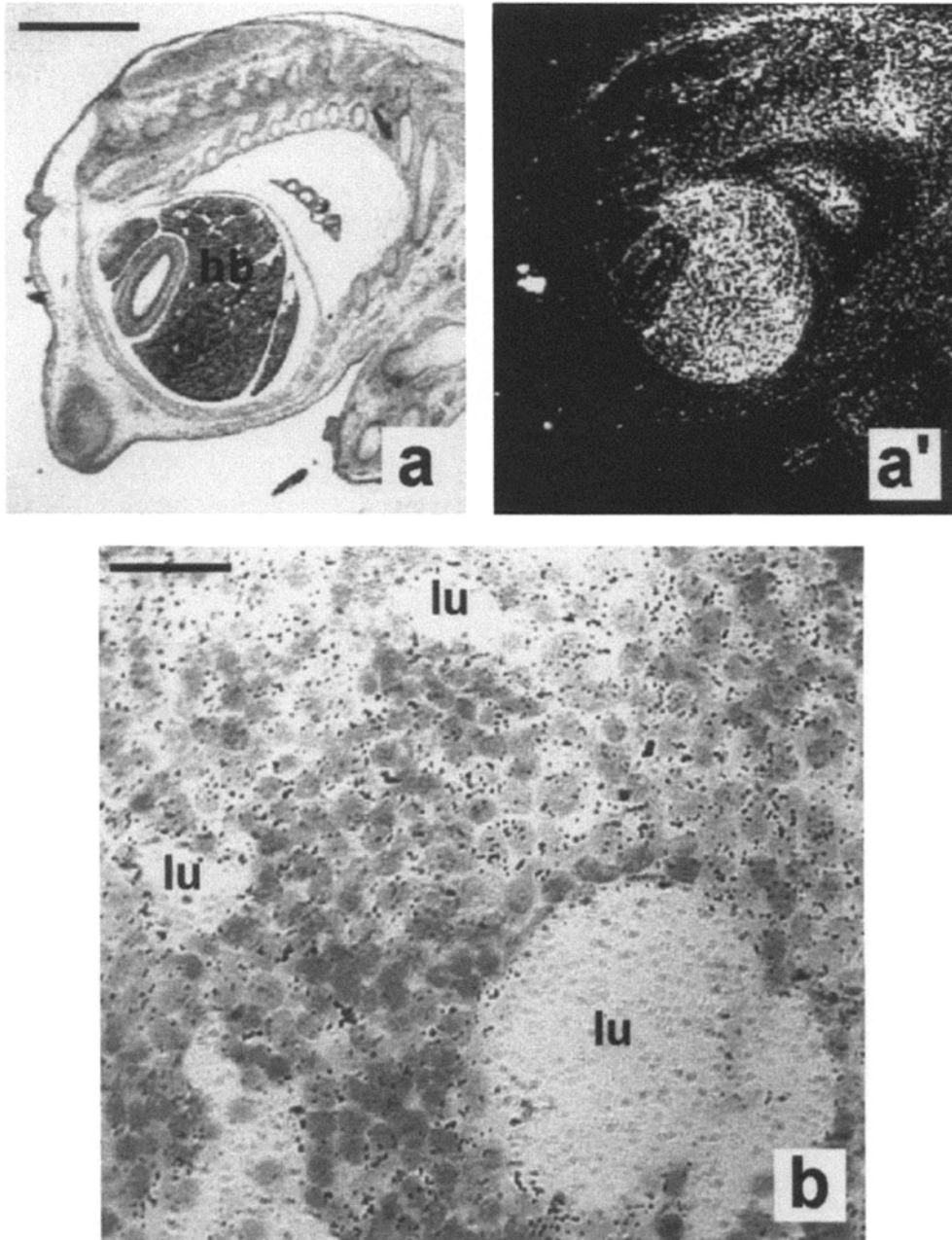
The HGF receptor expression in both endothelial and erythroid cells is consistent with their common ontogenetic origin, both lineages deriving from the same ancestors in yolk-sac blood islands (see Gilbert, 1988). The HGF receptor mRNA has actually been found in the hemopoietic tissue of extra-embryonal mesoderm (Simeone et al., manuscript in preparation).

Erythropoiesis is a complex process in which a specific genetic program is primed (commitment) and executed (maturation). Although much is known about maturation, most of the molecular events occurring during the commitment phase are still obscure (for a review see Takeshita and Benz, 1991). Growth and differentiation of erythroid precursor are regulated by humoral factors and by largely uncharacterized cell-cell interactions with bone marrow stroma, the so-called hemopoietic microenvironment (Metcalf, 1987). Erythropoietin has long been considered the major factor required for erythropoiesis (Koury and Bondurant, 1990), other factors being far less specific (IL-3, GM-CSF, TGF- $\beta$ ; Gasson, 1991; Miyajima et al., 1993; Sporn and Roberts, 1992). HGF represents a novel example of a humoral factor specifically active on erythropoiesis.



**Figure 6.** In situ analysis of *c-MET* (HGF receptor gene) expression in 10.5-dpc mouse embryos. (a) Toluidine blue-stained sagittal section showing the branchial arches (*ba*) and the heart ventricle (*hv*) containing circulating megaloblastic cells (*mb*). (b) Toluidine blue-stained sagittal section of the hepato/biliary primordium (*hb*) adjacent to the heart ventricle (*hv*), including nests of megaloblastic hemopoietic cells (*mb*). (a'-b') Dark field of the same section shown in *a* and *b*, hybridized with the *c-MET* antisense probe. Both circulating megaloblasts and hemopoietic nests in the liver express a high level of HGF receptor mRNA. In the hepato/biliary primordium, the epithelial cells are also positive. (c) High magnification of a sagittal section through the embryonic aorta. HGF receptor mRNA is expressed only in the megaloblastic cells filling the lumen.





**Figure 7.** In situ analysis of *HGF* expression in the hemopoietic liver of mouse embryo. (a) Toluidine blue-stained sagittal section of a 13.5-dpc embryo showing the hepato/biliary primordium (*hb*). (a') Dark field of the same section shown in *a* hybridized with the *HGF* antisense probe. (b) High magnification of a sagittal section through the hepato/biliary primordium of a 12.5-dpc embryo. The darker cells correspond to hemopoietic nests surrounding the lumen of blood vessels (*lu*).

We observed a potent synergism between *HGF* and *SCF*. In the presence of both factors, multipotent colonies were significantly increased, compared to colonies grown in the presence of *HGF* alone, while erythroid colonies were notably decreased. This observation indicates that the combination of the two factors preferentially recruits early multipotent progenitors rather than erythroid precursors. *SCF* is a known stimulator of early hemopoietic progenitors (Williams and Lyman, 1992). *HGF* seems to affect hemopoietic cells in a window of differentiation, including the transition from multipotent progenitors to committed erythroid precursors. In our experimental conditions, *HGF* was not synergistic with known cytokines active on hemopoietic precursors, such as *GM-CSF* and *IL-3*. This observation, obtained in the human hemopoietic system, does not confirm a previous report of synergism among *HGF*, *IL-3*, and *GM-CSF* in

promoting the growth of uncharacterized colonies from unfractionated murine bone marrow (Kmieciak et al., 1992). This discrepancy may be explained by species differences. Alternatively, since the mouse experiments were performed on total bone marrow cell suspensions (including lymphocytes and monocyte-macrophages), the *HGF* effect could have been indirect, mediated by production of hemopoietic cytokines by accessory cells.

The potent synergism between *HGF* and *SCF* gives interesting clues. (a) The *HGF* and *SCF* receptors are expressed in numerous and partially overlapping sets of target tissues, including epithelia, melanocytes, and hemopoietic cells (Kan et al., 1991; Rubin et al., 1991; Halaban et al., 1992; Matsumoto et al., 1991; Brizzi et al., 1991). (b) The *HGF* and *SCF* receptors are tyrosine kinases that transduce extracellular signals via complementary pathways. The cyto-

plasmic domain of the HGF receptor contains a multifunctional docking site that interacts with phospholipase-C- $\gamma$ , pp60<sup>c-src</sup>, the GRB-2/SoS complex and, less efficiently, with PI 3 kinase (Graziani et al., 1991; Ponzetto et al., 1994). The SCF receptor, on the other hand, binds very efficiently PI 3 kinase, while the interaction with other transducers is weak (Lev et al., 1991; Rottapel et al., 1991). (c) The two ligands, HGF and SCF, are associated to the cell surface: the SCF precursor is a transmembrane protein (Flanagan et al., 1991; Huang et al., 1992) and HGF is bound to cell surface heparansulphate glycosaminoglycans (Naldini et al., 1991a). Both factors may thus play a role in the juxtacrine interactions, occurring in the hemopoietic microenvironment.

HGF is a paracrine growth factor that governs mesenchymal-epithelial interactions. It is produced by nonparenchymal cells of the liver, kidney, and lung, while parenchymal (epithelial) cells in these organs express high levels of HGF receptor (Noji et al., 1990). A similar expression pattern is found during development of epithelial organs, where the factor is secreted by mesenchymal cells and the receptor is expressed by adjacent epithelial cells (Sonnenberg et al., 1993). We now suggest that HGF is a paracrine mediator of stromal-hemopoietic cell interactions both during embryogenesis and adult life. This factor may, therefore, be one of the long-sought molecules mediating developmental signals between microenvironment and hemopoietic progenitors.

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