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Stem Cell Review

Liver stem cells: when the going gets tough they get going

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Summary. The ability of the liver to regenerate is widely acknowledged, and this is usually accomplished by the entry of normally proliferatively quiescent hepatocytes into the cell cycle. However, when hepatocyte regeneration is impaired, small bile ducts proliferate and invade into the adjacent hepatocyte parenchyma. In humans and experimental animals these ductal cells are referred to as oval cells, and their association with defective regeneration has led to the belief that they are the progeny of facultative stem cells. Oval cells are of great biological interest since they may represent a target population for hepatic carcinogens, and they may also be useful vehicles for *ex vivo* gene therapy for the correction of inborn errors of metabolism.

The ability of oval cells to differentiate into hepatocytes has been demonstrated unequivocally. However, this process only occurs when the regenerative capacity of hepatocytes is overwhelmed, and thus, unlike the intestinal epithelium, the liver is not behaving as a classical continually renewing stem cell-fed lineage.

Keywords: Oval cells, liver, bile ducts, hepatocellular carcinoma, cholangiocarcinoma, stem cells.

Liver development

The liver primordium consists of endodermal and mesodermal components. Ventral foregut endoderm gives rise to hepatoblasts which form the parenchyma (hepatocytes), while both sinusoidal-lining cells and connective tissue components originate from the mesenchymal tissue which is invaded by the hepatic cords (Shiojiri *et al.* 1991). The primitive hepatoblasts which surround the portal mesenchyme form a double- layered cylinder of hepatocytes, the ‘ductal plate’ (see Figure 2a), which remodels and migrates into the portal mesenchyme to form the intrahepatic bile ducts (Van-Eyken *et al.* 1988a; Shiojiri *et al.* 1991). These cells synthesize the hepatocyte-specific proteins alpha-foetoprotein (AFP) and albumin as they migrate into the portal stroma and additionally begin to express the bile duct specific enzyme, gamma glutamyl transferase (GGT). Initially, in the intrahepatic bile ducts, intermediate filament expression is restricted to cytokeratins (CK) 8 and 18, but during the later stages of ductular morphogenesis, the ductal cells begin to express the characteristic biliary CKs 7, 8, 18, and 19 (Moll *et al.* 1982), though some continue to express hepatocyte traits such as AFP and albumin synthesis for the first 7–14 days after birth (Shiojiri *et al.* 1991). Thus, ductal plate cells transiently express both hepatocyte and ductal markers and can be

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considered to be oval cell equivalents. Indeed, the oval cell response which ultimately generates new hepatocytes is, in effect, a reversal of ontogeny. Those hepatoblasts not in contact with the portal mesenchyme differentiate into hepatocytes which form the liver cell plates, and in these cells the cytokeratin complement is restricted to 8 and 18.

In humans at 8–14 weeks gestation, bipotential progenitor cells may be both CK14- and CK19-positive, and commitment to the biliary lineage might be signalled by increased CK19 expression, loss of CK14 and transient expression of vimentin (Haruna *et al.* 1996). Short-lived expression of vimentin is also a feature of oval cells before they differentiate. In the mouse too, hepatoblasts from the liver diverticulum on day 9.5 of gestation are bipotential, and *in vitro* such cells can be made to differentiate into either hepatocytes or biliary cells (Rogler 1997).

Organization of the biliary tree

Periportal hepatoblasts give rise to bile ducts during liver development (the hepatocytogenetic theory of bile duct development), and in the adult the bile ducts remain connected to the hepatocyte parenchyma through a complex network of bile canaliculi (intralobular passages bound by parenchymal cells). The canalicular network drains the bile produced by the hepatocytes to the portal tract interface. The bile initially passes through ductules composed of a limiting plate hepatocyte and specialized duct cells (Steiner & Carruthers 1961), and more distally cholangioles (also called canals of Hering) lined exclusively by squamous ductular cells. From here bile passes into the peripheral or marginal interlobular ducts, and then into larger septal bile ducts (Millward-Sadler & Jezequel 1992), ultimately entering into the duodenum *via* the extrahepatic bile ducts.

The proliferative organization of the adult liver: to continually renew or not

In rapidly renewing epithelia (e.g. gut mucosa and epidermis), the stem cells represent the progenitors of the migrating and differentiating cells (Potten & Loeffler 1990; Potten *et al.* 1997). For the liver it has been suggested that the hepatic plates represent the trajectory along which cells migrate centrifugally from the periportal areas to the centrilobular regions, where they ultimately die by apoptosis (Zajicek *et al.* 1985). Following the fate of tritiated thymidine labelled cells in adult rat liver over a 5-week period, hepatocytes were thought to have moved a distance of 46 μm (approximately 1 cell) from the portal rim towards the hepatic vein. This observation led to the 'streaming liver ' hypothesis which has engendered considerable discussion (Grisham 1994; Correspondence 1995). Most significantly, studies utilizing genetic labelling with a *Escherichia coli* β-galactosidase gene do not support the 'streaming liver' hypothesis. For example, when rat hepatocytes are labelled *in vivo* at 24 h after a two-thirds partial hepatectomy (2/3PH) by an amphotropic retrovirus carrying the β-galactosidase gene coupled to a nuclear localization signal, then initially most labelled cells are located in the periportal and mid-zonal regions; but since the distribution of labelled cells did not change over the proceeding 15 months, this strongly argued against the streaming liver hypothesis (Bralet *et al.* 1994). A similar conclusion was drawn from studies of transgenic mice where the β-galactosidase gene was driven by a human α1-antitrypsin promoter (Kennedy *et al.* 1995). Here, neither postnatal growth nor a subsequent 2/3PH changed the distribution of labelled cells; the labelled cells simply presented as larger cell clusters with the passage of time.

Thus, it seems likely that in the unperturbed state, hepatocytes do not move in a unidirectional manner, but instead remain fairly static. Furthermore, the modest level of proliferative activity (approximately 3 cells/1000 in DNA synthesis at any one time) is probably a compensatory response to random 'wear and tear' cell loss.

Cell proliferation in response to wounding and other stimuli

The liver is able to mount a prompt proliferative response to hepatotoxic insult and other hyperplastic stimuli. These reactions may be categorized as follows:

- Regenerative or compensatory hyperplasia;
- Additive liver growth;
- Stem cell-driven (oval cell) reactions.

A wide variety of xenobiotics, e.g. paracetamol and carbon tetrachloride, cause parenchymal cell death. Such xenobiotics invariably, though not exclusively, cause centrilobular necrosis since the affected cells possess the enzymatic capacity to metabolize the compounds to their hepatotoxic metabolites. A useful model to study regeneration has been the rat liver after a 2/3PH. Resection triggers the hepatocytes in the remnant lobes to exit the G_0 phase and, after a lag of at least 15 h to enter DNA synthesis (Wright & Alison 1984; Alison 1986; Gerlach *et al.* 1997). Since this response actually occurs in quite separate lobes, this is strictly speaking viewed as a compensatory hyperplasia, though in practice the terms 'regeneration' and 'compensatory hyperplasia' are used synonomously. Of course, the reconstitution

of the liver architecture also involves the proliferation of all other cell types normally present in the liver, though their amplification tends to be delayed in comparison to hepatocytes. A second type of growth reaction occurs in response to a wide variety of xenobiotics which are not cytotoxic, but instead can be substrates (inducers) for the cytochrome P450 family of enzymes (phenobarbitone) or peroxisome proliferators such as the hypolipidaemic agent called nafenopin. These agents stimulate hypertrophy and/or hyperplasia, but withdrawal of such stimuli triggers a rapid reversion to normal liver size through induction of hepatocyte apoptosis.

The third category of liver growth does not involve hepatocytes, emanating instead from interlobular bile ducts (Figure 1). The extensive formation of biliary-derived ductules emerging from the portal tracts of rodents fed chemical carcinogens was first noted in 1944 (Opie 1944). Farber (1955) used the descriptive term of 'oval cells' to describe the cells which formed the expanding biliary network during such feeding regimes. The importance of such biliary hyperplasia was not appreciated until 1958 (Wilson & Leduc 1958) when it was shown by electron microscopy that oval cells, or as they called them, 'cholangioles', ultimately differentiated into cells which had all the morphological features of mature hepatocytes. Despite protracted scepticism, it is now quite clear that oval cells can differentiate into hepatocytes (Onoe *et al.* 1975; Evarts *et al.* 1987, 1989; Vandersteenhoven *et al.* 1990; Lemire *et al.* 1991; Novikoff *et al.* 1991; Nomoto *et al.* 1992; Dabeva & Shafritz 1993; Tarsetti *et al.* 1993; Factor *et al.* 1994; He *et al.* 1994; Sarraf *et al.* 1994; Sirica *et al.* 1994; Golding *et al.* 1995; Yavorkovsky *et al.* 1995; Alison *et al.* 1996, 1997; Yasui *et al.* 1997).

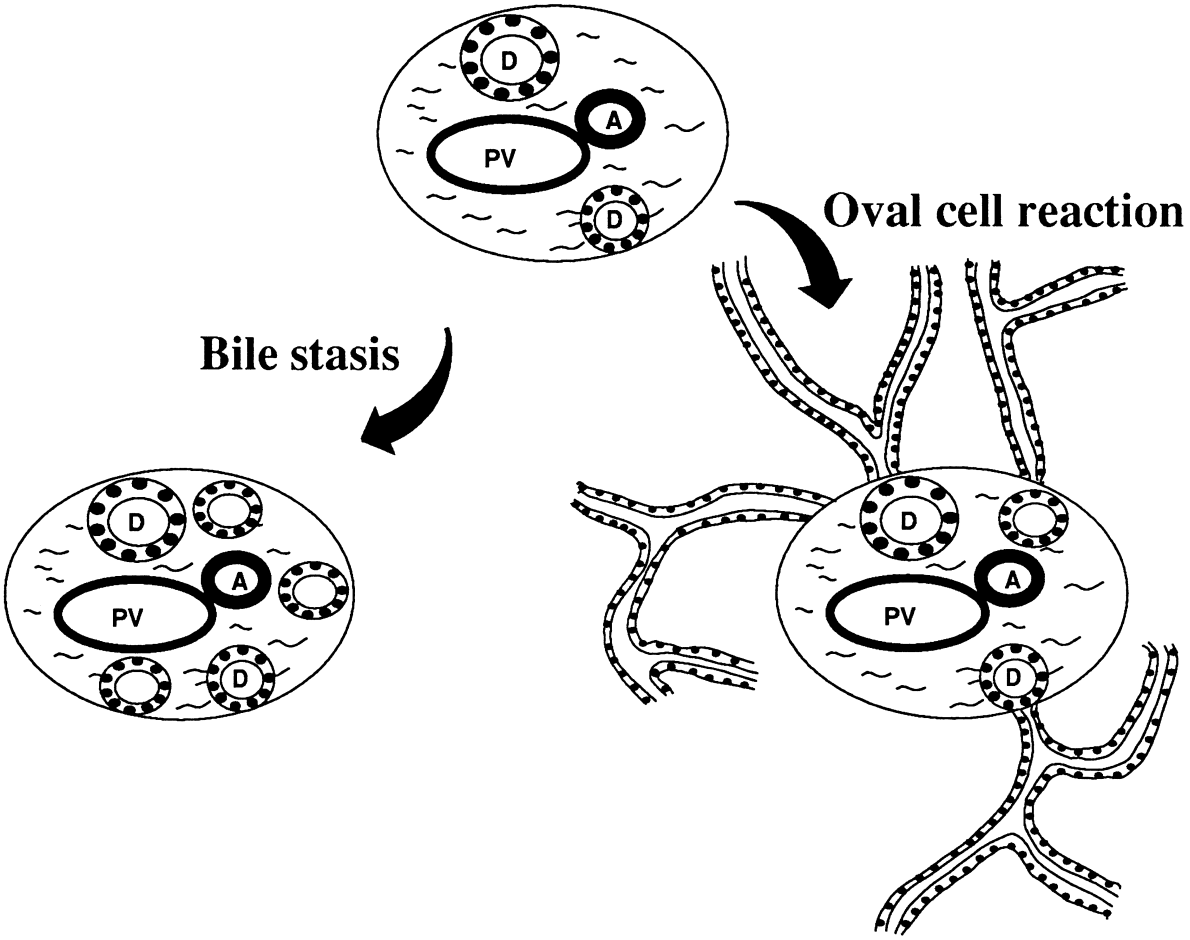

Figure 1. Schematic representations of hyperplastic biliary reactions in the liver. In oval cell reactions the branching ductules extend into the parenchyma. In contrast, bile stasis, induced experimentally by bile duct ligation, causes hyperplasia which increases the tortuosity of interlobular bile ducts that remain within the confines of the portal tract – hence an increase in ductular profiles. PV, portal vein; A, hepatic artery; D, interlobular bile duct.

Oval cells are the intralobular extensions of the portal biliary network

During embryological development, AFP transcription is the earliest example of liver-specific gene expression in determined endodermal cells (Shiojiri *et al.* 1991), and is followed one day later by albumin synthesis during which time formation of the hepatic cords begins (Nagy *et al.* 1994). The primitive intrahepatic bile ducts also express biliary epithelial markers in addition to these hepatocyte proteins, and so have been called 'transitional cells' (Fausto *et al.* 1993; Sell & Pierce 1994). Foetal liver-specific gene expression is recapitulated during oval cell proliferation by the re-emergence of transitional cell 'equivalents', represented by oval cells (Sell 1980; Sell *et al.* 1981; Germain *et al.* 1988; Lemire & Fausto 1991; Golding *et al.* 1995). Oval cells are considered to be the progeny of activated stem cells, a belief supported by the fact that they express markers thought to be characteristic of stem cells, including stem cell factor (SCF) and its receptor, c-kit (Fujio *et al.* 1994), Bcl-2 (Burt & MacSween 1993) and cytokeratin 14 (Thorgeirsson *et al.* 1993; Bisgaard *et al.* 1994; Haque *et al.* 1996). It is not clear if liver stem cells have a specific location, but there are several possible sites (Figure 2b). However, the simple fact remains that all biliary cells in the smaller interlobular ducts are heavily implicated, at least in the modified Solt-Farber procedure (see Figure 2b-d), while

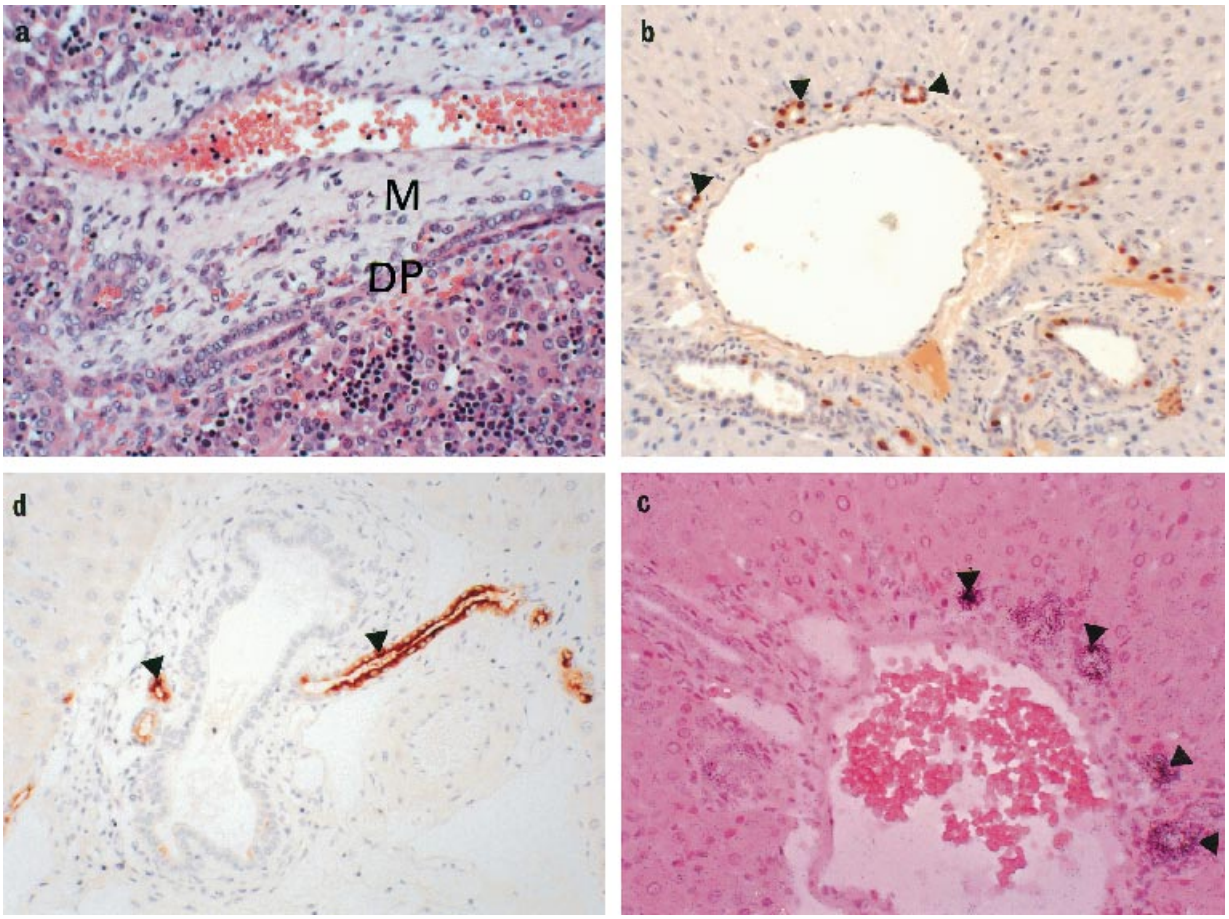

Figure 2. a, Foetal human liver demonstrating the migration of the ductal plate (DP) hepatocytes into the portal mesenchyme (M) to form the intrahepatic biliary network – oval cell responses are a reversal of this developmental process. b-d, characteristics of an early oval cell reaction induced in the rat liver using the AAF/PH protocol (see text for details). b, the first bromodeoxyuridine-incorporating cells after 2/3PH are mainly in the small interlobular bile ducts (arrow heads). c & d, likewise, these same cells (arrow heads) signal their commitment towards the hepatocyte lineage by massive upregulation of AFP mRNA and protein expression (demonstrated by an isotopically labelled antisense riboprobe to AFP mRNA and an anti-AFP antibody, respectively). Note the conspicuous lack of AFP expression in the larger ducts. Original magnifications ×200.

the larger ducts appear to be mere bystanders. Nevertheless, several studies have alluded to stem cells being a small subpopulation of ductular cells. Novikoff *et al.* (1996) made an ultrastructural analysis of oval cell proliferation during the Solt-Farber protocol, a model in which animals are fed the hepatotoxin 2-acetylaminofluorene (AAF) before and after a 2/3PH, and identified occasional 'blast-like cells' beneath the lining epithelial cells of biliary ductules. These cells were only 3–5 μm in diameter, smaller than the smallest cholangiocytes (6 μm) detected in rat liver (Alpini *et al.* 1996). These blast-like cells were not in contact with either the basal lamina or the lumen of the bile ductule, but were enveloped by neighbouring ductular cells. These cells are certainly possible candidates for the liver stem cells; they are unpolarised and proliferate, they have a very dense heterochromatic nucleus, do not possess any junctional complexes, and lack expression of any differentiation markers, e.g. CK19 or even the ubiquitously expressed cytoskeletal protein, actin.

Other opinions as to the site of the stem cells vary from small nondescript cells around cholangioles, the 'periductular cells' (Sell & Salman 1984), the cholangioles, sometimes called canals of Hering, terminal biliary ductules or transition ducts (Grisham & Porta 1964; Sell 1990; Lemire *et al.* 1991; Factor *et al.* 1994), and small interlobular ducts (Nomoto *et al.* 1992; Anilkumar *et al.* 1995; Golding *et al.* 1995). Having said this it is still likely that under particular circumstances any component of the intrahepatic biliary tree can give rise to oval cells (Lenzi *et al.* 1992; Golding *et al.* 1995). This is consistent with the observation that hepatocellular carcinomas can even develop from extrahepatic bile ducts (Park *et al.* 1991), suggesting multipotential stem cells exist throughout the biliary tree.

Models of oval cell activation

Irrespective of the experimental regime used, oval cells form cords and ductules continuous with interlobular bile ducts (Dunsford *et al.* 1985; Makino *et al.* 1988; Lenzi *et al.* 1992; Sarraf *et al.* 1994), see Figure 1, and seemingly similar bile ductular proliferation is frequently encountered in a variety of human liver diseases where substantial parenchymal damage occurs (Vandersteenhoven *et al.* 1990; De-Vos & Desmet 1992; Hsia *et al.* 1992; Koukoulis *et al.* 1992; Ray *et al.* 1993). Hepatocyte differentiation can even occur within the interlobular bile ducts in human liver (Nomoto *et al.* 1992), observations which have led to the belief that the small biliary cells which repopulate severely damaged liver parenchyma can function as a progenitor cell population for new hepatocytes. Certainly in rodents, early reactive bile ductules do not resemble hepatocytes (Figure 3a), but later acquire features of hepatocytes (see Figs 3d and 4).

The overriding principle operative in animal models is that some of the hepatic parenchyma is damaged (lost), but the ability of the surviving hepatocytes to regenerate is either totally nullified or at least severely compromised by the hepatotoxic insult. Generally speaking most of these chemical perturbations induce initially a fairly stereotyped intralobular ductular reaction (Figure 3a-c), but thereafter the efficiency of hepatocytic differentiation varies enormously. Model systems include feeding rats with furan (Elmore & Sirica 1991; Sirica *et al.* 1994), feeding mice with dipin (Engelhardt *et al.* 1990; Factor *et al.* 1994), or carrying out a 2/3PH on rats fed acetylaminofluorene (AAF) (Sarraf *et al.* 1994; Anilkumar *et al.* 1995; Golding *et al.* 1995). A choline-deficient ethionine-containing (CDE) diet (Hayner *et al.* 1984; Yaswen *et al.* 1985; Lenzi *et al.* 1992; Hiruma *et al.* 1993) is equally effective in inducing a ductular reaction. On the other hand, allyl alcohol-induced periportal necrosis causes proliferation of biliary cells which do not form typical ductular structures, yet they still rapidly differentiate into small hepatocytes (Yavorkovsky *et al.* 1995).

It is important to distinguish these authentic biliary proliferations from those derived from a ductular metaplasia of hepatocytes. Metaplasia is a common consequence of chronic parenchymal damage, and, for example, can be induced in guinea pigs by feeding α-naphthyl isothiocyanate (ANIT) (Bhathal & Christie 1969), where bile canalicular ectasia was considered responsible for the 'tubularization' of hepatic plates. In diseased human liver, a similar tubularization of hepatic plates is believed to result from a ductular metaplasia of hepatocytes, and not through hepatocytic differentiation of ductular cells (Uchida & Peters 1983; Van-Eyken *et al.* 1988b, 1989; Meybehm *et al.* 1993; Delladetsima *et al.* 1995), even though some metaplasias express dual biliary/hepatocyte phenotypes (Van-Eyken *et al.* 1988b, 1989; Vandersteenhoven *et al.* 1990).

Oval cell proliferation is also seen in $p21^{CIP1/WAF1}$ transgenic mice when overexpression is targeted to the liver, resulting in liver growth retardation (Wu *et al.* 1996). Oval cells are also prominent in the livers of Long-Evans Cinnamon (LEC) rats which have a defect in the *ATP7B* gene, resulting in the toxic accumulation of copper (Betto *et al.* 1996; Yasui *et al.* 1997). Premature death is common, and survivors develop chronic hepatitis and have a high incidence of hepatocellular carcinomas and cholangiocarcinomas.

One of the best understood models of oval cell

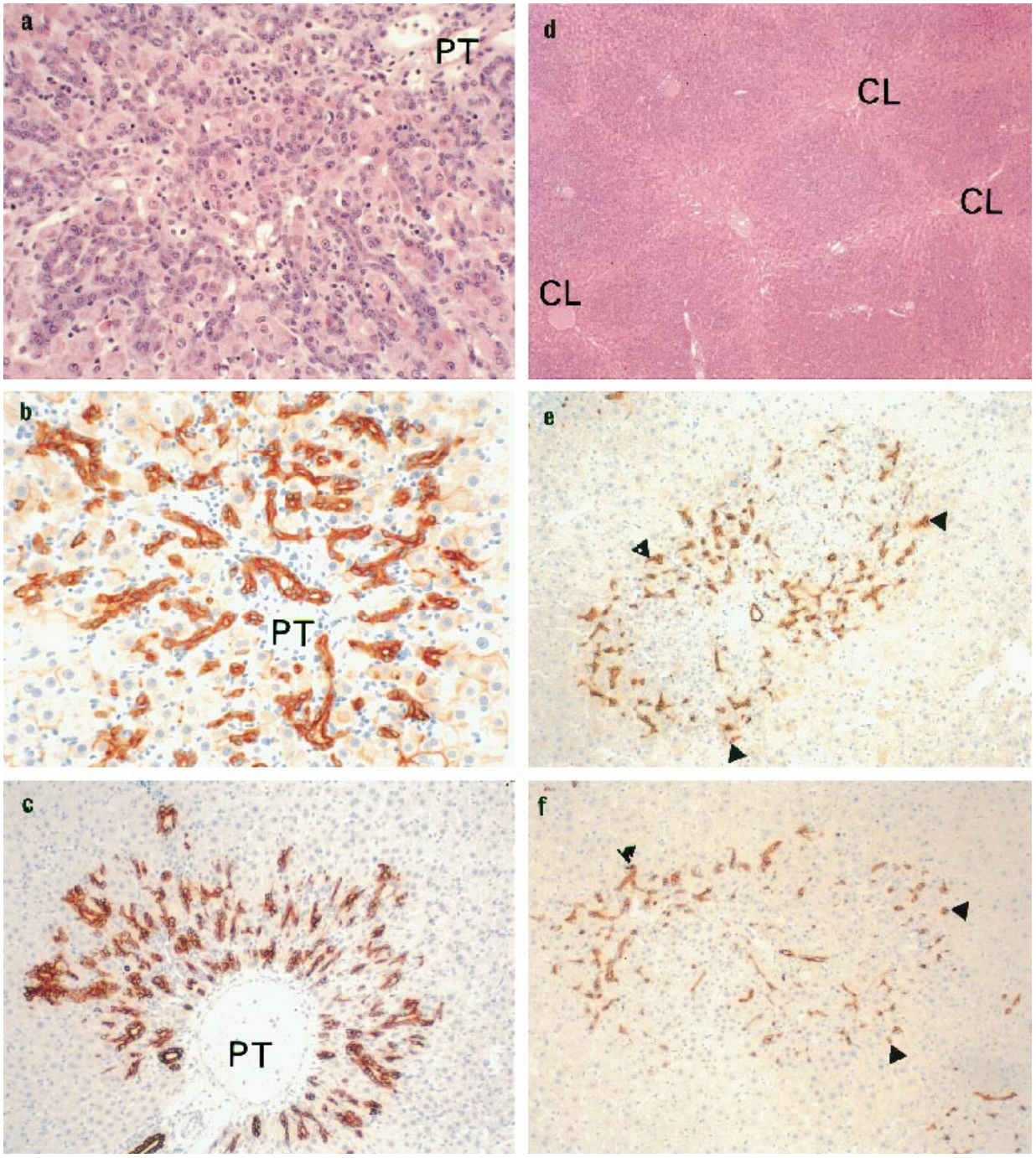

Figure 3. The differentiation of oval cell ductules into hepatocytes in the rat liver using the AAF/PH protocol; a-c, the initial phase, d-f, the later phase. a, within one week after 2/3PH oval cells extend from the portal tracts (PT) deep into the parenchyma as branching ductules (haematoxylin and eosin staining). b, these same cells demonstrate intense CK8 immunoreactivity typical of biliary cells, unlike the membranous expression seen in hepatocytes. c, they also express CK19 which is exclusive to biliary epithelia in the liver. d, from 2 to 3 weeks after 2/3PH these newborn cells loose their biliary phenotype and colonize the liver as basophilic hepatocytes which contrast with the more eosinophilic 'old' hepatocytes in the centrilobular (CL) areas. e & f, the differentiation of the ductular cells was accompanied by their loss of CK8 and 19 immunoreactivity, respectively, though some expression remained at the distal tips (arrow heads) of the ducts which had yet to differentiate. Original magnifications: a-c, e & f, ×100; d, ×40.

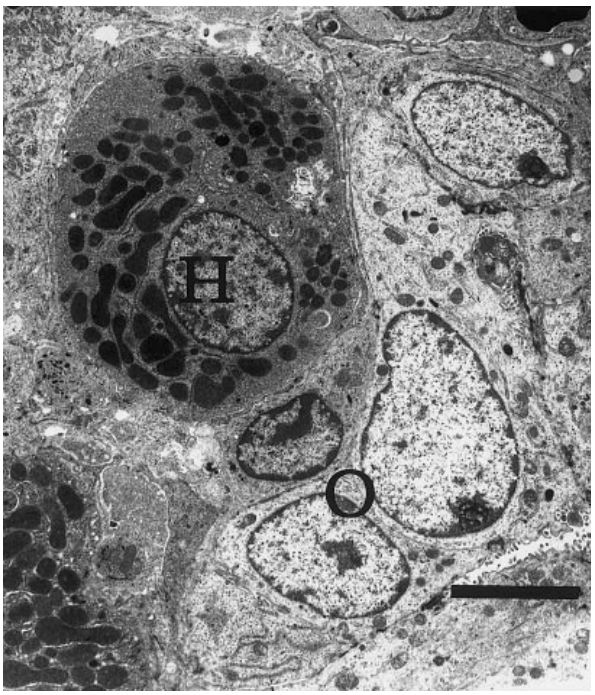

Figure 4. An electron micrograph of a 'juvenile' hepatocyte (H) with a high nuclear:cytoplasmic ratio, contrasting to a column of oval cell progenitors (O) with more euchromatic nuclei and distinctly fewer organelles. Bar, 7.5 μm.

activation is the modified Solt-Farber regime. Rats are subjected to a 2/3PH while being fed AAF. AAF is metabolised to its cytotoxic/mitoinhibitory *N*-hydroxy derivative by phase I metabolic enzymes (Kroese *et al.* 1990). Biliary cells and oval cells express very low amounts of phase I, and high levels of phase II enzymes compared with hepatocytes, and this favours detoxification over activation of hepatocarcinogens (Mathis *et al.* 1989). Therefore cultured oval cells, unlike hepatocytes, are resistant to the toxic effects of carcinogens (Ledda *et al.* 1983). Thus, in the presence of AAF, oval cells rather than hepatocytes proliferate after 2/3PH. In this model, [^{3}H]-thymidine labelled cholangioles are seen as early as 4 h after PH (Thorgeirsson *et al.* 1993) and proliferation is well underway by 24 h (Evarts *et al.* 1993; Thorgeirsson *et al.* 1993; Anilkumar *et al.* 1995; see Figure 2b). The larger septal bile ducts however, follow the time-course of biliary proliferation seen after an uncomplicated PH (Wright & Alison 1984), with significant numbers entering S-phase only 72 h after PH (Evarts *et al.* 1993; Thorgeirsson *et al.* 1993). Damaging the biliary system with methylene dianiline prevents oval cell proliferation in this model (Petersen *et al.* 1997), reinforcing the view that oval cells are derived from bile ductules.

Purely on kinetic grounds we can distinguish oval cell proliferation from the essentially intraportal bile duct proliferation accompanying a simple 2/3PH, which is probably activated by an expansion of lobular size (Grisham 1962; Fabrikant 1968). Furthermore, there are clear morphological and phenotypic differences between oval cell proliferation and some other bile duct hyperplasias. While oval cells radiate from the portal areas (see Figure 3a-c), invading deep into the hepatic parenchyma as tortuous arborizing ductules (Alpini *et al.* 1992; Sarraf *et al.* 1994), expressing hepatocyte proteins (Golding *et al.* 1995), bile duct ligation induces bile stasis and also biliary cell hyperplasia, but with the important distinction that such hyperplastic ducts remain within the confines of the portal space (Milani *et al.* 1989; Alpini *et al.* 1992; Lenzi *et al.* 1992; see Figure 1). In addition, these hyperplastic ducts do not differentiate into hepatocytes, neither do they express liver-enriched transcription factors which regulate AFP and albumin expression (Bisgaard *et al.* 1996), unlike AAF-induced oval cells. Moreover, while bile stasis in adult rat liver also fails to induce biliary expression of traditional oval cell 'markers' such as AFP, SCF and c-kit, these molecules are upregulated following bile duct ligation of very young rats (Omori *et al.* 1997). Perhaps this indicates the greater bipotentiality of early postnatal biliary epithelia, but the fact that these molecules were particularly upregulated in the smaller ductules, reinforces the opinion that the small ducts are the prime instigators of oval cell reactions (see Figure 2b-d).

Figure 2 illustrates the characteristics of a typical early oval cell reaction induced by the modified Solt-Farber procedure. The initial proliferative response is largely confined to the small interlobular bile ducts (Figure 2b), and these ducts rather than the larger radicals also begin expression of the oncofoetal glycoprotein α-foetoprotein (AFP) (Figure 2c,d), suggestive of hepatocyte lineage commitment. As the ducts invade the parenchyma they also express the mesenchymal intermediate filament, vimentin, in addition to the biliary CKs. Co-expression of CKs and vimentin is seen in many carcinomas, a combination of intermediate filaments considered to endow epithelial cancer cells with a more invasive and possibly metastatic phenotype (Chu *et al.* 1996; Gilles *et al.* 1996; Hendrix *et al.* 1997). An increased migratory phenotype is, of course, a useful attribute for oval cells. Likewise, cultured neonatal hepatocytes express both CKs and vimentin coincident with the acquisition of a more fibroblast-like morphology (Pagan *et al.* 1995); EGF promoted this coexpression, whereas the differentiating agent, dimethyl sulphoxide, inhibited the increase in vimentin (Pagan *et al.* 1997).

Ductular oval cells with an authentic biliary phenotype can eventually extend deep into the parenchyma (Figure 3a-c), but then differentiate into small hepatocytes, loosing biliary traits (Figure 3e-f, 4). They also cease expression of vimentin and AFP.

The influence of the extracellular matrix

Reactive ductules in human liver are surrounded by activated mesenchymal cells consisting primarily of myofibroblasts and perisinusoidal Ito cells (Burt & MacSween 1993). Following hepatic injury in man, Ito cells acquire a myofibroblast-like phenotype expressing desmin and α-smooth muscle actin (Schmitt-Graf *et al.* 1991; Arthur & Iredale 1994). Similarly activated Ito cells appear in rat livers damaged by carbon tetrachloride (Johnson *et al.* 1992), and in oval cell activated rat livers (Alison *et al.* 1993b; Sarraf *et al.* 1994; Anilkumar *et al.* 1995).

Ito cells are the first to proliferate in the AAF/PH model (Thorgeirsson *et al.* 1993), and are intimately associated with oval cells, enveloping them in a dense meshwork (Evarts *et al.* 1990). Ito cells are regarded as the principal source of extracellular matrix (ECM) proteins during hepatic regeneration both in rats (Johnson *et al.* 1992) and man (Griffiths *et al.* 1992); additionally they secrete metalloproteinases specific for the ECM, in particular for basement membrane proteins (Arthur *et al.* 1989). Secretion of matrix-busting enzymes may be critical for initiating oval cell invasion by 'clearing a path' through the damaged parenchyma to facilitate ductular proliferation, migration and morphogenesis, reactions promoted by Ito cell-produced HGF (see below). Certainly, after a 2/3PH, there is a considerable reorganization of the liver ECM with rapid rises in urokinase-type plasminogen activator (activator of plasminogen and HGF) (Kim *et al.* 1997). The oval cells themselves might also degrade matrix proteins, since primitive biliary cells migrating from the ductal plate can express matrix metalloproteinases during liver development in man (Terada *et al.* 1995). However, ECM degradation during oval cell migration may not always be so pressing as oval cells frequently migrate along the space of Disse (Betto *et al.* 1996; Alison *et al.* 1997). Ito cells also synthesize laminin as they penetrate clusters of normal regenerating hepatocytes, which might provide a stimulus for the ingrowth of endothelial cells to re-establish the sinusoidal vasculature (Martinez-Hernandez & Amenta 1995). So Ito cells amongst reactive biliary epithelia might function to stimulate both oval cell migration during the early stages of regeneration, as well as restoring the normal lobular vasculature after hepatocyte differentiation.

Ito cells may also insulate oval cells from the hepatocyte milieu during the early stages of cell migration, thereby preventing premature hepatocyte differentiation induced by the parenchymal microenvironment; cultured oval-like cells, when transplanted into the liver parenchyma of syngeneic rats swiftly integrate into the hepatic plates and acquire features of fully mature hepatocytes (Coleman *et al.* 1993; Grisham *et al.* 1993). The deterministic role of the hepatic microenvironment is further highlighted by the fact that it can even decrease the tumorigenicity of malignantly transformed oval-like cells (Coleman *et al.* 1993; Grisham *et al.* 1993). When these cells were transplanted subcutaneously they commonly formed highly aggressive, poorly differentiated tumours. On the other hand, those transplanted into the liver either lost the malignant phenotype or became more differentiated.

The close association of oval cells with stromal elements places them in a situation mimicking the biliary epithelium embedded in the portal mesenchyme. This may have a deterministic role in oval cell differentiation, retaining a biliary-like phenotype during the early stages of the regenerative response, which latterly breaks down, removing the 'biliary cell lineage restraint', permitting differentiation into hepatocytes**.**

Changes in matrix and intercellular adhesion very likely influence the dramatic tissue remodelling and alterations in gene expression which occur during oval cell responses. The influence of the ECM on gene transcription is highlighted by the well known rapid hepatocyte retrodifferentiation to a more primitive phenotype when cultured under simple conditions, a process which can be counteracted by addition of ECM proteins (Stamatoglou & Hughes 1994). For example, albumin synthesis is maintained when hepatocytes contact type IV collagen, but substitution of this protein with laminin results in the expression of a more primitive AFP-expressing phenotype, akin to limiting plate hepatocytes *in vivo* (Shah & Gerber 1990).

Growth factors controlling oval cell behaviour

The growth factors involved in oval cell reactions are essentially the same as those seemingly involved during normal hepatocyte regeneration. Nevertheless, the complexity of the remodelling process during ductular oval cell hyperplasia and differentiation clearly requires a sophisticated and highly coordinated temporal and spatial expression of both stimulatory and inhibitory growth factors, which in turn will influence cell-cell and cell–ECM interactions.

Parathyroid hormone related peptide (PTHrP) is believed to be important in the growth and differentiation

of several human tissues including liver. In man, PTHrP is expressed in reactive bile ductules of cholestatic and regenerating liver (Roskams *et al.* 1993a), as well as by cholangiocarcinomas (Roskams *et al.* 1993b). As growth factors such as EGF can rapidly induce PTHrP synthesis in cultured ductular cells, PTHrP could be a member of the early response gene family, and the peptide may have an autocrine role in bile ductular reactions (Roskams *et al.* 1995).

Oval cell proliferation and differentiation is mediated by complex growth factor loops which coordinate epithelial and stromal cell responses, highlighted by the expression of TGFβ by reactive bile ductules, a growth factor known to stimulate perisinusoidal cells to produce ECM proteins (Milani *et al.* 1991). TGFβ is also expressed by periductular Ito cells (Nagy *et al.* 1989; Evarts *et al.* 1990), and the abundance of this growth factor acting in an autocrine/paracrine fashion has been implicated in differentiation along the hepatocyte lineage, at least *in vitro* (Nagy *et al.* 1989). Ito cells also express TGFα, aFGF, HGF (Thorgeirsson *et al.* 1993) and stem cell factor (SCF) (Fujio *et al.* 1994). Oval cells express all of these growth factors apart from HGF (Evarts *et al.* 1992; Alison *et al.* 1993a), and the receptors for these ligands (Hu *et al.* 1993; Thorgeirsson *et al.* 1993; Fujio *et al.* 1994), and thus, may be autonomous in their requirement for mitogens and morphogens. Indeed, cultured hepatoblasts, putative embryological oval cell equivalents, are able to proliferate and migrate in serum free-conditions (Pagan *et al.* 1995), suggesting autocrine regulation.

HGF was originally called 'scatter factor' (Stoker & Perryman 1985) because of its ability to promote the scattering and spreading of a variety of epithelial cells *in vitro*. Significantly, HGF is a powerful stimulator of human biliary epithelial cells *in vitro* (Joplin *et al.* 1992; Strain *et al.* 1995) and, furthermore, HGF is the most potent known mitogen for hepatocytes *in vitro* (Strain *et al.* 1991). HGF is also a powerful morphogen, particularly during embryological tissue development (Barros *et al.* 1995), and therefore probably has a fundamental influence on oval cell behaviour (Alison *et al.* 1993b). The branching tubulogenesis of oval cells is undoubtedly augmented by HGF and TGFα, both of which have been shown to be critical in promoting and regulating ductular morphogenesis during epithelial tissue development (Barros *et al.* 1995), and may regulate this process *via* paracrine (Schirmacher *et al.* 1992; Hu *et al.* 1993) or autocrine (Burr *et al.* 1993) mechanisms. Indeed, HGF has been shown to induce glandular and ductular morphogenesis in a wide variety of epithelial cells growing in culture, including cell lines from colon, pancreas, mammary gland, prostate and lung (Brinkmann *et al.* 1995). In the AAF/PH model, aFGF mRNA expression by ductular oval cells coincides with their differentiation into hepatocytes (Marsden *et al.* 1992). In contrast, TGFβ and PTHrP which are also expressed during liver regeneration (Thorgeirsson *et al.* 1993), and by human reactive bile ductules (Roskams *et al.* 1993a), can inhibit ductal branching morphogenesis (Miettinen *et al.* 1994). HGF has, in addition, been shown to dissociate epithelial sheets to form mesenchymal-like cells, inducing a spindle cell-like morphology and increasing motility, migration and invasion, the latter phenomenon probably being accomplished by increased urokinase expression which mediates degradation of the ECM (Rosen *et al.* 1994).

HGF also plays a fundamental role in the control of proliferation and differentiation of erythroid progenitor cells in foetal liver, and acts in synergy with SCF (Galimi *et al.* 1994), an important effector of stem cell activation (Keshet *et al.* 1991). Both of these growth factors are expressed during oval cell proliferation (Thorgeirsson *et al.* 1993; Fujio *et al.* 1994), perhaps explaining why extramedullary haemopoiesis, probably from ordinarily resident pluripotential haemopoietic stem cells (Taniguchi *et al.* 1996), is such a common accompaniment to oval cell proliferation (Enomoto *et al.* 1978; Brill *et al.* 1993).

Oval cells are multipotential

In rodents, oval cells are certainly not restricted to differentiation along the biliary and hepatocyte cell lineages, and appear additionally to have the lineage potential of uncommitted gastrointestinal stem cells. For instance, they can differentiate into intestinal absorptive cells (Tatematsu *et al.* 1985), goblet cells (Tatematsu *et al.* 1985; Golding *et al.* 1995), and endocrine and Paneth cells (Karaki *et al.* 1991; Elmore & Sirica 1993). In humans, endocrine differentiation can occur in normal bile ducts (Kurumaya *et al.* 1989), in ductular hyperplasia accompanying regeneration after submassive necrosis (Roskams *et al.* 1991), and in cholangiocarcinomas (Roskams *et al.* 1993b).

Pancreatic differentiation/metaplasia has been observed in liver associated with oval cell proliferation (Thorgeirsson *et al.* 1993), and the human peribiliary glands which develop from the biliary ductal plate after birth, contain serous and mucus acini which can develop into ectopic exocrine pancreas (Terada & Nakanuma 1993). Conversely, hepatocyte (Rao *et al.* 1984, 1989) and goblet cell differentiation (Zalatnai & Schally 1990) can occur in pancreatic ductular and/or periductular cells in rodents, and pancreatic oval cells in culture exhibit a

ductular albumin-expressing phenotype (Ide *et al.* 1993). Similarly, cultured rat pancreatic ductal epithelial cells can undergo hepatocytic differentiation when transplanted subcutaneously or intraperitoneally into syngeneic rats (Chen *et al.* 1995). Furthermore, pancreatic acinar cell carcinomas expressing a combination of hepatocyte, intestinal and neuroendocrine markers have been found in man (Shinagawa *et al.* 1995), and hepatocyte differentiation can occur in adenocarcinomas of the renal pelvis (Ishikura *et al.* 1991) and stomach (Ishikura *et al.* 1986). The hepatoid stomach adenocarcinomas express a number of hepatocyte-specific markers, and in one case even bile secretion was evident (Ishikura *et al.* 1986). Interestingly, these tumours were always associated with intestinal metaplasia, a further example of deregulated gene expression.

This plasticity of cellular phenotypes is perhaps not surprising considering the liver, pancreas and intestine have a common embryological origin, and all three are connected *via* a continuous epithelium. Indeed, culture and transplantation of stem-like cells from normal liver has shown that these cells share epitopes with cells in the small ducts of the liver, pancreas, intestinal crypts and tracheobronchial epithelium (Grisham 1994). In fact, the default lineage commitment of the dorsal endoderm of the primitive gut (i.e. that which is not destined to become liver epithelium), appears to be the hepatic programme; AFP is expressed throughout the foetal gut villous epithelium (Tyner *et al.* 1990), but is later inhibited by the suppressive effects of adjacent endodermal components, which direct differentiation towards nonhepatic tissues of the gastrointestinal tract (Gualdi *et al.* 1996). It is interesting to note that transcription of the AFP gene is retained in a small number of enteroendocrine cells of the adult murine intestine (Tyner *et al.* 1990).

Are there markers on oval cells which are indicative of lineage commitment? Differential expression of a combination of AFP and CK14 maybe a useful indicator (Thorgeirsson *et al.* 1993). CK14 is not normally associated with the liver (Moll *et al.* 1982), but has been detected in a small population of biliary associated cells (Blouin *et al.* 1992). During the oval cell response using the AAF/PH model (Thorgeirsson *et al.* 1993), the newborn cells initially express CK14, and these cells give rise to two expanding oval cell populations consisting of cells expressing CK14 alone, or CK14 in combination with AFP. The latter phenotype appears to be restricted to differentiation along the hepatocyte lineage, while CK14 expression alone may be a marker of multipotential progenitor cells.

Oval cells clearly give rise to many different cell types, but it is uncertain as to whether there is a common multipotential stem cell, as in the bone marrow (see Graham & Wright 1997 – in this series of stem cell reviews), which gives rise to committed unipotential stem cells, or if there are lineage-specific stem cells scattered about within the biliary tree.

In vitro studies

Most cells isolated from normal or carcinogen-fed rat livers soon die in primary culture and cultures become dominated by rapidly growing colonies of small nonparenchymal epithelial cells traditionally called ‘liver epithelial cells’ (LECs) or ‘rat liver epithelial’ (RLE) cells (Tsao *et al.* 1984, 1985; Marceau *et al.* 1986; Blouin *et al.* 1992). Support for the existence of liver stem cells has received significant input from studies of cells derived from foetal and adult rat liver tissue (Grisham 1980; Hayner *et al.* 1984; Tsao *et al.* 1984; Grisham *et al.* 1993). These cells can share cholangiocyte and hepatocyte features, and closely resemble many of the cell lines that have been derived from oval cell activated rodent livers.

Other groups have propagated long-term cultures of small hepatocytes which generally do not express biliary cell characteristics such as CK7 and CK19 (Mitaka *et al.* 1993; Tateno & Yoshizato 1996). Cultured in the presence of nicotinamide and defined growth factor-containing medium, particularly EGF, these cells displayed appropriate hepatocyte traits – expression of CK8 and CK18, and production of albumin and transferrin. Such cells were considered to be committed progenitor cells rather than bipotential oval cells.

Cultured oval cells can certainly differentiate into hepatocytes when returned to their *in vivo* origins. Oval cells isolated from Long-Evans Cinnamon (LEC) rats will differentiate into albumin-producing hepatocytes when returned to the livers of LEC/Nagase analbuminaemic double mutant rats (Yasui *et al.* 1997). This is a particularly significant observation in the light of future prospects for *ex vivo* gene therapy (Figure 5), since Yasui *et al.* (1997). showed it was possible to genetically modify (expressed β-galactosidase after retroviral infection) isolated oval cells and return them *in vivo* where they functioned as hepatocytes.

Oval cells and the origins of primary liver cancers

Stem cells are important from the viewpoint of being the

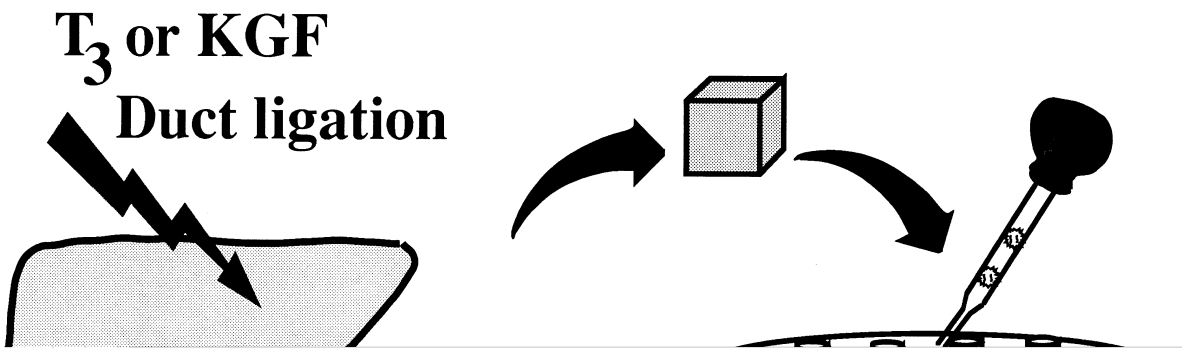

Figure 5. Possible strategies for gene therapy in the liver. Using a retroviral vector requires a proliferating target population, and triiodothyronine (T_3) or keratinocyte growth factor (KGF) are effective hepatocyte mitogens. Bile duct ligation renders the biliary epithelia hyperplastic, while the isolation of oval cells and their expansion *in vitro* could be another way of introducing new genes into hepatocytes.

most likely targets for carcinogenic agents, since their longevity assures a continued presence during the long latency between exposure and cancer development. However, the role of stem/oval cells in the histogenesis of primary tumours in the liver is still unresolved, and tumours could arise from the 'maturation arrest' of the differentiating stem cell progeny (Marceau 1990; Sell 1993a,b; Sell & Pierce 1994), or by dedifferentiation of mature liver cells. This question is still unresolved and will only be briefly covered here, but has been substantially reviewed elsewhere (Sell & Dunsford 1989; Aterman 1992; Sell 1993a,b). Both mechanisms are likely to operate, e.g. LEC rats exhibit extensive oval cell proliferation and invariably develop hepatocellular carcinomas and cholangiocarcinomas, conversely the yield of preneoplastic foci and tumours in rats exposed to chemical carcinogens after a 2/3PH is directly proportional to the level of hepatocyte proliferation (Wright & Alison 1984), and apparently unassociated with oval cell proliferation (Anilkumar *et al.* 1995).

Of relevance here is the large body of *in vitro* experimental evidence supporting the stem cell 'maturation arrest' hypothesis, in that cultured malignantly transformed oval cells are able to give rise to tumours with a whole range of phenotypes, highly suggestive of the multipotential nature of the target cells (Tsao *et al.* 1985; Tsao & Grisham 1987; Garfield *et al.* 1988; Steinberg *et al.* 1994). Similarly, it is possible to use extrinsic agents to switch a human cholangiocarcinoma cell line to one which has hepatocytic features (Enjoji *et al.* 1997), presumably reflecting the likely histogenesis of these two tumour types from a common precursor cell. In a similar vein, transplantation of transformed oval cells into syngeneic recipients can result in either hepatoblastomas, hepatocellular-carcinomas, cholangiocarcinomas, cholangiocarcinomas with intestinal features (Steinberg *et al.* 1994), intestinal adenocarcinomas (Marceau 1990), anaplastic tumours (Steinberg *et al.* 1994) and even sarcomas (Marceau 1990). Furthermore, the lineage commitment of oval cells can be altered by a single transforming oncogene, and the range of tumours derived from oval cells may reflect mutations in different combinations of cellular proto-oncogenes (Garfield *et al.* 1988).

Conclusions

Reactive biliary epithelium, usually organized into ductules, collectively known as oval cells, appear to be derived from facultative stem cells located in the cholangioles and small interlobular bile ducts. Both *in vitro* and *in vivo* studies have clearly demonstrated that oval cells have the potential to differentiate along many divergent pathways, and furthermore, after transformation and transplantation, they can similarly give rise to a range of tumour types, although their role in human hepatocarcinogenesis is unclear. Oval cell proliferation and differentiation can be readily induced in experimental animals, although the controlling mechanisms for these processes are poorly understood.

The selective harvesting and culture of both rodent (Alpini *et al.* 1997) and biopsied human (Strain *et al.* 1995) biliary epithelial cells is now possible, hopefully paving the way for *ex vivo* gene therapy of liver stem cell progeny for the correction of metabolic liver disorders (Figure 5). Also, selective *in vivo* gene transfer to the cholangiocytes of temporarily ligated rodent bile ducts has been performed (Cabrera *et al.* 1996), raising the potential for the application of a similar strategy to the human setting for the amelioration of the defective Cl^- and water secretion characteristic of cystic fibrosis.

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