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The efficiency of liver regeneration in response to the loss of hepatocytes is widely acknowledged, and this is usually accomplished by the triggering of normally proliferatively quiescent hepatocytes into the cell cycle. However, when regeneration is defective, tortuous ductular structures, initially continuous with the biliary tree, proliferate and migrate into the surrounding hepatocyte parenchyma. In humans, these biliary cells have variously been referred to as ductular structures, neoductules and neocholangioles, and have been observed in many forms of chronic liver disease, including cancer. In experimental animals, similar ductal cells are usually called oval cells, and their association with impaired regeneration has led to the conclusion that they are the progeny of facultative stem cells. Oval cells are of considerable biological interest as 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.

This review proposes that the liver harbours stem cells that are located in the biliary epithelium, that oval cells are the progeny of these stem cells, and that these cells can undergo massive expansion in their numbers before differentiating into hepatocytes. This is a conditional process that 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. We focus on the biliary network, not merely as a conduit for bile, but also as a cell compartment with the ability to proliferate under appropriate conditions and give rise to fully differentiated hepatocytes and other cell types.

Keywords: oval cells; liver; bile ducts; hepatocellular carcinoma; cholangiocarcinoma; stem cells

1. LIVER DEVELOPMENT

The liver primordium consists of endodermal and mesodermal components. Ventral foregut endoderm gives rise to hepatoblasts that form the parenchyma, whereas both sinusoidal-lining cells and connective tissue components originate from the mesenchymal tissue invaded by the hepatic cords (Shiojiri *et al*. 1991). The primitive hepatoblasts that surround the portal mesenchyme form a double-layered cylinder of hepatocytes, the 'ductal plate', which remodels and migrates into the mesenchyme to form the intrahepatic bile ducts (Van-Eyken *et al*. 1988*a*; Shiojiri *et al*. 1991). Rat hepatoblasts synthesize the hepatocyte-specific proteins alpha-foetoprotein (AFP) and albumin as they migrate into the portal stroma and, in addition, begin to express the bile-duct-specific enzyme, gamma glutamyl transferase (GGT) activity. 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), although 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, the hepatoblasts adjacent to the portal mesenchyme, which give rise to the intrahepatic bile ducts, transiently express both hepatocyte and ductal markers and can be considered to be oval cell equivalents. Hepatoblasts not in contact with the portal mesenchyme differentiate into hepatocytes that form the liver cell plates, although they continue to have GGT activity until birth. The hepatocyte cytokeratin complement is, of course, 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).

2. 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 via the complex anastomoses of the 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 (see figure 2). 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.

3. THE PROLIFERATIVE ORGANIZATION OF THE ADULT LIVER

There has been a considerable debate concerning the proliferative organization of the liver. In rapidly renewing epithelia (gut and skin), the stem cells represent the ancestors of the migrating pathways (Potten & Loeffler 1990), and in the liver too, 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, it was claimed that over 5 weeks hepatocytes moved a distance of 46 μm (*ca*. 1 cell) from the portal rim towards the hepatic vein. The so-called 'streaming liver' hypothesis has engendered considerable debate and not a little heat (Grisham 1994; Correspondence 1995), and models of hepatic organization have been based on the hypothesis (Sigal *et al*. 1992). Unfortunately, depicting the plates of the hepatic muralium as straight cords is misleading and, moreover, models that divide plates into proliferative, maturing and terminally differentiated compartments are too simplistic. All remaining hepatocytes can enter the cell cycle after a two-thirds partial hepatectomy (2/3PH). Furthermore, all hepatocytes are equally proliferation-competent by 48 h after surgery, and even the well-known periportal concentration of proliferative cells in the early post-operative period can be reversed by altering the direction of the afferent blood supply (Wright & Alison 1984). Studies using genetic labelling with an *Escherichia coli* β-galactosidase gene certainly do not support the 'streaming liver' hypothesis. For example, when rat hepatocytes are labelled *in vivo* at 24 h after a 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; as 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 reached 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 partial hepatectomy changed the distribution of labelled cells; the labelled cells simply presented as larger cell clusters with the passage of time.

4. GROWTH REACTIONS IN THE LIVER

Despite the very limited proliferative activity in the adult liver, probably resulting from 'wear and tear' loss, the liver is able to mount a prompt proliferative response to hepatotoxic insult and other hyperplastic stimuli. These reactions may be categorized as follows: (i) regenerative or compensatory hyperplasia; (ii) additive liver growth; and (iii) stem cell-driven (oval cell) reactions.

A wide variety of xenobiotics, e.g. paracetamol and carbon tetrachloride, cause parenchymal cell death. Such xenobiotics invariably, although not exclusively, cause centrilobular necrosis as the affected cells possess the enzymic 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 (Gerlach *et al*. 1997). Because this response actually occurs in quite separate lobes, this is strictly speaking viewed as a compensatory hyperplasia, although in practice the terms 'regeneration' and 'compensatory hyperplasia' are used synonymously. Figure 1*a* illustrates the spatial heterogeneity in hepatocyte proliferation at 24 h after 2/3PH, but at later times cell proliferative activity is randomly located across the liver (Wright & Alison 1984; Alison 1986). Of course, the reconstitution of the liver architecture also involves the proliferation of all other cell types normally present in the liver, although 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 that 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 (figure 1*b–d*), but withdrawal of the stimulus triggers a rapid reversion to normal size by invoking hepatocyte apoptosis.

A third category of liver growth does not involve hepatocytes, emanating instead from interlobular bile ducts. The extensive formation of biliary-derived ductules emerging from the portal tracts of rodents fed chemical carcinogens was first noted in 1944 (Opie 1944). Since then a number of experimental regimes have been used to induce bile ductular hyperplasia in the rodent liver (figure 1*e*), and these have been reviewed elsewhere (Aterman 1992; Burt & MacSween 1993; Sell 1994). Farber (1955) used the ambiguous term 'oval cells' to describe the cells that 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 that had all the morphological features of mature hepatocytes. As we shall see, the evidence that these oval cells can differentiate into hepatocytes is now overwhelming (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).

5. OVAL CELLS ARE THE PROGENY OF BILIARY-LOCATED, FACULTATIVE STEM CELLS

During embryological development, AFP transcription is the earliest example of liver-specific gene expression in determined endodermal cells (Shiojiri *et al*. 1991); it is followed one day later by albumin synthesis, during

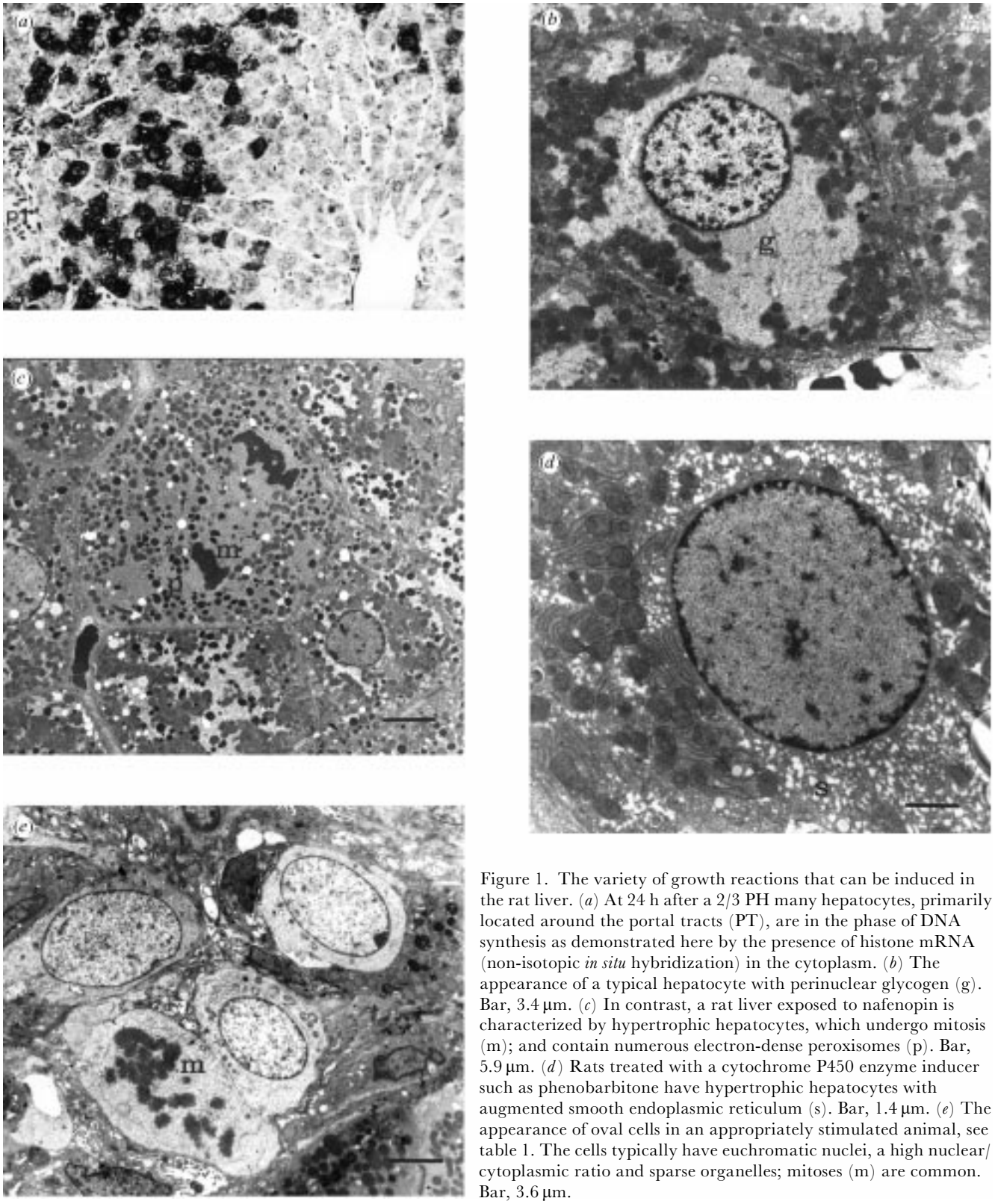

Figure 1. The variety of growth reactions that can be induced in the rat liver. (*a*) At 24 h after a 2/3 PH many hepatocytes, primarily located around the portal tracts (PT), are in the phase of DNA synthesis as demonstrated here by the presence of histone mRNA (non-isotopic *in situ* hybridization) in the cytoplasm. (*b*) The appearance of a typical hepatocyte with perinuclear glycogen (g). Bar, 3.4 µm. (*c*) In contrast, a rat liver exposed to nafenopin is characterized by hypertrophic hepatocytes, which undergo mitosis (m); and contain numerous electron-dense peroxisomes (p). Bar, 5.9 µm. (*d*) Rats treated with a cytochrome P450 enzyme inducer such as phenobarbitone have hypertrophic hepatocytes with augmented smooth endoplasmic reticulum (s). Bar, 1.4 µm. (*e*) The appearance of oval cells in an appropriately stimulated animal, see table 1. The cells typically have euchromatic nuclei, a high nuclear/cytoplasmic ratio and sparse organelles; mitoses (m) are common. Bar, 3.6 µm.

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), which are believed to remain in the adult liver as bipotential progenitors for both hepatocytes and biliary cells (Sell 1990; Sell *et al*. 1991; Shiojiri *et al*. 1991; Grisham *et al*. 1993; Blouin *et al*. 1995). Indeed, the lineage commitment of foetal hepatoblasts can be altered *in vitro* by manipulating specific culture conditions (Germain *et al*. 1988*b*; Marceau 1994; Blouin *et al*. 1995).

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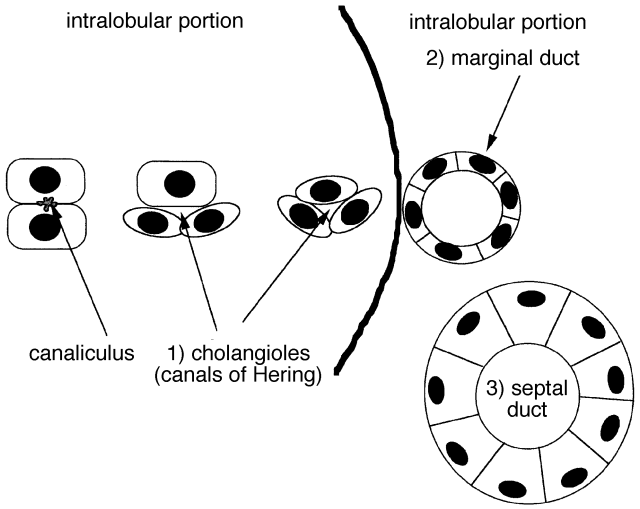

Figure 2. Possible locations of facultative liver stem cells within the biliary tree. Bile passes through canaliculi, which are channels between adjacent hepatocytes, and enters (1) cholangioles, which progressively become composed of specialized ductular cells, before feeding into (2) the small marginal interlobular ducts, which eventually coalesce into the larger (3) septal ducts. Cell kinetic and functional studies suggest that the stem cells are primarily in the cholangioles and small marginal ducts.

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*a*; Lemire & Fausto 1991; Golding *et al*. 1995). Oval cells represent the progeny of activated stem cells (Sell 1993*b*), a belief supported by the fact that they express markers considered characteristic of stem cells, including stem cell factor (SCF) (Fujio *et al*. 1994) 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 2). 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 4), while the larger ducts appear to be mere bystanders. Nevertheless, several studies have alluded to stem cells being a small sub-population of ductular cells. Novikoff *et al*. (1996) made an ultrastructural analysis of oval cell proliferation during the Solt–Farber protocol, 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 unpolarized 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. We have also demonstrated the presence of occasional CK19-negative and also intensely CK18-positive cholangiocytes in small bile ducts of rats treated by the modified Solt–Farber procedure, a model in which animals are fed

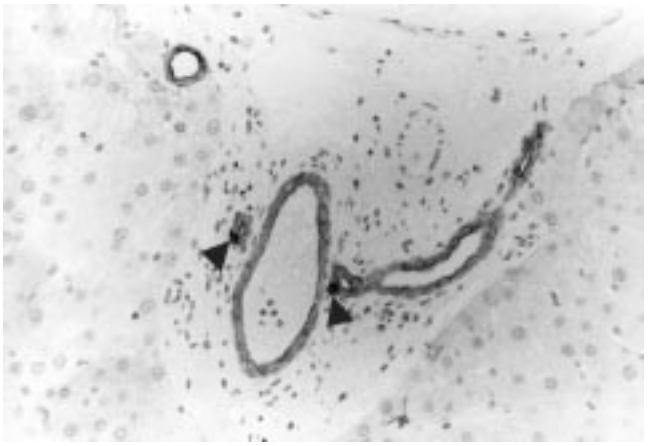

Figure 3. Photomicrograph illustrating two intensely CK18-immunoreactive biliary cells (arrows) within a portal tract; such cells could represent a distinct subpopulation of potential liver stem cells. Magnification ×200.

the hepatotoxin 2-acetylaminofluorene (AAF) before and after a 2/3PH (figure 3). A possible reason for the selective upregulation of CK18 in these rare cholangiocytes can be made from analogy with the cytokeratin profile of the intestinal epithelium. Flint *et al*. (1994) showed that CK8 and CK19 are uniformly distributed along the crypt–villus axis, but that CK18 is restricted to the proliferative crypt, whereas Calnek & Quaroni (1993) have shown that CK18 and CK19 mRNA are restricted to crypt cells as opposed to CK8 transcripts, which showed a uniform distribution along the crypt–villus axis. Interestingly, similarly intensely CK18-immunoreactive 'brush cells' with a speculated chemoreceptive function have been found randomly scattered among much weaker-staining epithelial cells of the pancreatic ducts (Hofer & Drenckhahn 1996). Thus, an abundance of CK18 in particular may signify a different proliferative potential among a hitherto morphologically similar population of ductular cells.

Various opinions exist as to the site of the stem cells. Cells situated within or in contact with the portal stroma (Yavorkovsky *et al*. 1995), small non-descript cells around cholangioles, the 'periductular cells' (Sell & Salman 1984; Sell 1993*b*), the cholangioles (sometimes called canals of Hering, terminal biliary ductules or transition ducts; see figure 2) (Grisham & Porta 1964; Sell 1990, 1993*b*; Lemire *et al*. 1991; Factor *et al*. 1994), and small interlobular ducts (Nomoto *et al*. 1992; Anilkumar *et al*. 1995; Golding *et al*. 1995) have all been mooted. 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 develop from extrahepatic bile ducts (Park *et al*. 1991), even though their embryological development is independent of intrahepatic bile ducts (Shiojiri *et al*. 1991).

6. 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 4), and seemingly

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

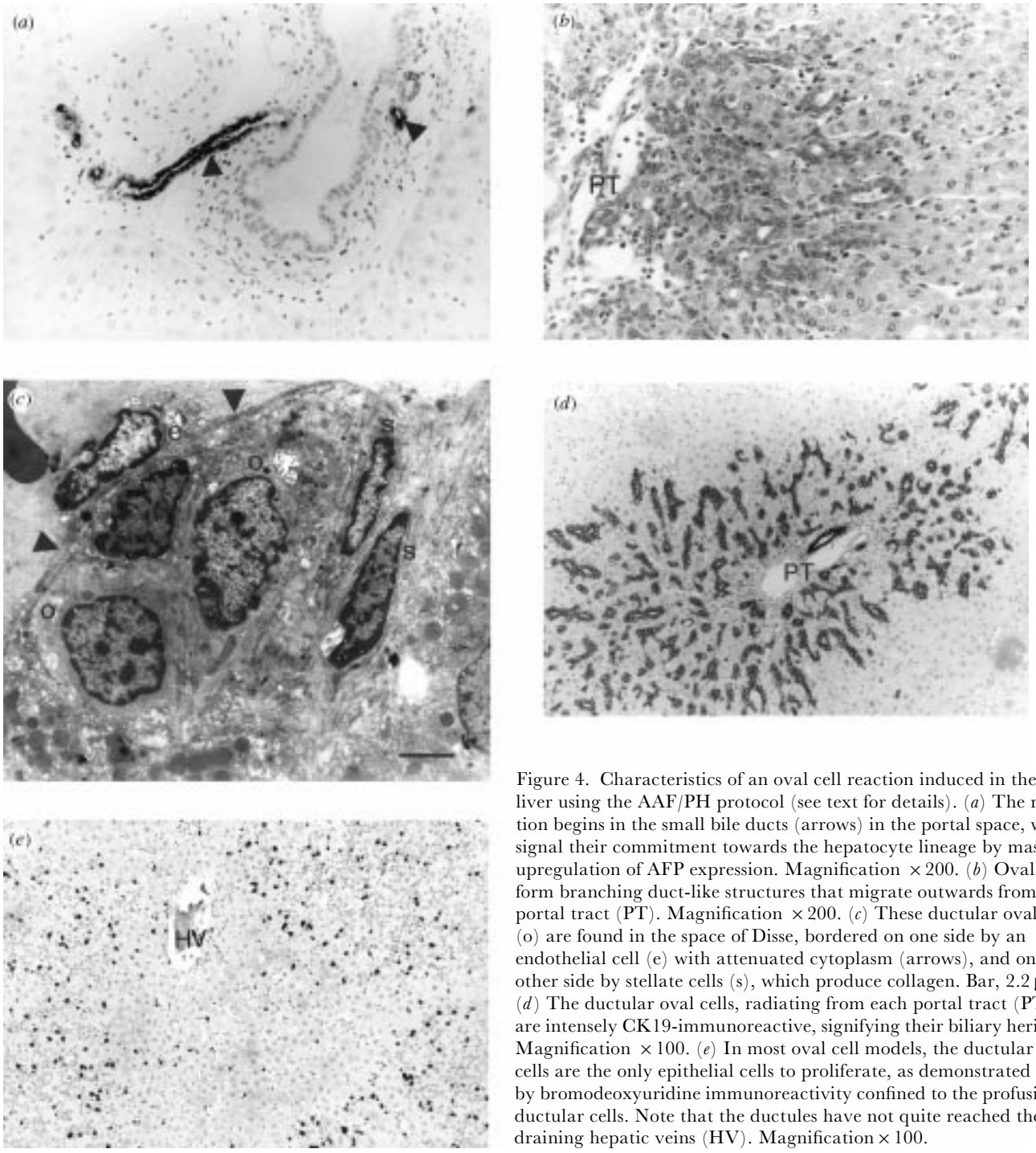

Figure 4. Characteristics of an oval cell reaction induced in the rat liver using the AAF/PH protocol (see text for details). (*a*) The reaction begins in the small bile ducts (arrows) in the portal space, which signal their commitment towards the hepatocyte lineage by massive upregulation of AFP expression. Magnification ×200. (*b*) Oval cells form branching duct-like structures that migrate outwards from each portal tract (PT). Magnification ×200. (*c*) These ductular oval cells (o) are found in the space of Disse, bordered on one side by an endothelial cell (e) with attenuated cytoplasm (arrows), and on the other side by stellate cells (s), which produce collagen. Bar, 2.2 µm. (*d*) The ductular oval cells, radiating from each portal tract (PT), are intensely CK19-immunoreactive, signifying their biliary heritage. Magnification ×100. (*e*) In most oval cell models, the ductular oval cells are the only epithelial cells to proliferate, as demonstrated here by bromodeoxyuridine immunoreactivity confined to the profusion of ductular cells. Note that the ductules have not quite reached the draining hepatic veins (HV). Magnification ×100.

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 that have led to the belief that the small biliary cells that 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 1*e*), but later acquire features of hepatocytes (see figure 5).

Table 1 summarizes the commonly adopted rodent models for oval cell proliferation; all produce similar early morphological changes, although the potential of the ductular cells to differentiate into hepatocytes varies greatly. For example, feeding rats with furan (Elmore & Sirica 1991; Sirica *et al*. 1994) induces massive biliary cell proliferation with a few cells expressing hepatocyte features. In contrast, feeding rodents dipin (Engelhardt *et al*. 1990; Factor *et al*. 1994), acetylaminofluorene (AAF) (Sarraf *et al*. 1994; Anilkumar *et al*. 1995; Golding *et al*. 1995), or a choline-deficient ethionine-containing (CDE) diet (Hayner *et al*. 1984; Yaswen *et al*. 1985; Lenzi *et al*. 1992; Hiruma *et al*. 1993), results in similar ductular

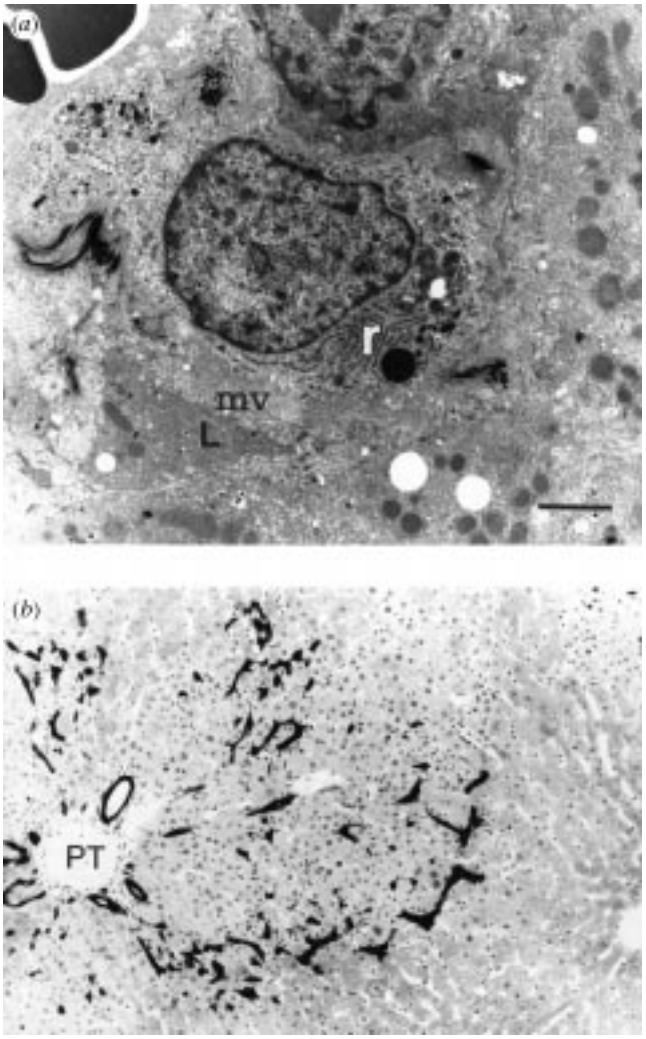

Figure 5. The differentiation of oval cell ductules into small hepatocytes. (*a*) Electron micrograph of a 'juvenile' hepatocyte in the space of Disse adjacent to the sinusoid lumen (L). In contrast to oval cell progenitors (see figure 1*e*), here there are organelles such as rough endoplasmic reticulum (r) and microvillus border (mv). Bar, 2 µm. (*b*) Small hepatocytes now surround each portal tract (PT) and expression of the biliary 'marker', CK19, is restricted to the periphery of these islands of new cells, where the biliary ductules have yet to differentiate into hepatocytes. Contrast with figure 4*d*, where the CK19-positive, intralobular biliary ducts are in obvious continuity with the 'parent' ducts in the portal tracts. Magnification ×200.

reactions with more substantial hepatocyte differentiation. Alternatively, allyl alcohol-induced periportal necrosis causes proliferation of biliary cells that do not form typical ductular structures, yet they still rapidly differentiate into small hepatocytes (Yavorkovsky *et al*. 1995). In contrast, ligation of the common bile duct (Sirica *et al*. 1985), feeding 4,4′-diaminodiphenylmethane or α-naphthyl isothiocyanate (ANIT) (Dunsford *et al*. 1985) induces bile duct proliferation with apparently no hepatocyte differentiation. 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 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*. 1988*b*, 1989; Meybehm *et al*. 1993; Delladetsima *et al*. 1995), even though some metaplasias express dual biliary/hepatocyte phenotypes (Van-Eyken *et al*. 1988*b*, 1989; Vandersteenhoven *et al*. 1990).

The characteristics of each ductular reaction are clearly dependent on the experimental regime used, primarily the administered hepatotoxin that impedes hepatocyte regeneration in the face of liver injury (Germain *et al*. 1988*a*). Each hepatotoxin could be acting in a highly specific manner resulting in the selective expansion of one or more ductal cell populations. For example, CDE diets induces cells of a 'transitional' biliary/hepatocyte phenotype, whereas rats fed on dimethylaminobenzidine (Germain *et al*. 1988*b*), or ANIT (Dunsford *et al*. 1985), results predominantly in a proliferation of cells committed to the biliary lineage. Feeding rats with furan induces a massive proliferation of ductular structures containing single 'hepatocyte-like cells' (Sirica *et al*. 1994). This suggests that furan induces a preferential proliferation of cells committed to the biliary phenotype, and/or inhibits proliferation/differentiation of hepatocyte precursor cells. The species, sex and strain of animal also have profound influences on the fate of ductular reactions (He *et al*. 1994), probably owing to different metabolic sensitivities to chemical carcinogens (Huitfeldt *et al*. 1990, 1991; Alpini *et al*. 1992; Yang *et al*. 1993). This is related to the differential expression of the various activating and detoxification systems, the phase I and phase II metabolic enzymes (Aterman 1992). For example, AAF used in the modified Solt–Farber regime (AAF/PH) is metabolized to the 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; Sirica *et al*. 1990). 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). 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).

Thus, 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

Table 1. *Experimental manoeuvres that cause biliary epithelial cell hyperplasia in the rodent liver, and the subsequent fate of these newborn cells*

regime	observations	reference
bile duct ligation		
ligation of the common bile duct of male rats for 1 day to 4 weeks	massive bile duct hyperplasia with moderate peri-biliary fibrosis and no hepatocyte differentiation or necrosis. Bile ducts have well-defined ductular profiles, do not penetrate parenchyma but remain confined to expanded portal stroma	Lenzi *et al.* (1992)
	as above, but occasional proliferating biliary cells express albumin and AFP mRNA	Alpini *et al.* (1992)
	as above, but did not express hepatocyte-enriched transcription factors or AFP mRNA	Bisgaard *et al.* (1996)
4,4′-diaminodiphenylmethane (DDPM)		
male rats fed basal diet containing 0.15% 4,4′-diaminodiphenylmethane for 1–12 weeks	as for bile duct ligation, with no expression of AFP or albumin	Sell (1983)
α-naphthyl isothiocyanate (ANIT)		
male rats fed casein diet containing 0.6% ANIT for 14–28 days	similar reaction to bile ligated livers, no AFP expression	Dunsford *et al.* (1985)
male rats fed standard diet containing 0.1% ANIT for up to 30 days	as above, but occasional cells in hyperplastic ducts express albumin and AFP mRNA	Alpini *et al.* (1992)
male rats fed ANIT by oral gavage (50 mg kg^{-1} body weight (BW) per day) for 3–20 days	necrosis of interlobular bile ducts within 3 days, followed by biliary cell proliferation and fibrosis by day 5. 'Indistinguishable' from normal bile duct hyperplasia	Carthew *et al.* (1989)
2-acetylaminofluorene alone		
male and female rats dosed by daily oral gavage of 10 mg kg^{-1} BW of either 2-AF, 2-AAF or N-OH-AAF for 1–9 days	no parenchymal necrosis. Proliferation of Hering duct cells and periductular cells, which express AFP, albumin and hepatocyte transcription factors within 24 h of carcinogen exposure. Potency of stimulus: N-OH-AAF > 2-AAF > 2-AF. No extensive proliferation and migration of these oval cells	Bisgaard *et al.* (1996)
2-acetylaminofluorene and CCl_4		
male rats fed a standard diet containing 0.02% 2-AAF for 2 weeks. CCl_4 (2 ml kg^{-1} BW) fed by oral gavage on day 7 and killed 1–14 days after CCl_4	classic zone-3 parenchymal necrosis with massive oval cell proliferation from portal tracts. Oval cells disappear after withdrawal of 2-AAF supplement	Ghoshal *et al.* (1983)
2-AAF in a choline devoid diet (CD/2-AAF)		
male rats fed CD diet supplemented with 0.05% 2-AAF for 12 days, returned to basal diet and killed up to 28 days after commencing the CD/2-AAF diet	essentially similar pattern of oval cell proliferation as for 2-AAF/PH regime. Albumin and AFP expression, but no evidence of complete hepatocyte differentiation	Sell (1983), Dunsford *et al.* (1985), Sell *et al.* (1981), Sell & Salman (1984)
2-acetylaminofluorene and partial hepatectomy(2-AAF/PH)		
oral gavage of male rats with 2-AAF for 5 days in first week, PH and feeding continued for further 4 days (average daily 2-AAF dose: 6.6 mg kg^{-1} BW). Rats killed up to 13 days after PH	no parenchymal necrosis. Gradual increase in number of oval cells after PH, with strong expression of albumin. [^{3}H]-thymidine initially incorporated exclusively in oval cells became 'transferred' to newly formed basophilic hepatocytes; i.e. the latter differentiated from the oval cells (the 'precursor–product relationship')	Evarts *et al.* (1987)
	oval cells express AFP and undergo intestinal metaplasia; metaplastic tissue did not express AFP	Bisgaard *et al.* (1994)
male rats fed a basal diet containing 0.02% 2-AAF for 2 weeks and PH administered at the midway point. Rats killed up to 9 weeks after PH	oval cell proliferation into parenchyma, with intestinal metaplasia common after 3 weeks. These metaplasias develop into cholangiofibrotic lesions. No hepatocyte differentiation	Tatematsu *et al.* (1985)
male rats fed 2-AAF (10 mg kg^{-1} BW) daily by oral gavage for 14 days, with PH performed at midway point. Rats killed up to 7 days after PH	massive oval cell proliferation with intestinal metaplasia by 1 week after PH. Oval cells express AFP and albumin, and differentiating CYP450-expressing hepatocytes develop from some of these ducts	Sarraf *et al.* (1994), Golding *et al.* (1995)

(*continued*)

Table 1. *(continued)*

regime	observations	reference
male rats fed 2-AAF at either 5 mg kg^{-1} or 2.5 mg kg^{-1} BW from 6 days prior to and up to 7 days after PH. Rats killed up to 14 days after PH	the higher of the two doses of 2-AAF produces a similar response as that observed using a 10 mg kg^{-1} dose (see above). The lower dose of 2-AAF gives a similar result up to day 7 after PH but without intestinal metaplasia, but by day 14 the majority of oval cells differentiate into small hepatocytes	Alison *et al.* (1996, 1997)
choline-devoid ethionine (CDE) diet		
male rats fed a standard choline-devoid diet supplemented with 0.05–0.1% ethionine for 1 day to 12 weeks	0.1% ethionine: proliferation of α-FP and of albumin-expressing, ductular oval cells which, unlike bile duct ligation, ANIT or DDPM treatment, do not commonly form well-defined ductular profiles. Hepatocyte differentiation very rare and no necrosis	Lenzi *et al.* (1992), Alpini *et al.* (1992)
	0.07% ethionine: oval cells proliferated as cords of cells initially, ducts by 2 weeks and some formed intestinal-like cells by week 5. Oval cells expressed both foetal and adult hepatocyte enzymes	Tee *et al.* (1994)
	0.05% or 0.1% ethionine: parenchymal necrosis, massive oval cell proliferation and cholangiofibrosis. Extent and onset of oval cell proliferation is greater and earlier at higher dose. No hepatocyte differentiation within ducts	Tarsetti *et al.* (1993)
	ultrastructurally, oval cells seen to migrate along sinusoids and also form intimate associations (desmosomal and tight junctions, canalicular formation, and interdigitation of microvilli) with neighbouring hepatocytes	Novikoff *et al.* (1991)
3′-methyl diaminobenzidine (3′-Me-DAB)		
male rats fed a standard diet containing 0.06% 3′-Me-DAB for up to 12 weeks	no parenchymal necrosis. Massive oval cell proliferation that penetrates parenchyma as ill-defined ductules. Cholangiofibrosis and intestinal-like differentiation after 12 weeks	Carthew *et al.* (1989)
	1–2% of proliferating oval cells expressing AFP	Germain *et al.* (1985)
	proliferation of oval cells accompanied by hyperplasia of small hepatocytes (derived from oval cells?) expressing the bile duct marker gamma glutamyl transpeptidase (GGT). By week 7, oval cell numbers decrease to be replaced by hyperplastic hepatocyte foci that express canalicular GGT	Suzuki *et al.* (1987)
galactosamine		
male rats given a single intraperitoneal (i.p.) dose of galactosamine (700 mg kg^{-1} BW) and killed 1–8 days after	parenchymal necrosis and proliferation of small clusters and ductular oval cells, expressing AFP and albumin. Transitional hepatocytes differentiate from these cells and lose biliary specific markers	Dabeva & Shafritz (1993), Dabeva *et al.* (1993)
two i.p. doses of galactosamine (750 mg kg^{-1} BW), 6 h apart and killed 1–10 days after	as above with oval cells expressing foetal hepatocyte enzyme markers and normal bile ducts. Small hepatocytes and 'hepatocytes lining atypical duct structures' present	Lemire *et al.* (1991)
dipin		
a single i.p. injection of dipin (60 mg kg^{-1} BW) into mice followed 2 h later with a PH. Livers examined up to 18 months thereafter	initial massive parenchymal necrosis, oval cell proliferation from which small foci of hepatocytes are derived. Differentiating hepatocytes occasionally seen in oval cell ducts. Damaged parenchyma replaced by 'newly formed hepatocytes'	Factor *et al.* (1994)

(continued)

Table 1. *(continued)*

regime	observations	reference
furan		
male rats fed furan (15–60 mg kg^{-1} BW) by oral gavage, once a day, 5 days a week for up to 13 weeks	liver necrosis and replacement by 'hyperplastic bile ductular structures' with abundant fibrosis. 'Ductular hepatocytic cells' expressing dual bile duct/hepatocyte phenotypes differentiating within some ducts. Development of intestinal-like glands and preneoplastic hepatocyte foci and hepatocellular carcinomas at later time points	Elmore & Sirica (1991, 1992, 1993), Sirica *et al.* (1994)
chloroform inhalation		
male and female rats exposed to chloroform vapour (300 p.p.m.) for 6 h per day, 5–7 days per week, for up to 13 weeks	parenchymal necrosis and regeneration by hepatocytes with biliary duct hyperplasia confined to portal stroma. Intestinal metaplasia with fibrosis developed from 6 weeks onwards	Jamison *et al.* (1996)
carbon tetrachloride inhalation		
inhalation of CCl_4 by mice, (no details of dose given)	classic centrilobular necrosis with AFP expressing oval cells that appear as small ducts or as small clusters of cells in the perinecrotic regions	Engelhardt *et al.* (1984)
diethylnitrosamine (DEN)		
three strains of male mice injected i.p. with 10–150 mg kg^{-1} BW DEN and examined 1–7 days thereafter	hepatic necrosis and oval cell proliferation. 'Transitional cells' (differentiating hepatocytes) observed within ducts. Different magnitudes of necrosis, oval cell proliferation and hepatocyte differentiation between strains	He *et al.* (1994)
long-term exposure to ethanol		
rats fed for 1–24 months on a totally liquid diet containing 5% ethanol	sparse numbers of very slowly proliferating oval cells expressing markers of foetal and mature hepatocytes. Similar changes as seen using the CDE protocol, yet over a much longer time frame; e.g. magnitude of response after 6–9 months on ethanol diet is similar to that after only 3 weeks on CDE regime	Smith *et al.* (1996)
allyl alcohol		
female rats injected with a single i.p. dose of allyl alcohol (0.62 mmol kg^{-1} BW) and killed after 6 h to 6 days	periportal necrosis induced within 6–12 h. Damaged parenchyma is reconstituted within 6 days by small hepatocyte-like cells (non-ductular oval cell progeny?) derived from 'intraportal stem cells'	Yavorkovsky *et al.* (1995)
diethylnitrosamine and *Clonorchis sinesis*		
Syrian hamsters infected with CS metacercariae and DMN (15 p.p.m.) in drinking water for 28 days	intense oval cell proliferation, followed by ductular dysplasia and development of cholangiocarcinoma	Lee *et al.* (1997)
***p21*$^{CIP1/WAF1}$ transgenic mouse**		
p21 overexpression targeted to the liver	retarded liver development with oval cell proliferation	Wu *et al.* (1996)
Long-Evans Cinnamon rat		
defect in Wilson disease gene (*ATP7B*) resulting in toxic accumulation of copper	acute hepatitis and death; survivors get chronic hepatitis accompanied by oval cell proliferation leading to development of hepatocellular carcinoma and cholangiocarcinoma	Betto *et al.* (1996), Yasui *et al.* (1997)

(figure 4*b*, *d*), invading deep into the hepatic parenchyma as tortuous arborizing ductules (Alpini *et al.* 1992; Sarraf *et al.* 1994), and 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). In addition, these hyperplastic ducts do not differentiate into hepatocytes, neither do they express liver-enriched transcription factors that regulate AFP and albumin expression (Bisgaard *et al.* 1996), unlike AAF-induced oval cells. Moreover, while bile stasis in adult 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.

Figure 4 illustrates the characteristics of a typical oval cell reaction with its demonstrable biliary heritage (figure

4*d*); the oval cells also express vimentin. 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 co-expression, whereas the differentiating agent, dimethyl sulphoxide, inhibited the increase in vimentin (Pagan *et al*. 1997).

Many studies have attempted to identify the first cells to proliferate at the onset of oval cell reactions, the rationale being that the initial 'proliferators' represent the stem cells. Cell proliferation may begin both in the cholangioles (Grisham & Porta 1964; Lenzi *et al*. 1992; Alison *et al*. 1996) and small marginal bile ducts (Evarts *et al*. 1993; Anilkumar *et al*. 1995), with concomitant expression of AFP and albumin (Golding *et al*. 1995). Some studies report that it is not the biliary epithelium that responds first, but the biliary-associated periductular cells (Sell 1990; Lenzi *et al*. 1992). These cells may give rise to oval cells themselves (Evarts *et al*. 1990), as well as components of the biliary stroma (Grisham & Porta 1964; Lenzi *et al*. 1992). Some periductular cells express AFP mRNA in normal liver (Alpini *et al*. 1992) and oval cell markers in carcinogen-treated livers (Evarts *et al*. 1990). They maybe Ito cells or myofibroblasts that co-proliferate with oval cells, and these have been distinguished from the latter by expression of desmin; on this basis their role as oval cell progenitors has been more or less discounted (Thorgeirsson *et al*. 1993). During foetal bile-duct development in humans, α-smooth muscle actin-positive Ito cells envelope the new ducts as they form from the ductal plate (Cocjin *et al*. 1996), emphasizing the close association between the two cell types.

Oval cell ductules eventually differentiate into small hepatocytes (figure 5*a*), loosing biliary traits (CK7 and CK19 expression, figure 5*b*), and ceasing expression of vimentin and the oncofoetal glycoprotein, AFP.

7. STROMAL INFLUENCES ON REACTIVE BILE DUCTULES

Reactive ductules in human liver are surrounded by activated mesenchymal cells consisting primarily of myofibroblasts and perisinusoidal Ito cells (Burt & MacSween 1993). After hepatic injury in humans, 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*. 1993*b*; 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 humans (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). The oval cells themselves might also degrade matrix proteins, as primitive biliary cells migrating from the ductal plate can express matrix metalloproteinases during liver development in humans (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; and see figure 4*b*). 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). Therefore, Ito cells among 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' and permitting differentiation into hepatocytes.

Cell–cell recognition and cell–ECM interactions are critical for cellular aggregation, segregation and migration. Contact with ECM components is fundamental in retaining cell polarity and regulating tissue-specific histogenesis and gene transcription, as well as maintaining tissue architecture (Rescan *et al*. 1993; Stamatoglou & Hughes 1994). Changes in matrix and intercellular adhesion very likely influence the pronounced tissue remodelling and alterations in gene expression that 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 that 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). The hepatic parenchyma is unique compared with other epithelia in that it does not rest on a true basement membrane, although the hepatocytes rest on a 'basement membrane-like matrix' consisting of collagen type IV, laminin, fibronectin and various proteoglycans (Stamatoglou & Hughes 1994). In addition to cell–matrix interactions, cell–cell associations are also likely to have an influence on the hepatocyte phenotype.

8. GROWTH FACTORS INVOLVED IN OVAL CELL ACTIVATION

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 humans, PTHrP is expressed in reactive bile ductules of cholestatic and regenerating liver (Roskams *et al*. 1993*a*), as well as by cholangiocarcinomas (Roskams *et al*. 1993*b*). The fact that growth factors such as EGF can rapidly induce PTHrP synthesis in cultured ductular cells has led to the belief that PTHrP is a member of the early response gene family, and that 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 that 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*. 1993*a*) 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*. 1993*b*). 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*. 1993*a*), 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).

9. THE MULTIPOTENTIALITY OF OVAL CELLS

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*. 1993*a*,*b*).

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 that 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 pancreatic ductal epithelial cells from rats 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 humans (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 non-hepatic 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).

Under normal conditions, remarkable similarities exist between the organs of the digestive system. For instance, like the intestine, goblet cells and brush border cells are found in the larger ducts of the pancreas, as are endocrine cells, which unlike those of the intestinal crypt, consist of cell foci (the islets of Langerhans). During embryological development the islet precursors appear as individual cells 'embedded' within the ducts (Slack 1995), and during situations that provoke chronic inflammation, not unlike those seen accompanying oval cell proliferation, new islets are observed budding from ductules in conjunction with ductular hyperplasia and goblet cell metaplasia. After selective ablation of the pancreatic acinar component, the remaining ducts will rapidly regenerate to replenish the lost tissue. In fact, the regenerative powers of the pancreas appear to be as great as those of the liver in that even after 90% of the organ is removed, the animal survives with subsequent regeneration of both exocrine and endocrine components (Slack 1995); evidently like liver stem cells, pancreatic stem cells also reside within the ductular network.

Are there markers on oval cells which are indicative of lineage commitment? Differential expression of a combination of AFP and CK14 may be 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, which gives rise to committed unipotential stem cells, or if there are lineage-specific stem cells scattered about within the biliary tree.

10. *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 non-parenchymal 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 liver tissue from foetal and adult rats (Grisham 1980; 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 (Hayner *et al*. 1984; Tsao *et al*. 1984; Marceau *et al*. 1986).

Other groups have propagated long-term cultures of small hepatocytes that generally do not express characteristics of biliary cells such as CK7 and CK19 expression (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 analbuminemic, double-mutant rats (Yasui *et al*. 1997).

11. OVAL CELLS AND CANCER

Stem cells are important from the viewpoint of being the most likely targets for carcinogenic agents, because 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 1993*a*,*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 1993*b*).

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 that 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).

12. CONCLUSIONS

Reactive biliary epithelium, usually organized into ductules and 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. In addition, 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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