Transgenic expression in the liver of truncated Met blocks apoptosis and permits immortalization of hepatocytes

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Hepatocyte growth factor induces proliferation, motility and differentiation of epithelial cells through the tyrosine kinase receptor encoded by the *MET* proto-oncogene. The cytoplasmic portion of Met (referred to as cyto-Met) is activated but only weakly transforming. In order to determine the effect of activated Met on hepatocytes, we have targeted truncated Met expression to the liver by incorporating the cDNA into a vector carrying the entire human *α -1-antitrypsin* transcriptional unit. Transgenic expression in the liver of truncated human Met, containing the regulatory and the catalytic cytoplasmic domains, renders hepatocytes constitutively resistant to apoptosis and reproducibly permits immortalization. The emerging stable cell lines are not transformed and maintain a highly differentiated phenotype judged by the retention of epithelial cell polarity and the expression of hepatocyte-enriched transcription factors as well as hepatic products.

Keywords: apoptosis protection/cell polarity/immortalized hepatocytes/Met

1991; Naldini *et al.*, 1991a,b). The pleiotropic responses triggered by HGF are mediated by the Met multifunctional docking site that, upon autophosphorylation, recruits multiple intracellular signal transducers (Ponzetto *et al.*, 1994).

It is well known that the expression of constitutively activated tyrosine kinases can lead to cell transformation, with concomitant loss of differentiated functions (Bishop, 1991). Constitutive (i.e. HGF-independent) activation of Met results either from overexpression of the intact receptor (Giordano *et al.*, 1989) or from truncation of the extracellular domain (Cooper *et al.*, 1984). In the latter case, following translocation, replacement of both the extracellular and the juxtamembrane domains by a dimerization motif generates an oncoprotein that is highly transforming *in vitro* and tumorigenic *in vivo* (Park *et al.*, 1986; Rodrigues and Park, 1993). By contrast, truncation of the extracellular domain alone generates a constitutively activated tyrosine kinase that is only weakly transforming (Zhen *et al.*, 1994).

The reproducible generation of immortalized hepatocyte-derived cell lines is a problem that has been resolved only partially, in spite of the development of transgenic mice in which expression of oncogenes or growth factors is targeted to the liver. While such mice permit the establishment of hepatocyte lines, the resulting lines may present transformed phenotypes and incomplete conservation of the hepatocyte differentiation programme (Antoine *et al.*, 1992; Wu *et al.*, 1994a,b).

Here we report that transgenic expression, directed to the liver, of a truncated Met protein has two major effects: it renders hepatocytes resistant to apoptotic stimuli and it is permissive for immortalization of hepatocytes. Moreover, the immortalized cell lines, obtained from explants of livers at different stages of development, show no impairment of their differentiation programme.

Introduction

Polypeptide growth factor receptors transduce signals controlling basic cell functions such as growth, differentiation, survival and, occasionally, motility (Barre *et al.*, 1992; Harrington *et al.*, 1994; Van der Geer *et al.*, 1994). Hepatocyte growth factor (HGF) elicits multiple responses in epithelial and endothelial cells, including stimulation of growth (Nakamura *et al.*, 1986; Michalopoulos, 1990; Rubin *et al.*, 1991; Bussolino *et al.*, 1992), extracellular matrix invasion (Weidner *et al.*, 1990; Giordano *et al.*, 1993), differentiation (Stern *et al.*, 1990; DeFrances *et al.*, 1992) and formation of tubular structures (Montesano *et al.*, 1991). Furthermore, it has a pivotal role in liver development, revealed by the observation of hepatocyte involution in HGF-null mice (Schmidt *et al.*, 1995). The HGF receptor is the transmembrane tyrosine kinase encoded by the *MET* proto-oncogene (Bottaro *et al.*,

Results

The transgene *AT-cyto-MET* encodes the cytoplasmic portion of Met, including the juxtamembrane and the catalytic domains, and the carboxy-terminal tail of the receptor. The *cyto-MET* open reading frame (ORF) is embedded into a non-conventional construct, based on an 18 kb genomic fragment containing the entire human *α -1-antitrypsin* (*AT*) gene (Figure 1A). In transgenic mouse liver, the *cis*-acting elements of the *AT* gene drive the expression of cyto-Met from 14.5 d.p.c. (days post-coitum) on and throughout adult life (Amicone *et al.*, 1995). Immunoprecipitation analysis of liver extracts revealed consistent expression of the transgene as a protein of the anticipated size (Figure 1B). The transgenic mice show no evidence of liver tumours during their entire life span.

Fig. 1. Structure and expression of the transgene. **(A)** Schematic representation of the *a-1-antitrypsin (AT)-cyto-MET* transgene: the fragment encoding the cytoplasmic domain of MET (amino acids 956–1390) was embedded in the 18 kb fragment containing the entire human *AT* gene. *Cyto-MET* is the only ORF because it is placed immediately after the ATG codon of *AT* and is provided with its own stop codon. The liver-specific transcription start site (start), *cyto-MET* and *AT* exons (white and black boxes respectively), the first (ATG) and the last (stop) codons are indicated. The figure highlights the relevant residues in cyto-Met protein: the negative Ser985 regulatory site, the tyrosine kinase domain with the major phosphorylation sites Tyr1234–1235 and the signal transducer docking site encompassing Tyr1349–1356 (Ponzetto *et al.* 1994). **(B)** Detection of transgenic cyto-Met protein. Proteins were solubilized from adult liver of three different transgenic lines, lanes 1–3 (#78, #42 and #36 respectively); from embryonic transgenic liver (day 15 #36), lane 4; from control wild-type mouse liver, lane 5; and from human hepatoma Hep3B cells stably transfected with the transgene, lane 6. Immunoprecipitation by specific anti-human Met antibody followed by Western blotting reveals the transgenic cyto-MET protein in lanes 1, 2, 3, 4 and 6, and the endogenous human β chain in lane 6. Smaller bands are due to splicing of the *AT* first exon to internal regions of cyto-Met (data not shown).

Transgenic mice are resistant to Fas-induced hepatocyte apoptosis

A major role of growth factors is to protect cells from programmed cell death or apoptosis (Raff, 1992; Raff *et al.*, 1993; Snider, 1994). To determine whether the expression of cyto-Met by hepatocytes had an influence on apoptosis induced by different stimuli, experiments were undertaken *in vivo* and *in vitro*. Since it is known that massive and lethal hepatocyte apoptosis can be obtained by injection of the Jo2 monoclonal antibody directed against the Fas receptor (Ogasawara *et al.*, 1993), transgenic mice were challenged with this stimulus. Figure 2A demonstrates that control animals are killed rapidly, as expected. However, the majority of transgenic animals

showed a total protection, and those that did succumb to the treatment did so only after a substantial delay. Histological analysis of the livers of control and transgenic animals, performed at 2, 4 and 5 h following anti-Fas injection (Figure 2B), provided further evidence of the protection conferred by expression of the transgene. In control animals, progressive damage was observed, starting at the 2nd hour with a few areas of inflammatory infiltration and culminating at the 5th hour with many necrotic and haemorrhagic areas. The cells appeared clearly damaged, with condensed cytoplasm and karyorrhexis and/or picnosis of the nuclei. On the contrary, in the transgenic liver, inflammatory infiltration was absent or scanty. A few damaged cells were detected at the 4th hour while only minor haemorrhages appeared at the 5th hour without major modification of the liver architecture.

Primary hepatocytes of transgenic mice are protected from apoptosis

Since hepatocyte primary cultures are less susceptible to Jo2 antibody (Ni *et al.*, 1994), owing perhaps to reduced expression of the Fas receptor, different criteria were used to assess the resistance of transgenic hepatocytes to apoptotic stimuli. Two established treatments to induce apoptosis of cultured cells are: (i) treatment with the broad-spectrum protein kinase inhibitor staurosporin (Jacobson *et al.*, 1994) and (ii) plating on poly-HEMA-coated dishes. The latter treatment prevents cell adhesion and is known to induce programmed cell death in anchorage-dependent cells (anoikia, Frish and Francis, 1994). Primary cultures of control and transgenic livers were prepared as described in Materials and methods, transferred once after 48 h, and challenged. In both assays, transgenic hepatocytes were protected from apoptosis (Figure 3A and B).

Staurosporin killing was reduced by 3- to 6-fold, depending upon the concentration used and the time of assay (8 or 24 h). Similarly, a 3-fold protection from poly-HEMA-induced apoptosis was recorded for the transgenic cells. The partial protection observed indicates that the apoptotic pathways are intact, but less readily triggered in transgenic cells. To assess more directly the role of cyto-Met in apoptosis protection of cultured hepatocytes, expression of the transgene was assessed in explanted hepatocytes.

Expression of cyto-Met in primary cultured hepatocytes

Since several liver-specific genes are known to be down-regulated in primary cell culture, we have tested directly the expression of the transgene and the production of the transgenic protein in primary cultured hepatocytes. Liver explants from transgenic homozygous embryos (#36, 16 d.p.c.) were kept in culture for 6 or 12 days and RNA was analysed by RT-PCR. Single-stranded cDNA was first obtained by reverse transcription of total RNA using a 3' human Met-specific reverse primer, then amplification was performed with primers complementary to the first exon of the *AT* gene (see Figure 1A) and ORF Met sequences. Figure 4A shows a Southern blot of the resulting RT-PCR products probed with an internal Met-specific third oligo. In addition, immunoprecipitation of primary culture extracts revealed consistent expression of the transgene as a protein of the anticipated size, although

Fig. 2. *In vivo* protection from apoptosis of transgenic hepatocytes. (A) Eight male mice of 8 weeks old in each group (wild-type or homozygotes of the transgenic #36, #42 and #78) were given intraperitoneal injections of Jo2 monoclonal anti-mouse Fas (Pharmigen; 3 µg/g body weight). Wild-type mice were all dead within 6 h while all transgenics remained healthy. Two transgenics died after 18 h and the remainder are still alive. (B) (Haematoxylin–eosin ×25). 2nd hour: in wild-type liver, a clear focus of inflammatory infiltration is detectable while only few inflammatory cells are present in the transgenic tissue. 4th hour: a huge necrotic area with many inflammatory cells in the wild-type liver is shown; on the contrary only few damaged hepatocytes are evident in the transgenic liver. 5th hour: almost all the hepatocytes appear damaged in the wild-type liver where a severe modification of the architecture, caused by haemorrhagic areas, is evident; only few and minor haemorrhages are present in the transgenic liver and both the hepatocytes and the organ architecture appear almost completely preserved.

our results suggest a down-regulation of the transgene expression when hepatocytes are isolated and cultivated (Figure 4B).

Transgenic hepatocytes are permissive for the establishment of cell lines

If transgenic hepatocytes are protected from apoptosis *in vivo* and in primary culture, and the transgene continues to be expressed when cells are placed in culture, these hepatocytes might permit the establishment of cell lines. When transgenic fetal livers were dissociated and grown *in vitro*, they did indeed give rise to cell lines, while a typical senescence crisis invariably was observed in cultures from the wild-type littermates.

Livers of animals of various ages and genotypes were subjected to mechanical dissociation and plated at high density on collagen-coated dishes. After overnight incubation, the majority of cells had attached. The semi-confluent cultures were washed and maintained without

transfer for several weeks. The epithelial monolayer that formed within 4 days was overgrown progressively by fibroblasts and undefined epithelial cells. After 5–6 weeks, a number of epithelial islands displaying hepatocyte-like morphology were visible in both transgenic and control cultures. However, it was only in the transgenic cultures that very small tightly packed cells were observed within the epithelial islands: it is these cells that give rise to immortalized lines. These islands were left on the primary dishes for another few weeks, during which time the neighbouring cells degenerated, permitting easy picking of each island, presumably containing the progeny of one cell. The recovered colonies were expanded slowly in dishes of increasing size, and frozen after ~25 cell generations. Passages are calculated from initial thawing. No failures to obtain established lines were encountered with the cyto-Met transgenics.

One or more immortalized cell lines per embryo or newborn animal, harvested from three different transgenic

lines, was obtained reproducibly (Table I). Out of 33 immortalized hepatocyte lines established, named MMH (from Met murine hepatocyte), nine have so far been kept in culture for >12 months and characterized. The cells of each of the nine lines grew as cobble-stone epithelial

monolayers (Figure 5) and did not display any features of transformation. They required collagen III for attachment and serum for proliferation. Each of the three growth factors, epidermal growth factor (EGF), insulin-like growth factor II (IGF-II) and insulin, was required for optimal proliferation and retention of the most 'hepatocyte-like' morphology. The nine characterized cell lines failed to grow in soft agar (Table I), and the four that have been tested were unable to form tumours in nude mice (data not shown). The generation time of each of the lines is ~24 h and, although plating efficiencies have not been measured, the cells recover rapidly and proliferate even when plated at low density after trypsinization. At early stages after they are obtained, the lines contain some metaphases with a diploid number of chromosomes, but this population is replaced rapidly by cells with a hetero-ploid chromosome number.

Characterization of the MMH cell lines: morphology

Morphological characterization has been carried out on both an embryonic and a newborn MMH line. Figure 5 shows a phase contrast image and immunofluorescence staining for ZO-1-decorated tight junctions and for cyto-keratins. ZO-1 is visible as a fine network on the membrane areas of cell-cell contact, indicating that the cells of both lines demonstrate simple epithelial polarity. The staining for cytokeratins reveals an abundant network of these intermediate filaments, outlining the epithelial growth

Fig. 3. *In vitro* protection from apoptosis of transgenic hepatocytes. (A) Primary cultures, prepared as described in Table I and transferred after 48 h to 24-well Costar microtitre plates (40 000 cells per well), were treated 16 h later with the indicated concentration of the apoptosis-inducing drug staurosporin, and cell death was assessed when indicated, using the trypan blue exclusion test on trypsinized cells. Each point was in triplicate. (B) Freshly prepared hepatocytes were treated as in (A), and plated on poly-HEMA-coated dishes to prevent cell attachment; after 24 h, cell viability was measured as in (A). For both (A) and (B), two different heterozygous transgenic families were used.

Fig. 4. Expression of cyto-Met in primary cultured hepatocytes. (A) Southern blotting of RT-PCR products. The reverse transcription reaction was performed on RNA extracted from homozygous embryonic liver explants kept in culture for 6 (lane 1) or 12 days (lane 2) from control wild-type mouse liver (lane 3) and from human hepatoma Hep3B cells stably transfected with the transgene (lane 4). One-tenth of the RT reaction was amplified and 15 of 50 μ l of the PCR products were loaded per lane and probed with an oligo not overlapping with PCR primers complementary to a single splice variant. (B) Detection of transgenic cyto-Met protein. Proteins were solubilized from two transgenic livers (lanes 1 and 2), from primary hepatocytes cultured for 6 or 12 days (lanes 3 and 4 respectively), from control wild-type liver (lane 5) and from human hepatoma Hep3B cells stably transfected with the transgene (lane 6). Immunoprecipitation by specific anti-human Met antibody followed by Western blotting reveals the transgenic cyto-MET protein in lanes 1–4 and the endogenous human β chain in lane 6.

Table I. Cumulative evaluation of immortalization events and soft agar colony formation from hepatocyte cultures of livers from fetal and newborn mice

Transgenic family	Stage of development	No. of transgenic livers put in culture	No. of epithelial islands at 40 days	No. of stable lines derived	No. of lines characterized	Efficiency of colony formation in soft agar
36	14.5 d.p.c.	3	8	8	4	$<1 \times 10^{-5}$
78	18 d.p.c.	2	4	4	2	$<1 \times 10^{-5}$
42	newborn	2	9	8	2	$<1 \times 10^{-5}$
36	day 2	10	2	2	0	–
78	day 3	3	2	1	1	$<1 \times 10^{-5}$
36	day 6	10	9	9	0	–
AT	newborn	3	0	0	–	–

The table summarizes the immortalization events obtained. The transgenic family AT carries the human α -1-antitrypsin gene. The positive control for the soft agar assay was the BW₁-J mouse hepatoma cell line (Cassio and Weiss, 1979) which forms colonies with an efficiency of 8×10^{-2} .

Fig. 5. Morphology of MMH cultures. Phase contrast and immunofluorescence micrographs of an embryonic and a newborn established hepatocyte line ($\times 380$). Upper images: a 14.5 d.p.c. hepatocyte line, showing immunofluorescence for the tight junction-associated ZO-1 protein (same field as phase contrast) and for cytokeratins. Lower panels: a newborn hepatocyte line; again, ZO-1 visualization is of the same field as the contrast image.

Fig. 6. Morphology of MMH cultures. (A and B) Electron micrographs ($\times 15\,000$) of apical (A) membrane of MMH cells (embryonic 14.5 d.p.c.) showing a differentiated bile canaliculus-like structure, sealed by tight and gap junctions, and of baso-lateral (B) membranes showing microvilli and pinocytosis vesicles. (C and D) Confocal analysis of immunostaining of MMH cells (newborn 1 day). (C) A three-dimensional reconstruction of ZO-1 protein staining, demonstrating a circle (arrow) that delimits a bile canaliculus. (D) Transverse section of double immunofluorescence of the Na^+/K^+ pump (red) and ZO-1 (green), showing the tight junction-associated protein ZO-1 localization restricted to the cellular surface precisely at the top of the cell layer and the pump at the baso-lateral region.

habit of the cells. The electron micrographs of Figure 6A and B highlight a bile canaliculus-like structure, sealed by tight junctions, as well as microvilli extruding from the baso-lateral-like domain and generating intercellular recesses. Figure 6C and D shows confocal microscopic images. Figure 6C is a three-dimensional reconstruction of ZO-1 staining, revealing the existence of occasional bile canaliculi. Figure 6D confirms the reality of polarization of these cells; they have been stained for a basolateral marker as well as ZO-1 and the cross-section demonstrates that ZO-1 is localized exclusively to the cell-cell contact at

the surface of the monolayer and that the sodium pump protein is confined to the baso-lateral domain.

Characterization of the MMH lines: hepatic functions

RNA was prepared from early and late passages of cell lines from four different liver stages, each maintained in complete growth medium or deprived of serum for 48 h. For comparison, RNA was prepared from fetal (18 d.p.c.) and adult liver. Blots were prepared and hybridized with probes for liver-enriched transcription factors and for

Fig. 7. Characterization of liver gene expression in MMH. Northern blot analysis of RNA extracted from four different MMH lines derived from embryonic or newborn liver as well as control embryonic (18 d.p.c.) or adult liver (E and A). Cultured cells were examined at early and late passages after the first thawing, in growth medium or after 48 h serum deprivation. Each lane was charged with 20 µg of total RNA. Probes used are indicated on the left. After hybridization, RNA amount (data not shown) was verified with a GAPDH probe.

hepatic functions (Figure 7). Among the transcription factors, HNF1- α , HNF4 and C/EBP- α are especially characteristic of hepatocytes. Transcripts for each of these factors are expressed in the different lines, although at variable levels. In some cases, the signal obtained is stronger at early than at late passage, in others a difference is observed between the cells maintained in serum or starved. Despite the variability, each of the lines examined expresses each of the factors. Concerning the hepatic functions, it is clear that the cell line showing the most consistent expression of each of the functions is from day 3 liver, as might be expected from the late expression of some hepatocyte genes.

Discussion

It is well known that the activated version of Met, like other tyrosine kinase receptors, can induce transformation of a variety of target cells (Sawyers and Denny, 1994). Expression of *cyto-MET* in transgenic hepatocytes does not result in tumour formation, but rather it renders the hepatocytes permissive for immortalization at all stages of development that have been tested. This was a totally unexpected finding. The fact that stable lines could be derived from different transgenic strains rules out the possibility that random inactivation of genes responsible for senescence or apoptosis had occurred by insertional mutagenesis (see Table I).

The most striking and uniform feature of the MMH cell lines is the absence of a transformed phenotype. Although

several laboratories have produced transgenic mice expressing an oncogene, or overexpressing a growth factor or its receptor, there are few reports concerning hepatocyte lines. Antoine *et al.* (1992) developed cell lines from tumorous liver tissue of a transgenic mouse expressing SV40 T antigen under control of the anti-thrombin III promoter: one of the two reported lines was 'well differentiated' but expressed nuclear T antigen and formed tumours in irradiated nude mice. The majority of cell lines isolated from mice overexpressing transforming growth factor- α (TGF- α) were also malignant (Wu *et al.*, 1994a), although one line remained untransformed and expressed a panel of hepatocyte-specific genes (Wu *et al.*, 1994b). The non-transformed phenotype of the hepatocyte lines from cyto-Met mice is certainly due to the only weakly transforming properties of the activated receptor used. The transgenic hepatocytes as well as the MMH lines are promising tools for the development of liver replacement models in mutant mice, since both should have a selective advantage (Overturf *et al.*, 1996).

We propose that the immortalization of the transgenic hepatocytes is a consequence of a cyto-Met-mediated protection from programmed cell death. There are provocative indications that inhibition of apoptosis may facilitate immortalization. Functional p53 and Rb are positive regulators of apoptosis. Viral proteins that bind p53 and Rb contribute to immortalization or are themselves immortalizing (Shay *et al.*, 1991), and fibroblasts from mice that are deficient in p53 are immortalized readily (Harvey *et al.*, 1993; Tsukada *et al.*, 1993). It is widely accepted that invalidation of the apoptosis mechanism is an important step in tumour development (Chiarugi *et al.*, 1994), and the same may be true of immortalization. Finally, it is easy to imagine that between the time of initiation of a cell culture and the emergence of a permanent line, multiple insults occur that result in apoptosis of cells that are poised for this event. Decreased sensitivity to apoptosis could facilitate immortalization by simply increasing the number of target cells for a rare, and presumably mutational, event.

The mechanism of cyto-Met-mediated protection from apoptosis remains to be clarified. The simplest explanation that takes into account the decreased sensitivity of transgenic hepatocytes to multiple apoptotic stimuli would be the continuous activation by cyto-Met of a signal transduction pathway, whose final effect is to prevent commitment to apoptosis. The molecular events linking growth factor receptors to the control of apoptosis are still largely unknown, although phosphatidylinositol-3-kinase appears to be involved in nerve growth factor-induced survival (Yao and Cooper, 1995). This signal transduction pathway is activated efficiently by Met (Graziani *et al.*, 1991; Ponzetto *et al.*, 1993). A second intriguing clue comes from the recent finding of interaction between the cytoplasmic portion of Met and the C-terminal region of Bag-1 (Bardelli *et al.*, 1996), a functional partner of Bcl-2, a key player in prevention of apoptosis (Vaux *et al.*, 1988; Hockenbery *et al.*, 1990; Nunez *et al.*, 1990; Takayama *et al.*, 1995).

MMH lines arise at a low frequency from a large number of primary cultured cells, similarly to the hepatocyte lines described by Antoine *et al.* (1992) and Wu *et al.* (1994b). Consequently, the immortalized lines are most probably

derived from a small number of transgenic cells that underwent some secondary event(s). Moreover, cells of some of the MMH established lines have ceased to express detectable amounts of the cyto-Met transcript (data not shown), analysed some 25–30 generations after initial plating. Since immortalization was never observed in cultures from wild-type livers, the expression of cyto-Met is apparently necessary to establish the immortal phenotype. However, the absence of cyto-Met in some of the immortalized cells implies that the protein is not required for maintenance of the immortalized state. Thus, transgene expression most likely represents the primary event associated with a multi-step phenomenon. A second hypothesis that would account for the rarity of the event is that cyto-Met expression alone is sufficient for immortalization, but that the number of susceptible target cells is very small, a candidate for this minor population being the ever-elusive liver stem cell (Sigal *et al.*, 1992).

Materials and methods

Animal experiments

Details on the construction and on the tissue-specific and temporal expression of the transgene have been published (Amicone *et al.*, 1995). For the *in vivo* induction of apoptosis, eight male mice of 8 weeks old in each group (wild-type or homozygotes of the transgenic lines #36, #42 and #78) were given intraperitoneal injections of Jo2 monoclonal anti-mouse Fas (Pharmigen; 3 µg/g body weight). Animal care and sacrifice was performed according to EEC guidelines.

Western blot

The liver samples and cultured cells were lysed in 10 mM PIPES pH 7.6, 100 mM NaCl, 5 mM MgCl₂, 300 mM sucrose, 5 mM EGTA, 1% Triton X-100, 2 mM phenylmethylsulphonyl fluoride (PMSF), 500 µg/ml leupeptin, 50 µg/ml pepstatin, 1 µg/ml aprotinin and 1 mM Na orthovanadate. Lysates were incubated with anti-human Met antibody (c-Met C-12, Santa Cruz Biotechnology) coupled to protein A-Sepharose. Immunoprecipitation was followed by Western blotting and development with ECL (Amersham).

Primary hepatocyte culture

Livers from fetuses (from 14.5 d.p.c.) or newborns (up to day 6) were dissected and mechanically dissociated (five strokes in a loose-fitting Potter), and plated at high density on collagen III (Sigma)-coated Nunc Petri dishes in RPMI 1640 containing 10% fetal calf serum (FCS) supplemented with 50 ng/ml EGF, 30 ng/ml IGF-II, 10 µg/ml insulin (Boehringer Mannheim) and antibiotics. After overnight incubation, the majority of cells had attached. The semi-confluent cultures were washed and maintained without transfer for several weeks. In some experiments, cells were inoculated and maintained for 2 days in hepatocyte attachment medium (Gibco-BRL), which reduced the fibroblast contamination.

Soft agar colony formation

The soft agar assay was performed by inoculating 1×10^5 cells of each line suspended in 1.5 ml of Dulbecco's modified Eagle's medium (DMEM) containing 10% FCS and 50 ng/ml EGF, 30 ng/ml IGF-II, 10 µg/ml insulin supplemented with 0.34% agar (Sigma) in 6 cm² tissue culture plates containing a 0.68% agar underlay. Cultures were kept for 20 days in 8% CO₂ at 37°C.

Histology

The liver samples were fixed in 4% (w/v) buffered paraformaldehyde overnight and embedded in paraffin. Sections, 4–8 mm thick, were stained with haematoxylin and eosin and observed under a light microscope.

In vitro test of apoptosis

A total of 40 000 cells were plated in each well of a 24-well Costar microtitre plate and, after 16 h, staurosporin (Sigma) was added at the indicated concentration. Each point was done in triplicate. Anoikia assay using poly-HEMA (Aldrich-Chemie) was performed according to Frish *et al.* (1994). Cells were trypsinized, stained with trypan blue and counted under the microscope.

Stable cell lines

Individual epithelial islands, presumably containing the progeny of one cell, were expanded slowly in collagen III-coated dishes of increasing size, and frozen after ~25 cell generations. Passages are calculated from initial thawing.

All the donor mice were heterozygous for the cyto-Met transgene except newborns, day 2 and day 6 (homozygous) and AT, which carries the human AT gene but is devoid of cyto-Met ORF. The mice used were progeny of founders described in Amicone *et al.* (1995) and Rther *et al.* (1987).

Immunofluorescence

Cells were fixed and treated as specified by Mvel-Ninio and Weiss (1981). Antibodies used were: monoclonal pan-cytokeratin (Sigma) diluted 1/100; ZO-1 rat monoclonal antibody (Stevenson *et al.*, 1986), supplied by Doris Cassio (Institut Curie, Orsay, France), used undiluted; mouse monoclonal anti- α subunit Na⁺/K⁺ ATPase (Gottardi and Caplan, 1993), supplied by Dr M.J. Caplan, Yale University, CT, used at 1/100; sheep anti-mouse IgG coupled to fluorescein isothiocyanate (FITC; Amersham) used 1/100; rabbit anti-rat FITC, Sigma, 1/100; and sheep anti-mouse Ig, Texas redTM-linked whole antibody, Amersham, 1/100. Confocal analysis was performed using a Leika based microscope with a Bio-Rad MRC 600 confocal imaging system. For thin sections, cells were fixed in Karnovsky solution 0.1 M cacodylate buffer pH 7.4 for 12–18 h at 4°C and post-fixed in 1% OsO₄. Cells were then dehydrated and embedded in epoxy resin. Thin sections for electron microscopy were counterstained with uranyl acetate and lead citrate.

RNA analysis

Single-stranded cDNA obtained from reverse transcription of 2.5 µg of total RNA using a reverse complement of nucleotides 4221–4245 of the ORF Met (oligo-1: TTTGACATAGTACTAGCACTATGAT, from GenBank accession No. K02212) was amplified by PCR according to the following protocol. Oligonucleotides used for PCR analyses were oligo-2 (CTCAGCTTCAGGCACCACCA) corresponding to nucleotides 11–30 of the exon 1 of the AT gene (from GenBank accession No. X54559) and oligo-1. Reactions were performed in a total volume of 50 µl. The reaction mixture first was heated to 95°C for 5 min, and then 40 cycles of 94°C for 30 s, 61°C for 30 s and 72°C for 1 min were carried out in a DNA thermal cycler (Perkin-Elmer-Cetus). Products were separated on a 1.5% agarose gel and then transferred to a nitrocellulose filter for hybridization with a third oligonucleotide (oligo 3: TGTAGACTACCGAGTACTT) corresponding to nucleotides 3054–3073 of ORF Met.

For Northern blot analysis, total cellular RNA was extracted from cells according to standard protocols. RNA samples (~20 µg per lane) were resolved by electrophoresis in agarose–1.85 M formaldehyde gels and blotted onto nylon membranes (Amersham). Blots were hybridized with α -³²P-specific probes (HNF1- α , Frain *et al.*, 1989; HNF1- β , De Simone *et al.*, 1991; HNF4, Sladek *et al.*, 1990; C/EBP- α , Landschulz *et al.*, 1988; C/EBP- β , Poli *et al.*, 1990; C/EBP- δ , Cao *et al.*, 1991; aldolase B, Skala *et al.*, 1987; albumin, Sargent *et al.*, 1979; β -fibrinogen, Crabtree and Kant, 1981; connexin 32, Paul, 1986) at 42°C in 50% formamide buffer (Hybrisol, Oncor).

Acknowledgements

We are grateful to Laura Pozzi for the production of transgenic animals and we thank Carmela Amedeo for cell culture. Work in the Italian laboratories was supported by AIRC Associazione Italiana Ricerca Sul Cancro. Work in the Paris laboratory was supported by the Biotechnology program of the EEC under contract number BIOT CT 93-0103. L.A. is the recipient of an AIRC postdoctoral fellow; G.S. was supported by a fellowship of the EEC under the Human Capital Mobility Program.

References

- Amicone, L., Galimi, M.A., Spagnoli, F.M., Tommasini, C., De Luca, V. and Tripodi, M. (1995) Temporal and tissue-specific expression of the MET ORF driven by the complete transcriptional unit of human A1AT gene in transgenic mice. *Gene*, **162**, 323–329.
- Antoine, B., Levrat, F., Valle, V., Berbar, T., Cartier, N., Dubois, N., Briant, P. and Kahn, A. (1992) Gene expression in hepatocyte-like lines established by targeted carcinogenesis in transgenic mice. *Exp. Cell Res.*, **200**, 175–185.

- Bardelli, A., Longati, P., Albero, D., Goruppi, S., Schneider, C., Ponzetto, C. and Comoglio, P.M. (1996) HGF receptor associates with the anti-apoptotic protein BAG-1 and prevents cell death. *EMBO J.*, **15**, 6025–6212.
- Barre, B.A., Hart, I.K., Coles, H.S.R., Burne, J.F., Voyvodic, J.T., Richardson, W.D. and Raff, M.C. (1992) Cell death and control of cell survival in the oligodendrocyte lineage. *Cell*, **70**, 31–46.
- Bishop, J.M. (1991) Molecular themes in oncogenesis. *Cell*, **64**, 235–248.
- Bottaro, D.P., Rubin, J.S., Faletto, D.L., Chan, A.M.L., Kmieciak, T.E., Vande Woude, G.F. and Aaronson, S.A. (1991) Identification of the hepatocyte growth factor receptor as the c-met proto-oncogene product. *Science*, **251**, 802–804.
- Bussolino, F. *et al.* (1992) Hepatocyte growth factor is a potent angiogenic factor which stimulates endothelial cell motility and growth. *J. Cell Biol.*, **119**, 629–641.
- Cao, Z., Umek, R.M. and McKnight, S.L. (1991) Regulated expression of three C/EBP isoforms during adipose conversion of 3T3-L1 cells. *Genes Dev.*, **5**, 1538–1552.
- Cassio, D. and Weiss, M.C. (1979) Expression of fetal and neonatal hepatic functions by mouse hepatoma–rat hepatoma hybrids. *Somat. Cell Genet.*, **5**, 719–738.
- Chiarugi, V., Magnelli, L. and Ruggiero, M. (1994) Apoptosis, senescence, immortalization and cancer. *Pharmacol. Res.*, **30**, 301–315.
- Cooper, C.S., Park, M., Blair, D.G., Tainsky, M.A., Huebner, K., Croce, C.M. and Van Woude, G.F. (1984) Molecular cloning of a new transforming gene from a chemically transformed human cell. *Nature*, **311**, 29–33.
- Crabtree, G.R. and Kant, J.A. (1981) Molecular cloning of cDNA for the alpha, beta, and gamma chains of rat fibrinogen. A family of coordinately regulated genes. *J. Biol. Chem.*, **256**, 9718–9723.
- DeFrances, M.C., Wolf, H.K., Michalopoulos, G.K. and Zarnegar, R. (1992) The presence of hepatocyte growth factor in the developing rat. *Development*, **116**, 387–395.
- De Simone, V., De Magistris, L., Lazzaro, D., Gerstner, J., Monaci, P., Nicosia, A. and Cortese, R. (1991) LFB3, a heterodimer-forming homeoprotein of the LFB1 family, is expressed in specialized epithelia. *EMBO J.*, **10**, 1435–1443.
- Frain, M., Swart, G., Monaci, P., Nicosia, A., Stampfli, S., Frank, R. and Cortese, R. (1989) The liver-specific transcription factor LF-B1 contains a highly diverged homeobox DNA binding domain. *Cell*, **59**, 145–157.
- Frish, S.M. and Francis, H. (1994) Disruption of epithelial cell–matrix interactions induces apoptosis. *J. Cell Biol.*, **124**, 619–626.
- Giordano, S., Ponzetto, C., Di Renzo, M.F., Cooper, C.S. and Comoglio, P.M. (1989) Tyrosine kinase receptor indistinguishable from the c-met protein. *Nature*, **339**, 155–156.
- Giordano, S., Zhen, Z., Medico, E., Gaudino, G., Galimi, F. and Comoglio, P.M. (1993) Transfer of motogenic and invasive response to scatter factor/hepatocyte growth factor by transfection of human MET protooncogene. *Proc. Natl Acad. Sci. USA*, **90**, 649–653.
- Gottardi, C.J. and Caplan, M.J. (1993) An ion-transporting ATPase encodes multiple apical localization signals. *J. Cell Biol.*, **121**, 283–293.
- Graziani, A., Gramaglia, D., Cantley, L.C. and Comoglio, P.M. (1991) The tyrosine-phosphorylated hepatocyte growth factor/scatter factor receptor associates with phosphatidylinositol 3-kinase. *J. Biol. Chem.*, **266**, 22087–22090.
- Harrington, E.A., Bennett, M.R., Fanidi, A. and Evan, G.I. (1994) C-Myc-induced apoptosis in fibroblast is inhibited by specific cytokines. *EMBO J.*, **13**, 3286–3295.
- Harvey, M. *et al.* (1993) *In vitro* characteristics of embryo fibroblasts isolated from p-53 deficient mice. *Oncogene*, **8**, 2457–2467.
- Hockenbery, D., Nunez, G., Millman, C., Schreiber, R.D. and Korsmeyer, S.J. (1990) Bcl-2 is an inner mitochondrial membrane protein that blocks programmed cell death. *Nature*, **348**, 334–336.
- Jacobson, M.D., Burne, J.F. and Raff, M.C. (1994) Programmed cell death and Bcl-2 protection in the absence of a nucleus. *EMBO J.*, **13**, 1899–1910.
- Landschulz, W.H., Johnson, P.F., Adashi, E.Y., Graves, B.J. and McKnight, S.L. (1988) Isolation of a recombinant copy of the gene encoding C/EBP. *Genes Dev.*, **2**, 786–800.
- Mével-Ninio, M. and Weiss, M.C. (1981) Immunofluorescent analysis of the time course of extinction, re-expression and activation of albumin production in heterocaryons and hybrids of rat hepatoma cells with mouse fibroblast. *J. Cell Biol.*, **90**, 339–350.
- Michalopoulos, G.K. (1990) Liver regeneration: molecular mechanisms of growth control. *FASEB J.*, **4**, 176–187.
- Montesano, R., Matsumoto, K., Nakamura, T. and Orci, L. (1991) Identification of a fibroblast-derived epithelial morphogen as hepatocyte growth factor. *Cell*, **67**, 901–908.
- Naldini, L., Vigna, E., Narsimhan, R.P., Gaudino, G., Zarnegar, R., Michalopoulos, G.K. and Comoglio, P.M. (1991a) Hepatocyte growth factor (HGF) stimulates the tyrosine kinase activity of the receptor encoded by the proto-oncogene c-met. *Oncogene*, **6**, 501–504.
- Naldini, L. *et al.* (1991b) Scatter factor and hepatocyte growth factor are indistinguishable ligands for the met receptor. *EMBO J.*, **10**, 2867–2878.
- Nakamura, T., Teramoto, H. and Ichihara, A. (1986) Purification and characterization of a growth factor from rat platelets for mature parenchymal hepatocytes in primary cultures. *Proc. Natl Acad. Sci. USA*, **83**, 6489–6493.
- Ni, R., Tomita, Y., Matsuda, K., Ichihara, A., Ishimura, K., Ogasawara, J. and Nagata, S. (1994) Fas-mediated apoptosis in primary cultured mouse hepatocytes. *Exp. Cell Res.*, **215**, 332–337.
- Nunez, G., London, L., Hockenbery, D., Alexander, M., McKearn, J.P. and Korsmeyer, S.J. (1990) Deregulated Bcl-2 gene expression selectively prolongs survival of growth factor-deprived hemopoietic cell lines. *J. Immunol.*, **144**, 3602–3610.
- Ogasawara, J., Watanabe-Fukunaga, R., Adachi, M., Matsuzawa, A., Kasugai, T., Kitamura, Y., Itoh, N., Suda, T. and Nagata, S. (1993) Lethal effect of the anti-Fas antibody in mice. *Nature*, **364**, 806–809.
- Overturf, K., Al-Dhalimy, M., Tanguay, R., Brantly, M., Ou, C.N., Finegold, M. and Grompe, M. (1996) Hepatocytes corrected by gene therapy are selected *in vivo* in a murine model of hereditary tyrosinaemia type I. *Nature Genet.*, **12**, 266–273.
- Park, M., Dean, M., Cooper, C.S., Schmidt, M., O'Brien, S.J., Blair, D.G. and Vande Woude, G.F. (1986) Mechanism of met oncogene activation. *Cell*, **45**, 895–904.
- Paul, D.L. (1986) Molecular cloning of cDNA for rat liver gap junction protein. *J. Cell Biol.*, **103**, 123–134.
- Poli, V., Mancini, F.P. and Cortese, R. (1990) IL-6DBP, a nuclear protein involved in interleukin-6 signal transduction, defines a new family of leucine zipper proteins related to C/EBP. *Cell*, **63**, 643–653.
- Ponzetto, C., Bardelli, A., Maina, F., Longati, P., Panayotou, G., Dhand, R., Waterfield, M.D. and Comoglio, P.M. (1993) A novel recognition motif for phosphatidylinositol 3-kinase binding mediates its association with the hepatocyte growth factor/scatter factor receptor. *Mol. Cell Biol.*, **13**, 4600–4608.
- Ponzetto, C., Bardelli, A., Zhen, Z., Maina, F., Dalla Zonca, P., Giordano, S., Graziani, A., Panayotou, G. and Comoglio, P.M. (1994) A multi-functional docking site mediates signalling and transformation by the hepatocyte growth factor/scatter factor receptor family. *Cell*, **77**, 261–271.
- Raff, M.C. (1992) Social controls on cell survival and cell death. *Nature*, **356**, 397–400.
- Raff, M.C., Barres, B.A., Burne, J.F., Coles, H.S., Ishizaki, Y. and Jacobson, M.D. (1993) Programmed cell death and the control of cell survival: lessons from the nervous system. *Science*, **262**, 695–700.
- Rodrigues, G.A. and Park, M. (1993) Dimerization mediated through a leucine zipper activates the oncogenic potential of the met receptor tyrosine kinase. *Mol. Cell Biol.*, **13**, 6711–6722.
- Rubin, J.S. *et al.* (1991) A broad-spectrum human lung fibroblast-derived mitogen is a variant of hepatocyte growth factor. *Proc. Natl Acad. Sci. USA*, **88**, 415–419.
- Rüther, U., Tripodi, M., Cortese, R. and Wagner, E. (1987) The human α -1-antitrypsin gene is efficiently expressed from two promoters in transgenic mice. *Nucleic Acids Res.*, **15**, 7519–7529.
- Sargent, T.D., Wu, J.R., Sala-Trepar, J.M., Wallace, R.B., Reyes, A.A. and Bonner, J. (1979) The rat serum albumin gene: analysis of cloned sequence. *Proc. Natl Acad. Sci. USA*, **76**, 3256–3260.
- Sawyers, C.L. and Denny, C.T. (1994) Chronic myelomonocytic leukaemia: tel-a-kinase what's all about. *Cell*, **77**, 171–173.
- Schmidt, C., Bladt, F., Goedecke, S., Brinkmann, V., Zschiesche, W., Sharpe, M., Gherardi, E. and Birchmeier, C. (1995) Scatter factor/hepatocyte growth factor is essential for liver development. *Nature*, **373**, 699–702.
- Shay, J.W., Pereira-Smith, O.M. and Wright, W.E. (1991) A role for both RB and p53 in the regulation of human cellular senescence. *Exp. Cell Res.*, **196**, 33–39.
- Sigal, S.H., Brill, S., Fiorino, A. and Reid, L.M. (1992) The liver as a stem cell and lineage system. *Am. J. Physiol.*, **263**, G139–G148.
- Skala, H., Vibert, M., Lamas, E., Maire, P., Schweighoffer, F. and Kahn, A. (1987) Molecular cloning and expression of rat aldolase C messenger RNA during development and hepatocarcinogenesis. *Eur. J. Biochem.*, **163**, 513–518.

- Sladek,F.M., Zhong,W.M., Lai,E. and Darnell,J.E.,Jr (1990) Liver enriched transcription factor HNF-4 is a novel member of the steroid hormone receptor superfamily. *Genes Dev.*, **4**, 2353–2365.
- Snider,W.D. (1994) Functions of the neurotrophins during nervous system development: what the knockouts are teaching us. *Cell*, **77**, 627–638.
- Stern,C.D., Ireland,G.W., Herrick,S.E., Gherardi,E., Gray,J., Perryman,M. and Stoker,M. (1990) Epithelial scatter factor and development of the chick embryonic axis. *Development*, **110**, 1271–1284.
- Stevenson,B.R., Siciliano,J., Mooseker,M.S. and Goodenough,D.A. (1986) Identification of ZO-1: a high molecular weight polypeptide associated with the tight function (zonula occludens) in a variety of epithelia. *J. Cell Biol.*, **103**, 755–766.
- Takayama,S., Sato,T., Krajewski,S., Kochel,K., Irie,S., Millan,J.A. and Reed,J.C. (1995) Cloning and functional analysis of BAG-1: a novel Bcl-2-binding protein with anti-cell death activity. *Cell*, **80**, 279–284.
- Tsukada,T. *et al.* (1993) Enhanced proliferative potential in culture of cells from p53-deficient mice. *Oncogene*, **8**, 3313–3322.
- Van der Geer,P., Hunter,T. and Lindberg,R.A. (1994) Receptor protein-tyrosine kinases and their signal transduction pathways. *Annu. Rev. Cell Biol.*, **10**, 251–337.
- Vaux,D.L., Cory,S. and Adams,J.M. (1988) Bcl-2 gene promotes haemopoietic cell survival and cooperates with c-myc to immortalize pre-B cells. *Nature*, **335**, 440–442.
- Weidner,K.M., Behrens,J., Vandekerckove,J. and Birchmeier,W. (1990) Scatter factor: molecular characteristics and effect on the invasiveness of epithelial cells. *J. Cell Biol.*, **111**, 2097–2108.
- Wu,J.C., Merlino,G., Cveklova,K., Mosinger,B.,Jr and Fausto,N. (1994a) Autonomous growth in serum-free medium and production of hepatocellular carcinomas by differentiated hepatocyte lines that overexpress transforming growth factor alpha 1. *Cancer Res.*, **54**, 5964–5973.
- Wu,J.C., Merlino,G. and Fausto,N. (1994b) Establishment and characterization of differentiated, non transformed hepatocyte cell lines derived from mice transgenic for transforming growth factor alpha. *Proc. Natl Acad. Sci. USA*, **91**, 674–678.
- Yao,R. and Cooper,G.M. (1995) Requirement for phosphatidylinositol-3 kinase in the prevention of apoptosis by nerve growth factor. *Science*, **267**, 2003–2006.
- Zhen,Z., Giordano,S., Longati,P., Medico,E., Campiglio,M. and Comoglio,P.M. (1994) Structural and functional domains critical for constitutive activation of the HGF-receptor (Met). *Oncogene*, **9**, 1691–1697.

Received on July 29, 1996; revised on September 24, 1996