Generation and characterization of p53 null transformed hepatic progenitor cells: oval cells give rise to hepatocellular carcinoma

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Oval cells are bipotential liver stem cells able to differentiate into hepatocytes and bile duct epithelia. In normal adult liver oval cells are quiescent, existing in low numbers around the periportal region, and proliferate following severe, prolonged liver trauma. There is evidence implicating oval cells in the development of hepatocellular carcinoma, and hence the availability of an immortalized oval cell line would be invaluable for the study of liver cell lineage differentiation and carcinogenesis. A novel approach in the generation of cell lines is the use of the p53 knockout mouse. Absence of p53 allows a cell to cycle past the normal Hayflick limit, rendering it immortalized, although subsequent genetic alterations are thought necessary for transformation. p53 knockout mice were fed a choline-deficient, ethionine-supplemented diet, previously shown to increase oval cell numbers in wild-type mice. The oval cells were isolated by centrifugal elutriation and maintained in culture. Colonies of hepatic cells were isolated and characterized with respect to phenotype, growth characteristics and tumorigenicity. Analysis of gene expression by Northern blotting and immunocytochemistry suggests they are oval-like cells by virtue of albumin and transferrin expression, as well as the oval cell markers alpha fetoprotein, M2-pyruvate kinase and A6. Injection into athymic nude mice shows the cell lines are capable of forming tumors which phenotypically resemble hepatocellular carcinoma. Thus, the use of p53 null hepatic cells successfully generated immortalized and tumorigenic hepatic stem cell lines. The results presented support the idea that deleting p53 allows immortalization and contributes to the transformation of the oval-like cell lines. Further, the tumorigenic status of the cell lines is direct evidence for the participation of oval cells in the formation of hepatocellular carcinoma.

Introduction

The p53 knockout mouse model provides a valuable tool for the generation of immortalized cell lines as functional p53 has been implicated in imposing the Hayflick limit. Normally a cell can undergo limited numbers of divisions before it senesces (the Hayflick limit), the number being specific to each cell type (1). It is hypothesized a cell counts each division by the gradual shortening of telomeres and, upon reaching a critical length, DNA stability is compromised and strand breaks occur. DNA damage signals p53 to induce G1 arrest through the increased levels of p21, which eventually forces the cell into G0.

p21 is reported to be up regulated in senescing cells (2–4) so, in the absence of p53, cells bypass the signal to senesce and obtain an extended lifespan. This theory is supported by an abundance of experimental evidence existing for the role of p53 in promoting senescence. Introducing mutant p53 to primary cultures of fibroblasts produces permanent fibroblast cell lines (5–7); in addition spontaneously immortalized fibroblasts were found to contain p53 mutations (5,6). Further, fibroblasts isolated from p53 null mouse embryo cells generate fibroblast cell lines more readily than from the wildtype (7–9).

It is not clear whether the loss of p53 alone is sufficient to cause cell immortalization. For a cell to be classified as immortal it needs to pass two barriers, these are the Hayflick limit and cell crisis. Lack of p53 can force a cell through the Hayflick barrier, however very few cells survive crisis, those that do are spontaneously immortalized. Mutations to key oncogenes may be necessary to render a cell immortalized and tumorigenic (10).

To date, a p53 null liver cell line has not been made, of particular interest would be the generation of a liver stem (oval) cell line. Oval cells are small, oval shaped epithelial cells identified in the liver during normal embryonic development (11) and in the adult liver only following severe, repetitive liver trauma. Evidence suggests the liver oval cell is at least bipotential, capable of differentiating into mature hepatocytes (12,13) or cholangiocytes (14,15) under different experimental regimes. Progenitor cells in the liver are identified by their ability to express markers characteristic of immature hepatocytes. Such markers include albumin, transferrin and alpha fetoprotein (AFP), all of which are expressed by the first primitive liver stem cells as well as oval cells (16). Other markers include the M2, immature isoform of pyruvate kinase (M2–PK) (11), the pi isoform of glutathione-S-transferase (17), and cytokeratin 19 (18). Antibodies to specific oval cell antigens are also used for identification, A6 is one such mouse oval cell marker (19).

Oval cells have been observed in various models of rodent experimental carcinogenesis, including exposure to DIPIN (20) and the choline-deficient, ethionine-supplemented (CDE) diet (21). They are also found in human liver pathologies, such as hepatitis C virus, hemochromatosis and alcoholic liver disease (22). These conditions are associated with an increased incidence of hepatocellular carcinoma or cholangiocarcinoma (23–25). There is increased evidence to suggest oval cells are the cellular targets for transformation in the development of hepatocellular carcinoma (26).

Overall, much remains to be learnt of the oval cell with

Abbreviations: PIL, p53 immortalized liver; AFP, alpha fetoprotein; PK, pyruvate kinase; PEPCK, phosphoenolpyruvate carboxykinase; TAT, tyrosine aminotransferase; G6-Pase, glucose-6-phosphatase; PAH, phenylalanine hydroxylase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; CDE, choline deficient ethionine supplemented diet.

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regard to its origin and fate, as well as its role in the formation of cancer. An oval cell line, phenotypically similar to the in vivo oval cell, would clearly be useful in studies to elucidate the origin, fate and contribution to cancer of these cells. We have exploited the absence of p53 in liver cells isolated from a p53 /– /– mouse to generate immortalized immature liver cell lines. The p53 null mouse was subjected to the CDE diet so that oval cells were induced to proliferate. The oval cells were then isolated and maintained in culture to generate cell lines. Five immortalized (three tumorigenic) cell lines morphologically similar to oval cells have been characterized and shown to stably express markers seen in immature hepatic cells. The p53 null cell lines formed are phenotypically similar to the in vivo oval cell. The production of tumorigenic oval-like cells lines is direct evidence of a link between oval cells and carcinogenesis.

Materials and methods

Mice

The p53 knockout mouse was a generous gift from Dr Tyler Jacks (MIT, USA), and was housed in a specific pathogen free animal facility. Female p53 +/+ mice were crossed with male p53 +/+ mice to generate p53 +/– and +/+ offspring. The p53 gene status was individually assessed by PCR screening (27).

Oval cell induction and isolation

Mice, 3–4 weeks old, were fed a CDE diet. This comprised a choline-deficient diet (product code TD 79246, Teklad, USA) and drinking water containing 0.165% (w/v) ethionine (Sigma, USA) (28). Ethionine given in the drinking water minimized the risk of exposure of the animal care providers to the carcinogen. Animals were killed 3 weeks after commencement of the diet. The livers were perfused in situ via the hepatic portal vein with perfusion buffer (KCl 0.4 g/l, NaCl 6.8 g/l, HEPES 4.8 g/l, NaHCO3 2.1 g/l, glucose 1.0 g/l, pH 7.4; (Sigma, USA)). Surgical anesthesia was obtained using 0.015 ml/g body weight of 2.5% Avertin. Two small pieces of liver were taken for histological and immunohistochemical analysis and immersed in Carnoy’s fixative, 2 h prior to paraaffin embedding or Cryoembed (Shandon-Elliot, USA) for frozen sections.

Liver cells were isolated by collagenase perfusion (29) and separated according to size and density by centrifugal elutriation (30). Four fractions were collected at flow rates of 18, 24, 40 and 50 ml/min and a fifth fraction was obtained by shutting down the rotor (from 3000 r.p.m.) while the flow rate was maintained at 50 ml/min. The majority of oval cells are present in fraction 3 with clusters of oval cells present in fraction 5.

Immunohistochemistry and cell scoring

Immunohistochemical analysis on CDE treated livers and P/L tumours was performed according to the method of Clement (31). In brief, paraffin sections were dewaxed and re-hydrated by passing through a series of decreasing alcohols and finally water. Endogenous peroxidases were blocked with 2.5% periodic acid followed by 0.02% sodium borohydride and sections were incubated with 10% fetal bovine serum (FBS) for 1 h, followed by the appropriate primary antibody for 1 h at room temperature; albumin (polyclonal rabbit), transferrin (polyclonal rabbit), M2-PK (Rockland, USA), PCNA (Leinco Technologies, USA). Frozen sections were used to detect A6 (19) and were post-fixed with acetone:ethanol (1:1) for 5 min, then blocked with hydrogen peroxide (3%) for 5 min at room temperature before incubating with the primary antibody overnight at 4°C. Negative control slides, where rabbit serum was replaced for primary antibody, were also performed. The sections were washed then incubated for 1 h at room temperature with the appropriate secondary antibody; anti-rabbit IgG-HRP (Sanofi Diagnostics Pasteur, France) for albumin and transferrin, anti-goat IgG-HRP (Rockland) for M2-PK, streptavidin-HRP (Amersham, UK) for PCNA, and anti-rat IgG-HRP (Amersham) for A6. Following this, sections were washed, then reacted with DAB for 5 min. All sections following staining were mounted in glycerol/gelatin and cover-slipped.

Cells positive for various antigens were scored over 20 fields at 40× magnification and expressed as a percentage of total cells per field. Scores for all animals were expressed as mean ± SE.

Culture conditions and cloning

Cells from fractions 3 and 5 were plated at a density of 2.5 × 10^6 cells onto 35 mm diameter plates which were coated in vitrogen collagen (Cohesion, USA) to encourage cell attachment (only used for the initial attachment).

Cells were plated in a Williams’ E (Gibco BRL, USA) based media containing 10% FBS and supplements as listed; 10 mM nicotinamide, 2 mM glutamine, 10^-7 M dexamethasone, 1 × ITS+ (Collaborative Biomedical, USA), 0.2 mM ascorbic acid, 20 mM HEPES, 1 mM Na pyruvate, 0.15% NaHCO3, 14 mM glucose, 20 ng/ml EGF (Collaborative Biomedical), 1.1% (v/v) fungizone (Gibco BRL), and 1.1% (v/v) penicillin/streptomycin (Gibco BRL).

The medium was changed 24 h after plating to Williams’ E plus supplements, without 10% FBS. Cells were incubated in a humidified atmosphere containing 5% CO2 at 37°C. Colonies of epithelial cells were identified by morphology under phase contrast illumination, colonies were marked and once of an appreciable size, selected and removed from the dish using a cloning ring and collagenase/dispose mixture. Cell colonies were plated onto 35 mm diameter plates (no collagen coating required) and maintained. Confluent dishes were subcultured using collagenase/dispose and the cells expanded into 25 cm² flasks. Cells were frozen at low passage (<15) for stocks and deliberately passaged frequently to obtain high passage cells (>30).

RNA isolation and northern analysis

Total RNA was isolated, from three confluent 75 cm² flask cell colony, using Trizol (Gibco BRL, USA) according to the manufacturer’s instructions. Samples of total RNA (15 µg) were electrophoresed through a 1% agarose gel containing 2.2 M formaldehyde (32) and then transferred to Genescreen Plus (NEN, USA) membranes by capillary blotting. Albumin, transferrin, AFP, TAT, PEPC, aldolase B, G-6-Pase, PAH, myc, rasG and GAPDH mRNAs were detected by hybridization with their respective cDNAs: albumin (3116; transcript); transferrin (347); aldolase B (38); pmG-6-Pase (39); PAH (40) mRNAs; and GAPDH (42); 3116 (33); transcript; (34); pRAF 87 AFP (35); TAT (36); pmPEPC (37); aldolase B (38); pmG-6-Pase (39); PAP (40) mRNAs; and pGAPDH-13 (43). All cDNA probes were labeled with [32P]dCTP (Amersham) using a Mega-Prime Kit (Amersham) and hybridized at 42°C for 24 h. The hybridized membranes were washed according to the manufacturer’s instructions before being exposed to a BAS IIIs phosphorimaging plate (Fuji, Japan). The phosphorimaging screen was scanned using a Fuji BAS 2500 phosphorimager. Quantitative analysis of the images was performed using NIH Image (National Institute of Health, USA), mRNA levels were expressed relative to GAPDH mRNA.

Immunocytochemistry

Cultured cells at low (<15) and high (>30) passage, were washed three times with phosphate buffered saline (PBS, pH 7.4) and fixated with a mixture of 4% paraformaldehyde/0.1% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.45) for 4 min at 4°C. Fixation was followed by three washes in PBS, and storage in PBS with thimerosal at 4°C. The indirect immunoperoxidase detection of proteins was performed (31). The fixed cells were washed three times in PBS. Endogenous peroxidases were blocked as previously described. Cells were blocked for 1 h with 10% goat serum in PBS containing 0.2% saponin (Sigma). Circular areas of cells on the same dish were demarcated by removal of surrounding cells with a cotton swab and circling of the cell colony with a wax pen. This enabled the same culture of cells to be tested with a variety of antibodies. Cells were incubated in rabbit anti mouse albumin, transferrin and AFP primary antibody for 1 h at room temperature, 10% goat sera was used as a non-immune sera. Subsequently, cells were washed with PBS/0.2% saponin three times for 15 min each. The cells were then incubated in the secondary antibody, peroxidase coupled-goat anti rabbit IgG, for 1 h at room temperature. The washing procedure was repeated, then the cells incubated in liquid diaminobenzidine for 5 min. The stained cells were washed in PBS and stored in PBS/thimerosal at 4°C.

Fig. 1. Comparison of the wildtype and p53 /– /– mouse liver 3 weeks after administration of the CDE diet. Haematoxylin and eosin staining of wildtype (A) and p53 /– /– livers (B) show increased numbers of small basophilic cells (closed arrows) around the peri-portal area of the liver. These small cells stain positive for several oval cell markers including M2-PK (C) and A6 (D) (open arrows). p53 /– /– /– livers only are shown. The same small cells express PCNA in p53 /– /– (E) and wildtype (F) liver (open arrows). The PCNA stained p53 /– /– /– liver (E) shows cytoplasmic staining in the mature hepatocytes (closed arrows). Scoring PCNA positive cells reports a two fold increase in proliferating cells, and further, there is a two-fold increase in M2-PK and A6 positive cells in the p53 /– /– mouse compared to the wildtype (G). Magnification bar = 100 µm.
Fig. 2. Photomicrographs of PIL-1 (A), PIL-2 (B), PIL-3 (C), PIL-4 (D) and PIL-5 (E) cell lines, at low passage (p10–15), under phase contrast microscopy (at two magnifications). Lipid droplets (closed arrows) and giant cells (open arrows) are present in all cell lines. The PIL-3 cells (C) have endothelial cell contamination (small thin arrow) and the PIL-4 cells (D) are contaminated with cells that appear morphologically similar to small hepatocytes (long thin arrow). Magnification bar = 100 µm.

Tumorigenicity assays
Cells ($8 \times 10^6$) were injected subcutaneously into syngeneic, athymic Arc/Swiss nude mice at 4–6 weeks of age. Animals were kept in a specific pathogen-free animal holding facility and examined daily for the formation of tumors. Animals were kept until the tumors were an appreciable size (~1 cm in diameter) and then killed by cervical dislocation. Control animals, that were mock injected (with no cells), showed no tumor development. Tumors were fixed in Carnoy’s solution (60% ethanol, 30% chloroform, 10% acetic acid) for 2 h for immunohistochemistry and some snap frozen and stored at −80°C for subsequent DNA isolation and PCR.

DNA isolation and PCR to confirm p53 status
Frozen tissue was homogenized gently in an appropriate volume of DNAzol (Gibco BRL) and DNA isolated according to manufacturer’s instructions. DNA was quantified and PCR performed (27). PCR products were visualized on a 2% agarose gel; the pGEM DNA standard (Promega, USA) was used to determine fragment sizes.
Cells from each colony were plated onto twenty-four, 30 mm diameter dishes on day 0. Following this, three plates were harvested per cell line, each day for 8 days. DNA estimations of all samples were carried out using the method of Hindegardner (44). Cells were suspended in 400 µl of Tris buffer (pH 7.4) then centrifuged, the pellet resuspended in 150 µl 5% TCA before heating to 60°C. Cell debris was removed by centrifugation and the DNA was reacted with DABA-2HCl for 45 min at 50°C. HCl was added to achieve acidic conditions for maximum fluorescence and the samples were mixed and read on a fluorophotometer with an excitation wavelength of 410 nm and emission wavelength of 505 nm. A standard curve was generated using 1 mg/mL calf thymus DNA, and treated in the same manner as the test samples. Population doubling times were calculated according to Kuchler (45).

### Results

**Oval cell proliferation is enhanced in CDE treated p53 knockout mice**

Following 3 weeks of administering the CDE diet, the p53 \(--/--\) mouse liver (Figure 1B) shows both a greater disruption of liver architecture and increased number of small, ovoid, basophilic cells when compared to the wildtype (Figure 1A). Both genotypes show deposition of lipid within the hepatocyte cytoplasm which is characteristic of a choline deficient diet.

Some small basophilic cells stain positively for the markers of oval cells, M2-PK (Figure 1C) and A6 (Figure 1D). Morphologically, these cells are similar to oval cells, having an ovoid nucleus and scant cytoplasm, and they are located peri-portal, as are oval cells. In addition to staining oval cells, the M2-PK antibody stains some endothelial cells in the sinusoidal spaces. These endothelial cells are easily distinguished by morphology from oval cells during cell scoring. There are many more PCNA positive cells (nuclear staining) in the p53 \(--/--\) liver (Figure 1E, 11.80%) compared with the wildtype (Figure 1F, 4.78%) and small oval-like cells constitute the majority (>90%) of the positive cells. Interestingly, there is cytoplasmic PCNA staining in hepatocytes of the p53 \(--/--\) liver which is not present in the wildtype. Quantification reveals a two-fold increase in the number of PCNA, M2-PK and A6 positive cells in the p53 null CDE treated liver compared with the wildtype (Figure 1G).

**Immortalized p53 null cell lines generated are morphologically similar to oval cells**

From cultures established at a density of 2.5 \(\times\) 10^6, 20 cell colonies were isolated. Of these, five were chosen for characterization as they were homogeneous and oval cell-like, based on morphology. Two of the cell lines were isolated from primary cultures of oval cells and have been named p53 immortalized liver (PIL) cells -1 and -2, respectively. The other three cell lines were isolated from mixed primary cultures of oval cells and hepatocytes and are designated PIL-3, -4 and -5. Under the specific conditions used in this study, stable cultures of oval cells isolated from wildtype CDE treated liver cannot be maintained past 4 weeks.

The PIL-1, -2 and PIL-5 cell lines are morphologically similar (Figure 2A, B and E). They are homogeneous and consist of small epithelial cells with a scant, granular cytoplasm. A small percentage (5–10%) accumulate lipid droplets in the cytoplasm and, overall, the cell nuclei are not prominent. A low number of multinucleated giant cells (5–10 nuclei) are present in all three cell colonies. Following passage, the cells adhere as small groups of about ten cells. These clusters grow and occupy ~70% of the plate surface and then begin to ‘pile up’ from the center of the cluster, before they float off the plate as spheroids.

The PIL-3 colony is heterogeneous with the majority of cells being epithelial and a small number of contaminating endothelial cells (Figure 2C), which are identifiable due to the circular structures they form. The epithelial cells increasingly appear more stellate as they grow and spread out, but when in clusters possess a similar morphology to the PIL-1, -2 and -5 cells. Again a small percentage of cells (5–10%) accumulate lipid in the cytoplasm.

The PIL-4 cells are readily distinguished, being a mixed population of immature and mature hepatic cells (Figure 2D). Clusters of small epithelial cells are present, as well as larger epithelial cells, which morphologically resemble small hepatocytes, with more prominent nuclei and a larger, granular cytoplasm.

All cell lines are shown to be p53 null by PCR (Figure 3). Further, they have been passaged in culture over 60 times and maintain their morphology. Population doubling times of the cell lines is between 53.6 and 63.2 h (Table I), similar to the reference tumorigenic fetal cell line, FRL-19 (46).

**All cell lines express various levels of liver specific genes**

Northern analysis on low passage cultures (Figure 4) shows that Northern analysis on low passage cultures (Figure 4) shows that all of the cell lines are hepatic. They express albumin (at low levels) and transferrin (more strongly) mRNA. Albumin mRNA decreases in all high passage number cultures (>30), while transferrin levels are maintained (Table II). At low passage number (<15) all cell lines express the immature (liver) marker AFP, which decreases in all cell lines to virtually nil (except PIL-4, which maintains low levels) after 30 passages. All cell lines also express low levels of PAH and aldolase B mRNA at low passage. mRNA for PAH decreases to undetectable levels after 30 passages, whilst aldolase B mRNA increases with time in culture. All cell lines are negative for PEPCK, G-6-Pase and TAT (except PIL-4, which expresses low levels of PEPCK).

<table>
<thead>
<tr>
<th>PIL Cell Lines</th>
<th>Controls</th>
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<tbody>
<tr>
<td>pGEM</td>
<td>1</td>
</tr>
<tr>
<td>375bp</td>
<td></td>
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<tr>
<td>375bp</td>
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</tbody>
</table>

Fig. 3. p53 genotyping of the five PIL cell lines determined by PCR. All cell lines show a single 375 bp band indicating they are all homozygous for the p53 gene knockout. Controls for the assay included a p53 +/+ control (375 bp band), a p53 +/- control (375 and 375 bp bands) and a p53 –/- control (575 bp band). Arrows indicate two fragment sizes. pGEM DNA standard was loaded in lane one.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Population doubling time (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PIL-1</td>
<td>66.1</td>
</tr>
<tr>
<td>PIL-2</td>
<td>62.7</td>
</tr>
<tr>
<td>PIL-3</td>
<td>53.6</td>
</tr>
<tr>
<td>PIL-4</td>
<td>58.5</td>
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<tr>
<td>PIL-5</td>
<td>63.2</td>
</tr>
<tr>
<td>FRL-19</td>
<td>59.8</td>
</tr>
</tbody>
</table>

Table I. Population doubling times of PIL cell lines
of TAT mRNA. Quantification of all high and low passage northern blots are shown in Table II, expression is relative to GAPDH.

Protein expression in the cell lines is similar to that observed in immature hepatic cells

The cell lines were stained with a panel of antibodies based on the mRNA expression (albumin, transferrin, AFP) as well as other oval cell markers (A6 and M2-PK). Their patterns of immunostaining are summarized in Table III. An example of the staining is illustrated in Figure 5, using the PIL-2 cell line since it is the most oval-like in phenotype. At low passage, all cell lines show strong, homogeneous and perinuclear staining for albumin and transferrin (Figure 5A and B). Albumin expression decreases when the cell confluency is high. All cell lines show cytoplasmic and heterogeneous staining of AFP (Figure 5C), except PIL-5 cells, which has some negative cells and also slightly positive cells. The membranous staining of the A6 antibody shows heterogeneity of expression in all cell lines, with strongly positive and negative cells, except the PIL-2 line in which all cells stain very strongly (Figure 5D). The PIL-2 cell line also stains very strongly for M2-PK (Figure 5E), whilst the other cell lines are negative with the exception of the PIL-3 cell line which is heterogeneous, having both negative and positive cells.

Three out of five cell lines are tumorigenic

Two experiments were performed to test the tumorigenicity of the PIL cell lines. In experiment one, the PIL-1 and -2 cell lines were injected at passage 17. Both the cell lines gave rise to tumors 65 and 113 days after injection, respectively. The tumorigenic control cell line, FRL-19 gave rise to tumors in 12 days.

The second experiment involved the injection of all five PIL cell lines at passage 32. Three (PIL-1, -2 and -5) of the five cell lines gave rise to tumors in 100% of nude mice injected. The FRL-19 cells produced three out of three tumors (of 1 cm diameter) in 19 days following injection. The PIL-1, -2 and -5 cell lines took 19, 90 and 27 days, respectively to form 1 cm diameter tumors. Table IV summarizes the time taken for the tumor to be visible and the time taken for the tumor to reach 1 cm. Whilst the PIL-5 cell colony initially gave rise to the tumor in the same time as the FRL-19 cell line, the growth of the tumor was considerably longer (8 days). The remaining two cell lines (PIL-3 and -4) have not generated identifiable tumors to date.

Tumors that arose from the PIL-1, -2 and -5 cells were heterozygous for the p53 gene (Figure 6), and express markers similar to the cell lines in culture. The tumors are similar to the PIL-1, -2 and -5 lines in vitro, as the cells of the tumors are morphologically similar (i.e. small with scant cytoplasm) to the original cells in culture (Figure 7A) and stain positively for albumin, transferrin, M2-PK and A6 (Figure 7B, C, D and E). Staining for the PIL cell markers in the tumors is heterogeneous with areas of the tumor being positive and others negative. Negative control slides for the immunohistochemistry show no staining.

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**Table II. Relative levels of mRNA in the PIL cell lines**

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>PIL-1</th>
<th>PIL-2</th>
<th>PIL-3</th>
<th>PIL-4</th>
<th>PIL-5</th>
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<td>Aldolase B</td>
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</table>

Levels of albumin, transferrin, AFP, PEPCK, TAT, G-6-Pase, aldolase B and PAH mRNA are normalized against GAPDH mRNA and expressed as a percentage of the in vivo adult liver expression of the marker (except AFP which is a percentage of E15 in vivo expression). Values include expression at low (<15) and high (>30) passage number. There was no expression of PEPCK and G-6-Pase mRNAs. ND: not detected.
All five PIL cell lines were analysed at the mRNA level for expression of the myc and ras oncogenes. There was no significant difference in the level of expression of these two oncogenes between the tumorigenic (PIL-1, -2 and -5) and non-tumorigenic (PIL-3 and -4) cell lines (result not shown).

Discussion

Oval cells reside in the liver in small numbers peri-portal, associated with the canals of Hering (47). Chronic liver damage induces the proliferation of these stem cells, which are at least bipotential, capable of differentiating into hepatocytes as well as cholangiocytes (11,48–51). Exposure of the liver to chemical carcinogens is sufficient to promote the appearance of oval cells; the CDE diet is one such regime (21,28). The CDE diet causes damage to existing hepatocytes and prevents them dividing to replace lost tissue; thus the oval cell population is recruited. In this study wildtype and p53 knockout mice were subjected to the CDE diet for 3 weeks to generate oval cells. Twice as many proliferating oval cells were induced in the p53 null liver, and we suggest that this is due to an increased proliferative response in the absence of normal cell cycle regulation. These p53 null oval cells may accumulate DNA mutations faster than the wild type due to a lack of G1 checkpoint control and time to repair mutations, culminating

![Fig. 5. Photomicrographs showing immunocytochemical localization of albumin (A), transferrin (B), AFP (C), A6 (D) and M2-PK (E) in the PIL-2 cell line, at low passage (p10–15). Cells were viewed and photographed under bright field illumination. Magnification bar = 100 µm.](image-url)
increased apoptotic response in the p53 null liver caused by the CDE diet and resulting in the leakage of nuclear proteins, such as PCNA, into the cytoplasm.

This study demonstrates the p53 null liver is useful for the derivation of hepatic cell lines. Following their isolation and maintenance in culture for ~12 months, 20 cell lines were generated, of which five p53 null lines were chosen for study based on their morphological similarity to oval cells. The extended life span and immortalization of a p53 null cell is an event reported in a small number of cell lineages (8,52–57). Immortalization of a hepatic cell by the absence of p53 is novel and allows the easy expansion of spontaneously arisen immortalized colonies of cells.

The p53 null immortalized oval-like murine cell lines (PIL) have a morphology similar to oval cells cultured in vitro, and to previously reported rat oval-like cell lines (26,58). The cells are small, with a scant cytoplasm, poorly defined nucleus and generally epithelial in appearance. The lines all contain giant cells with multiple nuclei and have lipid accumulation in the cytoplasm, characteristic of oval-like cell lines and hepatocellular carcinoma (HCC), respectively. The PIL cell lines have doubling times of, on average, 59.8 h, again similar to oval cell-like lines reported previously (26). The slow cycling time could be partially attributed to their culture medium which is optimized for oval cell maintenance, not proliferation, being serum free and supplemented with ITS+.

The PIL cell lines were characterized and shown to be hepatic and phenotypically immature. Albumin and transferrin are markers expressed in primitive hepatic cells, with expression maintained in the mature hepatocyte. All PIL cell lines express both albumin and transferrin mRNA and protein, although albumin expression decreases (except PIL-2) to be very low or nil by 30 passages.

Markers such as AFP, M2-PK and A6 are expressed in immature hepatic cells and levels decline to be undetectable as the cells mature. Oval cells in vivo express all these early hepatic markers. Variable expression of selected immature markers by the PIL cells suggests they are oval-like in phenotype. The PIL-2 cell line is the most similar to the oval cell in vivo. It expresses M2-PK and A6 strongly and stably (over 60 passages), although AFP expression is lost by 30 passages.

The PIL cells were also assayed for expression of more mature markers, including PEPC, G-6-Pase, TAT, PAH and aldolase B. These proteins are expressed primarily by the adult hepatocyte around the perinatal period of development and oval cells in vivo do not express them. Expression of selected mature hepatic markers in PIL cell lines is very low or absent. All low passage PIL lines express aldolase B and PAH mRNA. The expression pattern of oval-like cell lines often deviates from the in vivo expression, due to either the mechanism of immortalization affecting gene expression, or the culture conditions not being optimal for maintaining normal gene expression. It is plausible that immature liver cells (oval-like cells) may express these more mature mRNAs. Braun et al. report aldolase B expression in the LE/2 oval-like cells isolated from a two-week CDE rat liver (26).

The PIL oval-like cell lines are more stable than other oval-like epithelial cell lines produced to date. Pack et al. report maintenance of albumin production only up to passage 4, no GGT production after the first passage and the production of G-6-Pase (not reported in the PIL lines) (58). In the LE/2 and LE/6 lines, albumin and AFP protein were not detected by

---

**Table III. Immunocytochemical staining on the PIL cell lines at low passage (<15)**

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>PIL-1</th>
<th>PIL-2</th>
<th>PIL-3</th>
<th>PIL-4</th>
<th>PIL-5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Albumin</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Transferrin</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>AFP</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+/−</td>
<td>+/−</td>
</tr>
<tr>
<td>A6</td>
<td>++/−</td>
<td>++/−</td>
<td>+/+−</td>
<td>+/+−</td>
<td>+/+−</td>
</tr>
<tr>
<td>M2-PK</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

The PIL cell lines were stained with the immature liver markers; albumin, transferrin, AFP, A6 and M2-PK. Cells were scored as completely negative (−), positive (+) or strongly positive (++. Where cell colonies are expressing the proteins heterogeneously, they are designated positive and negative (+/−) or strongly positive and negative (++/−).

**Table IV. Tumor formation within 100 days**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Passage</th>
<th>Tumor formation (days)</th>
<th>Initial detection (days)</th>
<th>Tumor removal (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>PIL-1</td>
<td>17</td>
<td>3/3</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td>PIL-2</td>
<td>17</td>
<td>3/3</td>
<td>107</td>
</tr>
<tr>
<td></td>
<td>FRL-19</td>
<td>−</td>
<td>3/3</td>
<td>9</td>
</tr>
<tr>
<td>2</td>
<td>PIL-1</td>
<td>32</td>
<td>3/3</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>PIL-2</td>
<td>32</td>
<td>2/2a</td>
<td>48</td>
</tr>
<tr>
<td></td>
<td>PIL-3</td>
<td>31</td>
<td>0/3</td>
<td>−</td>
</tr>
<tr>
<td></td>
<td>PIL-4</td>
<td>31</td>
<td>0/3</td>
<td>−</td>
</tr>
<tr>
<td></td>
<td>PIL-5</td>
<td>31</td>
<td>3/3</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>FRL-19</td>
<td>−</td>
<td>3/3</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>No cells</td>
<td>−</td>
<td>0/3</td>
<td>−</td>
</tr>
</tbody>
</table>

Nude mice were injected with the PIL cell lines. Data given is the number of mice which produced tumors out of the three injected (Tumor formation), the time elapsed before the tumor was first detected (Initial detection), and the time taken for the tumor to be 1 cm in diameter (Tumor removal) were all recorded. The values given are mean numbers of days.

*The third animal died 63 days after injection – no tumor was reported in that time.

---

**Tumors arising in nude mice**

<table>
<thead>
<tr>
<th>PIL-1</th>
<th>PIL-5</th>
<th>PIL-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>pGEM</td>
<td>#1</td>
<td>#2</td>
</tr>
<tr>
<td></td>
<td>#3</td>
<td>#1</td>
</tr>
</tbody>
</table>

**Fig. 6.** PCR p53 genotyping of tumors developed in nude mice. Cells derived from tumors (PIL-1, -5 and -2) have a heterozygote p53 knockout genotype, shown by the presence of two bands (375 and 575 bp) for each tumor sample, denoted #1, #2, #3. A control p53 -/- sample is included (-/-) and pGEM DNA standard is indicated.
immunocytochemistry, although AFP mRNA was expressed at low levels (26).

We report three out of five PIL lines are transformed. The PIL-1 and -5 are highly tumorigenic forming tumors at the same rate (~19 days) as our reference tumorigenic fetal liver cell line, FRL-19 (46). The PIL-2 line also produced tumors, although only after 90 days. It is interesting to note the time taken for tumors to form from injection of the PIL-1 and -2 cell lines at the two different passage numbers (17 and 32). There is a decrease in the tumor latency with an increase in passage number, suggesting molecular changes occur in culture to produce the tumorigenic phenotype. As expected, all of the tumors formed had mixed cell populations, since the PIL lines are homozygote knockout and the tumor contains some cells contributed from the host nude mouse (p53 +/-).

The tumors also stained positively by immunohistochemistry for PIL cell line markers, indicating the tumors were derived from the injected cells. The tumors are classified as poorly differentiated hepatocellular carcinomas since their constituent cells express markers of immature hepatic cells.

In conclusion, this study documents the generation of p53 null hepatic cell lines with characteristics similar to the
in vivo oval cell. These PIL cell lines will be useful for the study of oval cells with regards to their differentiation and involvement in carcinogenesis. Oval cells have long been implicated as the cellular targets for transformation in the progression of hepatocellular carcinoma (26) and the tumorigenic status of three of the five PIL cell lines supports this role of oval cells in contributing to tumor formation. Specifically, it is direct evidence that oval cells are capable of producing poorly differentiated hepatocellular carcinoma. Further, the tumorigenic and non-tumorigenic PIL cell lines provide a means to dissect the molecular switches necessary for a cell to attain the cancerous phenotype.

Recent studies in primary embryonic fibroblasts suggest the cooperation of at least two oncogenes is necessary for a cell to be tumorigenic (10). Hanahan identified that lack of p53, coupled with telomerase expression, is critical for cell immortalization and, potentially, tumorigenesis. This results in the accrual of genetic alterations due to prolonged life span and lack of cycle control (59). Our results show both the myc and ras oncogenes are upregulated in all PIL cell lines, two oncogenes previously reported to be upregulated as a result of the CDE diet (60). Potentially this carcinogenic stimulation of oncogenes coupled with the lack of p53 is sufficient to transform the PIL cell lines.

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References


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