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Liver injury models for induction of hepatic oval cells in rodents

Abstract

Hepatic oval cells are progenitor stem cells residing in the liver that play an important role in liver regeneration when hepatocyte response to the injury is inadequate. On-going studies continue to characterize these elusive cells, understand their role in carcinogenesis, and determine their potential for therapeutics. Since oval cells proliferate only upon exposure to liver injury, a multitude of injury models have been developed in the past years to investigate their activity. These injury models comprise chemical, surgical and biological components. Depending on the chemical toxicity or lethality of injury, different extents and time required to induce oval cell responses are seen. Here, we review the various strategies available that can be used to induce hepatic oval cells in rodents.

Keywords: animal models, liver, liver regeneration, oval cells, rodentia

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Introduction

The liver is a remarkable organ with immense regenerative potential. Hesiod's *Theogony* describes the punishment meted out to Prometheus for restoring fire to humanity–his liver would be eaten by an eagle daily only for it to regenerate overnight before the ordeal is revisited. Modern science now affords us the opportunity to investigate why and how the liver regenerates. Most humans tolerate major hepatectomies with the remnant liver undergoing hypertrophy to maintain homeostasis. Animal experiments reveal that after a partial hepatectomy (PH), rats and mice regenerate their liver mass almost completely by 7 and 14days, respectively.[1–3]

Injury models to explore liver behavior with chemical induction of cirrhosis and carcinogenesis led to the work of Opie EL.[4] He recognized the development of biliary ductular cells proliferating around portal tracts of rats fed butter yellow (dimethyl aminoazobenzene). These cells were further characterized and given the name "oval cells" by Farber E.[5] Since then, numerous studies using a multitude of injury models in rodents have identified oval cells as hepatic progenitor or stem cells. We reviewed the MEDLINE database with a PubMed search using terms "hepatic oval cells" up till Dec 2013. Publications written in English pertaining to injury models and oval cells were reviewed in-depth. We begin with a concise summary of hepatic oval cells before reviewing the chemical and surgical strategies available to induce hepatic oval cells in rodents.

Hepatic oval cells

After massive injury, the liver regenerates by compensatory proliferation of hepatocytes.[6] However, when this pathway is inhibited or inadequate such as hepatocyte replicative senescence, hepatic oval cells may proliferate.[7] In such situations, oval cells constitute an available transit amplifying cell compartment to continue liver regeneration and repopulation.[8,9]

Oval cells appear as small cells approximately 1/3 the size of hepatocytes.[10] As so named, they are oval-shaped with unclear margins and scant, slightly basophilic-like cytoplasm.[5] The cell has a large round or oval-shaped blue-staining nuclei on routine hematoxylin & eosin staining, that contains a fine chromatin network and prominent nucleolus. In mice, oval cells have a diameter of 7-10μm.[11,12] In rats, they measure 7-12μm.[13–17] They are even slightly larger in hamsters at 7-14μm.[18] When oval cells proliferate, active DNA synthesis occurs and this can be observed using bromodeoxyuridine or 5-ethynyl-2'-deoxyuridine labeling.[15] Electron microscopy indicate that oval cells initially exhibit characteristics of undifferentiated cells with a high ratio of nucleus to cytoplasm with obvious nucleoli, rare cytoplasmic organelles with only few mitochondria, endoplasmic reticulum and some villus-like apophysis the cell surface.[10,19] The nuclei and cytoplasmic organelles resemble bile duct cells except that oval cells contain greater numbers of ribonucleoprotein granules.[20]

While the scientific literature on the origin of oval cells is still incomplete, oval cells are likely to originate from the embryonic ductal plate and reside facultatively at the canal of Hering.[12,21–23] Upon injury, they first appear near biliary ductules, spreading along portal tracts before infiltrating the rest of the liver.[5,24,25] Initially, they resemble proliferating bile duct cells, save that they do not form recognizable ductular profiles.[26] Cell surface markers have identified oval cells in normal rodent liver.[27] Normal mice have on average 8.07±5.02 oval cells per portal tract,[28] or 0.04% of total liver cells.[29] In normal rats, 2.5±0.5 x10^5 oval cells have been isolated per 100g body weight.[30]

Oval cell surface markers

A major impediment in studying oval cells is the lack of consistent and specific cellular markers. Identification begins with hematoxylin and eosin staining to visualize typical histological characteristics. In the past decade, developments in immunohistochemistry and hybridoma technology have defined the oval cell population

further.[31]Numerous cellular markers are reported to identify oval cell populations although only few have been specific to oval cells. This may be due to the progressive differentiation of oval cells in which the expression patterns of markers change. Due to the cross-reactivity of markers with other cell types, conflicting studies are often found positing for and against the same marker. CD44, for example, has been linked to oval cells,[32] undifferentiated oval cell phenotypes,[33] and small hepatocytes.[34] Thymocyte antigen 1 (Thy-1), a hematopoietic stem cell marker, is expressed on oval cells[35] although other studies report the opposite and that bone marrow cells,[36] myofibroblasts and activated stellate cells are Thy-1 positive.[37]

Nonetheless, some markers more commonly applied by the research community are described. Markers related to immature oval cell phenotypes include c-kit, stem cell antigen-1 (Sca-1), neural cell adhesion molecule and CD34[11,38–40] while markers for mature phenotypes share similarities to adult biliary cells or hepatocytes. Murine oval cell markers include A6, Cytokeratin 19 (CK19), OV-6 and M2-isozyme of pyruvate kinase (M_2PK).[41–45] For rats, markers include but are not limited to gamma-glutamyl transpeptidase (γGT), CK19, OV-6, BD-2, OC-2, Thy-1, β-catenin, M_2PK, alpha-fetoprotein (AFP), delta-like 1 homolog (Dlk-1), albumin and epithelial cell adhesion molecule. [16,26,32,35,37,46–52] Other animals in the *Rodentia* family share similar antigens on their oval cells. Oval cells in hamsters express CK19, AFP, albumin and OV-6[18,53] while the woodchuck expresses CK18.[54]

To isolate oval cells, they are first separated from hepatocytes through centrifugation with the oval cells partitioning into the non-parenchymal cell fraction. Thereafter, methods such as Nycodenz gradient separation, flow cytometry or immuno-magnetic sorting (using above-stated cell markers) are applied to isolate oval cells.[55] These methods allowed isolation of highly-pure fractions of oval cells of more than 95% with demonstrable proliferative potential.[35,56] Nevertheless to the lack of specific cell surface antigens, a combination of criteria is used to define oval cell populations during cell isolation.

Two of these criteria are described:

A. diameter of 10-15μm, presence of CK19, presence of γGT activity, presence of albumin, absence of peroxidase activity.[46]

B. diameter of 10-15μm, immunoreactivity to OV-6, expression of CK19 and placental glutathione S-transferase (πGST), presence of γGT activity, partial expression of albumin and AFP, and absence of peroxidase activity.[13]

Functions of hepatic oval cells and potential for clinical use

Oval cells are hepatic progenitor cells as they possess some stem cell characteristics. Stem cells are defined as undifferentiated cells capable of proliferation, self-maintenance, producing a large number of differentiated functional progeny, regenerating tissue after injury and flexibility in the use of these options.[57] As mentioned earlier, hepatic oval cells proliferate to regenerate the liver after injury. Established stem cell markers such as Sca-1,[11] CD34,[11,40] CD35;[11] and markers of hepatoblasts such as CD44,[32,33] Dlk-1[33,52,58] have been identified on oval cells.[59] Oval cells also demonstrate the side population phenotype characteristic of stem cells by their ability to efflux Hoechst 33342 dye.[60] Importantly, oval cells are able to differentiate into both hepatocytes and biliary epithelial cells.[16,37,61–64] Oval cells can also differentiate into other cells. For example, intestinal metaplasia has been observed in 0.02% N-acetyl-2-aminofluorene (2-AAF)/PH model.[65,66] Trans-differentiation of oval cells toward pancreatic beta-cell phenotype with production of pro-insulin[67] occurs when exposed to high glucose environments.[68,69]

In keeping with having properties of a stem-cell, oval cells have been reported to differentiate into cells outside an endodermal lineage. Oval cells have been found to express vimentin and N-cadherin indicating their ability to acquire mesenchymal properties,[32,37,40] and can differentiate into mesenchymal cells after being implanted subcutaneously into a host.[70] Deng et al reported oval cells undergoing neural trans-differentiation after being injected into brain tissue, adopting the morphology and antigenic phenotype of macro- and microglial cells and assuming immune-positivity for nestin.[71]

Stem cells, which possess the ability for tissue repair and regeneration, may be the key to treating severe and chronic illnesses. Hence, much interest lies in the potential of oval cells as a therapeutic agent to reconstitute injured livers.[72] Cultured oval cells have been propagated to 100 passages without malignant transformation, highlighting the possibility of an expandable cell-source for hepatocytes.[73] When transfused into a host, oval cells demonstrate liver-tropism and can repopulate up to 90% of the liver after PH.[37,74] Transplanting oval cells in rats improves liver function and survival after D-galactosamine (GaIN) induced fulminant hepatic failure[75] and ameliorates ischemia/reperfusion injury.[64] In rat liver transplantation, addition of oval cells prolonged median survival from 21 to 38days,[14] improved liver function and reduced rejection activity.[14,64] These findings suggest significant potential for oval cells as therapy for acute liver failure and modulating liver transplantation outcomes.[72]

Background on rodent liver injury models

The Solt-Farber protocol was the first consistent and reproducible method to induce oval cell proliferation.[76] This involved an intra-peritoneal dose of di-ethylnitrosamine (DEN) 20mg per 100g body weight at day 0 followed by a recovery period of 2weeks. Thereafter, 2-AAF was added in the basal diet and PH performed one week later. Oval cells were identified 30hours after PH.

Since then, variations to streamline this induction process, new toxicology tests yielding oval cells, and gene modification have led to a myriad of injury models. We propose an adaptation of Terblanche et al.[77] model for acute hepatic failure.

An ideal injury model to study oval cells would comprise:

i. a standardized animal model,

ii. an option for gene modification to investigate activation pathways,

iii. reproducible oval cell response,

iv. minimize mortality to allow studying long-term effects of oval cell activation and carcinogenesis,

v. minimal hazard to personnel.

We will first discuss the factors surrounding animal models followed by the injury agents.

Animal models

Species, gender and strain: Rats and mice are the two most common rodents used as they are easily standardized animal models. Few studies have used hamsters and woodchucks. Each species possesses different metabolic pathways and liver micro-environments. Between species, the same agent does not generate equivalent oval cell responses and cellular expression, possibly even of oval cell phenotype, as indicated

by discrepant markers.[52] Gender discrepancies have been observed in rats. In the pioneering work by Farber, oval cell responses after ethionine and 2-AAF were seen only in male Wistar rats while livers of corresponding females were normal.[5] Bisgaard et al.[78] also found that 2-AAF elicited smaller mitogenic responses in female Fischer-344 rats compared to males. This phenomenon may be due to female rats having a smaller enzymatic capacity to N-hydroxylate 2-AAF into N-hydroxy-2-AAF, the chemical that elicits the mitogenic effects of 2-AAF.[79] Notwithstanding, oval cell proliferation in female Wistar, Sprague-Dawley and Fischer-344 rats has been reported in other studies.[80–84]

Limited information is available on strain-related differences although one study by Wood et al.[85] compared oval cell responses between 2 strains of age-matched rats. The oval cell response in Fischer-344 rats was extensive but oval cells remained localized to the portal areas in Copenhagen rats. Further work is required to characterize the gender discrepancies within rats, the different rat strains and how they affect interpretation of oval cell responses.

Age: Consistent with other stem cell types, oval cells are subject to age-related quiescence and decline in function.[86] While experiments with mice ranging from 3 up to 12weeks have identified oval cells,[43,87,88] age-related discrepancies have been noted in terms of number of cells and proliferative ability. With regard to number of oval cells, conflicting evidence has been reported. Comparing mice older and younger than 8weeks undergoing an interval-feeding injury model, no oval cells were seen in mice younger than 8 weeks. It was suggested that this may be related to how oval cells respond to cumulative proliferative demand over extended periods of time.[3] On the other hand, studies exist reporting age dependent oval cell responses in the opposite direction, with younger animals demonstrating greater responses compared to older animals.[89,90] Concerning the actual proliferative ability of the oval cells isolated, evidence has been consistent in finding that oval cells from older mice have lower regenerative potential.[25,90] Cell lines cultured from mice 4weeks old were maintained for more than 30 passages, but those from mice 8weeks old were maintained for only 20 passages.[25] Therefore, the age of the animal may influence the extent of oval cell response as well as the ability of the oval cells to effect a regenerative response**.**

Injury agent

Basis and methods of oval cell induction: There are two differing hypotheses on how oval cells are activated. One hypothesis is the resistant hepatocyte model that involves the use of a toxic carcinogen.[76] The cytotoxic effect of the carcinogen causes hepatic cell death which selects for cells resistant to or acquire resistance to the toxicity and gain a proliferative advantage. Oval cells become selected and activated through this mechanism. Experiments by Anil kumar et al.[91] instead indicate that oval cells belong to a facultative stem cell compartment. The alternative two-hit hypothesis suggests that oval cells exist facultatively and that both hepatocytes and oval cells respond to the injury, except that hepatocytes proliferate preferentially. A "first hit" exhausts or inhibits hepatocyte responses while the "second hit" initiates the oval cell response.[92,93]

Cellular injury does not guarantee oval cell proliferation.[94] The site of injury within the liver (centri-lobular vs peri-portal) influences the time taken and magnitude of oval cell response. Chemicals such as N-acetyl-p-aminophenol (APAP) and carbon tetrachloride (CCl_4) which preferentially damage centri-lobular liver have been observed to induce quicker and greater oval cell responses.[28,92] This has been hypothesized to be due to oval cells residing in the peri-portal region. Hence, agents targeting centri-lobular regions spare the oval cells, allowing for more prolific responses.[28] Combining agents may result in synergistic effects. For example, allyl alcohol (AA) and CCl_4 were compared using dosages that resulted in equivalent levels of hepatic necrosis. When 2-AAF was added to these dosages of AA and CCl_4, the 2-AAF/CCl_4 model generated significantly greater and more persistent responses as compared to 2-AAF/AA.[92]

Classification of injury agents: Injury models can be broadly classified into chemical, surgical and biological, or a combination. The 2 most common injury methods are chemical agents (Table 1) and the combination of chemical agents with PH (Table 2). The high number of permutations of agents, dosage, delivery method, kinetics, and duration presents a challenge in providing a meaningful analysis. We have elected to discuss the published results of more commonly used agents.

Table 1 Chemical liver injury models in rodents

Model	Species	Selected references
CDE	Mouse, Rat	17,20,27,44,99,192
DDC	Mouse	11,12,52,100,101,106,193,194
GalN	Rat	94,111-115
DEN	Mouse	117–119
APAP	Mouse	28,52,127
CCl_4	Mouse, Rat	130,131
2-AAF	Rat	5,133
2-AAF/CCl_4	Rat	33,92,94,136,182
2-AAF/AA	Rat	92,136,139
Choline deficient diet/DEN/2-AAF	Hamster	53
3'-Me-DAB	Rat	5,51,144,188
Phenobarbital/Cocaine hydrochloride	Mouse	145
TCPOBOP	Mouse	45

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Table Continued....

Model	Species	Selected references
DDC/TCPOBOP	Mouse	101
Retrorsine/GalN	Rat	115
Fumosinin B_1	Rat	148,149
Ethanol	Mouse, Rat	7,151
High fat diet & ethanol	Mouse	150
Iron overload	Rat	152

CDE, choline-deficient diet supplemented with ethionine; 3'-Me-DAB, 3'-methyl-4-dimethyl aminoazobenzene; DDC, 3,5-diethoxycarbonyl-1,4-dihydrocollidine; Gain, d-galactosamine; DEN, di-ethyl nitrosamine; APAP, n-acetyl-p-aminophenol; Ccl4, carbon tetrachloride; 2-AAF, n-acetyl-2-aminofluorene; AA, allyl alcohol; TCPOBOP, 1,4 bis[2-(3,5-dichloropyridyloxy)] benzene.

Table 2 Combined chemical and surgical liver injury models in rodents

Model	Species	Selected references
DEN/PH	Rat	120
2-AAF/PH	Mouse, Rat	14,24,37,52,62,75,141,162,195
DEN/2-AAF/PH	Rat	76,85,121,122
Choline deficient diet/DEN/2-AAF/PH	Hamster	18
Dipin/PH	Mouse	42,146,147
Retrorsine/PH	Rat	32,196
Ethanol/PH	Mouse	45

DEN, di-ethylnitrosamine; PH, 2/3 partial hepatectomy; 2-AAF, n-acetyl-2-aminofluorene; dipin, 1,4-bis [n,n'-di(ethylene)-phosphamide]-piperazine.

Chemical

A. Choline-deficient diet supplemented with ethionine (CDE): Choline, a quaternary compound (trimethyl-β-hydroxy-ethylammonium), is an essential nutrient and precursor of the acetylcholine. It is needed to form cellular membranes and is an important methyl-group donor necessary for converting homocysteine to methionine.[95] Choline deficiency leads to inflammation and oxidative stress, causing steatohepatitis and liver fibrosis. Its involvement with methylation and epigenetic changes also causes genetic mutations and cancer when deficient.[96]

Choline-deficient diets can be supplemented with ethionine in drinking water to exacerbate their toxicity.[97] Ethionine is the ethyl antagonist of methionine and results in hypomethylation of hepatic DNA due to the accumulation of S- adenosyl ethionine.[98] This is the most commonly applied chemical injury model on both rats and mice.[20,44] Akhurst et al.[99] suggested that an ideal response using CDE in mice was achieved with 50% choline-deficient diet and 0.15% DL–ethionine for 2-3weeks. Full choline-deficient diets resulted in extensive hepatic steatosis while 50% choline-deficient diet maintained the mice in a good condition with minimal steatosis. Reducing ethionine resulted in fewer oval cells seen.[99] Peak oval cell response and plateauing of doubling time is seen at around 2weeks.[27,99]

B. 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC): DDC is a porphyrinogenic hepatotoxin that can be reconstituted at 0.1% in the diet to generate a prolific oval cell response at 3-6weeks.[52–100] It activates constitutive androstane receptor (CAR, NR113) that induces transcription of the cytochrome p450 2b gene in hepatocytes.[101] CAR also regulates endogenous energy metabolism by inhibiting key gluconeogenic genes that encode phosphoenoyl pyruvate carboxylkinase and glucose-6-phosphatase.[102,103]

DDC causes the accumulation of N-methylprotoporphyrin IX, a potent inhibitor of the enzyme ferrochelatase which converts protoporphyrin IX into heme.[104] This results in aggregation of protoporphyrin IX and porphyrin precursor δ-aminolevulinic acid.[105] Porphyrin crystals form within hepatocytes in the peri-portal regions,[101] causing hepatocyte, ductal and oval cell proliferation.[12] In contradistinction to other models where hepatocyte proliferation is inhibited, hepatocyte proliferation continues allowing the study of interactions between hepatocytes and oval cells.[106]

C. D-Galactosamine (GaIN): GaIN is a hexosamine metabolized through the galactose pathway in the liver.[107] GaIN sequesters available uridine which results in depletion of intracellular uridine derivatives in hepatocytes and loss of intracellular calcium homeostasis.[108] Cell membranes and organelles are disrupted, and RNA and protein synthesis are arrested.[77,109] GaIN liver injury in rats causes pan-lobular, focal hepatic necrosis that is microscopically and biochemically similar to that of viral hepatitis in humans.[110] GaIN is administered intra-peritoneally as a single dose to rats between 50-140mg per 100g body weight. Maximal oval cell proliferation is seen between 2-5days[94,111,112] and oval cells disappeared 7-10days after the injection.[113,114] GaIN can also be given 2weeks after retrorsine, a tumorigenic pyrrolizidine alkaloid, to generate greater numbers of oval cells.[115]

d. ***Di-ethylnitrosamine (DEN)***: DEN belongs to the family of carcinogenic N-nitroso compounds.[116] It causes alkylation of nucleic acids and proteins in hepatocytes resulting in cirrhosis and cancer.[117] DEN can be given per-oral or intra-peritoneally, and/or in combination with other agents.[47,76,118] Mouse models use DEN alone (intra-peritoneal)[119] while rat and hamster models use DEN (either per-oral or intra-peritoneal) in combination with 2-AAF and/or PH.[53,120,121] For rats, oral preparations are dosed at 1mg per 100g body weight[120] while the intra-peritoneal dose is 15-20mg per 100g body weight.[122,123] In the DEN/2-AAF/PH model, peak oval cell response is seen 7 and 14days after PH.[85,124]

e. ***N-acetyl-p-aminophenol (APAP):*** APAP is an aminobenzene that is metabolized by cytochrome p450 into the toxic metabolite N-acetyl-p-benzoquinone imine (NAPQI). NAPQI is usually conjugated by glutathione in the liver into harmless cysteine and mercapturic acid conjugates. When given in overdose quantities, glutathione stores are depleted and NAPQI covalently binds to DNA and cysteine residues on hepatic proteins leading to 3-(cysteine-S-yl) APAP adducts.[125] Changes in the liver may be detected within 30minutes of injury – sinusoidal endothelial cells swell and microvascular congestion occurs in the centri-lobular region.[126] Necrosis of hepatocytes develops at around 2hours.[126] Oval cell responses are detected as early as 3hours after injury and it is hypothesized that this rapid response is due to a centri-lobular form of injury.[28] For mice, APAP is given as a single intra-peritoneal dose between 25-100mg per 100g body weight[28,52] or in multiple doses at 30mg per 100g body weight.[127]

f. ***Carbon Tetrachloride (CCl4):*** CCl_4 is a halogenated alkane that is metabolized in the liver into trichloromethyl radical (CCl_3*) and chlorine. CCl_3* binds to numerous cellular molecules, produces reactive oxygen species and forms DNA adducts.[128] Similar to APAP, CCl_4 preferentially damages the centri-lobular region.[92] Studies in rats have found that CCl_4 alone cannot generate an oval cell response[94,114,129,130] although the converse has been reported.[131] In mice, few A6 positive oval cells were seen after chronic CCl_4 exposure.[130] CCl_4 is commonly combined with 2-AAF for use in rats. In 2-AAF/CCl_4 models, the oral dose of CCl_4 is 90mg per 100g body weight[81] while the intra-peritoneal dose is 150mg per 100g body weight.[36,83,132] Maximal oval cell responses after 2-AAF/CCl_4 are between days 7-9 after the start of the protocol.[36,83,132]

g. ***N-acetyl-2-aminofluorene (2-AAF):*** N-acetyl-2-aminofluorene (2-AAF) is an aromatic amine that becomes metabolized to N-hydroxy-2-AAF which is the potent proximate carcinogen.[78] It blocks proliferation of hepatocytes[94] and forms DNA adduct leading eventually to cancer.[133] After 1week of 0.02% 2-AAF in the diet, at least 95% of hepatocyte replicative response is inhibited when PH is performed.[134] The liver mass of 2-AAF/PH treated rats is arrested until 11days after PH (from 33 to 57%) compared to near complete regeneration in 7days after PH alone.[2] Since 2-AAF needs to be metabolically activated, various oval cell responses have been seen depending on the gender, strain and species.[5,78,79,135]

2-AAF mixed in the diet (constituting 0.02-0.04%) generates an oval cell response after 2-4weeks.[5,133] 2-AAF is frequently combined with PH, CCl_4 or AA in mouse and rat models.[62,76,92,136] These studies delivered 2-AAF at doses of 0.4-2mg per 100g body weight daily[33,52,66,137] or 1-1.5mg per day by gavage.[24,138] In recent years, 2-AAF is commonly administered with a time-released pellet implanted subcutaneously or intra-peritoneally, delivering 2.5mg per day.[35,139,140] In 2-AAF/PH models, 2-AAF is started for 4-6days prior to PH, omitted (if administered orally) on the day of PH, and resumed thereafter for another 4-10days.[37,62,75,141] Maximal oval cell proliferation after 2-AAF/PH is reported at 6-11days after PH.[15,37,52,142]

h. ***Others:*** Numerous other chemical agents are available. One example is 3'-methyl-4-dimethylaminoazobenzene (3'-Me-DAB), an azo dye with significant hepatocarcinogenic properties. Nearly 100% of rats develop hepatocellular carcinoma after 20weeks of 3'-Me-DAB diet.[143] Measuring serum AFP levels while on 3'-Me-DAB demonstrates two peaks: the first corresponds with the appearance of oval cells while the second indicates the development of hyperplastic nodules and hepatocellular carcinoma.[143,144] Cocaine hydrochloride is a hepatotoxin that, in contra-distinction to APAP and CCl_4, causes peri-portal injury.[145] TCPOBOP (1, 4 bis [2-(3,5-dichloropyridyloxy)]-benzene) activates the same CAR-ligand as DDC. Interestingly, there is preferential CYP2B10 induction in centri-lobular hepatocytes, as opposed to DDC.[101] Dipin (1, 4-bis [N,N'-di(ethylene)-phosphamide]-piperazine) is an alkylating drug that causes irreversible damage to DNA, RNA and proteins.[146] Dipin is combined with PH to induce oval cell proliferation although the time taken varies from 1-11weeks.[42,142,146,147] Fumosinin B_1, a mycotoxin produced by Fusarium moniliforme, is a non-genotoxic carcinogen that induces oval cell proliferation but allows for hepatocytes to proliferate, similar to DDC.[148,149] Diet modification using high fat or alcohol diets leads to steatohepatitis and oval cells responses can be observed after chronic exposure.[7,150,151] Chronic diet-induced iron overload also shows similar oval cell responses.[152]

Surgical

i. Partial hepatectomy (PH): The surgical procedures that can be performed on rodent livers to induce injury are listed in Table 3. The most commonly used procedure is PH, which involves removing 50-83.4% of the liver.[76,137,152] A widely described technique is the 70% or 2/3 PH model described by Higgins et al.[154] The left lateral and median lobes are approximately 72% of total liver weight.[155] PH alone does not always generate oval cells [114,156,157] and is used in combination with chemical agents. However, some studies have isolated oval cells in a pure PH model.[153,158,159] This is a unique opportunity to study oval cell responses in livers that are otherwise healthy and naïve to toxic injury. The modified Solt-Farber protocol uses 2-AAF/PH without DEN.[62,100,160] 2-AAF/PH can be used on mice and rats, and peak responses are between 7 to 11days after PH.[37,52,60,156,161,162]

Table 3 Surgical liver injury models in rodents

Model	Species	Selected references
PH	Mouse, Rat	[153,158,159]
Bile duct ligation	Rat	[164,165]
Portal vein branch ligation	Mouse, Rat	[166,167]
PH, 2/3 partial hepatectomy.		

ii. ***Bile duct or portal vein branch ligation:*** Bile duct ligation causes cholestatic liver injury and biliary ductal proliferation. Oval cell proliferation is not commonly associated with this model[156,157,163] although few studies have identified oval cells 7 and 10days post-ligation using M_2PK and CK19 as markers, respectively.[164,165] Ligating a portal vein branch causes ischemia, atrophy of the affected lobes and compensatory hypertrophy of non-affected lobes. Ligating the left portal vein branch produces a peak oval cell response after 7days.[166,167] Similar to PH alone, these ligation models allows the study of oval cells induced by non-carcinogenic injury.[167]

iii. ***Other surgical adjuncts:*** Parasympathetic stimulation is known to modulate oval cell responses. Vagotomized rats generated a smaller response due to the loss of stimulatory effects of acetylcholine via the muscarinic acetylcholine receptor type-3.[168] Inhibiting the sympathetic nervous system chemically with 6-hydroxydopamine or pharmacologically with prazosin generated heightened oval cell responses but less hepatic necrosis and steatosis.[169] These findings have implications on the role of oval cells in liver transplantation since donor grafts are denervated.

Biological: Biological injury models involve gene modification or the introduction of and infection or immune-mediated injury (Table 4). Gene modification generates animals which develop different forms of liver injury. Mutation of the Atp7b gene creates Long-Evans Cinnamon rats that have toxic accumulation of copper within the liver. Spontaneous hepatitis develops at 16weeks of age with concomitant oval cell proliferation.[170,171] Homozygous obese (ob/ob) mice develop steatohepatitis and oval cell responses similar to alcohol-induced models.[7,45] AL-uPA transgenic mice express hepatotoxic urokinase-type plasminogen activator in hepatocytes, generating a proliferative response from trans-gene deficient hepatocytes and oval cells.[172] Gene-transfer models with suicide genes injure the liver biochemically. One example is recombinant adenoviral vector AdCMVtk which contains herpes simplex virus thymidine kinase (HSV-tk) gene. When ganciclovir is administered to animals treated with AdCMVtk, HSV-tk phosphorylates ganciclovir into a cytotoxic derivative that causes severe liver injury and massive oval cell responses.[173] Adenoviral vectors can also introduce growth factors such as hepatocyte growth factor (HGF) and vascular endothelial growth factor (VEGF) to modulate oval cell behavior.[155,174] In the cases of HGF and VEGF, increased oval cell responses were seen after liver injury.[155,174]

Table 4 Biological liver injury models in rodents

Model	Species	Selected references
Long-Evans Cinnamon	Rat	170,171
Obese mutant (*ob*/*ob*)	Mouse	7,45
Urokinase-type plasminogen activator transgene	Mouse	172
Herpes simplex virus thymidine kinase gene	Rat	173
Taurine transporter knockout (*taut*-/-)	Mouse	175
Helicobacter hepaticus	Mouse	177,178
Woodchuck hepatitis virus	Woodchuck	54,179
Woodchuck hepatitis virus/ Aflatoxin B_1	Woodchuck	179,197
Fas receptor/CD95	Mouse	25

Gene knockout models can be used to induce liver injury or investigate pathways of oval cell activation. Taurine transporter knockout (taut-/-) mice develop non-specific non-steatotic hepatitis and oval cells could be identified in these mice beyond 1year of age.[175] Interleukin-6 (IL-6), WW45 and p53 knock-out models are some examples used with injury models described above to delineate oval cell signaling and carcinogenic pathways.[87,145,176] Infective hepatitis models include *Helicobacter hepaticus* infection in mice[177,178] and chronic woodchuck hepatitis virus in woodchucks.[54,179] Acute and chronic hepatitis develops in these animals, eliciting oval cell responses and subsequently, hepatocellular carcinoma. Direct immune-mediated injury can be induced by stimulating the Fas receptor/CD95 causing apoptosis of hepatocytes and fulminant hepatic failure.[180] Tsuchiya et al.[180] injected mice intra-peritoneally with 3 doses of 0.03mg per 100g body weight anti-mouse Fas every 2days and isolated oval cells after 5days.[25]

Dose, duration and delivery of injury agent: The dose of injurious agent should be titrated to deliver significant liver injury and yet minimize both short and long-term mortality. In the case of chemicals, we can introduce the agent at LD_{50} dose.[92,114] The LD_{50} dose by definition induces mortality in 50% of experimental animals and has served as a historical test to compare the toxicity between different agents. This may be appropriate for single dose agents such as GaIN and CCl_4. Other injury models such as choline-deficient ethionine supplemented (CDE) diet and 3, 5-diethoxycarbonyl-1, 4-dihydrocollidine (DDC) require sequential dosing, and their dosages have been derived empirically**.**

Extent of injury can be objectively assessed by measuring the transaminitis induced[145] or by quantifying the amount of hepatic necrosis on microscopy.[92] It should be noted that oval cells may not respond equally even if the extent of injury is comparable biochemically. One study compared GaIN with CCl_4 dose-adjusted to equivalent extents of hepatic necrosis and levels of serum aspartate aminotransferase.[94] In this study, GalN induced a generous oval cell response while CCl_4 did not.[94]

Depending on the injury agent, the first appearance of oval cells may be anytime between 1 day to 3weeks after inducing the injury[51,62,80,143,181] while peak response varies between 5days to 6weeks.[20,50,148,161] Cessation of the injury agent leads to termination of oval cell response[182,183] although changes to the liver such as cholangiofibrosis are not reversed once formed.[50] Prolonging exposure

to injury may allow greater oval cell proliferation but risks increased hepatic fibrosis. If the intent is to isolate oval cells, distorting the liver architecture may impede perfusion digestion methods thereby reducing yields.[46]

The method of delivering the agent is an important component of the experimental design that may lead to variable results.[184] Animal-dependent feeding (with the drug mixed in the diet or drinking water) is certainly influenced by the keenness of the animal to feed, but may also be influenced by the cage design leading to non-standardized oral-intake and dosing.[185] In this case, it was a cage that limited access to food. Alternative methods of gavage feeding, subcutaneously implanted time-release pellets, or intra-peritoneal injections addresses this problem of consistency in dosimetry. Gavage feeding delivers the drug directly to the stomach and is associated with some animal discomfort but is otherwise safe and avoids anesthetic agents that may interfere with the metabolic profile of the animal. The disadvantage is enterally-delivered drugs are subject to a first-pass effect with its associated issues of variable absorption and metabolism. This leads to variable effects and potentially a slower onset of action. Parenteral methods (subcutaneously-implanted pellet or intra-peritoneal injection) avoid the first-pass effect but are still subject to absorption (either through the subcutaneous tissue or peritoneum). They also involve a minor surgical procedure and anesthetic and risks infective or surgical complications that may compromise the experiment.[184] Another concern albeit unrelated to the animal is the hazard to personnel. Ethionine, for example, is an airborne carcinogen and can be potential risk to staff when administered in powder or pellet form. Administering ethionine in the drinking water instead will reduce this hazard for research personnel.[99]

Comparing oval cell responses: Last, the degree of oval cells responses can be compared between injury models. Four scoring systems were identified and listed in Table 5. Each system proposes reasonable methods of assessing oval cell response. Smaller scores indicate oval cells only around the portal tracts. Higher scores reflect greater proliferation and more extensive infiltration into liver parenchyma.[119,124,149,172] Based upon the articles and reports with negative findings identified in this review, the most reliable method appears to favor the use of 2-AAF/PH liver injury model as described above. In the absence of an available animal surgeon, the reliable chemical induction methods identified were choline-deficient diet with ethionine supplementation (CDE) or 3, 5-diethoxycarbonyl-1,4-dihydrocollidine (DDC).

Table 5 Oval cell scoring systems

Score	Park YN et al.[124]	He XY et al.[119]	Braun KM et al.[172]	Lemmer ER et al.[149]
0	None	Normal liver	-	None
1	Focal proliferation at peri-portal area	<5 oval cells (in 3cm^2 area) and only in portal areas	Absent/rare within diseased parenchyma	Confined to portal tracts
2	Focal proliferation in the lobule	>5 oval cells (in 3cm^2 area) or oval cells have migrated into parenchyma; and newly formed bile duct/hepatocytes were AFP or π GST positive	Intermediate response	Radiating out from portal tracts into fibrous septa
3	Sheet-like proliferation at the peri-portal area	Proliferation and migration of >10 oval cells around portal areas with some migration into zone 2	Intermediate response	Infiltrating liver parenchyma
4	Incomplete encircling of hyperplastic lesions	Groups of oval cells in zone 2 and necrotic areas and not limited to portal areas	Intermediate response	Diffuse proliferation
5	Complete encircling of hyperplastic lesions	-	Extensive oval cell presence throughout all zones of diseased liver	-

Liver injury models and carcinogenesis: The process of cancer induction and the entity cancer stem cells are comprehensive topics that warrant discourses of their own. Nevertheless, we briefly introduce some relevant issues. Many injury models inducing oval cell responses also form cancer nodules as chronic injury leads to acquisition of mutations over periods of hyper-proliferation. Oval cells have been suspected to be tumor-initiating cells or cancer stem cells.[186,187] Indeed, oval cell responses often correlate directly with the likelihood of tumor formation.[44,188] Cancerous and oval cells often co-express markers such as OV-6, c-kit, Glypican-3, and AFP as well.[39,111,114,122] Since much interest in oval cells come from the potential for therapy, this raises the concern of transplanting potentially cancerous cells.[189] It was reported that oval cell lines became tumorigenic and formed carcinomas when inoculated into rats and nude mice.[17,87] However, non-tumorigenic propagable oval cell lines have also been reported in literature.[73,190] The significance of oval cells in carcinogenesis needs to be further explored and perhaps, may lead to developments in targeted cancer therapy.[191–197]

Future direction

Oval cells may play an important role in liver regeneration. We summarized pertinent information on oval cells and reviewed various injury models used to generate them. Future work to characterize these cells will provide greater insight into the exact pathways of activation and translate toward therapeutic utility. Developing surgical methods to induce oval cells will allow research on oval cells naïve to toxic chemicals and recreate scenarios that better mirror clinical situations. Elucidation of malignant transformation pathways will determine the exact role oval cells play in carcinogenesis and may potentially contribute toward targeted therapy.

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Conflict of interest

Author declares that there is no conflict of interest.

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