Alpha-fetoprotein: origin of a biological marker in rat liver under various experimental conditions

W. D. KUHLMANN

Laboratory of Experimental Medicine and Immunocytochemistry
Institut für Nuklearmedizin, DKFZ Heidelberg, Germany

NOTES FROM LECTURES ON IMMUNOHISTOLOGY
Published in parts by
Virchows Arch. A Path. Anat. Histol. 393, 9-26, 1981
and
Int. J. Exp. Path. 87, 343-359, 2006

In the course of phylogenetic and ontogenetic development, proteins are synthesized which are characteristic for the fetus. The emerging protein patterns are associated with histogenesis and organ differentiation. Among those fetal molecules, alpha-1-fetoprotein (AFP) has become one of the most intensively studied substances. It is synthesized in the yolk sac, the gastrointestinal tract and the liver of the fetus from many species. After birth, the protein disappears from body fluids following almost complete suppression of the responsible genes.

Serological and immunohistological detection of AFP gene expression can serve to follow pathways of liver cell differentiation under various conditions. In our experimental design, the studied models included

- liver regeneration after partial hepatectomy (70% resection) in mice and rats,
- liver intoxication by carbon tetrachloride (CCl_4) in mice and rats,
- liver intoxication by N-nitrosomorpholine (NNM) in rats,
- chemical hepatocarcinogenesis by NNM in rats.

From all the injury and hepatocarcinogenesis studies it was deduced that cells at different levels of the hepatic lineage will become involved in both regenerative repair and generation of carcinomas. The role of adult hepatocytes and canalicular epithelial cells including the biliary epithelium as transit compartment in regenerative processes is evident. The cell types which respond are (a) the normal adult and differentiated hepatocytes; (b) the bipotential stem-like cells in the canals of Hering giving rise to populations of oval cells as progenitor cells. The possible role of multipotent progenitor cells of presumed extra-hepatic origin (hematopoietic stem cells, bone marrow) is discussed.

Postnatal AFP gene repression occurs with species and strain dependent strictness. When hepatocytes regenerate, AFP levels will rise. In partial hepatectomy and carbon tetrachloride injury, adult hepatocytes are prolific and regenerative. Stem cells with multilineage differentiation ability are not required in this type of liver regeneration. The concomitant rise of serum AFP is due to its synthesis by adult hepatocytes. The degree of AFP gene expression is also species and strain dependent.

In severe injury when regenerative capacity of hepatocytes is blocked by substances such as N-nitrosomorpholine at high doses, reconstitution of livers occur through biliary epithelial cells which are collectively named oval cells. They are derived most probably from the canals of Hering and the small interlobular bile ducts (as source of intraorgan stem cells). Oval cells exhibit multilineage differentiation potential. It is not yet clear, however, if the regenerated structures differentiate either from stem cells as defined by their capability of self-renewal and multiple differentiation or from lineage-committed cells. At least, oval cells are a population with differentiation potential to functionally mature hepatocytes and bile duct epithelia. Proliferating cells of the bile duct system reach to a level of differentiation with reactivation of foetal genes and significant AFP synthesis (as sign of reversal of ontogeny and of potential stemness).

Development of hepatocellular carcinomas is a multistep process. Tumors arise from a pool of differentiating oval cells or by dedifferentiation of mature hepatocytes because both cell types have stem-like properties. The lineage and phenotype of chemically induced liver carcinoma may arise from a single cell through genetic and epigenetic alterations; a clonal origin and expansion is probable. AFP resurgence is associated with the appearance of hepatocellular carcinoma and reflects a process of retrodifferentiation. Clonality and retrodifferentiation succeed in selecting cell populations with highest autonomy.

The lack of a unique stem cell marker in the research of stem cells in general and also in liver regeneration remains a problem. The possibility of lineage plasticity and transdifferentiation of potential hepatic stem cells is still a matter of controversy, but the value of stem cells seems to be considerable in either conventional studies of differentiation or in studies on self-renewing potential and in therapy.

Experimental models and procedures

Rats of the inbred strain BD X as well as mice of the inbred strains C3H/He and BALB/c/J were used. The genetic regulation of AFP was studied in a great variety of mouse strains (Olsson *et al.* 1977), and it was found that a single Mendelian gene called *Raf* (regulation of alpha-fetoprotein) was involved in the regulation of AFP synthesis under normal conditions.

Tab. 1. Experimental models

Experiments	Treatment	Period of study
Partial hepatectomy[a] 20 week-old mice 8 week-old rats	70% resection	1–7 days after operation
CCl_4 poisoning[b] 20 week-old mice 8 week-old rats	100 µl CCl_4/100 g body weight	1–7 days after oral ingestion
NNM poisoning[c] 8, 12 week-old rats	20 mg NNM/kg/day for 4 weeks	7–60 days from NNM start
Hepatocarcinogenesis 8, 12 week-old rats	6 mg NNM/kg/day for 12 weeks 20 mg/kg/day for 6 weeks	induction phase, hepatoma stage

[a] See Brues et al. (1936) for mice, Higgins and Anderson (1931) for rats
[b] Mode of CCl_4 application see Kuhlmann (1979b)
[c] Description of N-nitrosomorpholine (NNM) see Druckrey et al. (1967)

Liver slices were fixed in 99% ethanol-1% acetic acid for 12-15 hours at 0-4ºC and embedded in paraffin. For immunocytochemical staining, 5-7 µm thick sections were mounted an acetone cleaned slides, deparaffinated and passed into phosphate buffered saline (PBS).

All immunological and immunocytochemical methods including the preparation of anti-AFP antibodies, rabbit anti-mouse IgG, rabbit anti-rat IgG antibodies, sheep anti-rabbit IgG antibodies and the respective peroxidase conjugates have been described earlier (Kuhlmann 1975; Kuhlmann 1978; Kuhlmann 1979). AFP reacted sections were post-stained with haematoxylin or with Gomori's silver impregnation. In the latter case, sections were first photographed because AFP stain was lost during silver impregnation.

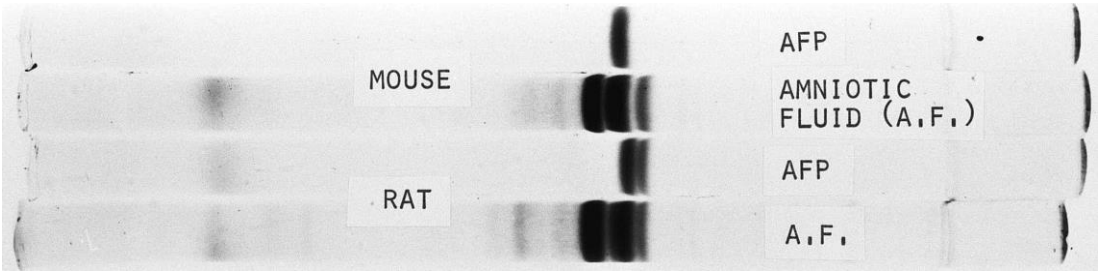

Fig. 1. Polyacrylamide gel electrophoresis of purified mouse and rat AFP; the respective amniotic fluids were also run in parallel.

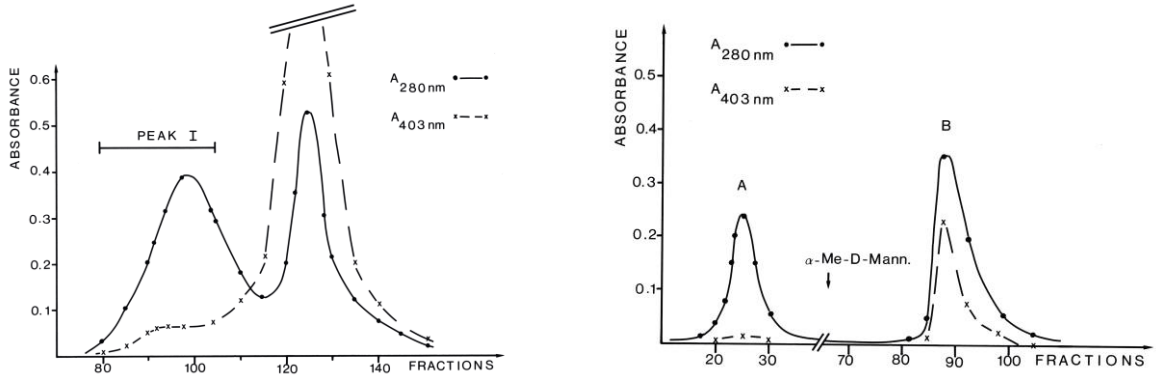

Fig. 2. Purification of peroxidase conjugated antibodies can be achieved on the basis of gel filtration and lectin bindings. In the latter case, we employ a combination of gel filtration and Concanavalin A binding; (a) first step: gel filtration on Sephacryl S-200, and peak I contains labelled and unlabelled antibodies; (b) second step: affinity chromatography of peak I (from the first step) by use of a Sepharose 4B-Concanavalin A column. The column flowthrough (peak A) contains unlabelled antibodies. Then, elution of the peroxidase labelled antibodies (peak B) is performed with 0.01 mol/L alpha-methyl-D-mannoside in starting buffer.

For routine histology, sections were stained with haematoxylin-eosin. Gomori's silver impregnation and toluidine blue staining were also performed. Glycogen accumulation was verified by PAS reaction (overnight starved animals).

All observations are summarized in Table 2; for details of AFP in mice see also a previous paper (Kuhlmann 1979).

Tab. 2. Summary of results

Experiments	AFP in sera[a] (μg/ml)	Cellular localization of AFP	Liver histology (main events)
Normal adult life			
BALB/c/J mice	0.5	–[b]	normal adult liver
C3H/He mice	0.1	–	
BD X rats	0.1	–	
Partial hepatectomy			
BALB/c/J mice	200	hepatocytes	regeneration from residual lobules
C3H/He mice	45		
BD X rats	0.9		
CCl_4 intoxication			
BALB/c/J mice	700	hepatocytes	centrolobular necrosis regeneration from residual hepatocytes
C3H/He mice	80		
BD X rats	5.5		
NNM intoxication			
BD X rats	1.5	canalic. epith. cells	massive liver necrosis prolif. of canalic. epith. cells
Hepatocarcinogenesis			
BD X rats			
1. induction phase			
6 mg NNM/kg/day	–	–	pre-hepatoma foci[c]
20 mg NNM/kg/day	1.8	canalic. epith. cells	NNM intoxication
2. hepatoma stage	4,000	hepatoma cells	manifestation of hepatocell. carcinoma

[a] Mean from at least 10 samples at days of peak concentrations
[b] not detected
[c] Description by Bannasch (1968) and J. Natl. Cancer Inst. 64, 179–206, 1980

Serological, histological and immunohistological results

Partial hepatectomy – In both strains of mices, AFP increase was slight at 24 hours after partial hepatectomy, then it rose steadily and reached a maximun on day 4; afterwards, serum AFP decreased rapidly. Significant strain differences were observed in mice with serum AFP levels 5 to 10 times higher in the BALB/c/J than in the C3H/He strain. In contrast to mice, partial hepatectomy in BD X rats led only to a slight increase in serum AFP.

Histological aspects of liver regeneration were similar in all animal species. Few mitoses were seen at 24 hours after hepatectomy, and mitotic peaks occurred on day 3 (about 24 hours before serum AFP peak was measured) followed by rapid decline. Through the days 2 to 4, strong immunoexpression of AFP occurred in portal and periportal hepatocytes of mice livers; some hepatocytes in centrolobular and intermediate zones were weakly AFP immunoreactive. Generally, AFP immunoexpression was stronger in BALB/cJ than in C3H/He mice. At any time, no cellular AFP was detected in BD X rats.

Carbon tetrachloride intoxication – Histotoxic patterns were similar in all animal strains. Cell and organelle oedema occurred within 24 h after poisoning followed by focal lesions with necrotic hepatocytes and cell infiltrates on the second day. The marginal zone between viable

and necrotic areas contained most of the dividing mature hepatocytes; the highest mitotic activity was between day 2 and day 6 after a single oral dose of CCl_4.

On all days and in all animals studied the AFP increase was always higher than after partial hepatectomy. Peak values were reached on day 4, afterwards AFP decreased again. In BALB/c/J and C3H/He mice, the slopes of AFP curves were in parallel but here AFP concentrations in sera of BALB/c/J mice also reached levels 10-fold higher or more than in C3H/He mice.

In BALB/c/J mice, few AFP-positive hepatocytes (in portal and periportal zones) were seen 24 hours after poisoning. On subsequent days, their number and staining intensity increased and reached a maximum on days 3 and 4, afterwards AFP positivity decreased. AFP was exclusively manifested in hepatocytes of nondamaged liver areas. Hepatocytes adjacent to the necrotic areas often contained the strongest AFP staining. In C3H/He mice, the histological distribution of positive hepatocytes was the same as seen BALB/c/J mice, but the number and staining intensity of AFP-positive hepatocytes were lower than in the latter.

In BD X rats, liver regeneration led also to an increase in serum AFP which, however, was much lower than in the mice. Rat liver regeneration was similar to that in mouse with a mitotic peak on day 2. AFP-positive hepatocytes were only detected on days 3 and 4 and occurred either as single stained cells or in small groups of cells in midlobular zones near to the necrosis and in portal areas.

NNM intoxication in early stages of hepatocarcinogenesis – In the course of low dose NNM feeding, no serum AFP elevation was measured in the carcinoma induction phase. In contrast, an increase of serum AFP was noted with the application of high NNM doses. At the time of the third bleeding (day 21), serum AFP levels ranged from 1.1 to 2.3 µg/ml and peak values were attained between days 21 and 35 from onset of NNM application. These concentrations were maintained until NNM feeding was stopped. Then, AFP levels dropped within 2 weeks and reached background concentrations as observed in control rats.

Histotoxic patterns were dose-dependent. In rats chronically fed low concentrations of NNM, necroses were rare and no proliferative activity occurred. Serum AFP remained at normal during the induction phase and no cellular AFP was detected.

In high dose NNM feeding experiments, extensive necrosis of hepatocytes developed within 14 days reaching portal areas. Then, inflammatory infiltrates were present and consisted mainly of histiocytes. From day 21, proliferation of small oval-shaped cells occurred within the zones of necroses exhibiting high [^{3}H]thymidine incorporation. Hepatocytes were not involved in [^{3}H]thymidine incorporation. When NNM feeding was stopped, necroses and oval cell proliferations decreased. Finally, livers showed heavy distortion of the original lobular architecture and a cirrhotic pattern.

When AFP reappeared for the first time, the fetal protein was detected in the cytoplasm of the proliferating oval cells. Their morphology corresponded to bile ductular cells. AFP-positive oval cells were seen either as small single cells or as strings of cells which formed rosettes and ductular structures with bile duct aspect. During the following weeks, the phenotype of AFP-positive oval cells changed and indicated the development towards small-sized hepatocytes. While their bile ductular feature disappeared, foci of hyperplastic appearance occurred. At his developmental stage, AFP-staining vanished even if cells were proliferating: in pulse-labelling as well as in pulse-chase labelling experiments with [^{3}H]thymidine, cells in

the hyperplastic areas were still actively engaged in DNA synthesis. In the later stages, nodules with a clear-cut hepatocytic phenotype were found.

Hepatocarcinogenesis (induction phase and hepatoma stage) – The induction time of hepatocellular carcinomas in the BD X rats varied from 90 to 150 days. They usually developed later with low dose NNM than with high dose NNM feeding. In some rats with the experiments of low NNM doses, AFP-producing carcinomas were observed as late as 200 days after the carcinogen ingestion was started. At the carcinoma stage and when significant amounts of serum AFP were detected for the first time in weekly bleedings, its concentrations were in the range of 0.04 µg to 10 µg AFP/ml; the minimum detectable concentration of AFP was estimated to be 5 ng/ml. AFP levels rose steadily during the following 10 to 12 weeks and reached serum levels which were usually found between 50 and 1,000 µg AFP/ml. In rare cases, concentrations even exceeded amounts of 4,000 µg AFP/ml serum. The dynamics of serum AFP levels over the time were highly heterogenous as controlled by weekly measurements: slow but continuous increases within a period of several weeks, and sometimes very steep increases over the time. Increases up to 10-fold within one week were also observed. At a given concentration, AFP could level off to reach plateau values, mainly in the order of 10^2 and 10^3 µg AFP/ml. These were maintained for a short time (one to two weeks) and then followed by further increases. Once AFP has appeared in the rats, AFP levels never dropped until the animals were sacrificed for histology. However, endpoint studies were not performed. For reasons of animal care, experiments were finished at least when rats showed signs of severe illness.

With the AFP appearance in serum, livers contained one or more distinct hepatocellular carcinomas. Some but not all of them would stain for AFP. AFP immunoexpression was always restricted to hepatocellular carcinomas with their neoplastic hepatocytes. Normal liver tissue, dysplastic foci and hyperplastic nodules did not stain for AFP. In serial tissue sections processed by conventional histological stains, AFP positive cells exhibited a basophilic character. They were free of glycogen (PAS negative). The high proliferation acti-vity was revealed by incorporation of [^{3}H]thymidine. Typically, up to 50% of the neoplastic hepatocytes became labelled in a 24 h pulse experiment while the percentage of labelled nuclei in normal hepatocytes stayed below 1%.

AFP in response to injury

Adult hepatocytes proved their inherent capacity for regeneration, and at no time was there progenitor cell activation which might have led to lineage regeneration. Thus, adult hepatocytes are believed to be functional stem cells as discussed earlier (Alison 1986; Fausto 2000). Furthermore, rising serum AFP levels and concomitant cellular AFP immuno-expression have shown that this function is coupled with induced AFP gene expression, with the extent of induced AFP gene expression being strongly species and strain dependent. The restriction of AFP immunostaining to hepatocytes underlined the reappearance of AFP as due to a cell-specific gene expression.

Because AFP was synthesized in small quantities before mitoses reached their maximum, a direct connection between AFP and DNA synthesis was not evident. On the other hand, some correlation between hepatocyte mitosis and amount of newly synthesized AFP will exist. In CCl_4 intoxication, moderate increase of AFP mRNA by the remaining hepatocytes was described and suggested to be linked to their reentry into the proliferative cycle (Tournier *et al.* 1988). Also, in Sprague-Dawley rat experiments with partial hepatectomy, moderate

increase in AFP gene expression was observed in the course of regeneration which could be attributed to replicative cycles (Bernuau *et al.* 1988). It seems then that the cell cycle *per se* is linked with the regulating principle of AFP gene expression, however, the degree of this linkage remains to be defined. The great difference in the AFP levels during the recovery phase (partial hepatectomy and carbon tetrachloride) of both mouse strains used in this study and between mouse and the BD X rat strain must have further reasons. In any case, the AFP levels did not merely reflect the number of hepatocytes engaged in repair because liver injury and subsequent regeneration occurred to the same extent under both experimental conditions (and irrespective of strains and species). Some mechanism inherent to carbon tetrachloride as hepatotoxin must further contribute to the expression of AFP (Taketa *et al.* 1975).

The biliary epithelium as progenitor compartment

Putative stem cells are supposed to participate in liver regeneration of injury models other than partial hepatectomy and carbon tetrachloride intoxication. To this aim, conditions must be chosen in which cell damage is very extensive or chronic so that regeneration by mature hepatocytes is impeded. For example, NNM (at least in sufficiently high doses) are substances by which the biliary system proved to possess regenerative capacity with multi-lineage differentiation potential. While normal quiescent bile ducts failed to stain for AFP, proliferating biliary epithelial cells led to a marked AFP immunoexpression, and the bile duct system was regarded as a reservoir of tissue-specific stem and progenitor cells.

During proliferation of bile duct progenitors (oval cells), these cells will reach to a level of cytodifferentiation with reactivation of fetal genes. Significant AFP synthesis by oval cells is then signalling a certain degree of retrodifferentiation (reversal of ontogeny) and a potential stemness. This observation suggests to us the existence of a mechanism different from the above described AFP gene activation in adult hepatocytes during regeneration after partial hepatectomy and CCl_4 induced liver injury: proliferation of cells from the biliary epithelium with concomitant AFP expression and the appearance of a new phenotype, i.e. the proliferation of oval cells. Under the microscope, the enhanced mitotic activity of biliary epithelial cells led to pictures of increased numbers of bile ductular cross-sections which may correspond to a higher amount of biliary ducts, extensive arborization of expanding ductules, or alternatively to prolongation of ductular structures. This phenomenon was related to the term oval cell proliferation that originates in the terminal branches of the bile ductular system and in the canals of Hering at the hepatocyte-biliary interface (Alison *et al.* 1997; Kuhlmann and Wurster 1980; Saxena *et al.* 1999; Roskams *et al.* 2004; Sell 2003). Support for the role of some intraportal stem cells in liver repair comes also from studies with allyl alcohol (Yavorkovsky *et al.* 1995). The authors concluded from their experiments that restitutive proliferation of periportal necrosis by allyl alcohol might be accomplished by proliferation of intra-portal cells whose progeny differentiate and eventually repopulate the necrotic zone. Although proliferation of surviving hepatocytes adjacent to the injured zones could not be ruled out, proliferation of periportal and intraportal “stem cells” was predominant to replace necrotic areas. The importance of hepatic oval cells to be facultative stem cells that arise as a result of certain forms of liver injury has also been found in mouse models (Petersen *et al.* 2003).

The significant role of bile ducts in restitutive response is strongly supported, and oval cells will function as facultative liver stem cells. This is in agreement with the concept that proliferating cells in the liver include the original tissue-determined stem cells which are represented in the adult organ by cells of the canal of Hering (Sell 2001). When oval cells can

be regarded as functional progenitors for hepatocytes and cholangiocytes which can differentiate into hepatocytes or bile duct epithelia, i.e. the mature forms of the two hepatic epithelial cell lines, then, at least the terminal branches of the bile ductular system and the canals of Hering harbor the *intrahepatic* or *ductular stem cells*. With respect to the same embryonic origin of bile ducts and hepatocytes, the biliary epithelium and its proliferating oval cells have a defined role in liver regeneration as transit and amplification compartment.

The fate of progenitor cells with respect to their differentiation into hepatocytes or bile ducts will be governed by the liver microenvironment. In this connection, the observed inflammatory stress with its associated cytokine secretions will certainly play a role. Cytokines were suggested in cross-regulation of epithelial and mesenchymal elements by the formation of a regenerative unit in which hepatopoiesis will take place (Craig *et al.* 2004). The importance of growth modulators and cytokine signalling to stimulate proliferation, migration and differentiation of liver cells is well established. For example, the onset of hepatocyte proliferation after partial hepatectomy was shown to be accompanied by increased NF-κB activity (FitzGerald *et al.* 1995). Also, NF-κB activity is required for HGF-induced proliferation in a hepatic stem-like cell line (Yao *et al.* 2004). Furthermore, at the time when hepatocyte proliferation is blocked by toxic agents and when oval cells begin to proliferate, an expression of stem cell factor (SCF) and its receptor (c-kit) can be observed in the oval cell compartment (Fujio *et al.* 1996). This suggests that the SCF/c-kit system in combination with other growth factor system, f.e. growth and transforming growth factors (e.g. HGF, EGF; TGF), cytokines (TNF, IL, interferon γ network) and signalling pathways, are involved in the activation of hepatic stem-like cells as well as in their expansion and differentiation (Hu *et al.* 1996; Lemaigre 2003; Lowes *et al.* 2003).

Stem cells of extra-hepatic origin in liver regeneration

Oval cells are considered as progeny of intrahepatic stem cells. Now, there is also some evidence that hematopoietic stem cells can contribute to the development of hepatocytes. Indeed, a link between hematopoietic and hepatic cells is likely, at least during fetal development where the liver is the principal hematopoietic organ (Timens and Kamps 1997). In adult life, hematopoiesis can reemerge in liver during extreme stress and, this phenomenon may suggest the existence of a common stem cell (Masson *et al.* 2004). In any case, the observation of transdifferentiation or so-called plasticity of adult stem cells is of considerable interest. Most experiments which could show plasticity usually used cells derived from bone marrow with some evidence of reprogrammed adult stem cells to differentiate into other cell types, e.g. into hepatocytes and other epithelial cells including hepatic oval cells (Bjornson *et al.* 1999; Petersen *et al.* 1999; Alison *et al.* 2000; Clarke *et al.* 2000; Lagasse *et al.* 2000; Anderson *et al.* 2001; Krause *et al.* 2001; Holden and Vogel 2002; Korbling *et al.* 2002; Oh *et al.* 2002; Schwartz *et al.* 2002; Wagers and Weissman 2004). Apart from these findings, recent data have shown that the sources of oval cells are endogenous liver progenitors and that they do not arise through transdifferentiation from bone marrow cells (Menthena *et al.* 2004).

Some support of relationship between liver lineage cells and bone marrow comes from facts inasmuch as hepatic oval cells and hematopoietic stem cells share common markers such as CD34, Thy-1 and C-kit mRNA and protein (Fujio *et al.* 1994; Omori *et al.* 1997; Petersen *et al.* 1998; Crosby *et al.* 2002). Furthermore, the relationship between hematopoietic stem cells and liver could be deduced from combined transplantion and liver injury studies: for example, after transplantation of male bone marrow into lethally irradiated syngeneic females, the male Y-chromosome could be observed in the hepatocytes of the female recipient animal after liver

injury; or in transplantation experiments with different donor/recipient expression of marker molecules where the marker molecules were detected in the recipient hepatocytes. Hence, an extrahepatic source for liver repopulation seems possible (Petersen *et al.* 1999; Alison *et al.* 2000; Theise *et al.* 2000a; Theise *et al.* 2000b).

An alternative explanation for the development of plasticity may be the formation of hybrids by spontaneous cell fusion which gives rise to heterokaryons (Terada *et al.* 2002). Transplantation experiments and cytogenetic analyses support the possibility that hepatocytes being derived from bone marrow will arise from cell fusion instead of differentation of haematopoietic stem cells. Such cells were able to divide and, also, the expression of previously silent genes became induced (Ying *et al.* 2002; Wang *et al.* 2003).

AFP in hepatocarcinogenesis

Irrespective of low- or high-dose NNM feeding, hepatocellular carcinomas developed in each experimental group. With AFP appearance in serum, livers contained one or more distinct hepatocellular carcinomas. Some but not all of them would stain for AFP. Immunoexpression of AFP was restricted to hepatocellular carcinomas with their neoplastic hepatocytes. Normal liver tissue, dysplastic foci and hyperplastic nodules did not stain for AFP. In serial tissue sections processed by conventional histological stains, AFP positive cells exhibited a basophilic character. They were free of glycogen (PAS negative). The high proliferation activity was revealed by incorporation of [^{3}H]thymidine. Typically, up to 50% of the neoplastic hepatocytes became labelled in a 24 h pulse experiment while the percentage of labelled nuclei in normal hepatocytes stayed below 1%.

The heterogenous character of AFP producing hepatomas was reflected by measurements of transient rises and plateaus in serum AFP levels and by the behaviour of immunohistological stainings: (a) AFP-positive and AFP-negative carcinomas occurred side by side in a given histological preparation; (b) more or less stained carcinomas were seen in a given animal.

Apart from dose-dependent acute toxicity which is followed by regeneration, a multistep process of hepatoma induction is started. In the toxic stages with NNM application, hepatocyte necrosis was accompanied by massive proliferation of ductular epithelial cells together with AFP reappearance. Since both NNM schedules lead to hepatocellular carcinomas, oval cell proliferation and transitory AFP synthesis cannot be regarded as a prerequisite for conversion to cancer. Merely, oval cells resulted from restitution of damaged liver due to acute toxic injury by high dose NNM feeding. However, these present findings do not exclude that oval cells can be also a target for hepatoma development.

In their early proliferation stage, oval cells and ductular-like cells were heavily engaged in DNA synthesis ([^{3}H]thymidine labelling experiments). Moreover, pulse-chase experiments gave evidence for their development into hepatocytes. While still forming ductular-like structures, the AFP-positive cells reached the appearance of small-sized hepatocytes. Finally, areas of hyperplasia and nodular structure were found in cirrhotic livers. At this stage, DNA synthesis has come to a standstill and glycogen accumulation occurred.

From the above we have seen that oval cells will function as facultative progenitor cells for hepatocytes and biliary tract cells. In experimental hepatocarcinogenesis they can also give rise to regenerated hepatocytes with a high risk for transformation, and, also, to foci of altered hepatocytes (Dunsford *et al*. 1989). The latter are usually considered to be preneoplastic (Pitot

1990) with growth advantage over normal cells (Rabes *et al.* 1982). These properties are indicators of distinct stages of carcinogenesis. The role of oval cells in the histogenesis of liver carcinomas, however, is still debated. Some reports have evidence of an important role of oval cells in this direction (Tian *et al.* 1997; Libbrecht and Roskams 2002), while other experiments (without signs of liver injury and oval cell proliferation) concluded that precursor lesions will not originate from oval cells. Then, early foci and nodules must be derived from resistant hepatocytes (Anilkumar *et al*. 1995). The same conclusion was drawn from experiments in which parenchymal necrosis and massive oval cell proliferation were produced, but the development of foci of altered hepatocytes and hepatocellular adenomas led to phenotypes without the expression of cytokeratin 19 (a marker for bile duct epithelia). Consequently, this observation was reported as not to support a precursor-product relationship between oval and parenchymal cells; only the hepatocyte cell lineage being involved in the devolopment of hepatocellular tumors (Steinberg *et al.* 1991).

Hepatocellular carcinoma, retrodifferentiation and clonality

At the carcinoma stage when AFP appeared in our rat sera, AFP was stained in the cytoplasm of cells which by routine histology were typical basophilic and PAS-negative neoplastic hepatocytes. AFP was unequivocally caused by the carcinoma cells, and these cells were easily distinguished by their size and shape from normal adult hepatocytes. Most often, both AFP-positive and AFP-negative hepatocellular carcinomas were observed as circumscribed areas within the liver of a given animal. Hence, not every carcinoma was an AFP producer. Moreover, AFP-positive and AFP-negative nodules could be observed side by side. In [^{3}H]thymidine pulse labelling experiments, both AFP-positive and AFP-negative carcinoma cells showed active proliferation. Based on the observations in the present study, we suggest that the AFP-positive population represents distinct neoplastic hepatocytes with a certain degree of differentiation and of clonal origin. Serum AFP levels are the result from synthesis, secretion and turnover of this marker, respectively, and the rising rates of serum AFP in the developmental course of hepatocellular carcinomas are indeed useful indicators for production and secretion of this oncofetal protein by the carcinoma cells. The heterogeneity of phenotypic cell markers and differences in growth rates of foci and precancerous nodules is known from most of the experimental models, and this heterogeneity will point to different cellular origins as well as to variations of malignant potency of preneoplastic lesions. In any case, the possibility of a random clonal origin of hepatocellular carcinomas from mature hepatocytes was definitively shown by a recently published method which used genetic labelling of hepatocytes (Bralet *et al.* 2002).

Neoplasia may be preceded or accompanied by molecular and morphologic patterns which are characteristic for cells with variable degree of maturity. Thus, fetal patterns of gene expression are observed which led to the hypothesis that the emergence of tumors correlates with a process of retrodifferentiation (Uriel 1976; Uriel 1979). Retrodifferentiation is inverse to differentiation, i.e. reversing the maturation process and programming of mature cells backwards along the normal developmental pathway. This formulation was inferred from the dynamics of fetospecific antigens and isozymic patterns during ontogenic and neoplastic growth. Although foetal antigens such as AFP are reexpressed in hepatoma cells, they are not considered as characteristic of malignancy because such molecules can emerge in tissues undergoing nonmalignant growth. Hence, the term “transitory cell antigens” appears to be more significant for these biomolecules which are usually restricted to a transient period of cell differentiation. Clonality and retrodifferentiation succeed in selecting cell populations

with highest autonomy and unresponsiveness to regulatory principles operating in normal organisms.

Finally, the development of hepatocellular carcinomas is a multistep process. Tumors arise from a pool of differentiating oval cells or by dedifferentiation of mature hepatocytes because both cell types have stem-like properties. The lineage and phenotype of chemically induced liver carcinoma may arise from a single cell through genetic and epigenetic alterations; a clonal origin and expansion is probable. AFP resurgence is associated with the appearance of hepatocellular carcinoma and reflects a process of retrodifferentiation. Clonality and retrodifferentiation succeed in selecting cell populations with highest autonomy.

The lack of a unique stem cell marker in the research of stem cells in general and also in liver regeneration remains a problem. The possibility of lineage plasticity and transdifferentiation of potential hepatic stem cells is still a matter of controversy, but the value of stem cells seems to be considerable in either conventional studies of differentiation or in studies on self-renewing potential and in therapy.

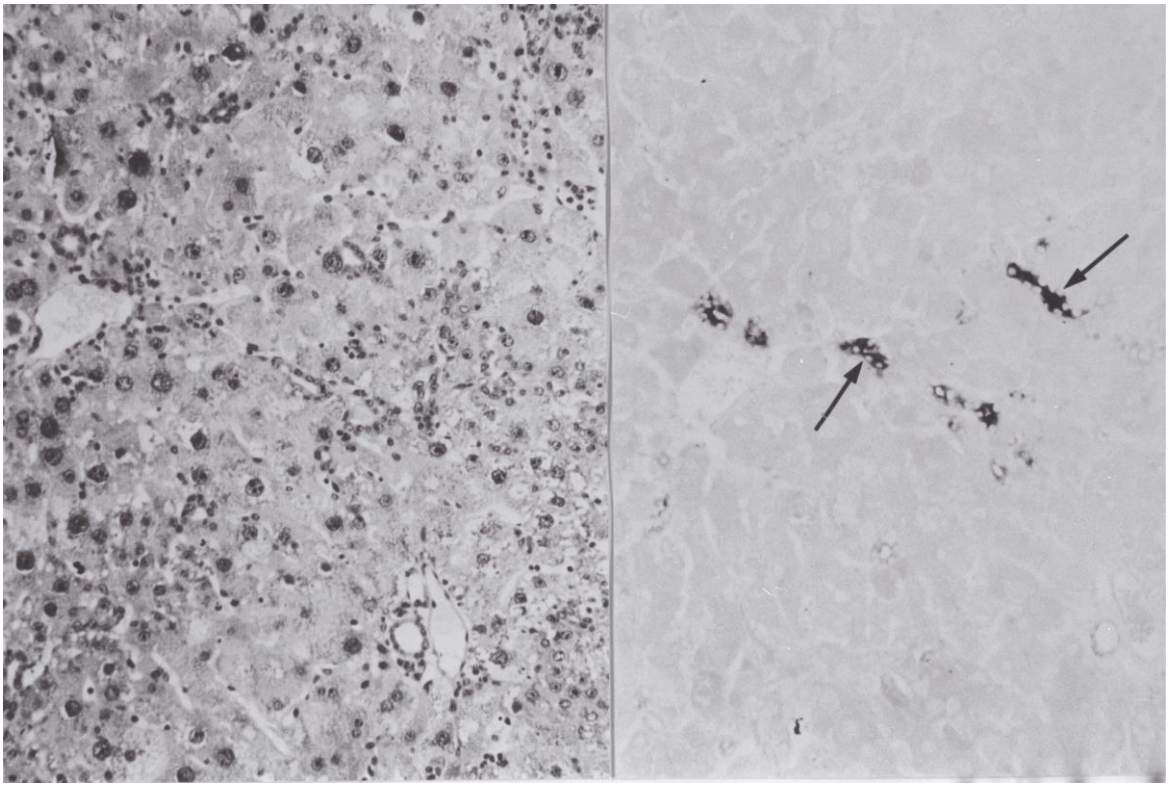

Fig. 3a-b. Liver from rat after 28 days of NNM feeding (20 mg/kg). Serial sections stained with HE (a) and for AFP (b). Note nests of small, oval-shaped cells (←) with cytoplasmic AFP staining.

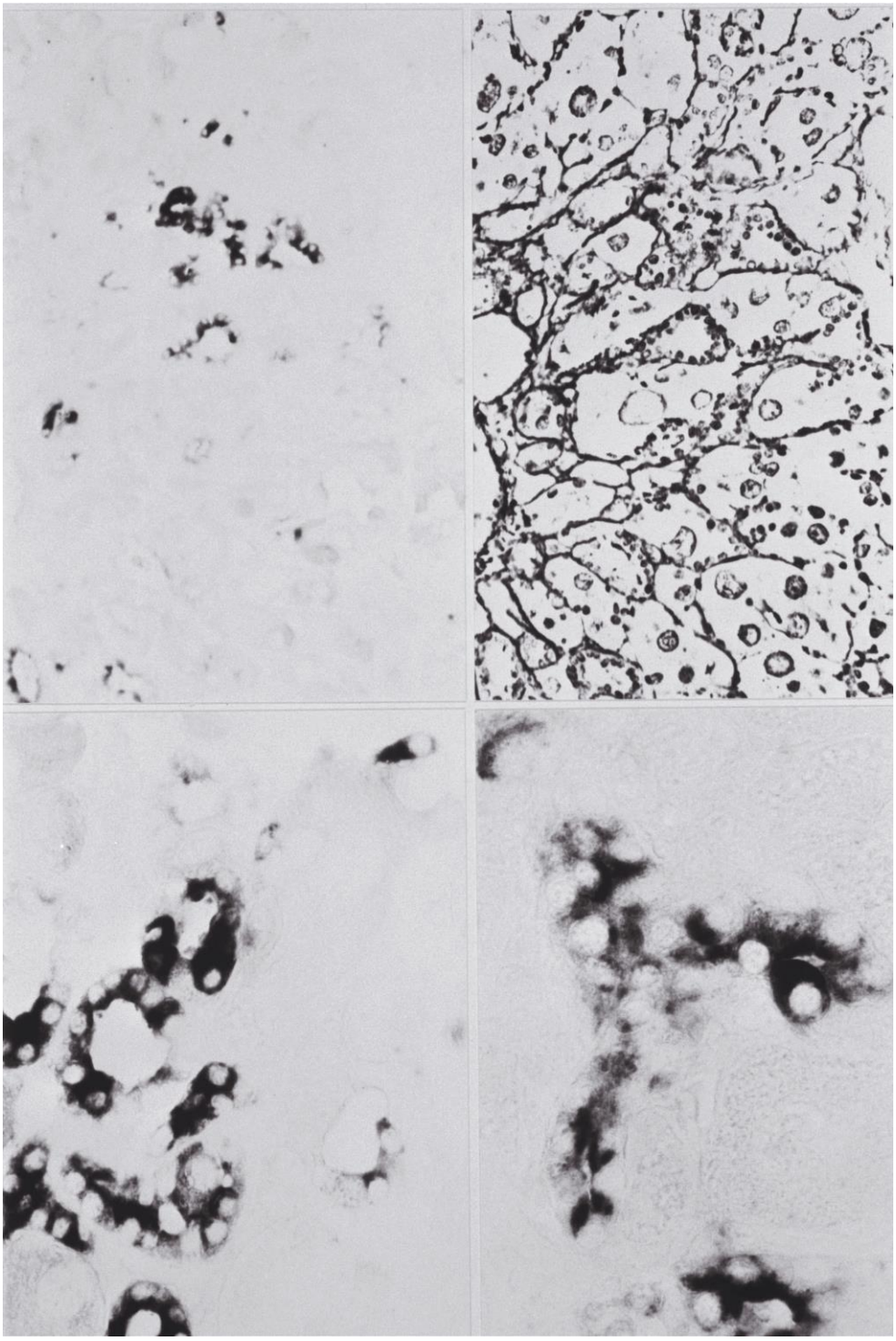

Fig. 4a-b. Liver from day 35 of NNM intoxication. Note localization of AFP in grouped cells which form canalicular epithelial structures. (a) Immunoperoxidase labelling of AFP. (b) Same preparation as (a) after Gomori's silver impregnation.

Fig. 5a-b. Higher magnification view of AFP staining in canalicular epithelial cells, bile duct epithelium.

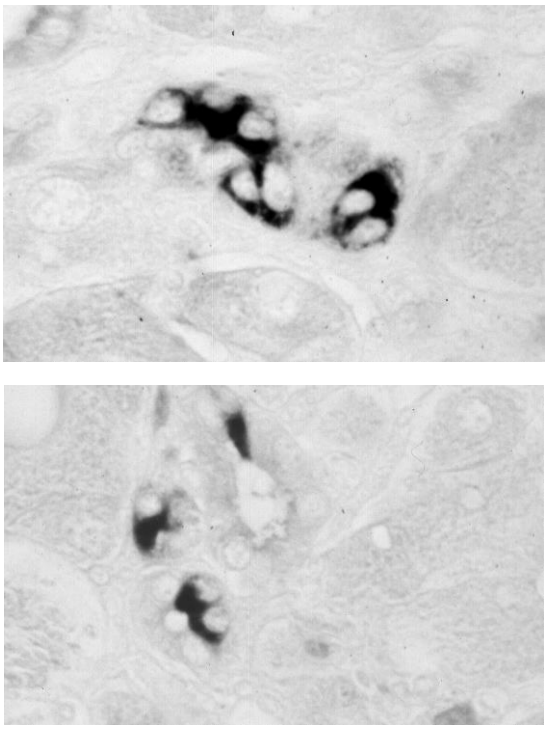

Fig. 6a-b. Liver from day 35 of NNM intoxication. Immunoperoxidase labelling of AFP; note nests of oval-shaped cells which form canalicular epithelial structures.

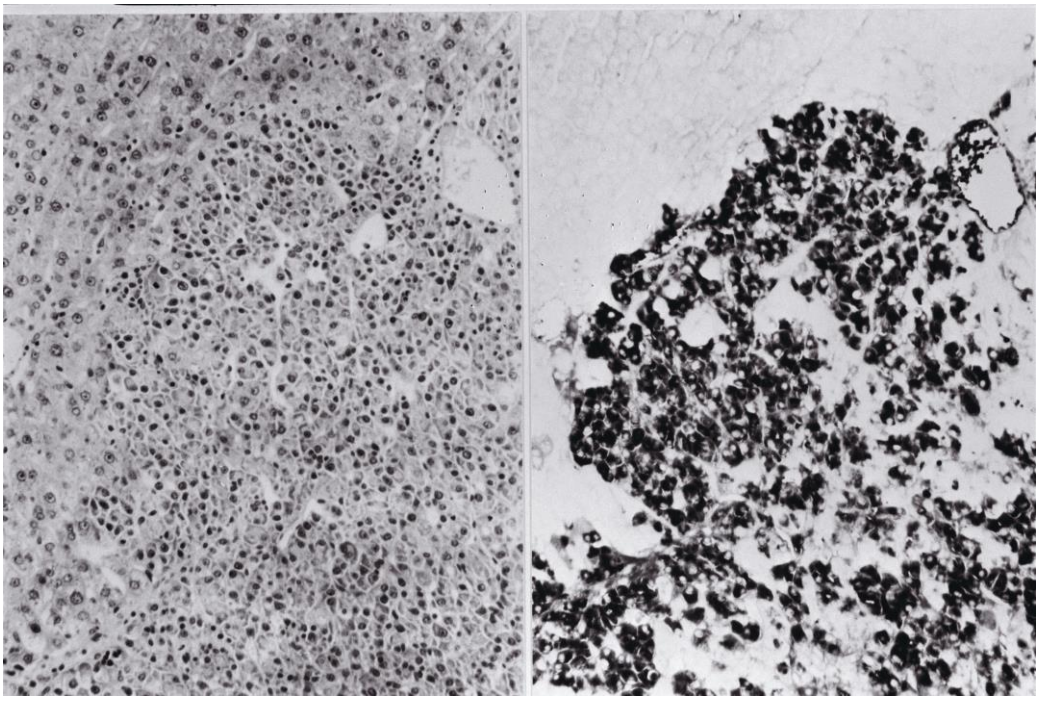

Fig. 7 a-b. Localization of AFP at the hepatoma stage. (a) Liver section stained by HE. (b) Serial section from same tissue and reacted for AFP; note AFP-staining in cells of hepatocellular carcinoma while normal hepatocytes do not stain.

References

Abelev GI. Alpha-fetoprotein in ontogenesis and its association with malignant tumors. *Adv. Cancer Res.* **14**, 295-358, 1971

Abelev GI et al. Production of embryonal α-globulin by transplantable mouse hepatomas. *Transplantation* **1**, 174-180, 1963

Alison MR. Regulation of hepatic growth. *Physiol. Rev*. **66**, 499-541, 1986

Alison MR et al. Wholesale hepatocytic differentiation in the rat from ductular oval cells, the progeny of biliary stem cells. *J. Hepatol.* **26**, 343-352, 1997

Alison MR et al. Hepatocytes from non-hepatic adult stem cells. *Nature* **406**, 257, 2000

Anderson DJ et al. Can stem cells cross lineage boundaries? *Nat. Med*. **7**, 393-395, 2001

Anilkumar TV et al. The resistant hepatocyte model of carcinogenesis in the rat: the apparent independent development of oval cell proliferation and early nodules. *Carcinogenesis* **16**, 845-853, 1995

Bade EG et al. Autoradiographic study of DNA-synthesis in the regenerating liver of the mouse. *Exp. Cell Res.* **44**, 676-678, 1966

Bakirov RD. Appearance of embryonal serum α-globulin in adult mice after inhalation of carbon tetrachloride. *Byull. Eksp. Biol. Med.* **2**, 45-47, 1968

Bannasch P. The cytoplasm of hepatocytes during carcinogenesis. *Recent Results in Cancer Research* **19**, 1-105, 1968

Bannasch P. Die Cytologie der Hepatocarcinogenese. In: Altmann HW et al. (eds.) *Handbuch der allgemeinen Pathologie*. Vol. 6, pp. 123-276, Springer Verlag, Berlin Heidelberg New York 1975

Bannasch P, Müller HA. Lichtmikroskopische Untersuchungen über die Wirkung von N-Nitrosomorpholin auf die Leber von Ratte und Maus. *Arzneim. Forsch. (Drug Res.)* **14**, 805-814, 1964

Bernuau D et al. In situ cellular analysis of alpha-fetoprotein gene expression in regenerating rat liver after partial hepatectomy. *Hepatology* **8**, 997-1005, 1988

Bjornson CR et al. Turning brain into blood: a hematopoietic fate adopted by adult neural stem cells in vivo. *Science* **283**, 534-537, 1999

Bralet MP et al. Demonstration of direct lineage between hepatocytes and hepatocellular carcinoma in diethylnitrosamine-treated rats. *Hepatology* **36**, 623-630, 2002

Brues AM et al. A quantitative study of cell growth in regenerating liver. *Arch. Pathol.* **22**, 658-673, 1936

Clarke DL et al. Generalized potential of adult neural stem cells. *Science* **288**, 1660-1663, 2000

Craig CE et al. The histopathology of regeneration in massive hepatic necrosis. *Semin. Liver Dis*. **24**, 49-64, 2004

Crosby HA et al. Progenitor cells of the biliary epithelial cell lineage. *Semin. Cell Dev. Biol.* **13**, 397-403, 2002

Delpré G, Gilat T. Revue générale. L'Alpha-foeto-protéine. Deuxième partie. *Gastroenterol. Clin. Biol.* **2**, 193-214, 1978

Dempo K et al. Immunofluorescent study on α-fetoprotein-producing cells in the early stage of 3'-methyl-4-dimethyl-aminoazobenzene carcinogenesis. *Cancer Res.* **35**, 1282-1287, 1975

Druckrey H et al. Organotrope carcinogene Wirkungen bei 65 verschiedenen N-Nitroso-Verbindungen an BD-Ratten. *Z. Krebsforsch.* **69**, 103-201, 1967

Dunsford HA et al. Different lineages of chemically induced hepatocellular carcinoma in rats defined by monoclonal antibodies. *Cancer Res*. **49**, 4894-4900, 1989

Engelhardt NV et al. Immunofluorescent study of alpha-foetoprotein (α fp) in liver and liver tumors. I. Technique of α fp localization in tissue sections. *Int. J. Cancer* **7**, 198-206, 1971

Engelhardt NV et al. Detection of α-foetoprotein in mouse liver differentiated hepatocytes before their progression through S phase. *Nature (Lond. New Biol.)* **263**, 146-148, 1976

Fausto N. Liver regeneration. *J. Hepatol*. **32**, 19-31, 2000

FitzGerald MJ et al. Rapid DNA binding by nuclear factor kappa B in hepatocytes at the start of liver regeneration. *Cell Growth Differ*. **6**, 417-427, 1995

Fujio K et al. Expression of stem cell factor and its receptor, c-kit, during liver regeneration from putative stem cells in adult rat. *Lab. Invest*. **70**, 511-516, 1994

Fujio K et al. Coexpression of stem cell factor and c-kit in embryonic and adult liver. *Exp. Cell Res*. **224**, 243-250, 1996

Gitlin D, Boesman M. Fetus-specific serum proteins in several mammals and their relation to human α-fetoprotein. *Comp. Physiol.* **21**, 327-336, 1967

Gitlin D, Boesman M. Sites of serum α-fetoprotein synthesis in the human and in the rat. *J. Clin. Invest.* **46**, 1010-1016, 1967

Gitlin D et al. Cellular distribution of serum α-fetoprotein in organs of the foetal rat. *Nature (Lond.)* **215**, 534, 1967

Goussev AI et al. Immunofluorescent study of alpha-foetoprotein (α fp) in liver and liver tumors. II. Localization of α fp in the tissues of patients with primary liver cancer (PLC). *Int. J. Cancer* **7**, 207-217, 1971

Graham RC, Karnovsky, MJ. The early stages of absorption of injected horseradish peroxidase in the proximal tubules of mouse kidney: ultrastructural cytochemistry by a new techni-que. *J. Histochem. Cytochem.* **14**, 291-302, 1966

Grisham JW. A morphologic study of deoxyribonucleic acid synthesis and cell proliferation in regenerating rat liver; autoradiography with thymidine-H^3. *Cancer Res.* **22**, 842-849, 1962

Guillouzo A et al. Light and electron microscope immunolocalization of alpha-fetoprotein in rat liver cells in vivo and in vitro. *Scand J. Immunol.* **8** (suppl.), 289-296, 1978

Heby O, Lewan L. Putrescine and polyamines in relation to nucleic acids in mouse liver after partial hepatectomy. *Virchows Arch. (Cell Pathol.)* **8**, 58-66, 1971

Higgins GM, Anderson RM. Experimental pathology of the liver: restoration of the liver of the white rat following partial surgical removal. *Arch. Pathol.* **12**, 186-202, 1931

Holden C, Vogel G. Stem cells. Plasticity: time for a reappraisal? *Science* **296**, 2126-2129, 2002

Hu Z et al. Expression of transforming growth factor alpha/epidermal growth factor receptor, hepatocyte growth factor/c-met and acidic fibroblast growth factor/fibroblast growth factor receptors during hepatocarcinogenesis. *Carcinogenesis* **17**, 931-938, 1996

Inaoka Y. Significance of the so-called oval cell proliferation during azo-dye hepato-carcinogenesis. *Gann* **58**, 355-366, 1967

Institute of Laboratory Animal Resources. Histologic typing of liver tumors of the rat. *J. Natl. Cancer Inst.* **64**, 179-206, 1980

Korbling M et al. Hepatocytes and epithelial cells of donor origin in recipients of peripheral-blood stem cells. *N. Engl. J. Med*. **346**, 738-746, 2002

Krause DS et al. Multi-organ, multi-lineage engraftment by a single bone marrow-derived stem cell. *Cell* **105**, 369-377, 2001

Kuhlmann WD. Purification of mouse alpha-1-fetoprotein and preparation of specific peroxidase conjugates for its cellular localization. *Histochemistry* **44**, 155-167, 1975

Kuhlmann WD. Immunocytological studies on alpha-1-fetoprotein producing cells under normal and pathological conditions. In: Peeters H (ed.) *Protides of the biological fluids*, vol. 24, pp. 269-276, Pergamon Press, Oxford 1976

Kuhlmann WD. Ultrastructural immunoperoxidase cytochemistry. *Progr. Histochem. Cytochem.* **10**, 1-57, 1977

Kuhlmann WD. Localization of alpha-1-fetoprotein and DNA-synthesis in liver cell populations during experimental hepatocarcinogenesis in rats. *Int. J. Cancer* **21**, 368-380, 1978

Kuhlmann WD. Ultrastructural detection of alpha-1-fetoprotein in hepatomas by use of peroxidase-labelled antibodies. *Int. J. Cancer* **22**, 335-343, 1978

Kuhlmann WD. Immuno-electron microscopy of α-fetoprotein during normal development of rat hepatocytes. *J. Ultrastruct. Res.* **68**, 109-117, 1979

Kuhlmann WD. Immunoperoxidase labelling of alpha-1-fetoprotein (AFP) in normal and regenerating livers of a low and a high AFP producing mouse strain. *Histochemistry* **64**, 67-75, 1979

Kuhlmann WD et al. A comparative study for ultrastructural localization of intracellular immunoglobulins using peroxidase conjugates. *J. Immunol. Meth.* **5**, 33-48, 1974

Kuhlmann WD, Wurster K. Correlation of histology and alpha-1-fetoprotein resurgence in rat liver regeneration after experimental injury by galactosamine. *Virchows Arch. A Pathol. Anat. Histol.* **387**, 47-57, 1980

Lagasse E et al. Purified hematopoietic stem cells can differentiate into hepatocytes in vivo. *Nat Med*. **6**, 1229-1234, 2000

Lannér M et al. Purification of enzyme-labelled conjugate by affinity chromatography. In: Hoffmann-Ostenhof O et al. (eds.) *Affinity chromatography*, pp. 237-241, Pergamon Press, Oxford 1978

Laurence DJR, Neville AM. Foetal antigens and their role in the diagnosis and clinical management of human neoplasms: a review. *Brit. J. Cancer* **26**, 335-355, 1972

Le Bouton AV. Extrusion of nascent albumin into the cytosol: an artifact. *Anat. Rec.* **190**, 457, 1978

Lemaigre FP. Development of the biliary tract. *Mech. Dev*. **120**, 81-87, 2003

Libbrecht L, Roskams T. Hepatic progenitor cells in human liver diseases. *Semin. Cell Dev. Biol*. **13**, 389-396, 2002

Lindahl G et al. Mouse alpha-fetoprotein: genetic studies. *Scand. J. Immunol.* **8** (suppl. 8), 209-212, 1978

Lowes KN et al. Oval cell-mediated liver regeneration: Role of cytokines and growth factors. *J. Gastroeneterol. Hepatol*. **18**, 4-12, 2003

Masson S et al. Potential of hematopoietic stem cell therapy in hepatology: a critical review. *Stem Cells* **22**, 897-907, 2004

Menthena A et al. Bone marrow progenitors are not the source of expanding oval cells in injured liver. *Stem Cells* **22**, 1049-1061, 2004

Miller LL, Bale WE. Synthesis of all plasma protein fractions except gamma globulins by the liver. The use of zone electrophoresis and lysin-ε-C^{14} to define the plasma proteins synthesized by the isolated perfused liver. *J. Exp. Med.* **99**, 125-132, 1954

Mohanty M et al. Cellular basis of induced α-fetoprotein synthesis by hepatocytes of adult mouse after hepatotoxic injury and partial hepatectomy. *Int. J. Cancer* **22**, 181-188, 1978

Nishioka M et al. Localization of α-fetoprotein in hepatoma tissues by immunofluorescence. *Cancer Res.* **32**, 162-166, 1972

Okita K et al. Localization of α-fetoprotein by immunofluorescence in hyperplastic nodules during hepatocarcinogenesis induced by 2-acetylaminofluorene. *Cancer Res.* **34**, 2758-2763, 1974

Olsson M et al. Genetic control of alpha-fetoprotein synthesis in the mouse. *J. Exp. Med.* **145**, 819-827, 1977

Omori N et al. Partial cloning of rat CD34 cDNA and expression during stem cell-dependent liver regeneration in the adult rat. *Hepatology* **26**, 720-727, 1997

Onoé T et al. Significance of α-fetoprotein appearance in the early stage of azo-dye carcinogenesis. *Gann Monogr. Cancer Res.* **14**, 233-247, 1973

Palmer PE, Wolfe HJ. α_1-Antitrypsin deposition in primary hepatic carcinomas. *Arch. Path. Lab. Med.* **100**, 232-236, 1976

Perova SD et al. α-Fetoprotein in rat sera after partial hepatectomy. *Byull. Eksp. Biol. Med.* **71**, 45-47, 1971

Petersen BE et al. Bone marrow as a potential source of hepatic oval cells. *Science* **284**, 1168-1170, 1999

Petersen BE et al. Hepatic oval cells express the hematopoietic stem cell marker Thy-1 in the rat. *Hepatology* **27**, 433-445, 1998

Petersen BE et al. Mouse A6-positive hepatic oval cells also express several hematopoietic stem cell markers. *Hepatology* **37**, 632-640, 2003

Pihko H, Ruoslahti E. Alpha fetoprotein production in normal and regenerating mouse liver. In: Masseyeff R (ed.) *L'Alpha-foetoprotéine*, pp. 333-336, INSERM, Paris 1974

Pitot HC Altered hepatic foci: their role in murine hepatocarcinogenesis. *Annu. Rev. Pharmacol Toxicol.* **30**, 465-500, 1990

Popper H et al. Ductular cell reaction in the liver in hepatic injury. *J. Mt. Sinai Hosp.* **24**, 551-556, 1957

Porter KR. The ground substance: observations from electron microscopy. In: Brachet J, Mirsky AE (eds.) *The cell*, pp. 621-675, Academic Press, New York London 1961

Post J et al. Responses of the liver to injury. Effects of age upon the healing pattern after acute carbon tetrachloride poisoning. *Arch. Pathol.* **70**, 314-321, 1960

Price JM et al. Progressive microscopic alterations in the livers of rats fed the hepatic carcinogens, 3'-methyl-4-dimethylaminoazobenzene and 4'-fluoro-4-dimethylaminoazo-benzene. *Cancer Res.* **12**, 192-200, 1952

Rabes H, Tuczek HV. Quantitative autoradiographische Untersuchung zur Heterogenität der Leberzellproliferation nach partieller Hepatektomie. *Virchows Arch. (Cell Pathol.)* **6**, 302-312, 1970

Rabes HM et al. Clonal growth of carcinogen-induced enzyme-deficient preneoplastic cell populations in mouse liver. *Cancer Res.* **42**, 3220-3227, 1982

Reynolds ES. The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. *J. Cell Biol.* **17**, 208-212, 1963

Roskams TA et al. Nomenclature of the finer branches of the biliary tree: canals, ductules, and ductular reactions in human livers. *Hepatology* **39**, 1739-1745, 2004

Ruoslahti E, Seppälä M. α-Fetoprotein in cancer and fetal development. *Adv. Cancer Res.* **29**, 275-346, 1979

Saxena R et al. Microanatomy of the human liver – exploring the hidden interfaces. *Hepatology* **30**, 1339-1346, 1999

Schultze B et al. Autoradiographische Untersuchung über die Proliferation der Hepatocyten bei der Regeneration der CCl_4-Leber der Maus. *Virchows Arch. (Cell Pathol.)* **14**, 329-343, 1973

Schwartz RE et al. Multipotent adult progenitor cells from bone marrow differentiate into functional hepatocyte-like cells. *J Clin. Invest*. **109**, 1291-1302, 2002

Sell S et al. Hepatocyte proliferation and α_1-fetoprotein in pregnant, neonatal, and partially hepatectomized rats. *Cancer Res.* **34**, 865-871, 1974

Sell S et al. Expression of an oncodevelopmental gene product (α-fetoprotein) during fetal development and adult oncogenesis. *Cancer Res.* **36**, 4239-4249, 1976

Sell S. The role of progenitor cells in repair of liver injury and in liver transplantation. *Wound Repair Regen*. **9**, 467-482, 2001

Sell S. The hepatocte: heterogeneity and plasticity of liver cells. *Int. J. Biochem. Cell Biol*. **35**, 267-271, 2003

Shinozuka H et al. Early histological and functional alterations of ethionine liver carcinogenesis in rats fed a choline-deficient diet. *Cancer Res.* **38**, 1092-1098, 1978

Smuckler EA et al. α-Fetoprotein in toxic liver injury. *Cancer Res.* **36**, 4558-4561, 1976

Steinberg P et al. Enzyme histochemical and immunohistochemical characterization of oval and parenchymal cells proliferating in livers of rats fed a choline-deficient/DL-ethionine-supplemented diet. *Carcinogenesis* **12**, 225-231, 1991

Sternberger LA. *Immunocytochemistry*. Prentice-Hall, Englewood Cliffs 1974

Stowell RE, Lee CS. Histochemical studies of mouse liver after single feeding of carbon tetrachloride. *Arch. Pathol.* **50**, 519-537, 1950

Taketa K et al. Different mechanisms of increased α-fetoprotein production in rats following CCl_4 intoxication and partially hepatectomy. *Ann. N. Y. Acad. Sci.* **259**, 80-84, 1975

Tchipysheva TA et al. α-Fetoprotein-containing cells in the early stages of liver carcinogenesis induced by 3'-methyl-4-dimethylaminoazobenzene and 2-acetylamino-fluorene. *Int. J. Cancer* **20**, 388-393, 1977

Teilum G et al. The histogenetic-embryologic basis for reappearance of alpha-fetoprotein in endodermal sinus tumors (yolk sac tumors) and teratomas. *Acta Pathol. Microbiol. Scand. (A)* **83**, 80-86, 1975

Terada N et al. Bone marrow cells adopt the phenotype og other cells by spontaneous cell fusion. *Nature* **416**, 542-545, 2002

Theise ND et al. Derivation of hepatocytes from bone marrow cells in mice after irradiation-induced myeloablation. *Hepatology* **31**, 235-240, 2000

Theise ND et al. Liver from bone marrow in humans. *Hepatology* **32**, 11-16, 2000

Tian YW et al. The oval-shaped cell as a candidate for a liver stem cell in embryonic, neonatal and precancerous liver: identification based on morphology and immunohisto-chemical staining for albumin and pyruvate kinase isoenzyme expression. *Histochem. Cell Biol.* **107**, 243-250, 1997

Timens W, Kamps WA. Hemopoiesis in human fetal and embryonic liver. *Microsc. Res. Tech*. **39**, 387-397, 1997

Tournier I et al. Cellular analysis of alpha-fetoprotein gene activation during carbon tetrachloride and D-galactosamine-induced acute liver injury in rats. *Lab. Invest*. **59**, 657-665, 1988

Tsuboi KK, Stowell RE. Enzyme alterations associated with mouse liver degeneration and regeneration after single carbon tetrachloride feeding. *Cancer Res.* **11**, 221-228, 1951

Uriel J. Cancer, retrodifferentiation, and the myth of Faust. *Cancer Res.* **36**, 4269-4275, 1976

Uriel J. Retrodifferentiation and the fetal pattern of gene expression in cancer. *Adv. Cancer Res.* **29**, 127-174, 1979

Wagers AJ, Weissman IL. Plasticity of adult stem cells. *Cell* **116**, 639-648, 2004

Wang X et al. Cell fusion is the principal source of bone-marrow-derived hepatocytes. *Nature* **422**, 897-901, 2003

Watanabe A et al. Differential mechanisms of increased α_1-fetoprotein production in rats following carbon tetrachloride injury and partial hepatectomy. *Cancer Res.* **36**, 2171-2175, 1976

Yao P et al. Hepatocyte growth factor-induced proliferation of hepatic stem-like cells depends on activation of NF-kappaB. *J. Hepatol*. **40**, 391-398, 2004

Yavorkovsky L et al. Participation of small intraportal stem cells in the restitutive response of the liver to periportal necrosis induced by allyl alcohol. *Hepatology* **21**, 1702-1712, 1995

Ying QL et al. Changing potency by spontaneous fusion. *Nature* **416**, 545-548, 2002

Yokoyama HO et al. Regeneration of mouse liver after partial hepatectomy. *Cancer Res.* **13**, 80-85, 1953