Int. J. Exp. Path. (1992), 73 99–114

Pathology of spontaneous and oncogene transformed rat liver epithelial cells and derived tumours in nude mice

A. Olufemi Williams, A.C. Huggett and S.S. Thorgeirsson

Laboratory of Experimental Carcinogenesis, Chemical and Physical Carcinogenesis Program, Division of Cancer Etiology, National Cancer Institute, National Institutes of Health, Bethesda, MD and Fogarty International Center, NIH, Bethesda, MD 20892, USA

Received for publication 22 April 1991
Accepted for publication 30 October 1991

Summary. The histological and ultrastructural features of oncogene transformed rat liver epithelial (RLE) cells in culture and spontaneously transformed RLE cells were studied. The tumours produced in nude mice by all the transformed cells were either moderately well differentiated carcinomas or sarcomatous tumours. Epithelial tumours were produced in the RLE cells after transduction of both v-raf and v-myc oncogenes whereas sarcomatous tumours were produced after transduction of v-raf alone. These data suggested that transformation of RLE cells with a single oncogene, v-raf, produced malignant tumours which were consistent with sarcomas while a combination of two oncogenes, v-raf and v-myc, produced an epithelial tumour, consistent with a carcinoma. The effects of these oncogenes on RLE cells indicate that they were able to differentiate and were capable of producing two morphologically distinct tumour types.

The possible role of v-myc in switching the sarcomatous lineage to an epithelial tumour lineage is considered to be significant and worthy of further studies. The epithelial tumour produced in RLE cells by combination of v-raf and v-myc is consistent with an embryonal type of hepatoblastoma. The trabecular type of liver cell carcinoma which resulted from spontaneous transformation of RLE cells illustrates the inherent potential of the RLE cell to undergo malignant change and strongly suggests that the RLE cells may be the precursor cells in the development of hepatocellular carcinoma in the rat.

Keywords: rat liver, oncogene, epithelial cells, transformation, morphology, tumour

During the past three decades, malignant transformation of the rat liver epithelial (RLE) cells has been the subject of several studies (Farber & Cameron 1980; Inaoka 1967; Tsao & Grisham 1987; Braun *et al.* 1987). The transformed cells are characterized by alterations in their morphological appearances irrespective of the nature of the transforming agents. Chemical carcinogens have been used quite extensively, and oncogenes have also been used to produce cellular transformation and immortalization (Tsao & Grisham 1987; Garfield *et al.* 1988; Sinha *et al.* 1987). The relationship between RLE cells

Correspondence: Dr Snorri S. Thorgeirsson, National Cancer Institute, NIH, Building 37, Room 3C28, Bethesda, MD 20892, USA.

and oval cells in rat hepatocarcinogenesis has been discussed in previous reports (Grisham *et al.* 1974; Sell & Leffert 1982; Tsao & Grisham 1987). Long-term cultures of RLE cell lines have been used as models to study cell transformation *in vitro* including the regulation of variable expressions of gamma glutamyl transpeptidase (GGT) in induced liver tumours (Miyazaki *et al.* 1983). In-vitro transformation of RLE cells using oncogenes (Garfield *et al.* 1988) or chemical carcinogens has been shown to correlate with tumorigenicity (Tsao & Grisham 1987). Oval cells have been isolated and transfected with transforming oncogenes with subsequent development of tumours (Braun *et al.* 1987, 1989). The histogenetic role of oval cells and RLE cells in experimental carcinogenesis appears to be significant (Braun *et al.* 1987, 1989).

Oval cells *in vivo* and RLE cells *in vitro* have been shown to be capable of synthesizing both albumin and alpha-foetoprotein in addition to expression of certain enzymes (Evarts *et al.* 1987a,b; Nagy *et al.* 1988, 1989). This is not only a significant finding in understanding the functional capabilities of these cells in liver carcinogenesis but supports their epithelial origin. Oval cells have been likened to stem cells (Grisham 1980) and their ability to differentiate into basophilic and fully differentiated hepatocytes is supportive of this hypothesis (Evarts *et al.* 1987a). A spectrum of tumours has been shown to result from transformed RLE cell lines and also from its single cell derived clonal subpopulations using recombinant retroviruses containing transforming oncogenes (Garfield *et al.* 1988). Furthermore, the morphology of RLE cells has been influenced by the effect of either one selected oncogene or a combination of oncogenes (Garfield *et al.* 1988).

The main object of this paper is to report the spectrum of morphological changes observed in RLE cells which have either been propagated in long-term cultures or transformed with selected oncogenes. Morphological changes in cell lines which underwent spontaneous transformation are also described. None of the transformed cells used in these studies was treated with chemical carcinogens. This study also describes the different histological types of tumours produced in nude mice following subcutaneous injection of RLE cells, transformed *in vitro*, with a selected oncogene (v-raf) or a combination of oncogenes (v-raf and v-myc). Tumours which developed in nude mice following injection of the spontaneously transformed RLE cells in culture are also described.

Materials and methods

The following pathological examinations were carried out on the RLE cells in culture and on the solid tumours produced in nude mice: (1) histology, (2) histochemistry, (3) cytology, (4) phase contrast microscopy, (5) transmission (TEM) and scanning electron-microscopy (SEM).

The clonal RLE cell lines were established as previously described (Garfield *et al.* 1988) and maintained routinely in HAMS F12 medium supplemented with 10% defined foetal bovine serum (Hyclone Labs, Logan, CT). Table 1 presents a summary of features of the different types of (transformed and control) cells used in the study.

For this study, 2×10^6 cells from all cultured cells which were spontaneously or oncogene-transformed were injected subcutaneously into male athymic nude mice in duplicate. When the tumours developed to the size of about 1.5–2 cm in diameter, the mice were sacrificed and the tumours excised. Each tumour was divided into several portions: one portion was fixed in Bouin's fluid, one portion was fixed in 3% glutaraldehyde, one portion was used for imprint cytology and another portion frozen for histochemical studies. The cells in culture were grown to confluence in T-150 flask, scraped with a rubber policeman into 15 ml centrifuge tubes, centrifuged into cell pellets and processed for electronmicroscopy. The cell pellets were fixed in 1.5% glutaralde-

Table 1. Summary of phenotypic properties of control and transformed RLE cells

Cell line	Passage	Soft agar growth‡		γGT§	Tumorigenicity		
		−EGF	+EGF		Incidence†	Latency (days)	Growth rate¶ (mm^2/day)
Control							
RLEϕ13	15	0.0	0.0	−	0/5	—	0.0
RLEC-2	43	0.0	0.0	−	0/5	—	0.0
B7(96)	38	0.0	0.0	−	0/5	—	0.0
v-raf/v-myc							
RJ2-14	43	44.1	46.9	+++	15/15	3	13.3
v-raf							
R3611-3	43	0.4	10.3	−	4/23	28	<1.0
R3611T-1	43	0.0	0.0	+	13/13	5	4.5
R3611T-2	43	0.0	46.1	++	19/19	4	9.3
R3611T-3	43	0.0	39.5	++	13/13	4	8.2
R3611T-4	43	0.0	0.0	+	12/12	8	4.9
R3611T-5	43	0.0	0.0	+	13/13	6	6.0
R3611T-7	43	0.0	8.3	++	10/10	4	8.1
Spontaneous							
C4T	38	33.7	33.6	+	5/5	8	13.8

* See Worland *et al.* (1990).
† Within 1 month.
‡ Colony efficiency (%); EGF (5 ng/ml).
§ Assessment of intensity of staining for γGT activity: −, no staining; +++, all cells show intense staining.
¶ Diameter × depth.

hyde, post-osmicated in 1% osmic acid, dehydrated through graded alcohols, embedded in Epon 810 resin, thin sectioned, and stained with lead and uranyl acetate. All tissues fixed in Bouin's fluid were embedded in paraffin wax and after sectioning, stained with haematoxylin and eosin, Masson's Trichrome, PTAH and PAS with and without diastase digestion. The transformed cells were grown in monolayer cultures using chamber slides, fixed in 95% ethanol and stained with both Giemsa and haematoxylin and eosin. The cytology imprints on microscopic glass slides were also fixed in 95% ethanol and stained by the Giemsa and Wright's method. Phase contrast microscopy of the cells was carried out on all the cell lines in tissue culture using an inverted phase microscope and photographed. Scanning and transmission electronmicroscopy were carried out on some cell cultures using platinum coating and lead/uranyl staining, respectively. GGT estimation was performed by the method of Rutenberg *et al.* (1969).

Results

All the nude mice injected with transformed clones of cells, either spontaneous or induced by oncogenes, developed subcutaneous tumours. On light microscopy, all the tumours could be classified into two major

histological groups based entirely on morphological characteristics of the predominant cell type in accordance with the histological typing of liver tumours in the rat (Institute of Laboratory Animal Resources 1979). The tumours observed were classified on light microscopy as either being sarcomatous when the predominant cell type was spindle shaped (Fig. 1) or carcinomatous when the cells were predominantly cuboidal or oval and arranged in trabecular pattern or in rows and cords (Fig. 2). On electronmicroscopy, one sarcomatous tumour (3611T-2) induced by v-raf had a few spindle shaped cells with demonstrable desmosomes between them (Fig. 3). This was in contrast to the epithelial features normally encountered in the v-raf and v-myc (RJ2-14) induced tumours (Fig. 4).

v-raf/v-myc Transformed RLE cells (RJ2-14) and tumours produced in nude mice

The tumours produced by injection of RJ2-14 cell line in nude mice were red, soft, highly vascular, friable and haemorrhagic. In a few areas, they were adherent to skin and fascia investing the muscle. Histological appearance of the tumours showed two patterns. In some areas, the tumour was highly cellular, monomorphic, and composed of round, oval or cuboidal cells with relatively high mitotic activity while in other areas, the tumour was less cellular but composed of rows or cords of round or oval cells in a loose oedematous or haemorrhagic stroma. Some of the thin-walled blood vessels would appear to have ruptured and destroyed the cellular areas of the tumour. Some

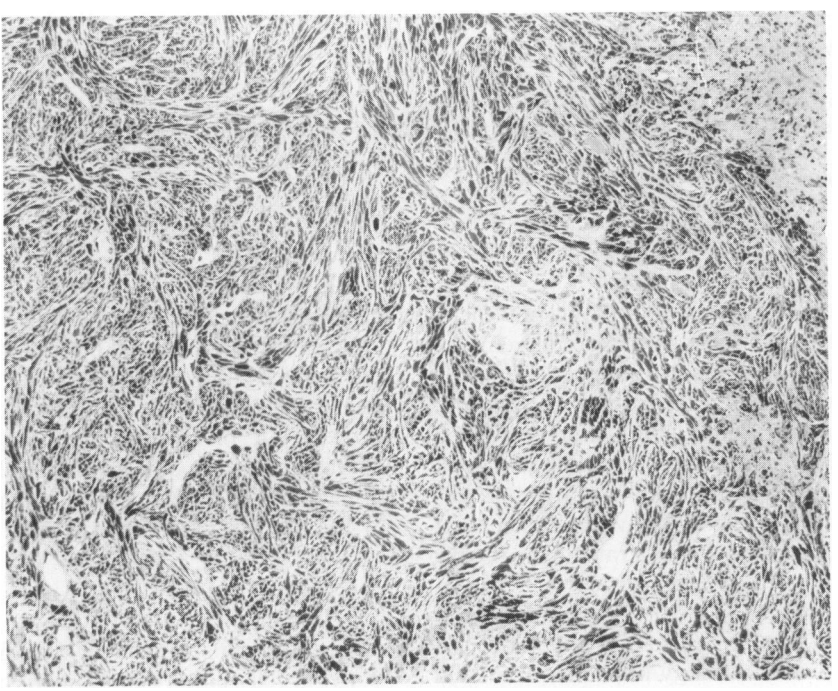

Fig. 1. Histological appearance of R3611T1 tumour, composed predominantly of spindle shaped cells arranged in bundles consistent with cells of mesenchymal origin. Note hypercellularity and a few mitotic figures. H & E. × 250.

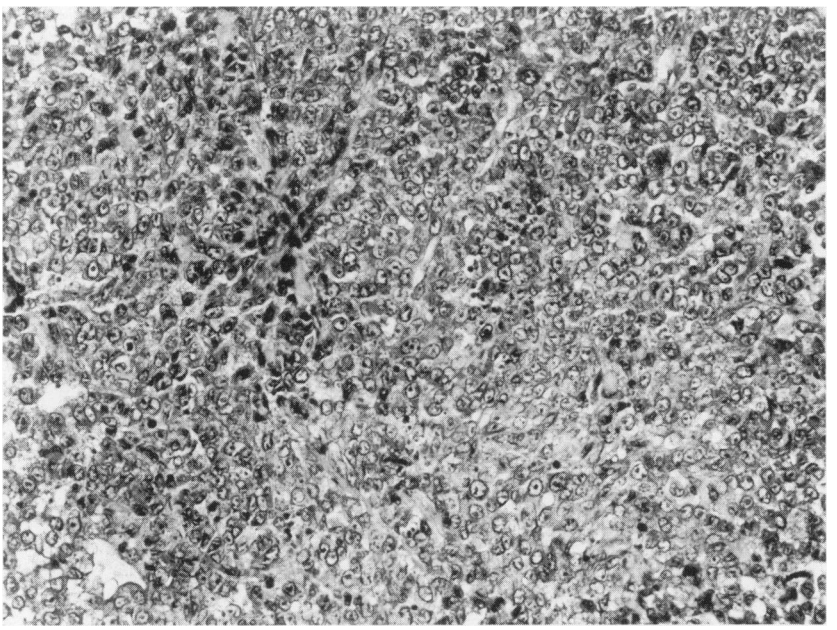

Fig. 2. Histological appearance of tumour cells in nude mice due to RJ2-14 cells. Note oval shape of epithelial looking cells, mitotic figures, formation of rows and cords, and hypercellularity of the tumour. H & E. × 125.

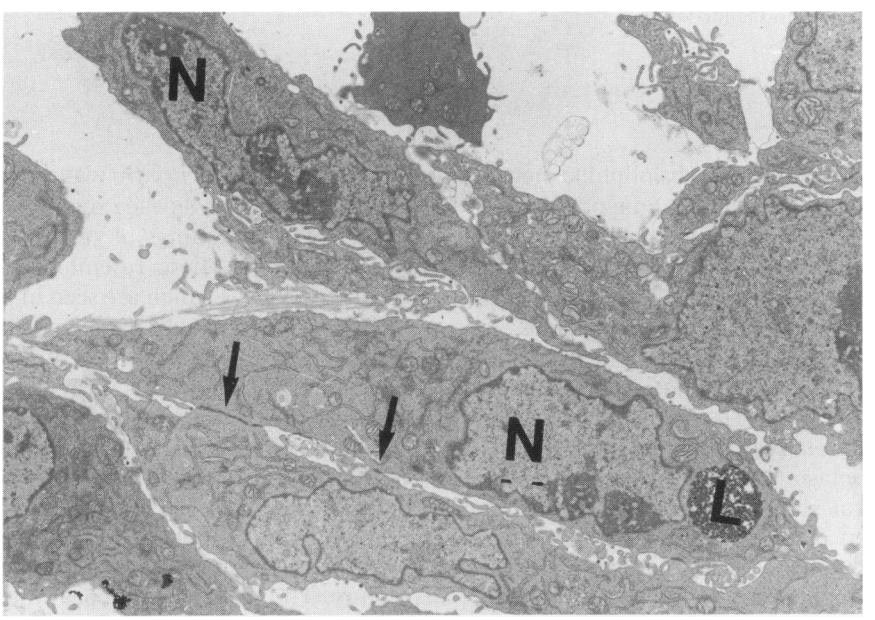

Fig. 3. Electronmicroscopic appearance of two spindle shaped cells with desmosomes in a predominantly epithelial tumour produced by RJ2-14 cells (v-raf and v-myc). Irregular extracellular spaces with microvilli are present between the two cells. Note other spindle shaped cells with elongated nuclei. Arrows point to desmosomes. N, Nucleus; L, lysosomal body. Uranyl acetate and lead. × 4000.

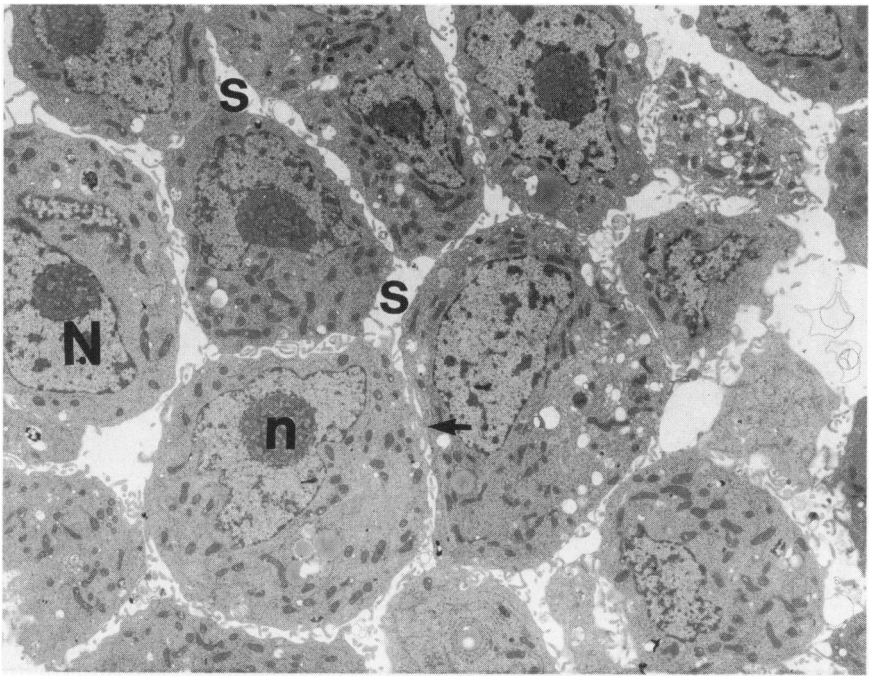

Fig. 4. Electronmicroscopic appearance of epithelial tumours produced by RJ2-14 cells in nude mice showing irregular extracellular spaces lined by irregular microvilli. Note oval, round or cuboidal shapes of cells. The absence of bile in the spaces is striking. S, Extracellular spaces; n, nucleolus; N, nucleus. Uranyl acetate and lead. ×4000.

tumour cells showed eosinophilic necrotic change but there was minimal or no inflammatory cellular reaction to them. In a few focal areas, there were attempts at pseudoglandular or trabecular formation. Ultrastructurally, there were desmosomes, few microvilli within irregular extracellular spaces reminiscent of biliary canalicular formations (Fig. 4). The nuclear–cytoplasmic ratio was relatively high in some cells and the cytoplasm contained few organelles. Some oval shaped cells with fenestrations were seen on transmission electronmicroscopy and in some areas they were arranged in rows and cords.

The ultrastructural features of the transformed cells *in vitro* were morphologically similar to those of solid tumours produced in the nude mice. One of the striking features of these epithelial cells *in vitro* was the irregular silhouette of their surface membranes produced by outpouchings of variable size and shape (Fig. 5). These resembled foot processes or pedicels which are seen in unicellular organisms capable of movement. They were not seen in the solid subcutaneous tumours. The morphological features of some of the RJ2-14 cells in culture were similar to the cells on imprint cytology. The tumours were predominantly composed of cuboidal or oval cells on imprint cytology. Similar appearances of these RJ2-14 cells were also observed in the monolayer tissue culture. The histological appearance of this epithelial tumour and its ultrastructural features were consistent with those of a highly vascular hepatoblastoma–embryonal type (Evarts *et al.* 1987b; Okuda & Ishak 1987).

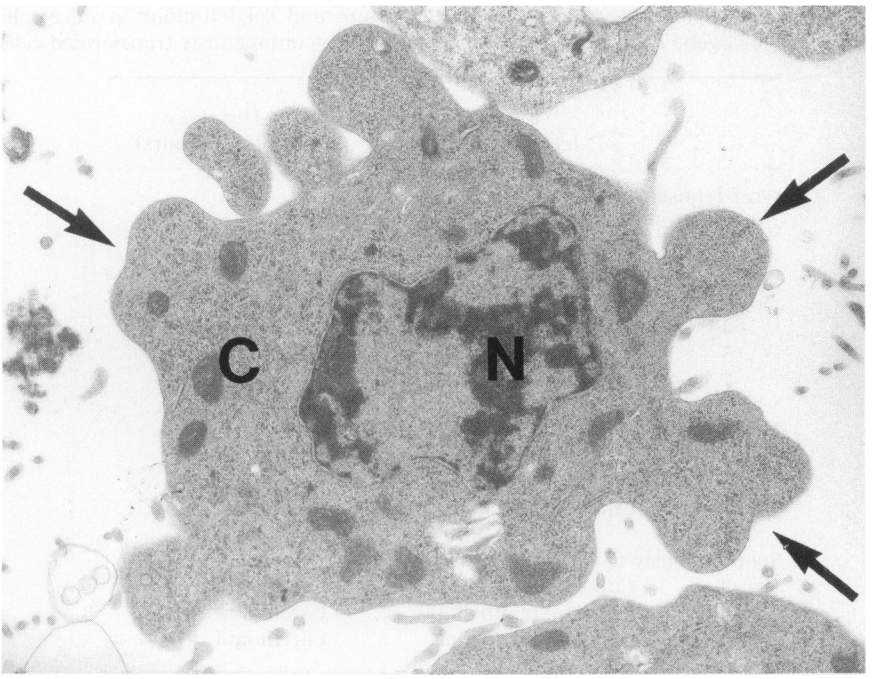

Fig. 5. Electronmicroscopic appearance of RJ2-14 cells in culture showing an irregularly shaped nucleus and very few cytoplasmic organelles. Note irregularity of cell shape due to superficial nodules which look like foot processes. Intranuclear cytoplasmic inclusions of cytoplasmic origin are present. N, Nucleus; C, cytoplasm. Arrows point to pedicels (foot processes). Lead and uranyl acetate. × 9750.

v-raf Transformed RLE cells and tumours produced in nude mice

All the six cell lines injected into the nude mice produced tumours which were highly cellular, relatively avascular and were predominantly composed of spindle shaped cells (Table 2). The tumours grew fast and infiltrated overlying skin and underlying subcutaneous fat and muscle (Fig. 6) but no evidence of local or distant metastases was seen before or after the animals were sacrificed. The tumours were firm in consistency, whitish in colour and in some areas appeared to be partially encapsulated. Histologically they were composed of highly cellular spindle shaped cells with varying degrees of mitotic activity, invasiveness and differentiation. A few tumours were undifferentiated, had no pattern and had a high mitotic index while some formed storiform, plexiform or fasciculate patterns. There were no areas of bone or cartilage formation but areas of myxomatous degeneration and necroses were seen in some tumours. Collagen production was minimal within the tumour but this was seen in the upper dermis where there was tumour infiltration.

Ultrastructurally, the predominant cell type in all the tumours was spindle shaped (Fig. 7). These cells varied in length and width and there was considerable variation in their nuclear/cytoplasmic ratio. A few cells contained liposomes and/or lysosomal bodies but there were few organelles in the cytoplasm. In three cell lines which did not grow in soft agar (3611T1, 3611T4, 3611T5) but expressed GGT, there was

Table 2. Light microscopic appearances of cells in culture and solid tumour in mice: phenotypic expressions in the oncogene transformed cells and also in the spontaneously transformed cells

	Cytology (cells in culture)	Histology (solid tumours)
v-raf Transformed*		
R3611T		
1	Spindle shaped	Sarcoma
2	Spindle shaped	Sarcoma + Carcinoma
3	Spindle shaped	Sarcoma
4	Spindle shaped	Sarcoma
5	Spindle shaped	Sarcoma
7	Spindle shaped	Sarcoma
v-raf/v-myc Transformed†		
RJ2-14	Cuboidal to oval (epithelial)	Carcinoma
RJ2-15	Cuboidal to oval (epithelial)	Carcinoma
RJ2-17	Cuboidal to oval (epithelial)	Carcinoma
Spontaneously transformed‡		
C4T	Cuboidal (epithelial)	Carcinoma
C3T	Cuboidal (epithelial)	Carcinoma
B3T	Cuboidal (epithelial)	Carcinoma

* Example illustrated in Figs 1 and 7.
† Example illustrated in Figs 2 and 4.
‡ Example illustrated in Figs 9 and 10.

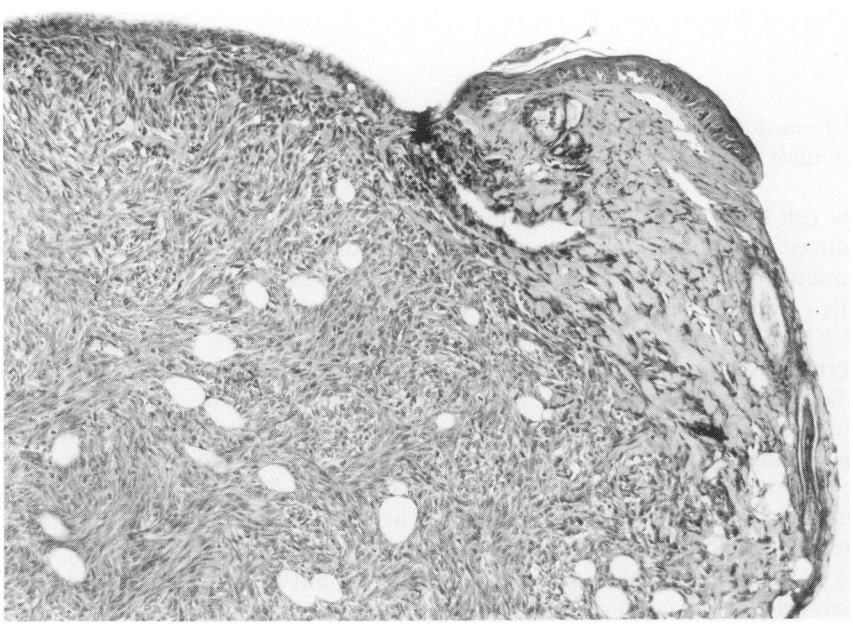

Fig. 6. Histological appearance of highly cellular spindle shaped tumour cells produced by 3611T-2 cells (v-raf only) infiltrating skin and adnexal structures of nude mice. Note ulceration of skin in the upper left half. H & E. × 125.

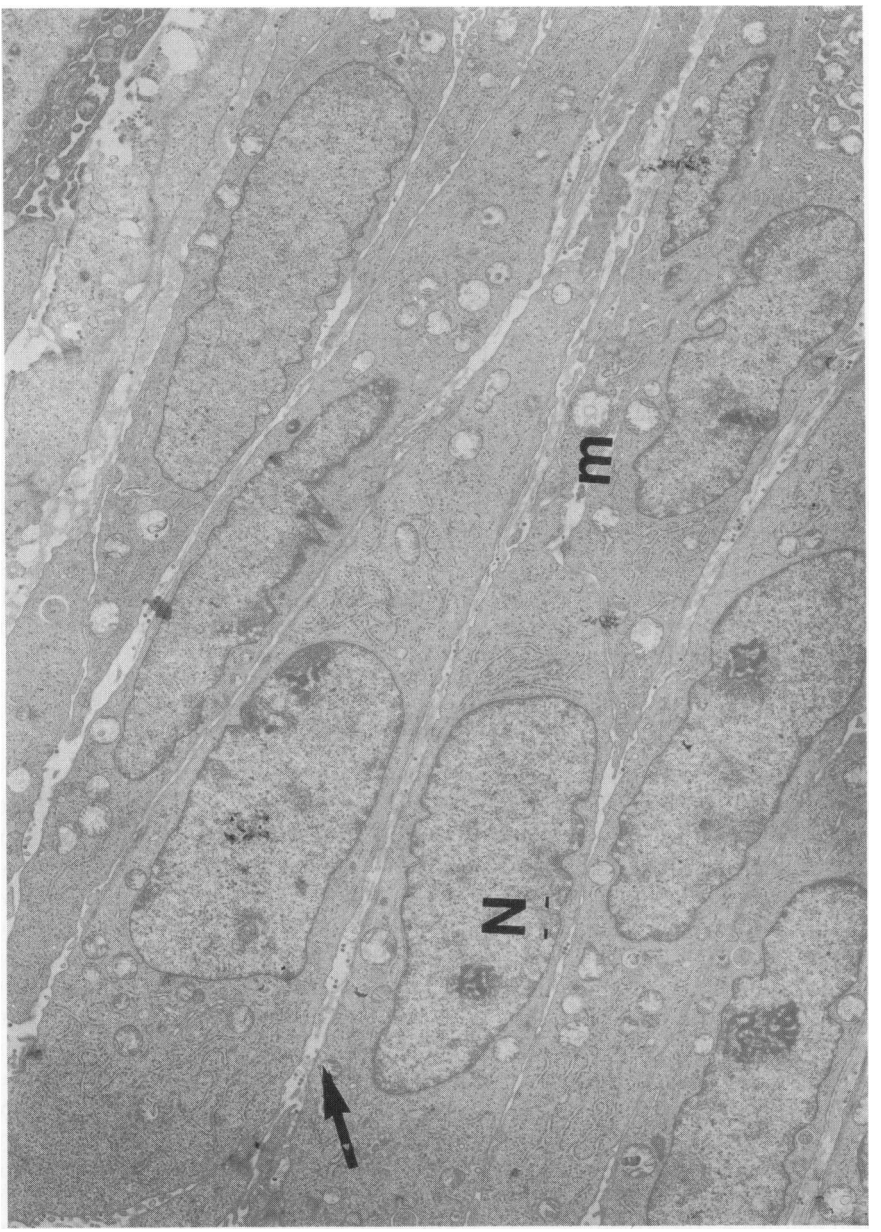

Fig. 7. Electronmicroscopic appearance of spindle shaped tumour cells showing the typical spindle shape phenotype of v-raf transformed RLE cell (R3611T-1). N, Nucleus; m, mitochondria; arrow points to cell membrane. Lead and uranyl acetate. × 3500.

Table 3. Electron microscopic (TEM and SEM) appearances of cells and solid tumours

Cell type	Morphologic type and diagnosis of tumours
v-raf Transformed RLE	
R3611T	
1	Spindle shaped cells—sarcoma (Figs 1 and 7)
2	Spindle shaped cells and desmosomes (carcino-sarcoma)
3	Spindle shaped cells—sarcoma
4	Spindle shaped cells—sarcoma
5	Spindle shaped cells—sarcoma
7	Spindle shaped cells—sarcoma
v-raf/v-myc Transformed RLE	
RJ2-14	Moderately differentiated carcinoma (epithelial) (Figs 2 and 4)
Spontaneously transformed RLE	
C4T	Well differentiated carcinoma (epithelial) (Figs 9 and 10)

proliferation and dilatation of the smooth endoplasmic reticulum some of which contained flocculent but weakly osmiophilic material. Desmosomes and extracellular space formations were not present. In the remaining three cell lines which grew on soft agar (3611T2, 3611T3, 3611T7) and which expressed relatively high levels of GGT, the spindle shaped cells showed no dilatation of their endoplasmic canals. Extracellular spaces with surface villi or desmosomes were also not seen. In only one cell line (3611T2), a few desmosomes were seen between morphologically spindle shaped cells (Fig. 3). The phenotypic appearance of the five tumours (3611T1, 3, 4, 5, 7; Tables 2 and 3) was consistent with variable stages of malignancy. The sixth tumour (3611T2) which, morphologically, was spindle shaped but had focal areas of epithelial features such as desmosomes and extracellular space formation, was considered to be a mixed tumour consistent with a carcino-sarcoma (Table 2) or could be a carcinoma with spindle shaped epithelial component.

The appearance of these v-raf transformed cells in monolayer cultures was essentially spindle shaped with varying degrees of mitotic activity (Fig. 8). Imprint cytological appearance of all the tumours was similar to their apearances in culture and consistent with malignant spindle cell tumours or 'fibrosarcoma'. The scanning electronmicroscopic appearance confirmed these to be spindle shaped cells.

Spontaneously transformed RLE cells

The spontaneously transformed RLE cell lines, C4T, C3T and B3T, produced tumours in nude mice which were grossly haemorrhagic. On light microscopy, the tumours were composed of cuboidal cells which were well differentiated and arranged in trabecular pattern (Fig. 9). The individual cuboidal cells were relatively large and had vesicular nuclei with prominent nucleoli. Mitotic figures were common and focal areas of necrosis were seen with inflammatory cellular reaction. Ultrastructurally, the tumour cells showed evidence of epithelial origin such as desmosomes and extracellular spaces with microvilli (Fig. 10). Similar cuboidal shaped cells were seen on imprint cytology of the tumours and also in monolayer culture of the cells. In some areas, a pseudoglandular pattern was seen on imprint but the cells were cuboidal and

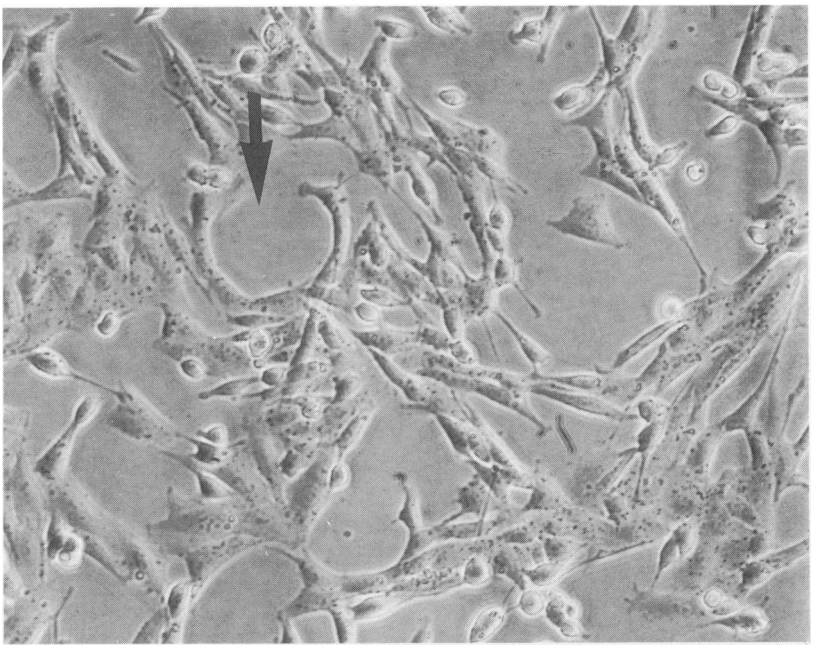

Fig. 8. Phase contrast microscopical appearance of 36 1 1T-1 cells (v-raf only) in culture showing spindle shaped cells. Note the presence of a horseshoe appearance (arrow) of cells. × 210.

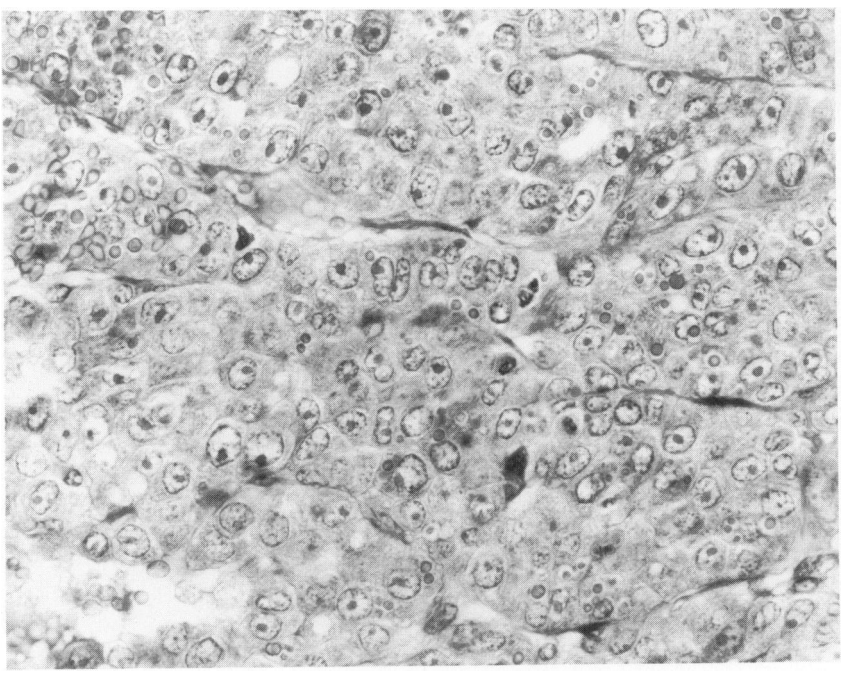

Fig. 9. Histological appearance of spontaneously transformed C4T cell line showing trabecular pattern of growth in solid tumour produced when injected into nude mice. Note large vesicular nuclei with prominent nucleoli in the rows of epithelial cells. Mitotic figures are also present. H & E. × 500.

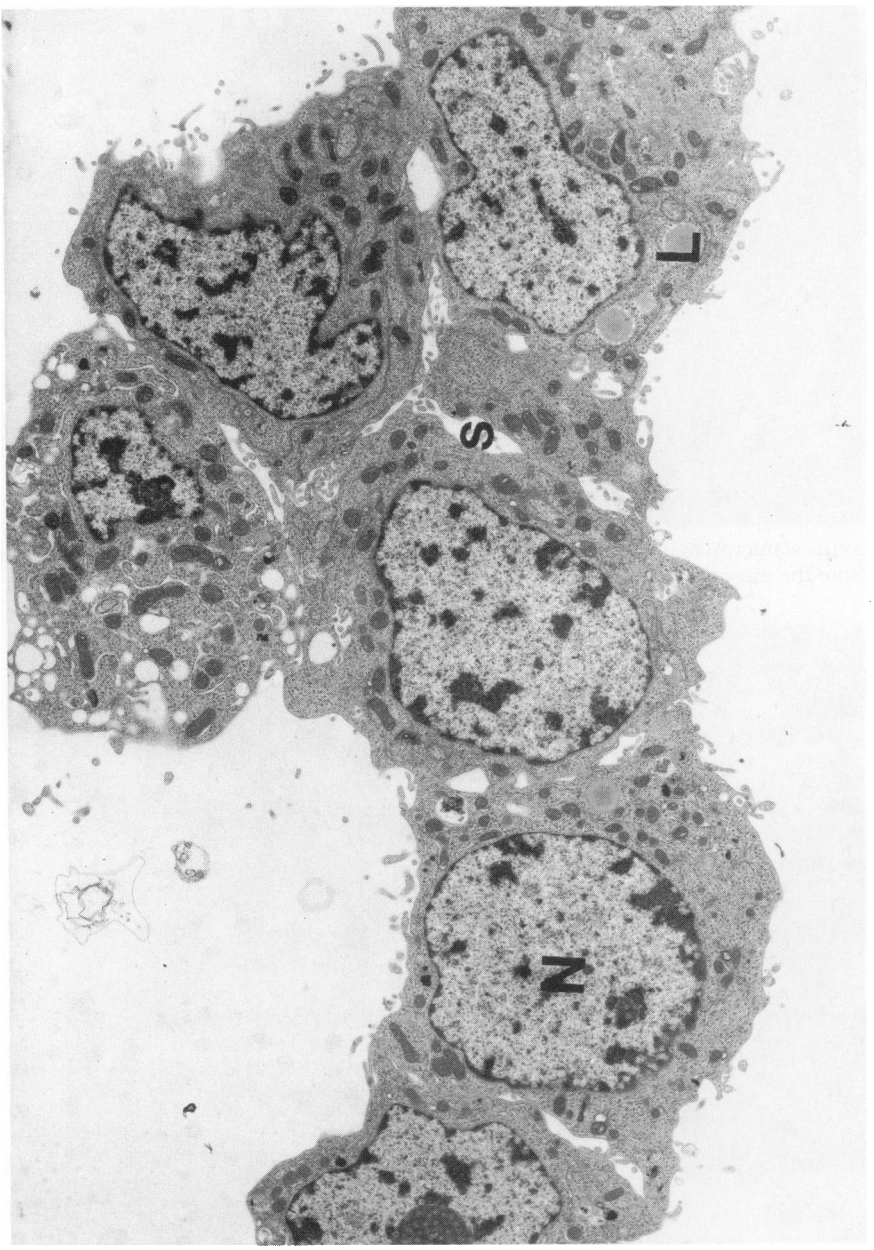

Fig. 10. Electronmicroscopic appearance of tumour produced by C4T cell line in mice showing cuboidal shape morphology. Note extracellular spaces with irregular microvilli reminiscent of bile canalicular spaces (S). L, Lipid body; N, nucleus. Lead and uranyl acetate. × 5000.

arranged in a trabecular pattern consistent with hepatocellular carcinoma. There was no secretion of bile or mucin in any of the solid subcutaneous tumours. Histochemical stain for bile canaliculi, using carcinoembryonic antigen as a marker, was negative in all the tumours.

Control RLE cells

The light microscopic appearances of the control RLEϕ13, RLEC2, B7(96) cells revealed predominantly cuboidal or oval morphology. This was also confirmed at the ultrastructural level.

Discussion

For this study, cloned RLE cell lines transformed with v-raf alone or a combination of v-raf and v-myc oncogenes, were used (Garfield *et al.* 1988) (Table 1). The RLE cells transformed with a combination of v-raf and v-myc were small and cuboidal with prominent nuclei and scanty cytoplasm. They appeared to be consistent with an embryonal type of hepatoblastoma (Okuda & Ishak 1987).

The combined effects of two oncogenes, v-myc and v-raf, which produced a rat hepatoblastoma, would suggest that the expression of v-raf is modulated by v-myc towards an epithelial lineage since v-raf oncogene alone transforms the RLE cell into a predominantly spindle shaped cell morphology. Since the v-raf oncogene-transformed cells were capable of producing a sarcomatous pattern in the mice and v-raf/v-myc oncogenes acting in combination produced an epithelial type of tumour, it is suggested that v-myc may either be cooperating or responsible for potentiating the phenotypic alterations observed in the epithelial (hepatoblastoma) tumours.

One striking histological feature of the hepatoblastoma produced in nude mice was the hypervascularity, the haemorrhagic necroses of tumour cells and the presence of cells resembling rat liver endothelial cells. In view of the vascularity, which is a feature of hepatoblastoma, it was not surprising that occasional liver endothelial cells were encountered on electronmicroscopy. The hepatoblastoma produced by a combination of v-raf/v-myc transformed RLE cells appeared to be less differentiated when compared with the spontaneously transformed tumours (C4T, C3T, B3T). This observation suggests that other factors, in addition to or independent of c-myc and/or c-raf activation, may be required for cellular differentiation in the spontaneously transformed cell lines.

The v-raf oncogene alone was able to transform RLE cells into sarcomatous tumours with the notable exception of one tumour cell line (3611T2). There was no correlation between the morphology of the transformed cells and the expression of GGT, or the growth pattern in soft agar in the presence or absence of EGF.

It is conceivable that the cellular heterogeneity observed in hepatocarcinogenesis may be due to the action of a single oncogene or of a combination of oncogenes or even possible additive effects of suppressor genes (Garfield *et al.* 1988; Sinha *et al.* 1987; Braun *et al.* 1987). It is evident from this study that the RLE cell is capable of being transformed by oncogenes with subsequent differentiation into different cell types. These transformed cells when transplanted into nude mice are capable of reproducing themselves by forming well recognized tumour patterns.

The current view on cellular lineages in hepatocarcinogenesis suggests that there is a sequence of cellular alterations which may start with stem cells or oval cells (Tsao & Grisham 1987; Grisham 1980). These changes have been observed with chemical carcinogens (Tsao & Grisham 1987) and with oncogenes (Garfield *et al.* 1988; Sinha *et al.* 1987; Braun *et al.* 1987). The effects of oncogenes on cell lineages have been the subject of several studies in the past (Adams *et al.* 1985; Alexander *et al.* 1987). Lineage switches have also been demonstrated in Eu-myc transgenic B cells into macrophages by the combined expression of myc and raf oncogenes (Klinken *et al.* 1988). This has

clarified the question of commitment of a cell to a single lineage. The phenomenon of 'lineage infidelity' has been exemplified by haemopoietic cells which are either 'biphenotypic' or have bipotential or multipotential capacities. For example, some lymphoid tumours are biphenotypic as shown by B lymphocytes that are ultimately derived from a haemopoietic stem cell that also generates myeloid and erythroid elements (Klinken *et al.* 1988; Greaves *et al.* 1986). The lineage commitment to the lymphoid phenotype is attributable to deletions in the variable region of the immunoglobulin gene and this takes place at a particular stage in ontogeny of the cells.

In this study, the untransformed RLE cell can be likened to a biphenotypic cell which is committed by v-raf oncogene to the spindle shaped morphologic cell lineage. This lineage switch, however, is blocked by the concommitant expression of v-myc oncogene which prevents a change to the mesenchymal phenotype, thereby retaining the epithelial morphology of the cells and resembling an embryonal type of hepatoblastoma. Similar series of molecular events have been shown in previous experiments using lymphoid and myeloid lineages in mice (Adams *et al.* 1985; Alexander *et al.* 1987).

In the case of spontaneously transformed RLE cells which gave rise to a well differentiated hepatocellular carcinoma, it is possible that malignant change had taken place in a clone of stem cells which were multipotential but committed to a well differentiated hepatocellular lineage. The lineage fidelity can be breached when the progenitor cell is transduced with a single or a combination of oncogene(s) such as v-raf or v-raf/v-myc combination. However, all the spontaneously transformed cell lines derived from RLEϕ13, so far examined, gave rise to well differentiated hepatocellular carcinomas. The absence of a spindle shaped cellular component in the tumours produced by the spontaneously transformed cell lines was striking. This suggests that spontaneous transformation to spindle shaped phenotype (mesenchymal) is uncommon. This hypothesis may explain the preponderance of spontaneously occurring malignant epithelial tumours (hepatocellular carcinomas) and a relatively small proportion of primary hepatic tumours with mesenchymal components such as the hepatoblastoma, sarcomas and also mixed tumours of the liver composed of variable proportions of epithelial and mesenchymal components (Okuda & Ishak 1987; Kuwano *et al.* 1984; World Health Organization 1975). Further studies need to be carried out to characterize these phenotypes, using their biosynthetic abilities and markers capable of determining the presence or absence of intermediate filaments.

Although the observations reported here differ from other studies which used single or multiple treatments with chemical carcinogens, the development of cellular lineages in the evolution of malignancy suggests similar or common molecular events for pathogenesis irrespective of the nature of the operating or triggering agents or methodology (Diwan *et al.* 1989). The histopathological features described in this study and other previous studies are morphologically similar to the well defined spectrum of human and rat liver malignancies in early or adult life including hepatoblastoma (World Health Organization 1975), hepatocellular carcinoma (World Health Organization 1975; Nakashima & Kojiro 1987), hepatic sarcoma (Chang *et al.* 1983), sarcomatous change in primary liver cancer (Chang *et al.* 1983; Kakizoe *et al.* 1987; Nagamine *et al.* 1978) and spindle cell carcinomas (Baltifora 1976). This study suggests that transformed rat liver epithelial cell provides a suitable model for studying liver cell carcinogenesis and also provides possible explanations for the phenotypic expressions of liver cells exposed to transforming agents.

Acknowledgement

Dr Olufemi Williams was a Fogarty Scholar-in-Residence of the National Institutes of Health, Bethesda, MD, USA.

References

ADAMS J.M., HARRIS A.W., PINKERT C.A., CORCORAN L.M., ALEXANDER W.S., CORY S., PALMITER R.D. & BRINSTER R.L. (1985) The c-myc oncogene driven by immunoglobulin enhancers induces lymphoid malignancy in transgenic mice. *Nature* **318**, 533–538.

ALEXANDER W.S., SCHRADER J.W. & ADAMS J.M. (1987) Expression of the c-myc oncogene under control of an immunoglobulin enhancer in Eμ-myc transgenic mice. *Mol. Cell. Biol.* **7**, 1436–1444.

BALTIFORA H. (1976) Spindle cell carcinoma—ultrastructural evidence of squamous origin and collagen production by the tumour cells. *Cancer* **37**, 2275–2282.

BRAUN L., GOYETTE M., YASWEN P., THOMPSON N.L. & FAUSTO N. (1987) Growth in culture and tumourigenicity after transfection with the ras oncogene of liver epithelial cells from carcinogen-treated rats. *Cancer Res.* **47**, 4116–4124.

BRAUN L., MIKUMO R. & FAUSTO N. (1989) Production of hepatocellular carcinoma by oval cells: cell cycle expression of *c-myc* and P53 at different stages of oval cell transformation. *Cancer Res.* **49**, 1554–1561.

CHANG W.W., AGHA F.P. & MORGAN W.S. (1983) Primary sarcoma of liver in the adult. *Cancer* **51**, 1510–1517.

DIWAN B.A., WARD J.M. & RICE J.M. (1989) Promotion of malignant 'embryonic' liver tumours by phenobarbital. *Carcinogenesis* **10**, 1345–1348.

EVARTS R.P., NAGY P., MARSDEN E. & THORGEIRSSON S.S. (1987a) A precursor-product relationship exists between oval cells and hepatocytes in rat liver. *Carcinogenesis* **8**, 1737–1740.

EVARTS R.P., NAGY P., MARSDEN E. & THORGEIRSSON S.S. (1987b) In situ hybridization studies on expression of albumin and α-fetoprotein during the early stage of neoplastic transformation in rat liver. *Cancer Res.* **47**, 5469–5475.

FARBER E. & CAMERON R. (1980) The sequential analysis of cancer development. *Adv. Cancer Res.* **31**, 125–226.

GARFIELD S., HUBER B.E., NAGY P., CORDINGLEY M.G. & THORGEIRSSON S.S. (1988) Neoplastic transformation and lineage switching of rat liver epithelial cells by retrovirus-associated oncogenes. *Mol. Carcinogenesis* **1**, 189–195.

GREAVES M.F., CHAN L.C., FURLEY A.J.W., WATT S.M. & MOLGAARD H.V. (1986) Lineage promiscuity in hemopoietic differentiation and leukemia. *Blood* **67**, 1–11.

GRISHAM J.W. (1980) Cell types in long-term propagable cultures of rat liver. *Ann. N.Y. Acad. Sci.* **349**, 128–137.

GRISHAM J.W., THAL S.B. & NAGEL A. (1974) Cellular derivation of continuously cultured epithelial cells from normal rat liver. In *Gene Expression and Carcinogenesis in Cultured Liver.* Eds L.E. Gerschenson & E. Thompson. New York: Academic Press, pp. 1–23.

INAOKA Y. (1967) Significance of the so-called oval cell proliferation during azo-dye hepatocarcinogenesis. *Gann* **58**, 355–366.

Institute of Laboratory Animal Resources (1979) *Histologic Typing of Liver Tumours of the Rat.* National Research Council, National Academy of Sciences, Washington, D.C.

KAKIZOE S., KOJIRO M. & NAKASHIMA T. (1987) Hepatocellular carcinoma with sarcomatous change. *Cancer* **59**, 310–316.

KLINKEN S.P., ALEXANDER W.S. & ADAMS J.M. (1988) Hemopoietic lineage switch: v-raf oncogene converts Eμ-myc transgenic B cells into macrophages. *Cell* **53**, 857–867.

KOJIRO M., SUGIHARA S., KAKIZOE S., NAKASHIMA O. & KIYONATSU K. (1989) Hepatocellular carcinoma with sarcomatous change. *Cancer Chemother. Pharmacol.* **23** (**Suppl. 1S**) 4–8.

KUWANO H., SNODA T., HASHIMOTO S. & ENJOJI H. (1984) Liver cancer with osteoblast-like giant cells. *Cancer* **54**, 837–841.

MIYAZAKI M., WAHID S., MIYANO K., YABE T. & SATA J. (1983) Expression of gamma-glutamyltranspeptidase in cultures of spontaneously and chemically transformed rat liver cells. *Int. J. Cancer* **32**, 373–377.

NAGAMINE Y., SASAKI K. & KAKU K. (1978) Hepatic sarcoma associated with hepatoma. *Acta Pathol. Jpn* **31**, 815–824.

NAGY P., EVARTS R.P., MARSDEN E., ROACH J. & THORGEIRSSON S.S. (1988) Cellular distribution of c-myc transcripts during chemical hepatocarcinogenesis. *Cancer Res.* **48**, 5522–5527.

NAGY P., EVARTS R.P., MCMAHON J.B. & THORGEIRSSON S.S. (1989) Role of TFG-β1 in normal differentiation and oncogenesis in rat liver. *Mol. Carcinogenesis* **2**, 345–354.

NAKASHIMA T. & KOJIRO M. (1987) *Hepatocellular Carcinoma. An Atlas of its Pathology.* Tokyo: Springer Verlag International.

OKUDA K. & ISHAK K.G. (EDS) (1987) *Neoplasms of the liver.* Tokyo/New York: Springer-Verlag.

ROSS R. & GREENLEE T.K. (1966) Electron microscopy: attachment sites between connective tissue cells and desmosomes in transitional cells going epithelial and mesenchymal. *Science* **153**, 997–999.

RUTENBERG A.M., KIN H., FISCHBEIN J.W., HANKER

J.S., WASERKRUG H.L. & SELIGMAN A.M. (1969) Histochemical and ultrastructural demonstration of γ-glutamyltranspeptidase activity. *J. Histochem. Cytochem.* **17**, 517–526.

SELL S. & LEFFERT H.L. (1982) An evaluation of cellular lineages in the pathogenesis of experimental hepatocellular carcinoma. *Hepatology* **2**, 77–86.

SINHA S., HOCKIN L.J. & NEAL G.E. (1987) Transformation of a rat liver cell line: neoplastic phenotype and regulation of gamma glutamyl transpeptidase in tumour tissue. *Cancer Lett.* **35**, 215–224.

TSAO M.S. & GRISHAM J.W. (1987) Hepatocarcinomas, cholangiocarcinomas, and hepatoblastomas produced by chemically transformed cultured rat liver epithelial cells. A light- and electron-microscopic analysis. *Am. J. Pathol.* **127**, 168–181.

WORLAND P.J., HAMPTON L.L., THORGEIRSSON S.S. & HUGGETT A.C. (1990) Development of an in-vitro model of tumour progression using *v-raf* and *v-raf/v-myc* transformed rat liver epithelial cells: correlation of tumourigenicity with the down-regulation of specific proteins. *Mol. Carcinogenesis* **3**, 20–29.

WORLD HEALTH ORGANIZATION (1975) *Histological Typing of Liver Tumours of the Liver and Gall Bladder.* Geneva: WHO.