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Transgenic mouse models in carcinogenesis: interaction of c-*myc* with transforming growth factor α and hepatocyte growth factor in hepatocarcinogenesis

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1 Overexpression of the c-*myc* oncogene is associated with a variety of both human and experimental tumours, and cooperation of other oncogenes and growth factors with the *myc* family are critical in the evolution of the malignant phenotype.

2 Double transgenic mice bearing fusion genes consisting of mouse albumin enhancer/promoter-mouse c-*myc* cDNA and mouse metallothionein 1 promoter-human transforming growth factor (TGF-α) cDNA were generated to investigate the interaction of these genes in hepatic oncogenesis and to provide a general paradigm for characterizing the interaction of nuclear oncogenes and growth factors in tumourigenesis.

3 Coexpression of c-*myc* and TGF-α as transgenes in the mouse liver resulted in a tremendous acceleration of neoplastic development in this organ as compared to expression of either of these transgenes alone. The two distinct cellular reactions that occurred in the liver of the double transgenic mice prior to the appearance of liver tumours were dysplastic and apoptotic changes in the existing hepatocytes followed by emergence of multiple focal lesions composed of both hyperplastic and dysplastic cell populations.

4 These observations suggest that the interaction of c-*myc* and TGF-α, during development of hepatic neoplasia contributes to the selection and expansion of the preneoplastic cell populations which consequently increases the probability of malignant conversion.

5 We have now extended these studies and examined the interaction of hepatocyte growth factor (HGF) with c-*myc* during hepatocarcinogenesis in the transgenic mouse model. While sustained overexpression of c-*myc* in the liver leads to cancer, coexpression of HGF and c-*myc* in the liver delayed the appearance of preneoplastic lesions and prevented malignant conversion. Similarly, tumour promotion by phenobarbitone was completely inhibited in the c-*myc*/HGF double transgenic mice whereas phenobarbitone was an effective tumour promoter in the c-*myc* single transgenic mice.

6 The results indicate that HGF may function as a tumour suppressor during early stages of liver carcinogenesis, and suggest the possibility of therapeutic application for this cytokine. Furthermore, we show for the first time that interaction of c-*myc* with HGF or TGF-α results in profoundly different outcomes of the neoplastic process in the liver.

Keywords transgenic mouse oncogenes suppressor genes carcinogenesis

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Introduction

Neoplastic development is a multistep phenomenon involving alteration in the genetic material of the cells [1, 2]. The genetic alterations that predispose to the development of neoplasia may involve activation of protooncogenes, inactivation of tumour suppressor genes and/or inappropriate expression of growth factors [3]. Accumulation of these genetic changes eventually leads to the emergence of malignant cell populations capable of devastating the host organism. Whether there exists a preferred sequence of genetic alterations in tumour development [4] or a number of non-ordered combinations of genetic changes may be capable of generating the same or a similar malignant phenotype remains to be determined.

The transgenic mouse system provides a unique model for assessing the effects of oncogenes acting either alone or in combination with other oncogenes within the organism [5, 6]. It therefore provides a research tool for exploring interactions of some of the genetic alterations observed in the fully-developed tumour phenotypes. This may be particularly important in the interaction of growth factors and nuclear oncogenes [3]. The *myc* gene family (c-*myc*, N-*myc* and L-*myc*) is an important member of the nuclear oncogenes. The *myc* oncoproteins act as sequence-specific transcription factors that regulate a variety of genes important in normal cellular growth and differentiation processes [7, 8]. Aberrant expression of all three members of the *myc* gene family has been implicated in the development of a wide variety of both experimentally-induced and naturally-occurring tumours [9]. Deregulation of c-*myc* expression is frequently observed in experimentally-induced hepatocellular carcinoma in rodents, as well as in primary human liver tumours [10–13]. Similarly, expression of transforming growth factor-α (TGF-α), a potent hepatotrophic mitogen synthesized in hepatocytes during regeneration, is frequently observed in both human tumours and in transformed cultured cells [14], including those of the liver [15, 16]. TGF-α is structurally and functionally related to epidermal growth factor (EGF), and exerts its effects via the EGF receptor [17–19]. Liver tumours occur in c-*myc* transgenic mice as a result of the selective expression of c-*myc* in the liver directed by the albumin enhancer/promoter or the α1-antitrypsin promoter [20, 21]. However, the tumour incidence in these c-*myc* transgenic mice is relatively low with a long latency period [20, 21]. Similarly, transgenic mice in which TGF-α is under the transcriptional control of the metallothionein promoter and is expressed at high levels in the liver and other tissues, develop liver tumours after a latency period of 10 to 15 months [22–24].

Although TGF-α and EGF seem to act as powerful liver tumour promoters, the role played by hepatocyte growth factor (HGF) in the development of hepatocellular carcinoma (HCC) is less clear. HGF, initially identified in the serum of partially hepatectomized rats as a potent mitogen for hepatocytes in culture [25, 26], is expressed in mesenchymal cells of different tissues, including non-parenchymal liver cells [27–29]. HGF induces motility [30–32], proliferative activity, and morphogenesis in hepatocytes and in many other extrahepatic cell types [33, 34]. Moreover, HGF is a potent angiogenic factor *in vitro* and *in vivo* [35, 36], and is involved in haematopoiesis [37] and local regulation of fibrinolysis and coagulation [38, 39]. The pleiotropic effects of HGF imply a physiological function as an essential paracrine and endocrine modulator of mesenchymal-epithelial interactions during development and repair/regeneration of tissues [34, 37, 40–44]. This modulatory activity is mediated by the HGF receptor encoded by the c-*met* protooncogene that is expressed in most of the epithelial tissues including the liver [45–47]. Numerous reports have shown expression of HGF and/or its receptor in tumoural tissues and cell lines [48–53], suggesting that the HGF/c-*met* dependent signalling may also be involved in neoplastic development, conceivably during tumour progression [54–58]. Nevertheless, the role of HGF during hepatocarcinogenesis is still controversial.

It has, however, been demonstrated that HGF can inhibit the growth of many transformed cell types, including HCC cell lines, by a cytostatic mechanism [59–63]. Also, intraportal infusion of HGF *in vivo* inhibits cell proliferation in neoplastic liver nodules [64]. It is also noteworthy, that peroxisome proliferators are able to promote clonal expansion of initiated hepatocytes by cooperating with EGF and/or TGF-α, but not with HGF [65]. Furthermore, in contrast to the TGF-α transgenic mice, no neoplastic transformation occurred in recently established transgenic mice overexpressing HGF in the liver [66]. These observations suggest that the involvement of HGF/c-*met* signal transduction system in the neoplastic process of the liver differs in a fundamental way from that of the TGF-α/EGFR system. Guided by this notion, we wished to characterize the effects of HGF upon the neoplastic process in a transgenic mouse model in which we had earlier established the impact of TGF-α on carcinogenesis [67].

Methods

Transgenic mice

The c-*myc* and c-*myc*/HGF transgenic mice were generated by using the mouse c-*myc* and the h-HGF recombinant DNA constructs as reported before [62, 67]. Several lines of both c-*myc* and h-HGF transgenic mice were developed, displaying similar phenotype [62, 66, 67]. Animal housing and animal care were in accordance with NIH guidelines. The transgenic offspring were identified by DNA dot blot and Southern analysis of tail DNA using nick-translated c-*myc* probe, or by PCR using the oligonucleotides 5′-AACATCCGAGTTGGTTACTGCTCCC-3′ (from nt 1111 to nt 1135) and 5′-ACCTGTTTGCGTTTCTCCTCTCCTC-3′ (from nt 1691 to 1667 nt) as primers (Bioserve Biotechnologies, Laurel, MD, USA) to amplify a 581 bp h-HGF product.

Macroscopical and histological analyses

The mice were sacrificed every month until they were 9 months old and then every second month until 16 months of age. Body weights were recorded and livers removed, weighed and examined for macroscopic lesions. For morphological, immunohistochemical, and molecular analysis, parts of the livers were fixed in 10% phosphate buffered (pH 7.4) formalin, or promptly frozen in liquid nitrogen and stored at -80° C. All fixed tissues were routinely embedded in paraffin and sections stained with haematoxylin and eosin (H&E). Two sections from each lobe of non-tumourous liver and all grossly visible tumours were examined. The histopathological diagnosis of hepatocellular adenoma (HCA) or HCC was independently done by three investigators on H&E-stained tissue sections obtained from all grossly visible tumours.

Northern blot analysis

Total RNA was isolated by guanidine isothiocyanate and centrifugation in caesium chloride gradient. Poly(A)$^+$ RNA was selected by oligo (dT)-cellulose chromatography. mRNA (10 μg from each sample) was fractionated in a 1% agarose/2.2 M formaldehyde gel, transblotted onto nylon membranes, and hybridized to ^{32}P-labelled probes with the Stratagene QuikHyb Hybridization Solution (Stratagene, La Jolla, CA, USA) according to the manufacturer's instructions. The probes utilized were: (a) a 847-bp fragment of human HGF gene corresponding to the coding region from bp 122 to 969 (kindly provided by R. Zarnegar, Department of Pathology, University of Pittsburgh School Medicine, Pittsburgh, PA, USA); (b) a 1.9-kb fragment containing the full coding sequence of mouse c-*myc* cDNA, 62 bp of the 5′, and 11 bp of the 3′ non-coding sequences, plus the rabbit β-globin 3′ non-coding sequence, as contained in the pLEC1 construct used to generate the *myc* transgene [67]; (c) a 985 bp fragment of rat TGF-β1 cDNA. The cDNA probe coding for rat cytoplasmic β-actin was used as an internal standard. After washing off the unspecific binding, membranes were exposed to Kodak XAR film or a phosphor screen and scanned with the phosphoimager to quantify the mRNA expression (Molecular Dynamics, Sunnyvale, CA, USA).

Immunohistochemistry and in situ *hybridization*

Immunohistochemical localization of the TGF-α protein in the liver was performed on deparaffinized sections. After inactivation of the endogenous peroxidase (30 min treatment with 0.3% H_2O_2 in methanol), the sections were treated with 0.05% saponin in phosphate buffered saline (PBS) for 2 h, followed by digestion with 0.1% trypsin in PBS for 10–15 min. After rinsing in PBS, sections were stained for TGF-α by the avidin-biotin-peroxidase method using the ABC kit from Vector Laboratories. The antibody (a gift from Dr L. Gentry) was a rabbit anti-rat pro-TGF-α antiserum (1 : 1000 dilution) raised against residues 137–159 of the c-terminal peptide of the cytoplasmic domain of pro-TGF-α [68].

The demonstration of TGF-α transcripts by *in situ* hybridization was accomplished by using anti-sense and sense human TGF-α riboprobes derived from 310 bp fragment of the 5′ end of the cDNA inserted into the pGEM3 transcription vector (Promega), as earlier described [22]. This probe hybridized with human TGF-α but only weakly with the murine gene product. Similarly, c-*myc* anti-sense and sense riboprobes were constructed by inserting a 870 bp fragment of murine c-*myc* cDNA into the pGEm4z transcription vector (Promega). The *in situ* analysis of TGF-α and c-*myc* transcripts were performed with the appropriate anti-sense and sense riboprobes as described [69–71].

Tumour promotion by phenobarbitone (PB)

Mice were given PB 0.05% in their food pellets starting at 3 weeks of age. Ten to 12 transgenic mice and five wild type mice (wt) were sacrificed at each of the indicated time points and further analysed as described for the untreated animals.

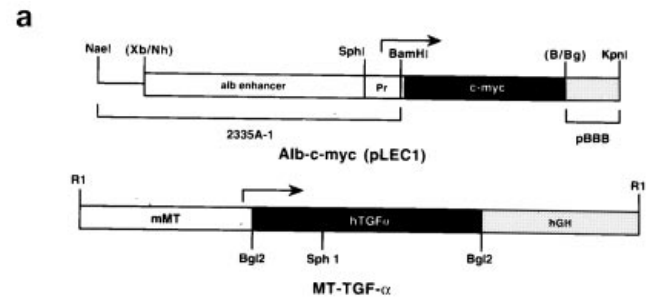
Results

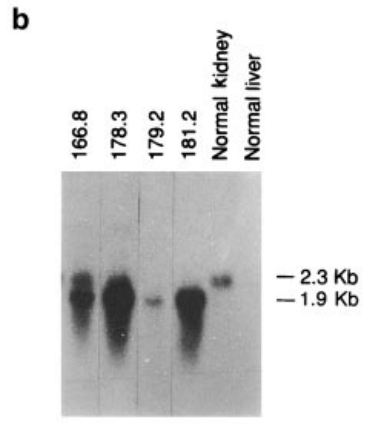
*Generation of Alb/c-*myc *and Alb/c-*myc*-MT/TGF-α (c-*myc*/TGF-α) double transgenic mice*

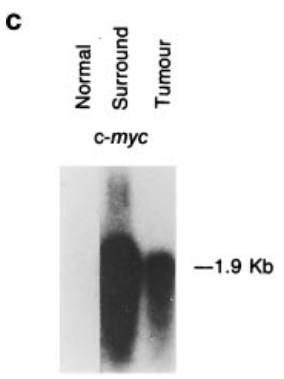
Eight founder animals bearing the Alb/c-*myc* construct (pLEC-1) were identified from which four lines were developed (Figure 1(b)). Three of the four lines (166.8, 178.3 and 181.2) contained 5–10 head-to-tail copies of the transgene, whereas line 179.2 had 1–2 copies of the transgene (data not shown). High levels of the expected transcript size (approximately 1.9 kb) of the c-*myc* transgene were found in livers of the three high copy number lines, whereas expression of the transgene was considerably lower in line 179.2 bearing the 1–2 copies of the Alb/c-*myc* construct (Figure 1(b)). Expression of the Alb/c-*myc* transgene was not detected in tissues other than liver (data not shown). The double transgenic mice were generated by crossing the c-*myc* mice (lines 166.8, 178.3, 181.2, and 179.2) with the TGF-α mice (line MT42, 22).

Development of hepatic neoplasia and expression of transgenes

The male mice were selected from the double c-*myc*/TGF-α transgenic mice and divided into two groups. One group was maintained on 50 mM of $ZnCl_2$ in the drinking water starting at 3 weeks of age, while the other was kept without the zinc supplement. The animals were then killed at 3, 6, 10 and 16 weeks after starting the zinc supplement. A minimum of 12 animals were analysed for each time point. The data reported here are those from a cross between line 166.8 and MT42, but all the lines bearing multiple Alb/c-*myc*







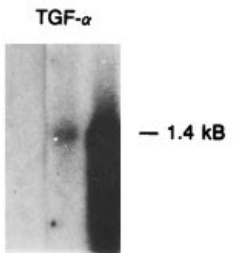


Figure 1 (a) Structure of transgenes; plasmid constructs are described in [67] and in Methods. (b) Transgene expression in four of the Alb/c-*myc* mouse lines. Northern blot analysis was performed on 20 μg of total RNA isolated from liver and electrophoresed through 0.9% agarose gel containing 12% formaldehyde, probed with nick-translated c-*myc* mouse cDNA, and washed as previously described [31]. (c) Expression of c-*myc* and TGF-α transgenes in a hepatocellular carcinoma and adjacent grossly normal liver. Northern blot analysis was performed as described in (b); nick-translated cloned human TGF-α cDNA was used to probe for the TGF-α transgene expression [32]. Equal gel loading was confirmed by ethidium bromide staining of the gels (data not shown). Data from Murakami *et al.* [67] (with permission).

copies gave essentially the same results when crossed with the MT42 line. After 3 weeks of zinc treatment the first histological indication of neoplastic development became evident and was characterized by the appearance of huge dysplastic hepatocytes around blood vessels displaying compact nuclei and condensed chromatin, indicative of apoptosis (Figure 2(c)). By the sixth week 80% of the mice displayed these lesions. Foci of distinct dysplastic cells displaying numerous mitotic figures were commonly found throughout the liver lobule by week 10 (Figure 2(d)). At this time intravascular spread of the tumour cells was first observed in close association with these dysplastic foci (Figure 2(e)), but no distant metastasis was detected. After 16 weeks of zinc treatment, over 70% of the double transgenic male mice carried single or multifocal nodules of which 25% histologically consisted of well-differentiated hepatocellular carcinomas displaying either pseudoglandular or trabecular pattern (Figure 2(f)). No neoplastic lesions were observed in either the c-*myc* or the TGF-α single transgenic mice during the corresponding time period. In contrast, the appearance of preneoplastic and neoplastic lesions in the double transgenic mice maintained without the zinc treatment was delayed by 6–8 weeks (data not shown).

Different patterns of expression of the TGF-α and *myc* transgenes were observed. Whereas expression of the c-*myc* transgene coincided with that of albumin (Figure 3(e)) [33, 34], TGF-α expression was most intense in the hepatocytes surrounding the central vein (Figure 3(a), (b)). Similarly, TGF-α was frequently expressed strongly in the dysplastic cells and tumours (Figures 1(c) and 3(c), (d)), while expression of the c-*myc* transgene was either absent or greatly reduced in the tumours (Figure 1(c)). However, high expression of the c-*myc* gene was occasionally seen in the early focal lesions (Figure 3(f)).

Inhibition of hepatocarcinogenesis by HGF in c-myc *transgenic mice*

Homozygous female C57Bl/6J × CBA/J mice bearing the pLEC1 mouse albumin enhancer/promoter c-*myc* fusion gene were crossed with male heterozygous FVB transgenic mice expressing a human HGF (h-HGF) cDNA under the control of the albumin enhancer/promoter. As expected, approximately 50% of the mice coexpressed c-*myc* and h-HGF (c-*myc*/HGF), and the other 50% only expressed c-*myc* in the same final background. (C57Bl/6J × CBAJ) × FVB hybrid were used as control animals. Due to the higher incidence of tumours only male animals were analysed. During the study eight or 10 mice from each group were killed monthly between the first and the ninth month of age, whereas 12 mice from each group were used at 10, 12, 14 and 16 months of age. As a measure of liver growth and the possible appearance of tumour masses the liver weight/body weight ratio was recorded (Figure 4(a)). In both transgenic lines during the first year of life the liver weight was moderately higher than that of control mice. However, the liver weight of c-*myc* transgenic

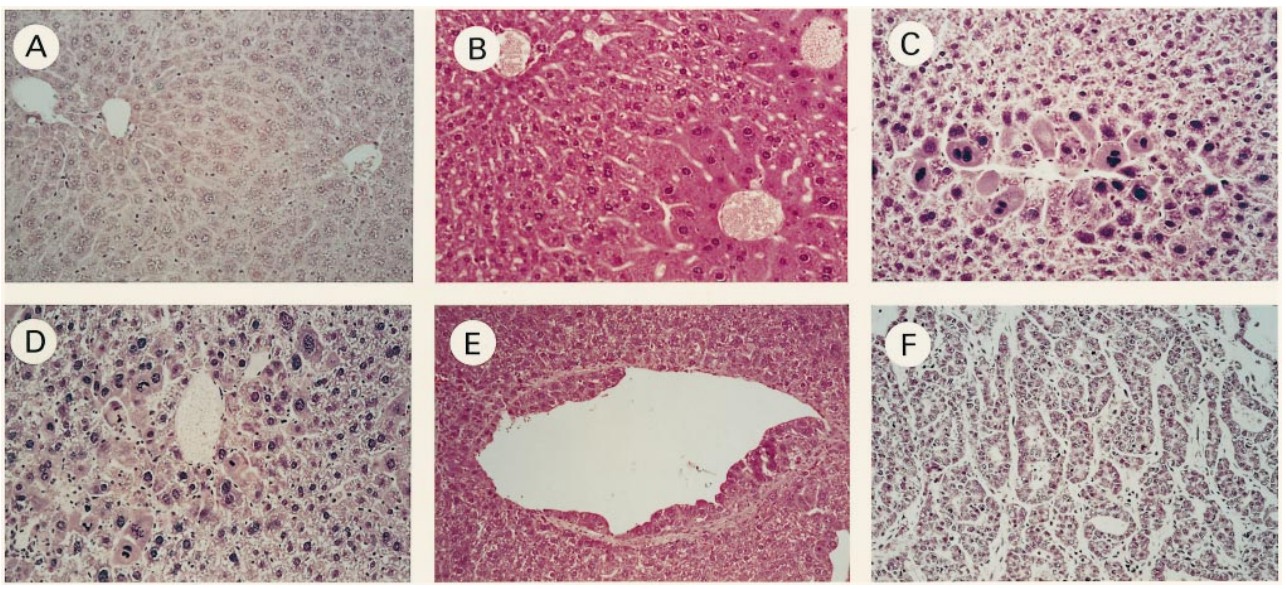


Figure 2 Histologic analysis of double transgenic mouse livers. Tissues were fixed in Bouin's for 1 to 4 h or in 10% neutral buffered formalin for 24 h, transferred to 70% ethanol, then processed, embedded in paraffin, cut at 5 μm, and stained with haematoxylin and eosin according to standard methods. (a) Liver of a 5-month-old nontransgeneic mouse showing normal hepatic structure (75×). (b) Liver of a 5-month-old Alb/c-*myc* transgenic mouse. The basic liver structure is preserved in the transgenic liver. Note, however, the enlarged hepatocytes around the central vein, a feature characteristic for all the Alb/c-*myc* transgenic lines (75×). (c) Liver of a double transgenic mouse 3 weeks after starting the zinc treatment is characterized by the appearance of huge dysplastic hepatocytes around blood vessels displaying compact nuclei condensed chromatin, indicative of apoptosis and few mitotic figures (75×). (d) A dysplastic lesion in a 10-week-old double transgenic mouse, typical for the multifocal lesions seen at this stage. The lesions are characterized by large dysplastic cells with numerous mitotic figures (75×). (e) Intravascular spread of tumour cells in a 16-week-old double transgenic mouse. Similar intravascular invasion was also seen in association with dysplastic lesions (panel d) at earlier time points (56.25×). (f) Liver tumour in a 17-week-old double transgenic mouse. The tumour is a well-differentiated pseudoglandular hepatocellular carcinoma (75×). Data from Murakami *et al.* [67] (with permission).

mice increased significantly after 12 months of age reaching 10% of the body weight by 16 months, reflecting the formation of large tumour masses. In contrast, the liver weight of c-*myc*/HGF mice older than 12 months did not differ from that of younger animals.

The onset of histologically detectable hepatic abnormalities occurred in the c-*myc* transgenic line at 4 months of age and consisted of perivascular dysplastic cells. Subsequently a progression from mild to severe dysplasia was observed, and all the single transgenic c-*myc* mice had large dysplastic hepatocytes by the age of 14 months. Dysplastic cells were detected in 15% of the c-*myc*/HGF double transgenic mice at 12 months of age and at 16 months 67% of the mice were affected by mild hepatic dysplasia. The appearance of preneoplastic lesions (foci of cellular alteration; [72]) in the c-*myc* transgenic line occurred as early as 7 months in 30% of the animals and foci were present in all the mice between 12 and 16 months. In contrast c-*myc*/HGF double transgenic mice showed preneoplastic foci only in mice older than 1 year and more than 50% of animals were still not affected at 16 months of age (data not shown).

The first benign hepatic neoplasms (HCA; [72]) were detected at 8 months in the c-*myc* transgenic mice, while the first HCC appeared at 10 months (Figure 4(b)). The incidence of both types of tumours continued to grow rapidly in older c-*myc* animals. In the c-*myc*/HGF chimeric line the onset of lesions was seen at 13 months (Figure 4(b)). Histological examination showed that, even at 16 months of age, these double transgenic c-*myc*/HGF mice were affected only by small benign lesions composed by cells resembling hepatocytes. Furthermore, at each time point the number of c-*myc*/HGF mice carrying benign lesions was always lower than that observed in the c-*myc* single transgenic mice affected by HCA and/or HCC (Figure 1(b)). In addition to displaying earlier onset and higher incidence, the tumours arising in the c-*myc* line were larger and more numerous than HCA appearing in the c-*myc*/HGF line, which were never more than two per liver (data not shown). The HCC growing in the c-*myc* transgenic animals were either of the trabecular or of the solid histological type, varying from well differentiated to poorly differentiated tumours with cell polymorphism, atypia and areas of haemorrhagic necrosis. These tumours ended up becoming confluent and replacing most of the liver parenchyma. None of the lesions described above was detected in the wild type mice throughout the time course of this study. Taken together these data suggest that hepatocytes overexpressing c-*myc* progressively develop HCC but the coexpression of HGF inhibits the appearance of preneoplastic lesions and prevents the progression towards malignant phenotypes.

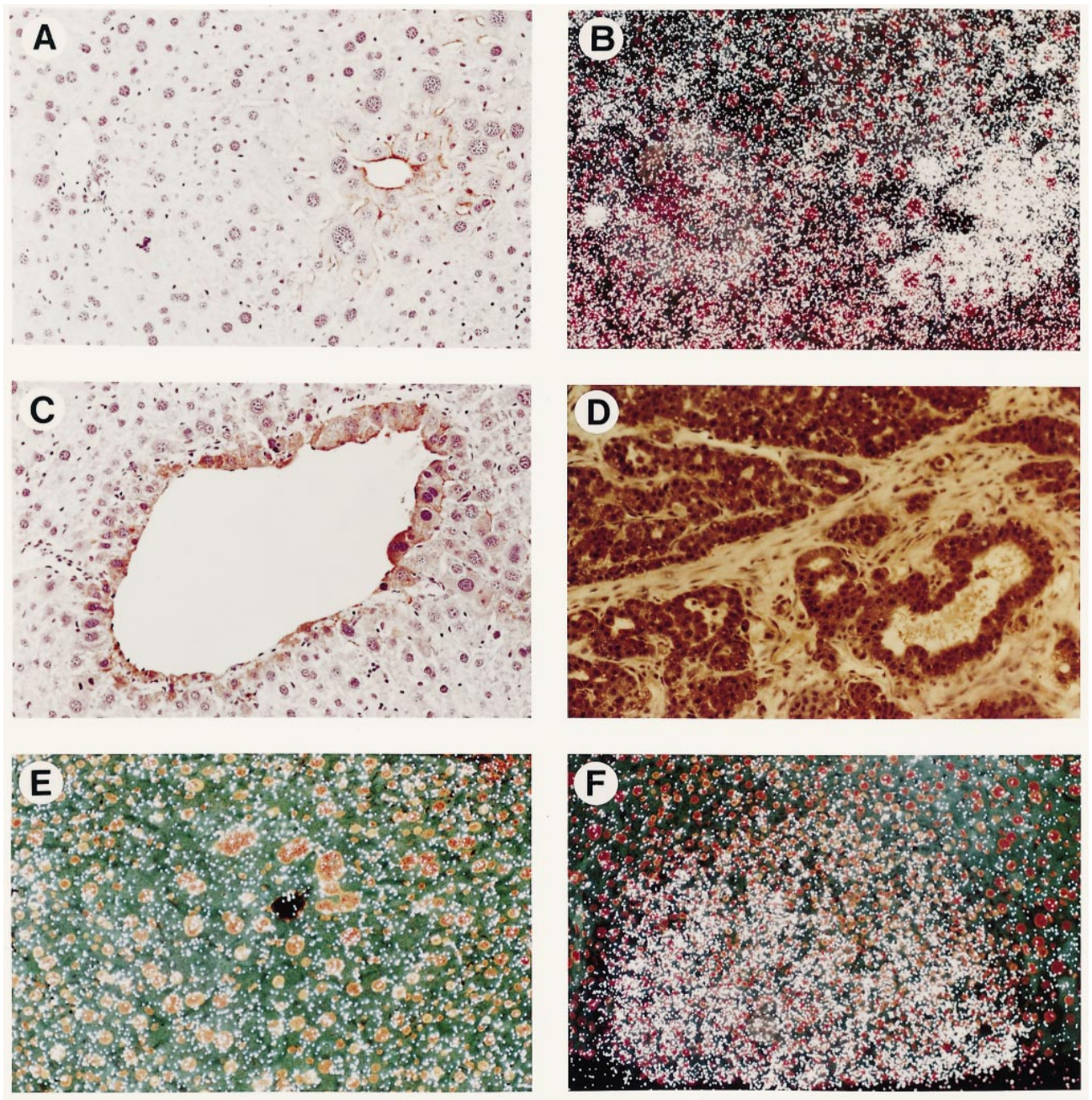


Figure 3 Cellular distribution of transcripts and proteins of the transgenes in livers of double transgenic mice. (a) Immunohistochemical demonstration of pro-TGF-α in a 3-week-old non-neoplastic liver of a double transgenic mouse. Note the predominant distribution of pro-TGF-α in the cell membrane and the most intense staining in the large hepatocytes surrounding the central vein (85 ×). (b) *In situ* hybridization with anti-sense human TGF-α riboprobe in corresponding section from the same liver as shown in panel (a) showing good agreement between the distribution of TGF-α transcripts and protein (dark field, 85 ×). Panels (c) and (d) show intense staining for pro-TGF-α in intravascular-spreading tumour cells and in hepatocellular carcinoma, respectively (42.5 ×). (e) *In situ* hybridization with anti-sense murine c-*myc* riboprobe in the 3-week-old non-neoplastic liver of a double transgenic mouse shown in panels a and b (dark field, 85 ×). (f) *In situ* hybridization with anti-sense c-*myc* riboprobe in liver section from a 10-week-old double transgenic mouse on zinc treatment showing high level of c-*myc* transcripts in a hyperplastic focus (dark field, 85 ×). Data from Murakami *et al.* [67] (with permission).

Inhibition of phenobarbitone tumour promotion by HGF

Phenobarbitone (PB) is considered a potent non-genotoxic liver tumour promoter in rodents treated with chemical carcinogens. Although several mechanisms by which PB promotes liver carcinogenesis have been proposed [73–76], the notion that initiated hepatocytes have a distinct growth advantage over the mitoinhibited normal hepatocytes has recently attracted increased interest. PB has also been shown to promote growth of transplanted HCC [77] and more recently to collaborate with TGF-α in accelerating hepatocarcinogenesis of TGF-α transgenic mice [78]. Increased plasma levels of HGF have been found in rats treated with PB and this observation has generated the hypothesis that HGF could trigger proliferation in preneoplastic cells during PB promotion [79, 80]. Since this hypothesis is in apparent conflict with our data we wished to test if PB

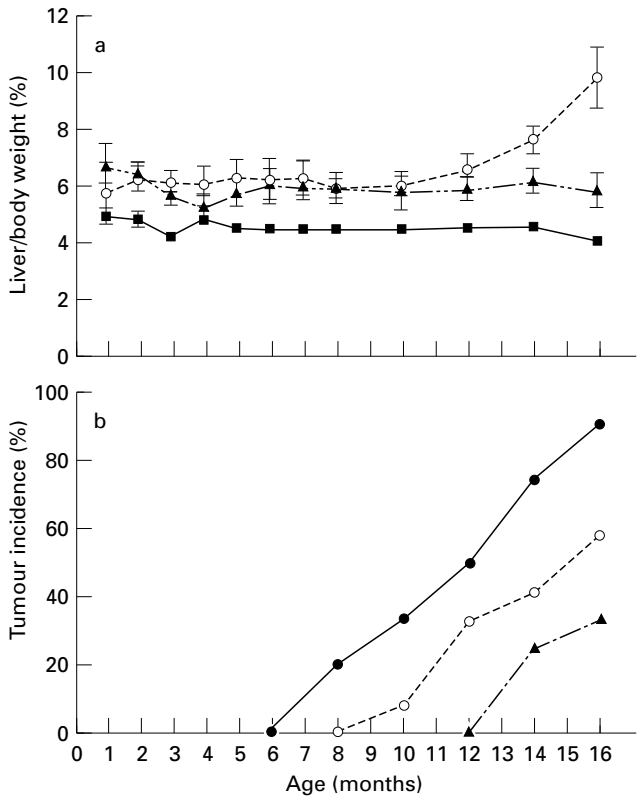


Figure 4 Liver growth and neoplastic development in c-*myc* (○) and c-*myc*/HGF (▲) transgenic mice. (a) Liver weight (% of body weight) during the ontogenesis of wild type (w.t., ■) and transgenic mice. Each time point represents mean value ±s.e. mean of 8–12 mice. (b) Time course and incidence of hepatic neoplastic lesions expressed as percentage of total number (8–12) of mice examined per time point. ● c-*myc* HCA; ○ c-*myc* HCC; ▲ c-*myc*/HGF HCA.

was capable of promoting to the same extent the formation of liver tumours in the c-*myc* and c-*myc*/HGF transgenic mice.

PB was administered in the diet to both transgenic lines and to wild type mice from 3 weeks to 10 months of age. The PB treatment resulted in an acceleration and a significant increase of the HCA and HCC incidence in c-*myc* transgenic mice as compared with the untreated c-*myc* mice (Table 1). The HCA and HCC appeared 2 months earlier, relative to untreated mice, and by 10 months of age 100% and 40% of PB-treated c-*myc* mice had HCA and HCC, respectively. These results suggest that hepatocytes overexpressing c-*myc* respond to PB promotion in a manner similar to hepatocytes initiated by chemical carcinogens. However, in striking contrast with the single transgenic c-*myc* mice, no tumour formation was detected in the c-*myc*/HGF mice after 10 months of PB treatment (Table 1). We therefore hypothesize that there is either no production of initiated hepatocytes in c-*myc*/HGF mice over the first 10 months of life or more likely that HGF can suppress the growth of these initiated cells.

Discussion

In this report we demonstrate that coexpression of the c-*myc* and TGF-α transgenes in mouse liver leads to a tremendous acceleration of neoplastic development in this organ as compared with that observed in mice expressing either c-*myc* or the TGF-α transgene alone [20–24]. Earlier studies with transgenic mice bearing the albumin enhancer/promoter c-*myc* fusion gene revealed mild to severe hepatocellular dysplasia by 2 months, no hepatic nodules when examined at various ages up to 1 year, and three of five mice examined between 15 and 18 months of age had hepatocellular adenomas [20]. Results from our own c-*myc* transgenic lines are in agreement with these earlier studies showing only dysplastic lesions at an early age (2–3 months) and appearance of hepatocellular adenomas between 14 and 18 months of age (data not shown). The TGF-α transgenic mice, in contrast to the c-*myc* mice, do induce multifocal, well-differentiated hepatocellular carcinomas between 10 and 15 months of age [22, 23]. Although the cellular and molecular mechanism by which TGF-α induces hepatocellular neoplasia is at present unknown, the data from the double c-*myc*/TGF-α transgenic mice suggest that involvement of c-*myc* and possibly other nuclear oncogenes may be an essential component in the growth factor induced neoplasia. The two distinct cellular events that precede the formation of liver tumours in the double transgenic mice are the initial dysplastic changes in the existing hepatocytes (mainly found in zone III) and the appearance of multiple focal lesions composed of both hyperplastic and dysplastic cell populations (Figure 2(c), (d)). Similar preneoplastic changes have been observed in livers of transgenic mice bearing a fusion gene consisting of MUP-SV40 T antigen [35]. These results suggest that the mechanism by which the SV40 T transgene alone and the c-*myc*/TGF-α transgene combination initiate the transformation process, eventually resulting in hepatic neoplasia, may be similar.

The high levels of both TGF-α transcripts and protein

Table 1 Tumour promotion by phenobarbitone (PB)

Age	*wt-PB*	*c*-myc	*c*-myc-*PB*	*c*-myc/*HGF*	*c*-myc/*HGF-PB*
			Incidence of HCA		
6 months	0/5 (0%)	0/10 (0%)	5/10 (50%)	0/10 (0%)	0/10 (0%)
8 months	0/5 (0%)	2/10 (20%)	8/10 (80%)	0/10 (0%)	0/10 (0%)
10 months	0/5 (0%)	4/12 (33%)	10/10 (100%)	0/12 (0%)	0/10 (0%)
			Incidence of HCC		
8 months	0/5 (0%)	0/10 (0%)	2/10 (20%)	0/10 (0%)	0/10 (0%)
10 months	0/5 (0%)	1/12 (8%)	4/10 (40%)	0/12 (0%)	0/10 (0%)

observed in the early dysplastic lesions (Figure 3) indicate a central role for the growth factor in expanding the preneoplastic cell population, and thus increase the probability of malignant conversion. This notion is further supported by the observation that events independent of the c-*myc*/TGF-α transgene expression appear to be required prior to the formation of liver tumours as evidenced by a remarkable heterogeneity in the expression of the transgenes in neoplastic nodules and hepatocellular carcinomas [82]. Whether the growth promoting effects of TGF-α are specific in this context or other growth factors known to stimulate growth of liver cells can substitute for TGF-α remains to be elucidated.

Although coexpression of c-*myc* with either SV40 T antigen or mutated H-ras in related transgenic systems has been shown to accelerate hepatocarcinogenesis as compared with that seen with the individual oncogenes [20], we believe our data to be the first to demonstrate synergistic effects of growth factors and nuclear oncogenes in neoplastic development in the transgenic model [67]. Since metallothionein-directed expression of TGF-α in transgenic mice induces, in addition to the liver, uniform epithelial hyperplasia in pancreas, breast, stomach and intestine [22–24], it is now feasible to test if coexpression of c-*myc* and TGF-α in these organs has the same or similar effects as observed in hepatic oncogenesis in the double transgenic model.

The results of our study show that the sustained overexpression of c-*myc* in the liver indeed leads to cancer. More importantly, our data reveal that the interaction of the nuclear oncogene c-*myc* with different growth factors acting via tyrosine kinase receptors can result in a profoundly different outcome of the neoplastic process. While coexpression of TGF-α in the c-*myc* transgenic mice is capable of accelerating the appearance of HCC [67], no HCC develops in the c-*myc*/HGF mice. Furthermore, the c-*myc* and *myc*/HGF transgenic lines display striking differences in the rate at which preneoplastic and neoplastic lesions develop in the liver, as well as in the characteristics of these lesions. It should be emphasized that these two transgenic lines are of the same genetic background, indicating that the observed differences are due to the expression of h-HGF.

The discovery that no malignant conversions of the neoplastic liver lesions were found in the c-*myc*/HGF double transgenic is of particular importance. The observation that the level of c-*met* expression is decreased in both the preneoplastic lesions and tumours suggests that intact HGF/c-*met* signal transduction system is required for effective suppression of the neoplastic process in this transgenic mouse model. In this context it is interesting to note that in a recent study by Grigioni *et al.* [83] on the relationship between c-*met* expression and malignant grade of human HCC the authors found an uneven distribution of the c-*met* expression in HCC in contrast to homogeneous pattern of c-*met* expression in normal liver and benign lesions. Based on the present data, it is possible to interpret the results of Grigioni *et al.* as indicating that in human HCC at least a percentage of the cells are not subject to the HGF/c-*met* dependent suppression and could therefore proliferate regardless of serum HGF levels. However, the c-*met* protooncogene has been shown to be overexpressed in a certain percent of different types of carcinoma [48, 50–54, 57] and sarcoma [58] as well as being an efficient transforming agent of NIH3T3 cells [49, 84]. It, therefore, seems likely that c-*met* could play an important role in malignant and metastatic phenotypes of tumours different from those generated in our c-*myc* transgenic mice.

The c-*myc*/HGF transgenic mice, in contrast to the c-*myc* mice, are completely resistant to the phenobarbitone promotion. These data suggest that the HGF/c-*met* system is most effective in inhibiting, possibly via apoptosis, the expansion of the initiated cell population. However, further work is needed in order to properly address questions regarding which stage(s) of liver carcinogenesis is most sensitive to the HGF/c-*met* dependent inhibition.

The present results are also supported by previous findings *in vitro* [60, 62, 63] showing that HGF selectively blocks the growth of transformed hepatocytes and stimulates the proliferation of the normal ones. HGF can, in light of its complex spectrum of activities, be considered as an homeostatic liver modulator essential for the development of this organ [43, 44] as well as for hepatic regeneration and repair [34, 40]. Our results indicate that, in addition to all these important functions, HGF may, at least in the liver, also act as a tumour suppressor.

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