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Ha-ras^{Val12} but not *p53*^{Ser247} leads to a significant neoplastic transformation rate of the putative rat liver stem cells (oval cell)

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In order to test the controversially discussed hypothesis that oval cells are part of a liver stem cell compartment and can give rise to cholangiocellular as well as hepatocellular carcinomas in the course of liver carcinogenesis, we transfected an oval cell line established in our laboratory with an oncogenically activated genomic *Ha-ras* clone (pUC EJ 6.6), carrying a valine at position 12 instead of the wild-type glycine, or a rat *p53* cDNA mutated by site-directed mutagenesis at codon 247, which corresponds to codon 249 in the human *p53*. This codon is of particular interest since it represents a mutation hotspot observed in hepatocellular carcinoma especially in regions with high aflatoxin B_1 exposure. Independent *Ha-ras*^{Val12} and *p53*^{Ser247} recombinant clones were subcutaneously injected into syngeneic newborn rats and the resulting tumours were analysed histopathologically. Each of two *p53*^{Ser247} clones gave negligible tumour yields (one tumour out of 13 injected animals), whereas each of two *Ha-ras*^{Val12} clones gave marked tumour yields (four tumours out of 13 and seven out of 12 treated animals, respectively). In addition, the *p53*^{Ser247}-induced tumours appeared only after 11 months and were small, whereas the *Ha-ras*^{Val12}-induced tumours appeared already after 6–8 weeks and grew rapidly. Histopathological analysis of the tumours revealed only undifferentiated carcinomas. Interestingly, one tumour that arose upon injection of *Ha-ras*^{Val12}-transfected cells stained positive for albumin, showing at least a partial hepatocytic differentiation.

Introduction

The differentiated liver parenchymal cells, although normally quiescent, start to divide upon treatment with certain carcinogens, which may induce mutations in critical genes leading to the onset of a sequence through preneoplastic foci and nodules to hepatocellular adenoma and carcinoma (1,2). The preneoplastic foci can be subdivided into clear and acidophilic (glycogen-storing), mixed-type and basophilic foci. Although it is widely accepted that this sequence originates from hepatocytes, carcinogenesis protocols exist in which the regimen exerts a toxic effect on hepatocytes, not allowing them to divide. Instead, a massive proliferation of oval cells can be observed (3–5). We have shown that these non-parenchymal epithelial cells are precursors of cholangiocellular carcinoma (1,2), but it remains controversial whether they can also give rise to hepatocellular carcinoma. If this were the case then the oval cells (or their as yet unidentified progenitors) would be good candidates for a liver stem cell. Similar (oval cell-like) cells have been recently described in human liver (6,7).

The role of oval cells as the precursors for cholangiocellular carcinoma has been clearly demonstrated. Upon treatment of rats with the hepatocarcinogenic choline deficient/DL-ethionine supplemented (CDE) diet (1,2) or *N*-nitrosomorpholine at high doses (8) a direct sequence could be demonstrated leading from oval cell proliferation to cholangiofibrosis and finally to cholangiofibroma and carcinoma. Under the experimental conditions of a CDE diet, which leads to extended necroses of parenchymal cells, not only cholangiocellular but also hepatocellular carcinomas arise. Thus, this experimental system is well suited for studying the role of oval cells in the genesis of these two tumour types.

The hypothesis that oval cells can also differentiate along the hepatocytic lineage is further supported by the demonstration of differentiated hepatocytic functions in isolated oval cells, i.e. synthesis of albumin and tyrosine aminotransferase activity upon treatment with differentiation inducing agents and expression of alpha-fetoprotein (9,10). In addition, treatment of a non-parenchymal liver epithelial cell line, phenotypically similar to oval cells, with MNNG and subsequent subcutaneous injection of these cells into newborn rats led to the formation of diverse liver tumours including a very small number of hepatocellular carcinomas (11). A strong argument in favour of the oval cell as a precursor for hepatocytes can be seen in the demonstration that, after administration of 2-AAF to rats in combination with partial hepatectomy, ^{3}H-labelled thymidine (which is incorporated into actively replicating DNA) appeared at an early stage in oval cells and at a more advanced stage in the DNA of preneoplastic basophilic hepatocytes (4). This result implies the differentiation of oval cells into hepatocytes.

In a previously published study we succeeded in transforming oval cells *in vitro* by keeping them at confluence for an extended period of time (12). Tumours that appeared upon s.c. injection of these cells into newborn rats exhibited a heterogeneous pattern with a cholangiocellular and solid morphology, whereas a hepatocellular differentiation could not be observed. The approach in the current study was to transform oval cells by transfection of tumour-related genes and to examine the differentiation pattern of these cells in a tumorigenicity test.

Materials and methods

Cell culture

Oval cells were cultured as previously described (13). Briefly, cells were cultured in Dulbecco's minimum essential medium:Ham's F10 (1:1) (Biochrom, Berlin, Germany), supplemented with 5% fetal calf serum (FCS), 1 ng/ml insulin (Sigma Chemical Co., Deisenhofen, Germany), 1 μg/ml hydrocortisone (Sigma), 100 μg/ml penicillin and 100 μg/ml streptomycin and maintained at 5% CO_2 in a humified (89%) incubator at 37°C. For transfection only low-passage cells were used as it is known that transfection efficiency drops dramatically as the passage number increases (14). In this work cells in passage 11 were used as recipients for the plasmid constructs.

Cell staining

Cells were plated on cover slips and allowed to adhere overnight. Cells were fixed in acetone/ethanol (1:1) for 10 min at –20°C. Blocking was performed

Abbreviations: MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; RT–PCR, reverse transcription–polymerase chain reaction.

with 1% bovine serum albumin in phosphate buffered saline. pAb 240 (mutant *p53*-specific in cell staining; Dianova, Hamburg, Germany) was used at a concentration of 10 μg/ml and incubated for 1 h. Peroxidase-conjugated goat anti-mouse antibody was used for detection with H_2O_2 and 3-amino-9-ethylcarbazol as substrate (Sigma).

Histology and immunohistochemistry

Sections of formalin-fixed tumour samples were stained with haematoxylin/eosin for light microscopic examination of tumour structure. Immunohistochemistry was performed by staining the sections with antibodies against *p53* and albumin. For staining paraffin-embedded tumour specimens with mutant *p53*-specific pAb 240 (Dianova) antigen retrieval was performed by heating the material in the microwave oven for 10 min in the presence of 6 M urea, pH 6.0, prior to primary antibody incubation

Characterization of the transformed phenotype

Growth kinetics. Twenty thousand cells were seeded in triplicate into 35 mm Petri dishes (Greiner, Nürtingen, Germany) and counted after trypsinization on day 3, 4 and 5 in a haemocytometer in the presence of 0.4% Trypan blue (Gibco BRL, Eggenstein, Germany).

Soft agar assay Soft agar assay was performed according to MacPherson and Montagnier (15). Twenty thousand cells in 2 ml of the standard culture medium containing 20% v/v FCS with 0.3% agar (Difco, Detroit, MI) were laid over a 1 ml basal layer of 1% w/v agar in 35 mm Petri dishes. After 1 week, 0.4 ml of fresh medium was added and colony formation was determined on day 14.

Tumorigenicity testing. One million cells were subcutaneously injected into newborn rats (Sprague–Dawley, Interfauna, Tuttlingen, Germany). Animals were palpated once a week and killed upon appearance of tumours approximately 1 cm in diameter and at the latest after 12 months. Tumour tissue was placed into formalin and processed for histopathological analysis.

Plasmids

Plasmid pBR6 coding for wild-type rat *p53* was a generous gift from C.Caron de Fromentel and pUC EJ 6.6 (genomic clone of *Ha-ras*^{Val12}) was obtained from the American Type Culture Collection (ATCC).

Site-directed mutagenesis

Site-directed mutagenesis was performed by a PCR/mismatched primer method as previously described (16). The template for PCR was the plasmid pBR6, containing the cDNA coding for wild-type rat *p53*. Briefly, two independent PCR reactions were performed. PCR 1: upper primer: 5′-ATT CTG CCC ACC ACA GCG ACA-3′ (Pos. 115–132); lower primer (mutation primer): 5′-CGT GAT ACC TGC AAG GAT GGG **ACT** GCG GTT CAT GCC CCC CA-3′ (Pos. 773–749); underlined is an artificially introduced restriction endonuclease type IIS recognition sequence for *Bsp*MI, in bold the sequence that introduces the mutation, which in the sense direction has to be read as AGT, i.e. serine. PCR 2: upper primer: 5′-GGC ATG ACC TGC CGG CCC ATC CTT ACC AT-3′ (Pos 763–779); underlined is the *Bsp*MI site; lower primer: 5′-CTT TCT TGA TCA TTG GTT TTT-3′ (Pos. 1180–1160). All primers were synthesized on a Gene Assembler Plus (Pharmacia Biosystems, Freiburg, Germany) and the lower primer in PCR 1 was additionally purified by HPLC. The conditions for PCR were: 5 min 94°C, addition of 2.5 u Pfu-polymerase (Stratagene, Heidelberg, Germany), 1 min 94°C, 1 min 30 s annealing temperature (PCR 1: 66°C, PCR 2: 52°C), 2 min 72°C After 16 cycles additional incubation was performed at 72°C for 5 min to complete chain elongation. PCR products were purified with the Jetpure PCR product purification kit (Genomed, Bad Oeynhausen, Germany) and sequentially digested with AccI, BspMI (which removes its own, artificially introduced recognition sequence) and BglII. The two amplicons were ligated via their sticky ends generated by BspMI digestion and then subcloned into pBR6, previously cut with BglII/AccI thereby exchanging the mutated against the wild-type sequence. The wild-type and mutated *p53* full length cDNA were subcloned into the eukaryotic expression vector pMPSV (generous gift from H.Hauser; 17).

Transfection

Eighteen hours before transfection, cells were seeded at a density of 7×10^5 cells per culture dish. Both, the pMPSV-based recombinant vectors and plasmid PUC EJ 6.6 were each mixed with plasmid LK 444 (generous gift from L.Kedes; 18) containing the neomycin resistance gene as a selection marker in a ratio of 20:1. The preformed DNA–calcium phosphate precipitates, containing a total of 20 μg plasmid DNA or 20 μg of salmon sperm DNA in 500 μl transfection buffer (5.5 mM dextrose, 21 mM HEPES, 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4 and 0.125 M $CaCl_2$, final pH 7.08), were added dropwise into the medium and the cells were allowed to stay for 4 h in the humified (89%) incubator at 37°C, thereafter a glycerol shock was performed for 2 min. Twenty-four hours later cells were split 1 15 and at 48 h post-transfection incubated with selection medium, containing 300 μg/ml G 418 (Gibco BRL). Selection medium was replaced every 3 days and colonies were picked 3 weeks later with the use of cloning cylinders. Clones were transferred into 75 ml flasks and propagated.

Polymerase chain reaction

Aliquots of 1 μg of genomic DNA were isolated with the Easy-DNA kit (Invitrogen, Leek, Netherlands) and amplified with 0.5 u Taq-Polymerase (Gibco-BRL) in a buffer containing 20 mM Tris–HCl, pH 4.8, 50 mM KCl, 2 mM $MgCl_2$, 0.5 μM of each primer and 50 μM of each dNTP. The primers used for amplification were: 5′-GAC AGG CAG ACT TTT CGG CAC AGC-3′ (upper primer) and 5′-TCT CCC AGG ACA GGC ACA AAC ACG-3′ (lower primer). Conditions for PCR were as follows: 1 min 94°C, 1.5 min 60°C (annealing), 2 min 72°C (extension) for 30 cycles. Further incubation at 72°C for 5 min was performed to allow completion of the PCR products.

Sequencing

Sequencing was performed on an automated sequencer by thermal cycling using fluorescent dideoxyterminators (Applied Biosystems, Foster City, CA).

Western blot analysis

Sub-confluent cells were harvested by trypsinization and sonified with a Branson cell disruptor. Protein content was determined by the Bradford assay (Bio-Rad, München, Germany). Aliquots of 50 μg of total protein were separated on a 10% SDS gel along with biotinylated molecular weight standards (Amersham, Braunschweig, Germany). Loading control was performed by staining the blot with Ponceau S. The proteins were transferred overnight onto Hybond™ ECL membranes (Amersham) and detected with the chemiluminescence assay system (Amersham). The first antibody used was pAb 240 (Dianova).

Northern blot analysis

Total RNA was isolated from cell lines as previously described (19) Aliquots of 10 μg were separated on a 1% agarose–formaldehyde (0.41 M)–MOPS gel, transferred downward onto nylon membrane (Nytran, Schleicher and Schüll, Dassel, Germany) (20), covalently bound by UV irradiation (0.5 J/cm^2) and hybridized overnight to a radiolabelled rat *p53*-specific cDNA probe by conventional procedures (21). After washing, the blots were exposed to Kodak X-OMAT AR-5 film with an intensifying screen at –70°C overnight. Equal loading of RNA samples was verified by ethidium bromide staining.

Results

Transfection of mutant p53 (p53^{Ser247}) into OC/CDE 6 cells

In rats it is codon 247 that corresponds to human codon 249, both lying in the evolutionarily highly conserved domain IV (22). Preliminary experiments were performed in order to characterize the endogenous *p53* in our oval cell preparation. Sequencing of an RT–PCR amplicon spanning codon 247 revealed the wild-type sequence coding for arginine. Site-directed mutagenesis at codon 247 was performed by a mismatch primer/PCR method (16). The success of the mutation was confirmed by dideoxy sequencing and no other alterations than the desired one were found.

The success of the transfection was verified by genomic PCR with intron spanning primers, thus differentiating between the endogenous genomic (538 bp amplimer) and the transfected sequence (218 bp amplimer; Figure 1a). Northern and Western blot analyses (Figure 1b and c) demonstrated a higher expression level of *p53* in the transfected as compared with the untransfected cells (where *p53* expression was just above the detection limit of these two methods), thereby reflecting the constitutive expression of the transfected sequence. Demonstration of the mutant phenotype of the transfected *p53* was confirmed by immunohistochemical cell staining (Figure 2a and b). As can clearly be seen, the antibody pAb 240, which is mutant-specific under non-denaturing conditions and is diagnostic for mutant-conformational *p53* when detected in the cytoplasm (23), reacts only with the transfected cells, whereas the original oval cells do not stain positively with this antibody. Furthermore, the exclusive cytoplasmic staining

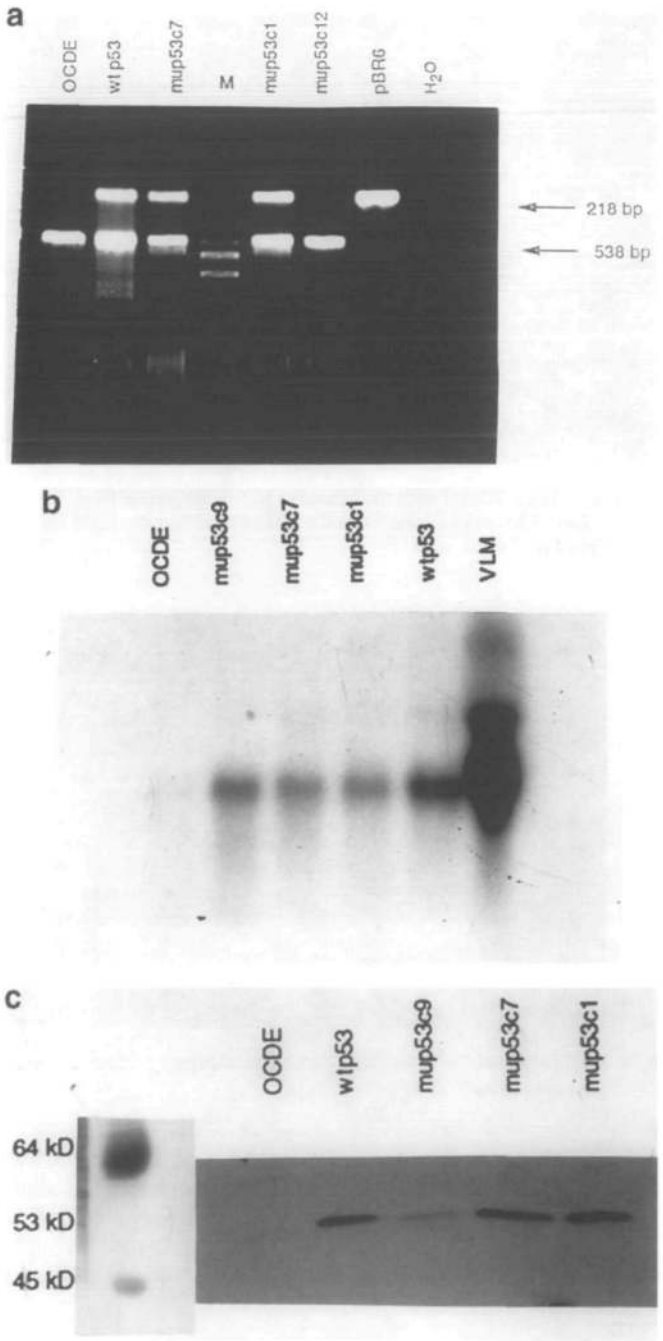

Fig. 1. *Identification of successfully transfected cells.* (**a**) Agarose gel electrophoresis of PCR products obtained with intron-spanning primers differentiating between the endogenous *p53* gene and the transfected *p53* cDNA. Amplification with the cDNA as the template yields an amplicon of 218 bp whereas the genome-derived amplicon is 538 bp long (for PCR conditions see Materials and methods). OCDE: untransfected oval cells; wt *p53*: oval cells transfected with the wild-type *p53* cDNA; mu *p53*c1, 7 and 12: independent oval cell clones transfected with the *p53* cDNA mutated at position 247; M: pBR 322 digested with the restriction endonuclease Alu I as a molecular weight standard. (**b**) Northern blot analysis of mock-transfected (OCDE), wt *p53*-transfected and mu *p53*-transfected oval cells VLM is a SV40-transformed cell line and served as a positive control for *p53* mRNA. (**c**) Western blot analysis using the monoclonal antibody pAb 240 at a dilution of 1:500. Abbreviations for cell clones are the same as in (b).

is in agreement with the postulated mechanism of mutant *p53* inactivating the wild-type counterpart by oligomerization and thereby preventing its entry into the nucleus. With the exception

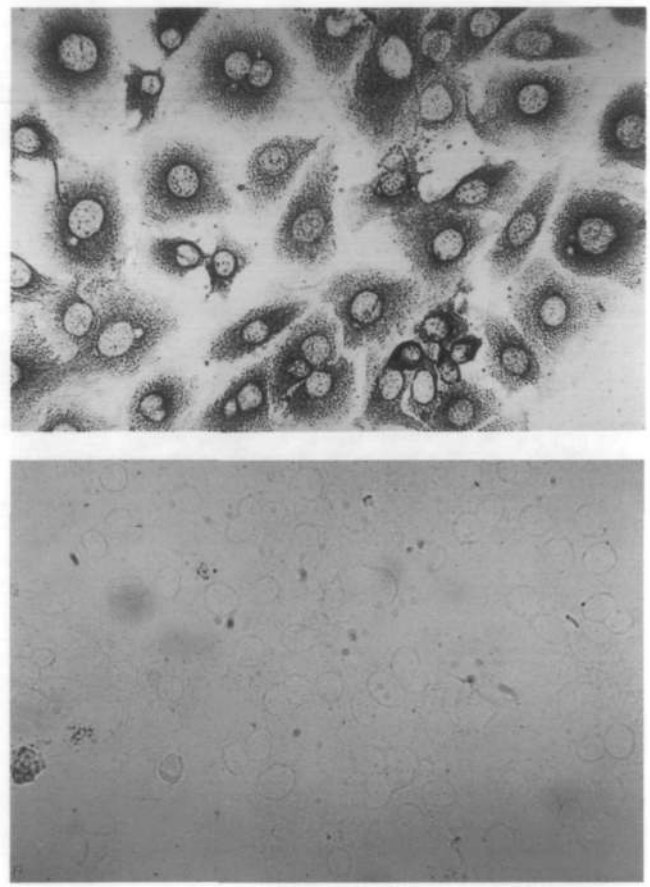

Fig. 2. Immunohistochemical staining of (**a**) oval cell clone mu *p53*c7, *transfected with the rat p53 cDNA mutated at codon 247*, 400×; and (**b**) mock-transfected oval cell clone, 400×. The anti-*p53* antibody (pAb 240) stains exclusively in the cytoplasm.

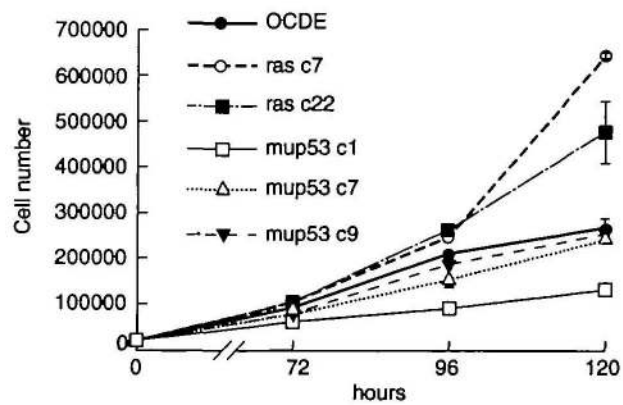

Fig. 3. Growth kinetics of normal and transfected oval cells. Twenty thousand cells were seeded in triplicate into Petri dishes and counted on days 3, 4 and 5. Whilst three clones, transfected with the mutant *p53* cDNA (mu *p53* c1, c7 and c9) did not exhibit an enhanced proliferative capacity, the *Ha-ras*^{Val12}-transfected clones *ras* c7 and c22 started to grow rapidly from day 4 on. In some cases the error bar is not visible because of very low deviation among the three counts.

of one clone (mu *p53*c1), which grew significantly slower than the control cells, the mu *p53*-transfected cells showed a similar proliferation rate as the control cells (Figure 3). Two clones, which exhibited focal growth on the Petri dishes but were negative in the soft agar assay (Table I), were assayed for their tumorigenic potential by s.c. injection into syngeneic newborn rats. A control was performed with mock-transfected

Table I. Colony formation on Petri dishes, growth in soft agar and tumorigenicity of cell clones *ras* c7, *ras* c22 (*Ha-ras*^{Val12}-transfected oval cells), wt *p53* (wild-type *p53*-transfected oval cells) and mu *p53* c1 and mu *p53* c7 (mutant *p53*-transfected oval cells)

Transgenic cell line	Colony formation in culture	Growth in soft agar	Tumour incidence (tumours formed/ animals injected)
ras c7	+++	+	4/13
ras c22	+++	+	7/12
wt *p53*	–	–	0/13
mu *p53* c1	+	–	1/13
mu *p53* c7	+	–	1/13

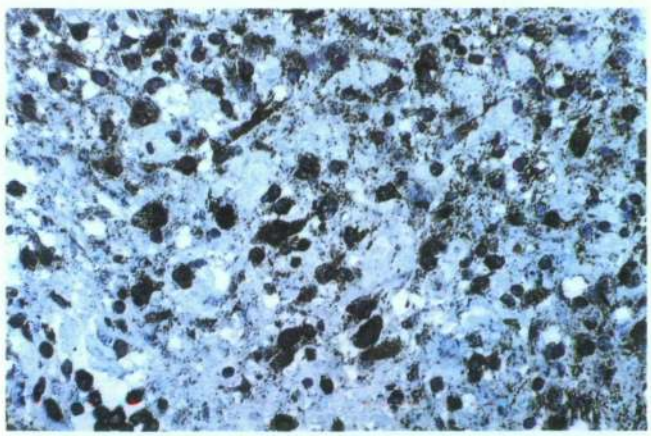

Fig. 4. Immunohistochemical staining of a thin section from a tumour derived from oval cells transfected with the mutant *p53* cDNA, 400×. The mutant *p53* expressed by these cells is detected only in the cytoplasm.

oval cells. The two independent *p53*^{Ser247} clones each yielded one tumour out of 13 injected animals (Table I). The histopathological analysis revealed only anaplastic, undifferentiated tumours. Staining of paraffin sections with the monoclonal antibody pAb 240 demonstrated exclusive cytoplasmic staining of the mutant *p53* conformation (Figure 4).

Transfection of an activated Ha-ras (Ha-ras^{Val12}*)*

Oval cells were transfected with the genomic *Ha-ras*^{Val12} clone, representing an oncogenically active form of the human *c-Ha-ras*. Interestingly, an altered phenotype (piling up) was already apparent very early after the transfection procedure, when the G418 resistant colonies were less than 1 mm in diameter. These clones were suspected to be transformed and selected for further investigation. Efforts to verify expression of the transfected *Ha-ras* gene by Western blotting failed, as the antibody used did not differentiate between wild-type and mutant *ras*, although otherwise stated by the manufacturer. Western blots with recombinant *ras*^{Gly12} and *ras*^{Val12} revealed that the antibody reacted equally well with either one of them (data not shown). Thus, RT–PCR was performed in order to demonstrate the expression of the transfected sequence at the mRNA level. Sequencing of the amplicons verified expression of the transfected, oncogenically active form of *Ha-ras* (data not shown). The *Ha-ras*^{Val12} positive clones showed an enhanced proliferative capacity as compared with controls (Figure 3), formed colonies on the Petri dishes and were strongly positive in the soft agar assay (Table I). Subsequent injection into syngeneic newborn rats led to the rapid formation of tumours within 6–8 weeks (Table I). The tumours were

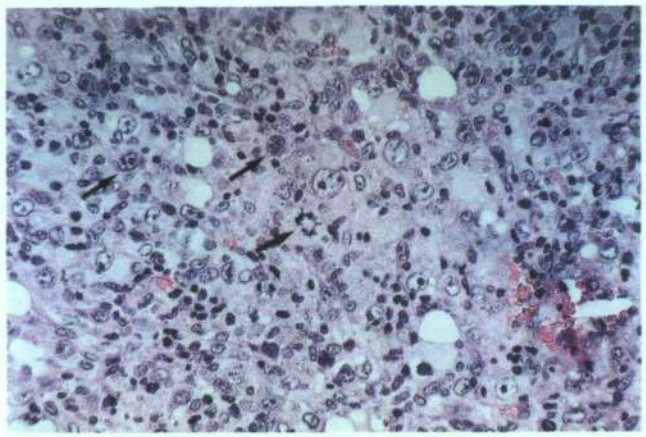

Fig. 5. Haematoxlin/eosin stained paraffin section from a tumour derived from oval cells transfected with the activated *Ha-ras* oncogene (*Ha-ras*^{Val12}), 200×. Arrows indicate mitotic figures that reflect the rapid and massive growth of the tumour.

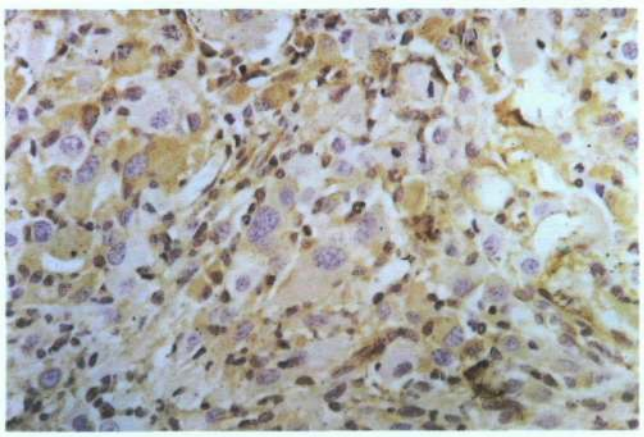

Fig. 6. Paraffin section from the same tumour as in Figure 5 incubated with an anti-albumin antibody, 400×.

epithelial in nature, rich in mitotic figures (Figure 5) and exhibited a highly malignant growth. No morphological signs of either cholangiocellular or hepatocellular differentiation were detected. However, one of the tumours stained strongly positive for albumin (Figure 6). This latter result shows at least a partial hepatocytic differentiation.

Discussion

Up to the present time the neoplastic transformation of oval cells has been achieved by three procedures, namely: (i) by leaving the cells in confluence for an extended period of time; (ii) by treating the cells with the direct alkylating agent MNNG; and (iii) transfection of viral homologues of proto-oncogenes. Formation of differentiated hepatocellular carcinoma upon s.c. injection of oval cells transformed by long-term culture of the cells in the confluent state has been described by Braun *et al.* (24), whereas the same protocol performed by another group resulted only in the formation of anaplastic carcinomas (25). Treatment of oval cells with MNNG led to the appearance of a very small number of hepatocellular carcinomas (11). Transfection of *v-Ha-ras* (carrying a valine at position 12 instead of glycine) led to the formation of differentiated hepatocellular carcinoma (26,27). In contrast to this result, transfection of an alternate *v-Ha-ras* construct by another

group only yielded undifferentiated tumours, whereas in that same study transfection of a *v-raf/v-myc* hybrid led to the formation of tumours with differentiated trabecular structures resembling hepatocellular carcinoma (28,29).

The controversial results concerning the capacity of oval cells to differentiate along the cholangiocellular and the hepatocellular lineages during experimental rat liver carcinogenesis prompted us to perform gene transfer experiments with an oval cell line established in our laboratory and to analyse the tumours that developed upon s.c. injection into syngeneic newborn rats. The activated *Ha-ras* oncogene (*Ha-ras*^{Val12}) was chosen in order to evaluate the aforementioned discrepancy concerning the differentiation potential of oval cells upon transfection with this oncogene. A mutant *p53* (*p53*^{Ser247}) was chosen because epidemiological and experimental evidence have revealed many human tumours to be associated with mutational hot spots in the *p53* tumour suppressor gene (30). These tumour-associated mutations mostly occur in higly conserved domains of the protein, leading to functional inactivation of the protein, to transdominant negative effects on wild-type *p53* or to as yet more or less undefined gains of function contributing to cellular transformation. In particular geographic regions with a high incidence of hepatocellular carcinoma, a specific G→T transversion in codon 249 of human *p53* (equivalent to codon 247 in rat *p53*) is found in these hepatic tumours (31,32). This transversion leads to an Arg→Ser exchange in the polypeptide chain and is supposed to be due to chronic exposure to the hepatocarcinogen aflatoxin B_1. This correlation was first demonstrated in epidemiological studies and the relevance of this specific mutation has been substantiated experimentally by incubating HepG2 hepatoma cells with aflatoxin B_1, which led to adduct formation and finally to a miscoding G→T transversion in codon 249 (33). In addition, there is evidence that the codon 249 mutation does not only lead to a loss of function as shown by reporter gene assays (34) but also confers a 'new phenotype', i.e. increased proliferation and survival (35), implying that this mutation may be intrinsically oncogenic. On the basis of these observations we introduced a *p53* mutated exactly at the homologous position of the rat protein into oval cells. Interestingly, OC/CDE 6 cells transfected with the mutated *p53* cDNA showed a cytoplasmic staining and a similar proliferative rate as the wild-type OC/CDE 6 cells. This is in contrast to the finding that tumour cells expressing mutated *p53* often show on the one hand enhanced nuclear staining (36) and on the other hand an enhanced proliferation rate (35). Furthermore, strong nuclear staining has almost always been interpreted as being due to mutations in the *p53* gene. That this is not a compelling conclusion has unequivocally been demonstrated in the case of mammary carcinoma where *p53* nuclear staining does not correlate with mutations (37). Almost exclusive cytoplasmic staining has also been observed in hepatocellular carcinomas induced by a choline-devoid diet in male Fischer 344 rats when using the antibody pAb 240 and this pAb 240-positive phenotype showed a good correlation with *p53* mutations (38). A possible explanation for the cytoplasmic localization of certain *p53* mutants may be self-aggregation due to constitutive overexpression or mutant *p53*-specific complex formation with proteins other than wild-type *p53*, e.g. hsc 70 heat-shock protein (39). It may be that by this mechanism enough wild-type *p53* escapes the transdominant negative effect of the mutant protein in our cellular system. In addition, one could argue, that, in order to enhance proliferation in a given cell, the mutated *p53* protein must be present in the nucleus to exert an as yet unidentified gain of function.

Our results demonstrate that (i) oval cells, at least in our experimental approach, do not have the potential to differentiate into the major histotypes of liver cancer, i.e. cholangiocellular and hepatocellular carcinoma; (ii) that *p53*^{Ser247} (corresponding to the mutational hotspot at position 249 in human *p53*) has no or a very weak transforming potential when compared with *Ha-ras*^{Val12} with respect to tumour incidence, growth rate and size; and (iii) although transformation of oval cells with the genomic *Ha-ras*^{Val12} and subsequent s.c. injection did not lead to the formation of hepatocellular carcinoma a strong staining for albumin in the tumour cells could be detected, showing at least a partial hepatocytic differentiation.

The contradictory results concerning the fate of oval cells during hepatocarcinogenesis encountered when studying the literature and considering our own results might possibly emerge from the fact that the oval cell preparations used in different laboratories differ in their commitment status. Oval cells that are isolated at early stages of the hepatocarcinogenic process may still have the potential to differentiate into the hepatocytic as well as into the bile ductular lineage, whereas oval cells isolated at later stages may have lost this potential. Furthermore, the nature of the regimen leading to the onset of oval cell proliferation, e.g. the CDE diet or AAF gavage in combination with partial hepatectomy, may be critical in influencing the lineage commitment of oval cells. Experiments are ongoing attempting to differentiate between these possibilities.

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