

Mapping of genes associated with liver regeneration from oval progenitor cells

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Mapping of genes associated with liver regeneration from oval progenitor cells

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Abstract

Regeneration of the adult rat liver in response to severe damage can be accomplished through the activation and differentiation of multipotential nonparenchymal progenitor cells (oval cells). Despite considerable research efforts during the past years, only little is known about the implicated molecules, and thereby the mechanisms, which control the actions of oval cells. In this study, Suppression Subtractive Hybridization (SSH) and Signal Peptide Selection (SPS), was applied on liver cDNA libraries generated from rats exposed to feeding with 2-acetylaminofluorene (2-AAF) and a 70% partial hepatectomy (PHx), in order to map genes that are highly expressed when oval cells are activated and recruited for liver regeneration.

From the SSH library, 29 known and five novel unique cDNA sequences were identified. Additionally four sequences matched expressed sequence tags (ESTs). A subset of the identified genes; interferon- γ receptor α subunit (IFN- γ R α), gp91phox, interleukin-1 β (IL-1 β), lymphocyte function associated molecule-1 α (LFA-1), eukaryotic initiation factor-2-associated 67-kd protein and α -fetoprotein (AFP), constitutes a part of the gene network connected to interferon- γ (IFN- γ). The study was therefore extended to include; IFN- γ , the IFN- γ receptor β subunit (IFN- γ R β), three secondary response genes of IFN- γ , i.e., IL-1 β -converting enzyme (ICE), intercellular adhesion molecule-1 (ICAM-1), and urokinase-type plasminogen activator receptor (uPAR), as well as interleukin-18 (IL-18), a cytokine which induces expression of IFN- γ by T lymphocytes. Expression analysis of all the genes was performed by Northern blotting and immunohistochemistry. Increased expression of the entire examined gene network was detected in response to oval cell activation by 2-AAF and the expression increased further when extensive proliferation of oval cells was induced by a subsequent PHx. During simple regeneration after PHx, only

modulation of IL-1 β and ICE was observed. The expression of the IFN- γ receptor complex, uPAR, ICAM-1, IL-18 and ICE could be localized to the ductular structures of oval cells. These results indicate that IFN- γ mediated events may be particularly important when cells in the bile ductules must respond to liver damage by production of oval cells.

SPS combined with Northern blot analysis was introduced in the present study in order to focus on genes encoding secreted and membrane-bound proteins specifically associated with regeneration from oval cells. Twenty two unique cDNAs encoding eighteen known membrane-bound or secreted proteins, and four novel proteins were identified. Northern blot analysis in various experimental models of liver damage and regeneration showed that one transcript encoding leukocyte-common antigen-1 (CD45) and the four novel transcripts were specifically associated with progenitor cell activity of the oval cell phenotype. Additional expression analysis of genes linked to the function of CD45, (i.e. Thy-1, the chemokine receptor CXCR4, and SDF-1, the ligand for CXCR4) further indicated a role for CD45 in oval cell-based liver regeneration.

One of the novel cDNAs from the SPS library, aocp1 (associated with oval cell proliferation 1), appeared especially interesting as its expression was highly restricted to the 2-AAF and 2-AAF/PHx protocols. Northern blot analysis of multiple adult rat tissues revealed that aocp1 was exclusively expressed in the testis, indicating its putative role in both oval cell based liver regeneration and spermatogenesis.

Dansk resumé

Beskadiges leveren i den voksne rotte voldsomt, kan det regenerative respons foregå via aktivering og differentiering af multipotente ikke-parenchymale stamcelle-lignende celler (kaldt ovale celler). Trods omfattende forskning indenfor de seneste år vides kun lidt om de implicerede molekyler, og dermed hvilke mekanismer, der kontrollerer aktiviteterne af ovale celler. I det foreliggende studie blev Suppressions Subtraktiv Hybridisering (SSH) og Signal Peptid Selektion (SPS) anvendt på cDNA biblioteker, genereret fra rotter eksponeret for fodring med 2-acetylaminofluorene (2-AAF) og en 70 % partiel hepatektomi (PHx), med det formål at kortlægge gener der er højt udtrykt når ovale celler aktiveres og rekrutteres ved leverregeneration.

Fra SSH biblioteket blev der identificeret 29 kendte og 5 hidtil ukendte unikke cDNA sekvenser. Yderligere 4 sekvenser var identiske med "udtrykte sekvensender" (expressed sequence tags (ESTs)). Nogle af de identificerede gener: interferon- γ receptor α subunit (IFN- γ R α), gp91phox, interleukin-1 β (IL-1 β), lymphocyte function associated molecule-1 α (LFA-1), eukaryotic initiation factor-2-associated 67-kd protein and α -fetoprotein (AFP), udgør en del af det gen-netværk der er forbundet til interferon- γ (IFN- γ). Derfor blev studiet udvidet til at inkludere: IFN- γ , IFN- γ receptor β subunit (IFN- γ R β), 3 sekundære respons gener til IFN- γ , mere præcist IL-1 β -converting enzyme (ICE), intercellular adhesion molecule-1 (ICAM-1) samt urokinase-type plasminogen activator receptor (uPAR), og ligeledes interleukin-18 (IL-18), et cytokin der inducerer ekspressionen af IFN- γ fra T lymfocytter. Ekspressionsanalyse af alle disse gener blev foretaget ved Northern blotning og immunohistokemi. For hele det undersøgte gen-netværk blev der påvist øget ekspression som respons på oval celle aktivering via 2-AAF og ekspressionen øgedes yderligere når omfattende celledeling af ovale celler blev

induceret ved en efterfølgende PHx. Under simpel regeneration efter en PHx var det kun ekspressionen af IL-1 β og ICE der blev ændret. Ekspressionen af IFN- γ receptor komplekset, uPAR, ICAM-1, IL-18 og ICE kunne lokaliseres til de rørstrukturer der består af ovale celler. Disse resultater indikerer at påvirkninger der er en følge af IFN- γ 's tilstedeværelse kan være af stor vigtighed når celler i galdegangene responderer på lever beskadigelse via produktion af ovale celler.

SPS kombineret med Northern blot analyse blev introduceret i det foreliggende studie med formålet at fokusere på de gener der koder for secernerede og membranbundne proteiner, som specifikt er associeret med regeneration fra ovale celler. 22 unikke cDNA'er, kodende for 18 kendte membranbundne eller secernerede proteiner og 4 nye proteiner, blev identificeret. Northern blot analyse i forskellige eksperimentelle modeller for leverskade og -regeneration viste at et af de kendte transkripts, kodende for leukocyte-common antigen-1 (CD45), samt de 4 nye transkripts, specifikt var associeret med aktivitet af celler med oval celle fænotype. Opfølgende ekspressionsanalyse af gener der er forbundet med funktionen af CD45, (her Thy-1, kemokin receptoren CXCR4, og SDF-1, liganden for CXCR4) indikerede yderligere en rolle for CD45 i leverregeneration baseret på oval celler.

Et af de nye cDNA'er fra SPS biblioteket, aocp1 (associated with oval cell proliferation 1), fremstod som specielt interessant, da ekspressionen var helt begrænset til 2-AAF og 2-AAF/PHx protokollerne. Northern blot analyse af adskillige væv fra voksne rotter viste, at aocp1 udelukkende blev udtrykt i testis, hvilket kunne indikere at aocp1 spiller en rolle både i leverregeneration baseret på ovale celler og ved spermatogenese.

Papers

This thesis is based on the following articles, referred to as Paper I and Paper II in the text, and additional data presented herein.

- I. Bisgaard HC, Müller S, Nagy P, Rasmussen LJ and Thorgeirsson SS. 1999.
Modulation of the gene network connected to interferon- γ in liver regeneration from oval cells. *Am. J. Pathol.* 155, 1075-1085.

- II. Müller S, Tygstrup N, Rasmussen LJ and Bisgaard HC.
Selective cloning of genes encoding secreted and membrane-bound proteins with putative roles in liver regeneration from oval cells.
Has been submitted to *Am. J. Pathol.* July, 2000.

Abbreviations

2-AAF	2-acetylaminofluorene	LIFR	Leukemia inhibitory factor receptor
AA	Allyl alcohol	LPS	Lipopolysaccharide
AFP	α fetoprotein	NF- κ B	Nuclear factor κ B
aocp	Associated with Oval Cell Proliferation	NPC	Nonparenchymal cells
APAP	Acetaminophen	PAI-1	Plasminogen activator inhibitor-1
BDL	Bile duct ligation	Pfu	plaque forming units
BMTx	Bone marrow transplantation	PHx	Partial hepatectomy (70%)
cDNA	Complementary DNA	PCR	Polymerase chain reaction
cdk	Cyclin dependent kinase	SCF	Stem cell factor
CHO	Chinese hamster ovary	SDF-1	Stromal cell derived factor-1
CK	Cytokeratin	SSH	Suppression Subtractive Hybridization
DPPIV	Dipeptidyl peptidase IV	SPS	Signal Peptide Selection
ECM	Extracellular matrix	STAT1, -3	Signal transducer and activator of transcription protein 1 or 3
EGF	Epidermal growth factor	TGF- α	Transforming growth factor- α
EGFR	Epidermal growth factor receptor	TNF- α	Tumor necrosis factor- α
EST	Expressed sequence tag	TNFR-1	Tumor necrosis factor receptor-1
FLHSC	Fetal liver hematopoietic stem cell	uPA	Urokinase-type plasminogen activator
GalN	D-galactoseamine	uPAR	Urokinase-type plasminogen activator receptor
GGT	γ -glutamyl transpeptidase	UTP	Uridine nucleotides
HGF	Hepatocyte growth factor	WLTx	Whole liver transplantation
HNF	Hepatocyte nuclear factor		
ICAM-1	Intercellular adhesion molecule-1		
ICE	IL-1 β -converting enzyme		
IL(-6,-18)	Interleukin-6, -18		
IFN- γ	Interferon- γ		
LFA-1	Lymphocyte function-associated molecule-1 α		
LIF	Leukemia inhibitory factor		

1. Introduction

1.1. Organization of the hepatic parenchyma

In adults, the liver performs myriad functions. It is the main producer of blood plasma proteins and a major site for metabolism. These activities include synthesis and secretion of acute phase proteins, binding proteins for steroid hormones, lipoproteins and clotting factors like fibrinogen, digestive functions via bile production and secretion, organic metabolism including conversion and production of glucose, cholesterol metabolism and metabolic alterations of foreign organic molecules by the microsomal enzyme system, e.g. hydroxylation reactions.

The main cell type of the adult liver is the hepatocyte which carries out most of its functions, but the organ further include: Cholangiocytes lining the Canals of Hering; fenestrated endothelial cells which are unique among endothelial cells due to their large cytoplasmic gaps that allow maximal contact between blood and hepatocytes; Kupffer cells, which correspond to macrophages localized in hepatic sinusoids; and Ito cells located under the sinusoids, the fat storing cells which are unique to the liver and synthesize connective tissue proteins and secrete several growth factors (Gressner, 1995).

In the adult, rat hepatocytes are stacked into plates of cells which are one-cell wide and up to 20 cells long, and which bifurcate and merge freely (McCuskey, 1993). Epithelial cells of bile ducts, ductules and hepatocytes form a continuous cellular array; ductules are joined at the portal ends of plates to bile canaliculi which occupy the intercellular space between adjacent hepatocytes and the canalicular network drains the bile produced by the hepatocytes to the portal tract interface (Figure 1).

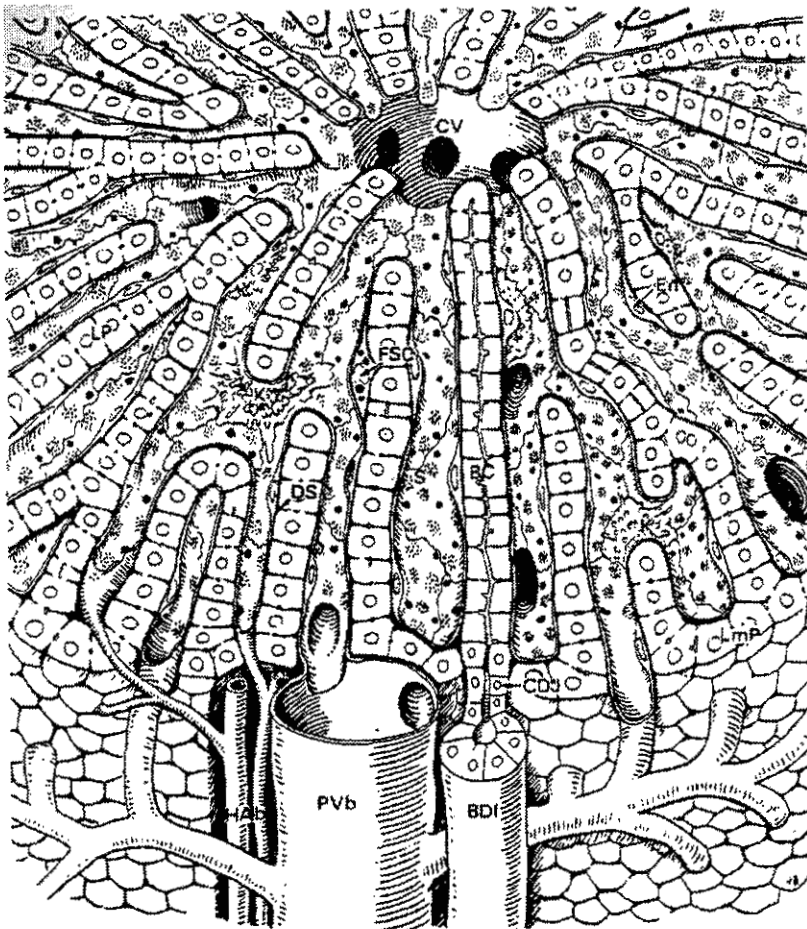


Figure 1. Part of a microsegment of a liver parenchyma. CV, central vein; K, Kupffer cell; FSC, fat storing cell (Ito cell); BC, bile canaliculus; En, endothelial cell; S, sinusoid with fenestrations; DS, space of Disse (separates the hepatocytes from the sinusoidal wall); Hab, hepatic artery branch; PVb, portal vein branch; CDJ, caniculo-ductular junction (Canals of Hering); BDI, bile ductule; LmP, limiting plate; LP, liver plate. From Muto and Yamamoto (1978).

The continuous mass of hepatocytic plates is organized in lobular structures called hepatic lobules, which in a 2-dimensional view is organized like a wheel, which is interpenetrated by two distinct vascular beds; a central vein and six sets of portal triads. The portal triads (also known as portal tracts or portal spaces) are composed of microscopic branches of three vessels: A portal vein, a hepatic artery and a bile ductule. The portal vein brings blood to the liver from the intestine, the stomach, the pancreas, the gallbladder and the spleen and this blood is mixed with the highly oxygenated arterial blood in the sinusoids and the mixed blood flows towards the central vein. The bile ductule carries the bile produced by hepatocytes away to the larger bile ducts.

Despite extensive studies for more than a century, the liver anatomy is still not fully understood. The overall organization of the organ is well described, but at the level of microanatomical interfaces, the relationships between the vascular tree, the biliary system and the hepatic parenchyma still appears inadequately explained. Thus, the definition of an independent functional unit is presently debated. The status on this subject is reviewed by Saxena *et al.* (1999).

1.2. Liver development

During gastrulation the endoderm forms the primordium of the digestive tract. In addition to the pharynx, the esophagus, the stomach and the intestines, it also gives rise to many associated glands, including the salivary glands, the pancreas and the liver. The endoderm forms the epithelial components of these structures while the supporting muscular and fibrous elements arise from the mesoderm. In the developing liver the ventral foregut endoderm gives rise to primitive hepatoblasts, whereas sinusoidal-lining cells and connective tissue components originate in the mesenchymal tissue invaded by the liver cords (Shiojiri *et al.*, 1991). By use of *in situ* hybridization and immunohistochemical techniques, Shiojiri *et al.* (1991) conducted comprehensive studies to examine cell lineages during rat liver development. Differentiation was monitored by use of the markers α -fetoprotein (AFP) and albumin (hepatocyte specific), gamma glutamyl transferase (GGT) (bile duct specific) and cytokeratins (CK's) (adult hepatocytes contain only CK8 and CK18, while duct cells in addition contain CK7 and CK19 (Moll *et al.*, 1982; Van Eyken *et al.*, 1987)). It was shown that the endodermal epithelial cells constituting the adult liver (hepatocytes and bile ducts), all originate from AFP producing, bipotential hepatoblasts. The hepatoblasts which surround the portal mesenchyme form a double-

layered cylinder which remodels and migrate into the mesenchyme to form the intrahepatic bile ducts (Van Eyken *et al*, 1988; Shiojiri *et al*, 1991). The hepatoblasts express AFP, later also albumin and GGT, as they migrate into the portal stroma. Initially, intermediate expression is restricted to CK8 and -18, but during later stages of ductular morphogenesis, the ductal cells begin to express CK7 and -19 as well (Moll *et al*, 1982). In contrast, hepatoblasts which are not in contact with the portal mesenchyme differentiate into hepatocytes which form the liver cell plates and CK expression is restricted to CK8 and -18.

1.3. The phenomenon of liver regeneration

The liver of an adult individual is normally a mitotically quiescent organ. The mass of the liver is, however, precisely regulated and in case of cellular loss, it demonstrates a remarkable regenerative capacity. This characteristic has been known by mankind all the way back to the ancient Greeks as it emerges from the myth of Prometheus. Prometheus stole the secret of fire from the gods of the Olympus and brought it to the human beings. As a punishment the gods condemned him to have a portion of his liver eaten by an eagle daily. The liver regenerated overnight and the torture could continue for ever. A consistent model for studying the phenomenon of liver regeneration was introduced by Higgins and Anderson (1931). In this model, two-thirds of a rat liver is removed by surgery (partial hepatectomy, PHx), which, in the terminology used by Higgins and Anderson, corresponds to the median and left lateral lobe. The residual lobes are left behind intact and enlarge to make up for the reduced liver mass, a process which is completed within 1-2 weeks. The process of regeneration in the PHx model differ from that seen in other regenerating tissues, e.g. bone marrow or skin, as it

apparently does not involve progenitor or stem cells. Resection triggers the hepatocytes in the remnant lobes to exit the G₀ phase and they enter DNA synthesis about 12 hours after PHx, while the other cells of the liver enter into DNA synthesis about 24 hours later (Michalopoulos and DeFrances, 1997).

However, in real life liver mass reduction is caused by toxic injuries from agents like viruses and chemicals rather than by surgery, and some of these injuring agents do not only cause mass reduction, but also impair or block the proliferative capacity of hepatocytes. As described more thoroughly below, the organ may then turn to an alternative strategy of regeneration by proliferation, migration and differentiation of progenitor cells forming atypical structures initially continuous with the biliary tree (Alison, 1998). In rodents, these progenitor cells are called oval cells due to morphological appearance of the nucleus (Faber, 1955). They share the characteristics of fetal hepatoblasts and possess a multi-potent progenitor cell potential which, *in vivo*, include differentiation along hepatocyte and bile duct lineages, but also intestinal type epithelium (Alison *et al*, 1998; Lemire *et al*, 1991; Evarts *et al*, 1996; Tatematsu *et al*, 1985).

Elucidation of the mechanisms which control the actions of oval cells has become an important issue as similar atypical ductular reactions have been identified in many forms of human chronic liver diseases (Lowes *et al*, 1999) and due to the ability of oval cells to replace hepatocytes (Evarts *et al* 1996). Identification of the key molecules which control oval cell actions could influence on future treatment strategies in the clinic, as liver progenitor cells as therapeutic targets would make it possible to e.g.: initiate the process of oval cell differentiation which fail in chronic liver diseases; activate oval cells and their developmental potential as an alternative to transplantation or stop the

proliferation of ductular cells in situations of uncontrolled growth, e.g. in response to acetaminophen (APAP) induced injury (Lowes *et al*, 1999).

1.4. Regeneration from oval progenitor cells

The subject of regeneration from liver stem cells has been controversial for decades, partly because no one so far has been able to demonstrate neither the localization nor the exact nature of such cells. It is, however, well established, that injuries which impair the proliferative capacity of hepatocytes may result in proliferation of epithelial (oval) cells, in which fetal liver-specific gene expression is recapitulated. The oval cells themselves are considered to represent the progeny of liver stem cells, as they are associated with impaired regeneration, and because they express markers identified as characteristic of stem cells, e.g. stem cell factor (SCF), its receptor *c-kit* and cytokeratin 14 (Fujio *et al*, 1994; Bisgaard *et al*, 1994). Markers for oval cells are shown in Table 1.

Rats have been used most extensively to generate experimental protocols which initiate oval cell proliferation (reviewed by Alison *et al*, 1998). These include feeding with a choline-devoid ethionine diet (Tee *et al*, 1994); 2-acetylaminofluorene (2-AAF) administration (Bisgaard *et al*, 1996); D-galactosamine (GalN) exposure (Lemire *et al*, 1991); feeding with a 3'-methyl diaminobenzidine containing diet (Carthew *et al*, 1989); long term exposure to ethanol (Smith *et al*, 1996); toxic accumulation of copper due to defect in Wilson disease gene (Long-Evans Cinnamon rat) (Betto *et al*, 1996); and 2-AAF in a choline devoid diet or combined with PHx, carbon tetrachloride (CCl₄) or allyl alcohol (AA), (Sell, 1983; Evarts *et al*, 1987; Petersen *et al*, 1998 b). Central to all these models is the extensive destruction and/or compromised function of hepatocytes, coupled with the apparent inability of the residual hepatocytes to proliferate. However, only the models of D-

Marker(s)	Notes	References
AFP, albumin	AFP synthesis ceases upon hepatocytic differentiation	Goldin <i>et al.</i> (1995), Alison <i>et al.</i> (1997)
CK's 7, 8, 18 and 19	Highly expressed biliary cytokeratins. Hepatocytes express CK8 and -18.	Alison <i>et al.</i> (1996)
CK14	Only expressed by a minority of oval cells	Fujio <i>et al.</i> (1994)
Vimentin	Mesenchymal intermediate filament expressed by migrating oval cells	Alison <i>et al.</i> (1997)
OV6, OC2, OC3	Cytoskeletal proteins in oval cells. OV6 labels most oval cells, OC2 and OC3 label sub-populations	Hixon <i>et al.</i> (1997)
BD1	This is a negative marker as it recognizes mature biliary epithelia but does not immunostain oval cells	Yang <i>et al.</i> (1993)
GGT	Both bile duct epithelial and oval cells stains for this marker	Yang <i>et al.</i> (1993)
Thy-1	Cell membrane-bound glycoprotein also expressed by haemopoietic cells	Petersen <i>et al.</i> (1998 a)
SCF/c-kit	SCF and its receptor is implicated in survival, proliferation and differentiation of stem cells in hematopoiesis, gametogenesis and melanogenesis.	Fujio <i>et al.</i> (1996)
CD34	A glycoprophosphoprotein which selectively identifies essentially all lineages of hematopoietic stem/progenitor cells	Omori <i>et al.</i> (1997)

Table 1. Markers for oval cells. Antigens expressed by oval cells which can be detected immunocytochemically. The antigens are not expressed by oval cells only, but many are very highly expressed and can be used to highlight the presence of oval cells in histological sections and can be utilized in cell sorting. Modified from Alison (1998).

Galactosamine (GalN) administration or administration of the carcinogen 2-AAF in combination with PHx, have been documented to result in extensive oval cell proliferation, migration and finally differentiation into hepatocytes (Lemire *et al.*, 1991; Dabeva and Shafritz, 1993; Evarts *et al.*, 1987; Evarts *et al.*, 1996).

Irrespective to the localization of facultative stem cells, the oval cells in the GalN and 2-AAF/PHx models originate in portal zones in the regions of terminal bile ductules and then emerge from the portal regions, invade the entire lobular parenchyma and differentiate (Grisham and Thorgerirsson, 1996). By light microscopy studies Sarraf *et al.* (1994) showed that oval cells in the 2-AAF/PHx protocol initially expand from, and are continuous with, the biliary tree. By detection of AFP and CK19 expression it has subsequently been shown, that it is the smaller interlobular ducts that are heavily implicated, rather than the larger ducts (Alison *et al.*, 1998). The area from which the oval

cells appear to expand, and possible differentiation lineages, identified by *in vivo* and *in vitro* studies, is shown in Figure 2.

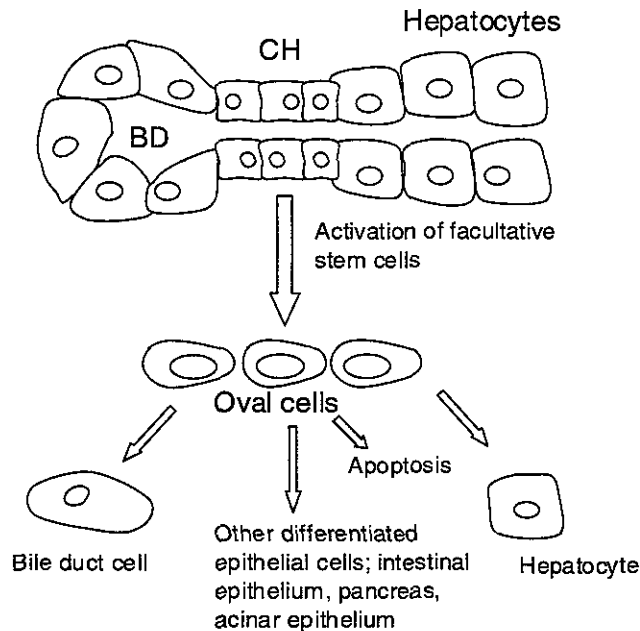


Figure 2. Schematic representation of the localization from which the oval cells expands. The scheme shows the developmental options that the oval cells possess, including differentiation into hepatocytes and biliary epithelium. BD: Bile ductule; CH: Canal of Hering. Redrawn and modified from Grisham and Thorgeirsson (1996).

Recent studies in rats, mice and humans have shown that extrahepatic derived cells of bone marrow origin also can contribute as a source of hepatocytes and bile duct cells in liver regeneration and renewal (Petersen *et al*, 1999; Theise *et al*, 2000 a; Theise *et al*, 2000 b). In the study by Petersen *et al* (1999), three approaches were used in conjunction with the 2-AAF/ CCl_4 protocol: i) bone marrow transplantation (BMTx) from male rats into lethally irradiated females and detection of donor cells in the recipients by means of DNA probes to the Y chromosome; ii) BMTx from dipeptidyl peptidase IV-positive (DPPIV⁺) male rats into DPPIV⁻ females and detection of DPPIV⁻ expressing cells in the recipient animals and; iii) whole liver transplantation (WLTx) with L21-6 antigen expressing recipients of livers from rats that did not express the L21-6 antigen. In summary, all approaches confirmed that a subpopulation of the oval cells and/or hepatocytes and ductular structures derived from the donor bone marrow cell population

and in case of approach iii), a widespread staining for L21-6 was detected as a result of the influx of oval cells that originated from an extrahepatic source of the recipient rats. The contribution of donor derived hepatocytes in approach i) and ii) was quantified at a time when the oval cells had started to differentiate into hepatocytes and was estimated to about 0.14% and 0.16% of the total hepatocytes, respectively. The proportion of Thy-1+ oval cells expressing the Y marker in approach i) was calculated to about 0.1%.

An approach based on lethal irradiation of female mice which received whole bone marrow transplants from male donors was used in the study by Theise *et al* (2000 a) but a protocol directed towards liver specific injury was not included in these experiments. Y-chromosome positive hepatocytes constituted about 1 to 2 % of total hepatocytes in animals sacrificed 2 month or longer posttransplantation, indicating that stem cells of bone marrow origin may take part in normal tissue renewal in the liver. Liver specimens from human recipients of therapeutic bone marrow liver transplants have also been analyzed for marrow-derived hepatocytes and biliary epithelium cells (cholangiocytes) (Theise *et al*, 2000 b). The pathological cases included chronic myelogenous leukemia, primary sclerosing cholangitis, alpha-1 antitrypsin deficiency and hepatitis C. Engraftment of Y-chromosome positive hepatocytes and cholangiocytes was detected in all specimens, with adjusted values ranging from 4 to 43 % and 4 to 38 %, respectively, showing that in humans too, hepatocytes and biliary epithelium cells can be derived from extrahepatic stem cells, probably of bone marrow origin.

1.5. Activation of oval cells

As described above, several experimental protocols, which provoke a regenerative response involving oval cells, exists. The mechanisms which activate the oval

cells and the reason why only the 2-AAF/PHx (or 2-AAF + an alternative injury) and GalN protocols results in a complete regenerative process, is, however, poorly understood. The nature of injuries in the noncarcinogenic GalN protocol and the carcinogenic 2-AAF/PHx protocol are quite different. When introduced into rats at concentrations higher than 200 mg/kg, GalN causes a series of metabolic reactions to occur in the hepatocytes, most notably a complete consumption of all uridine nucleotides (UTPs). The UTP trapping results in blocked RNA synthesis, followed by inhibition of protein synthesis, changes in plasma membranes and finally hepatocyte death (Decker and Keppler, 1972; Decker and Keppler, 1974; Bachmann *et al*, 1977). The nonparenchymal cells are not targets for UTP trapping and oval cells start to replicate at day 1 (Lemire *et al*, 1991). Hepatocytes recover in the GalN protocol at day 3 and thereby contribute to the restoration of normal liver structure which is completed within only 7 –10 days (Lemire *et al*, 1991).

The tissue destruction and oval cell activation is obtained in the GalN protocol by use of one or two (6 hours apart) intraperitoneal doses (Dabeva and Shafritz, 1993; Lemire *et al*, 1991), while 2-AAF in all variations of the 2-AAF/PHx protocol is administrated daily for at least 9 days and PHx is typically performed at the midway point (reviewed by Alison *et al*, 1998). Hepatocytes do not contribute to the tissue restoration in these protocols and whether oval cells differentiate into hepatocytes or undergo intestinal metaplasia appears to depend on the amount of 2-AAF administrated. DNA synthesis in oval cells and Ito cells is seen already at four hours after PHx (Thorgeirsson *et al*, 1993), but proliferation of oval cells is not extensive for the first several days after PHx and the period prior to differentiation into hepatocytes is prolonged when compared to the GalN protocol. Significant differentiation in the 2-AAF/PHx protocol is typically seen between the 11th and 14th day after PHx. 2-AAF is metabolized into the cytotoxic/mitoinhibitory *N*-

hydroxy derivative by phase I metabolic enzymes in hepatocytes (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, which favors detoxification over hepatocarcinogen activation in these cell types (Mathis *et al*, 1989; Sirica *et al*, 1990). By use of [³H] thymidine labeling of DNA synthesis, it has been shown *in vivo* that 2-AAF alone elicits a mitogenic response in ductal and nondescript periductal cells, whereas DNA synthesis in hepatocytes is blocked (Bisgaard *et al*, 1996). The carcinogen exposure also induced expression of AFP, albumin and transcription factors which regulate their expression (Hepatocyte Nuclear Factor (HNF) 1 β and HNF3 γ), reflecting the presence of oval cells within the population of induced ductular cells.

Progression through cell cycle checkpoints are mainly regulated by the levels and activities of different cyclin/cyclin dependent kinase (cdk) complexes. *p53* (the size of the protein is 53 kDa) is one of the molecules which can mediate G₁ cell cycle arrest by activation of cdk inhibitor *p21* synthesis (El-Deiry *et al*, 1994). Cyclin/cdk complexes are also known to be involved in *p53*-mediated G₁ arrest induced by TGF- β (Ewen *et al*, 1995), which is a potent inhibitor of hepatocyte proliferation. It has recently been found that *p53* expression increases in response to 2-AAF treatment (Ohlson *et al*, 1998). Cyclin E/cdk2 complex formation has been identified as critical for G1/S phase progression as it enables transition to the S phase by acting as histone H1 kinase (Dulic *et al*, 1992). It was further shown by Ohlson *et al* (1998) that the cell cycle related increase in cdk2 is not seen in 2-AAF treated rats. Finally, immunofluorescence studies have shown that cyclin E positive signals are restricted to oval cells, while hepatocytes remain negative (Trautwein *et al*, 1999). These studies by Ohlson *et al* (1998) and Trautwein *et al* (1999) thereby point at two molecular explanations for the 2-AAF mediated inhibition of hepatocyte proliferation;

Induction of *p53* expression resulting in elevated *p21* expression and lack of cyclin E and cdk2 expression. Both mechanisms arrest hepatocytes in the G₁ phase.

The combination of 2-AAF treatment with PHx results in a dramatic increase in the expression of several growth factors when compared with 2-AAF treatment alone (Thorgeirsson *et al*, 1993). The presence of these growth factors appears to be essential for oval cell survival. 2-AAF treatment alone does not result in a net increase of oval cells, as the number of apoptotic NPCs in the periportal areas also increases, thereby retaining the normal liver morphology (Bisgaard *et al*, 1996). A direct mitogenic effect of the 2-AAF metabolites on the hepatic ductal cell compartment can not be excluded, but the stimulation could also be mediated through the mitoinhibitory effect on the hepatocytes. The latter alternative might reflect a common mechanism in the 2-AAF/PHx and GalN protocols. Despite their different mode of actions, both of these protocols cause situations of hepatocyte specific mitoinhibition combined with a liver mass reduction which provides a growth stimulus.

1.6. A mitogenic effect of growth factors implies priming of target cells

Whether regeneration is accomplished in response to PHx treatment or chemical injury, numerous growth factors are involved. Among these, the epidermal growth factor (EGF), the hepatocyte growth factor (HGF) and the transforming growth factor- α have been demonstrated to act as complete mitogens for hepatocytes in culture and are considered to play very important roles in rat liver regeneration (Michalopoulos, 1990). Direct infusion of these growth factors into the liver of normal rats does not, however, cause a major wave of DNA synthesis (Webber *et al*, 1994; Nagy *et al*, 1996). A significant DNA synthesis response is obtained though, if growth factor infusion is

preceded by: A 30 % PHx (which does not by itself induce a wave of DNA synthesis) (Webber *et al*, 1994); collagenase treatment (a mixture of bacterially produced matrix metalloproteinases which perfuse the liver) (Liu *et al*, 1994); or administration of 2-AAF (Nagy *et al*, 1996), demonstrating that a priming stimulus must be provided in order to effectuate the mitogenic potential of growth factors. Within minutes after PHx, the transcription factor Nuclear factor κ B (NF- κ B) is transiently activated by dimerization of the preexisting p50/NF- κ B1 and p65/RelA subunits (FitzGerald *et al*, 1995; Cressman *et al*, 1994). This activation is mediated by signaling through tumor necrosis factor receptor type 1 (TNFR-1), as demonstrated by PHx in TNFR-1 deficient mice, in which DNA synthesis is severely impaired and the expected increase in NF- κ B binding fail to occur (Yamada *et al*, 1997). The NF- κ B downstream signaling pathway involves transactivation of the cytokine interleukin-6 (IL-6) which leads to increased binding of, most importantly, the transcription factor signal transducer and activator of transcription protein 3 (STAT3). Injection of IL-6 in TNFR-1 deficient animals before PHx restores STAT3 binding to normal levels and corrects the DNA synthesis defect (and thereby the deficiency in regeneration), but it does not effect the NF- κ B binding (Yamada *et al*, 1997). Similarly it has been shown, that disruption of the IL-6 gene in mice results in absence of STAT3 and impaired liver regeneration in the PHx model, a situation which could be reversed by a single dose of IL-6 (Cressman *et al*, 1996). In outline, the described activation events result in sensitivity of the hepatocytes to the effect of the growth factors, which enable the activation cascade of immediately early and subsequently delayed early genes characterizing the G₁ phase to continue (Haber *et al*, 1993; Taub, 1996). The above indications of the cytokine tumor necrosis factor- α (TNF- α), as the initial factor acting as a primer to sensitize hepatocytes to the proliferation effect of growth factors have recently been further supported by Webber

et al (1998). Infusion of HGF and TGF- α into the portal vein of normal rats in concert with TNF- α , transiently activated NF- κ B and STAT3 and increased the proliferative response of hepatocytes to HGF and TGF- α by fourfold. Though the sequence of events for initiation of regeneration in the PHx protocol; TNF- α \rightarrow TNFR-1 \rightarrow NF- κ B \rightarrow IL-6 \rightarrow STAT3 \rightarrow remaining immediately early genes \rightarrow delayed early genes \rightarrow DNA synthesis, seems likely, it is not known whether TNF- α is the sole initiating factor in the PHx model of regeneration. The observation that regeneration is severely impaired in TNFR-1 deficient mice may indicate that TNF- α is one among multiple factors required, though these are not sufficient by themselves to elicit a full regenerative response. The initial response to PHx by the release of preexisting cytokines like TNF- α and IL-6 from Kupffer cells (Michalopoulos and DeFrances, 1997) and the activation of transcription factors by posttranslational modifications, explain the speed by which the liver responds to this type of injury. Stimulation of cytokine production can be induced by various means, e.g. by endotoxin, free radicals or viruses (Simpson *et al*, 1997). The mechanism which actually starts the process in response to PHx (or other liver injuries) is, however, not known.

The regenerative response to PHx does not significantly involve oval cells. Transiently, the transcription of SCF, *c-kit* and AFP exceeds detectable levels, but there is no evidence indicating that the oval cells contribute significantly to the regeneration of the liver mass (Thorgeirsson *et al*, 1994; Grisham and Thorgeirsson, 1996). It has been hypothesized that PHx treatment involves activation of both hepatocytes and the facultative liver stem cells (Thorgeirsson, 1996). In case of hepatocyte-specific mitoinhibition, the activation of the stem cells and their progeny (oval cells) are sustained, and a similarly sustained expression of growth factors, including those known to be involved in response to PHx treatment, results in the generation of the differentiated cell

lineages needed for regeneration. This scenario may partly explain the progress in the 2-AAF/PHx protocol, but it certainly does not account for the mechanism which activates oval cells in the GalN protocol. On the contrary, the much faster response of oval cells seen in the GalN protocol indicates, that a mechanism which actively primes the oval cells for the proliferative effect of growth factors, exists.

TNF and IL-6 are also involved in regeneration from oval cells, but the data illustrating the importance of the cytokines in this context are conflicting. Dexamethasone is a well-known inhibitor of TNF and IL-6. In a study by Nagy *et al* (1998), the effect of dexamethasone on rats exposed to PHx, 2-AAF/PHx and bile duct ligation (BDL) was examined. Pretreatment with dexamethasone suppressed expression of both TNF and IL-6 after PHx and significantly reduced the proliferative response of hepatocytes, but the failed response could be rescued by IL-6 administration. Long term dexamethasone treatment prior to induction of oval cells by the 2-AAF/PHx protocol turned out to be lethal. If animals in the 2-AAF/PHx protocol were administrated dexamethasone 24 hours before they were killed, bromouracil (BrdU) positive oval cells decreased approximately 10 fold compared to 2-AAF/PHx controls. Dexamethasone treatment prior to BDL did not influence on the regenerative response and it was concluded that dexamethasone effectively inhibits the proliferative response of hepatocytes and oval cells, but not bile duct cells. However, a possible synergistic effect of 2-AAF and dexamethasone was not examined, e.g. by a 2-AAF/dexamethasone/BDL experiment or rescue of 2-AAF/dexamethasone/PHx treated animals by IL-6 administration. In a recent study by Rosenberg *et al* (2000), the regeneration from periportal liver injury induced by phenobarbital feeding and cocaine injection (induce proliferation of hepatocytes in middle and central zones and proliferation of intraportal oval cells) was examined in normal and

IL-6 knockout mice. It was demonstrated that IL-6 knockout mice compensated for a reduced hepatocyte proliferation by increased oval cell proliferation, resulting in timely repair of the liver injury. These results show that IL-6 differently influences on the proliferative capacity of hepatocytes and oval cells.

Our present knowledge regarding mechanisms for priming of oval cells is clearly insufficient. The fact that oval cells do not contribute to regeneration from PHx treatment could reflect that additional priming mechanisms/factors are necessary in order to activate oval cells. Recruitment of such factors could be controlled by hepatocytes, and if so, only in situations of compromised proliferative capacity of hepatocytes. The liver could thereby ensure that the slower process of regeneration from cell types incapable of immediately conducting liver cell functions are only activated in emergency situations.

1.7. Growth factors involved in regeneration from oval cells.

Due to the tremendous regenerative capacity and the existence of a simple and well functioning experimental approach (PHx), the regenerating liver has been widely used to identify and study the role of growth modulators in regeneration. Several important factors involved in regeneration from PHx treatment have been characterized (Michalopoulos and DeFrances, 1997), but the high degree of redundancy and pleiotrophic effects of many of these factors have complicated the elucidation of their functions and how the complex interplay between them is organized and controlled. Expression-analysis of cytokines, growth factors and their respective receptors in the context of regeneration from oval cells in the 2-AAF/PHx model, has been performed for acidic fibroblast growth factor (aFGF), leukemia inhibitory factor (LIF), TGF- α , EGF, and SCF as well as HGF and the urokinase-type plasminogen activator (uPA), urokinase-type plasminogen activator

receptor (uPAR), plasminogen activator inhibitor-1 (PAI-1) system which is involved in the release, conversion and regulation of pro-HGF to its active form (Marsden *et al*, 1992; Hu *et al*, 1995; Evarts *et al*, 1993; Omori *et al*, 1996; Evarts *et al*, 1992; Nagy *et al*, 1996; Fujio *et al*, 1994; Hu *et al*, 1993; Bisgaard *et al*, 1998).

The proliferative activity in the 2-AAF/PHx protocol is concentrated on oval cells (and Ito cells) and this is in accordance with the general picture which emerges from the study of growth modulators and their receptors. Whereas the activity of growth modulators in response to PHx treatment (at least in the initial phase) mostly expands from hepatocytes (and Ito cells), and are directed towards hepatocytes, this activity pattern changes in the 2-AAF/PHx protocol, in which growth modulators mostly are expressed by oval cells and Ito cells and are directed towards oval cells. Studies of SCF, LIF and HGF have identified especially interesting individual characteristics as described in the following.

SCF was originally identified as an important hematopoietic cytokine (Zsebo *et al*, 1990). SCF and its receptor *c-kit* are expressed in fetal liver, which is a site of hematopoiesis during fetal life, but the transcripts are not detected in adult liver (Fujio *et al*, 1994). They differ from the remaining factors mentioned above, as their expression does not increase significantly in response to PHx treatment. Expression of SCF and its receptor *c-kit* is, however, dramatically induced in response to the 2-AAF/PHx protocol (Fujio *et al*, 1994). Expression of mRNA for both ligand and receptor increased from 12 hours after the partial hepatectomy with a peak of SCF at day 4, and with a more gradual increase of *c-kit* transcripts, which stayed elevated until a large portion of the oval cells started to differentiate. The SCF transcripts were expressed by oval cells and Ito cells but transcripts for the *c-kit* receptor were restricted to oval cells. The induction of SCF and *c-kit*

shortly after the liver mass reduction by PHx in the 2-AAF/PHx protocol may reflect a role of this cytokine system in the early development of oval cells. This has been further indicated in a recent study by Matsusaka *et al* (1999). The role of *c-kit* in the 2-AAF/PHx protocol was examined by use of Ws/Ws rats, whose *c-kit* tyrosine kinase activity was severely impaired owing to a deletion in the kinase domain. Compared to normal rats, the development of oval cells was significantly suppressed at day 7, 9 and 13 after PHx. However, the expression of oval cell markers and the proliferative activity was detected at equal levels in Ws/Ws and normal rats, indicating that the receptor mediated signal transduction only plays a minor role in determining the phenotype and in the proliferative activity of oval cells.

LIF is a polyfunctional cytokine with several biological characteristics similar to TNF, IL-1 and IL-6 (Gearing, 1993; Hilton and Gough, 1991; Kurzrock *et al*, 1991). Receptors for LIF (LIFR) exist in low- and high-affinity forms and conversion to the latter form is conferred by the signal transducing subunit gp130, which LIF shares with IL-6 type cytokines, reflecting a functional redundancy (Kishimoto *et al*, 1995). For the LIF/LIFR/gp130 system, a phenotypic difference between hepatocytes and oval cells in their signaling through gp130 has been indicated. A well described response of hepatocytes exposed to LIF is the expression of acute phase proteins. By separation of parenchymal and nonparenchymal cells combined with *in situ* hybridization analysis, Omori *et al* (1996) showed that α 1-acidglycoprotein (α 1-AGP) (representing acute phase proteins) expression was restricted to hepatocytes in the 2-AAF/PHx protocol. The LIF, LIFR and gp130 transcripts were mainly found in nonparenchymal cells, including oval cells. These observations indicate that the function of the LIF/LIFR/gp130 system differ significantly between hepatocytes and oval cells.

HGF was originally identified in the serum of rats exposed to PHx treatment as a potent mitogen for normal hepatocytes in culture (Michalopoulos *et al*, 1984; Nakamura *et al*, 1984), but has also been recognized as a motogen as well as a morphogen factor (see Naldini *et al* (1995) for references). The receptor for HGF is *c-met* (Naldini *et al*, 1991), which, like several other growth factor receptors, e.g. EGFR and the receptors for aFGF, transduce its signal through a tyrosine kinase activity. In the liver, HGF is not produced by hepatocytes, but by nonparenchymal sinusoid-lining cells, principally Ito cells. The HGF transcript is expressed in low levels in normal liver (Hu *et al*, 1993), but the level increases dramatically in response to PHx treatment, starting from 3-6 hours after PHx, and peaks at 12 hours after PHx (Zarnegar *et al*, 1991). The increase of HGF in plasma of rats exposed to liver injuries (PHx and CCl₄ administration) is even faster, with a peak of 17 fold increase just 2 hours after PHx (Lindroos *et al*, 1991). In the 2-AAF/PHx protocol, an increase in the HGF transcript level is seen 1 day after PHx, with a peak at day 6 and a decrease after day 13 (Hu *et al*, 1993). *c-met* expression also increased gradually with a peak around day 9 to 13. *In situ* hybridization further showed that the HGF transcripts were localized in Ito cells, whereas the transcript for *c-met* were strongly expressed by oval cells, indicating that the HGF/*c-met* system functions by a paracrine mechanism. Hu *et al* (1993) did not observe an increase in HGF and *c-met* transcripts in response to 2-AAF treatment alone. HGF is secreted as an inactive single-chain precursor (pro-HGF), which binds to the cell surface or the ECM, presumably via its affinity for heparin-like glycoaminoglycans, and it also binds to the *c-met* receptor, but without triggering its kinase activity (Naldini *et al*, 1995). Conversion of pro-HGF to an active heterodimeric molecule requires proteolytic activity, a process which takes place in the extracellular environment. Plasminogen activators (PAs) and inhibitors (PAIs) are thought to be key participants in

the balance of proteolytic and antiproteolytic activities that regulate matrix turnover (Vassalli *et al*, 1991). In the liver exposed to PHx treatment, *de novo* appearance of active uPA and uPAR in homogenates has been documented as early as 1 minute after surgery (Mars *et al*, 1995). Western blot analysis and zymography further showed that the resulting enhanced activity of uPA was mainly due to a dramatic increase in uPAR expression. uPA appeared to be the primary agent that activated pro-HGF, as cleavage could be prevented by antibody against uPA. In studies using tissue cultures it has been shown that activation of pro-HGF involves the formation of a stable complex between pro-HGF and uPA bound to the *c-met* receptor (Naldini *et al*, 1995). By use of immunohistochemistry, Northern blot analysis and zymography Bisgaard *et al* (1998) established, that the plasminogen activator/plasmin system is also involved in regeneration in the 2-AAF/PHx protocol. Plasminogen activation was located over the expanding oval cell population, but not over the mitoinhibited mature hepatocytes, thereby providing an effective mechanism by which HGF activation can be specifically targeted to the oval cell surface. A summary of oval cell growth modulating molecules are shown in Figure 3.

1.8. The extracellular matrix and the local microenvironment

The bile duct structures in the portal tracts are surrounded by matrix components which form a classic basement membrane, containing type IV collagen and laminin, and which are associated with the cholangiocytes and the adjacent hepatocytes too. The ECM gradually changes across the liver acinus and the basal lamina is replaced with components such as fibrillar collagens and fibronectin (Martinez-Hernandez and Amenta, 1993). The plasminogen activator/plasmin cascade is involved in degradation of type IV collagen and fibronectin (Gold *et al*, 1992; MacKay *et al*, 1990) and may thereby

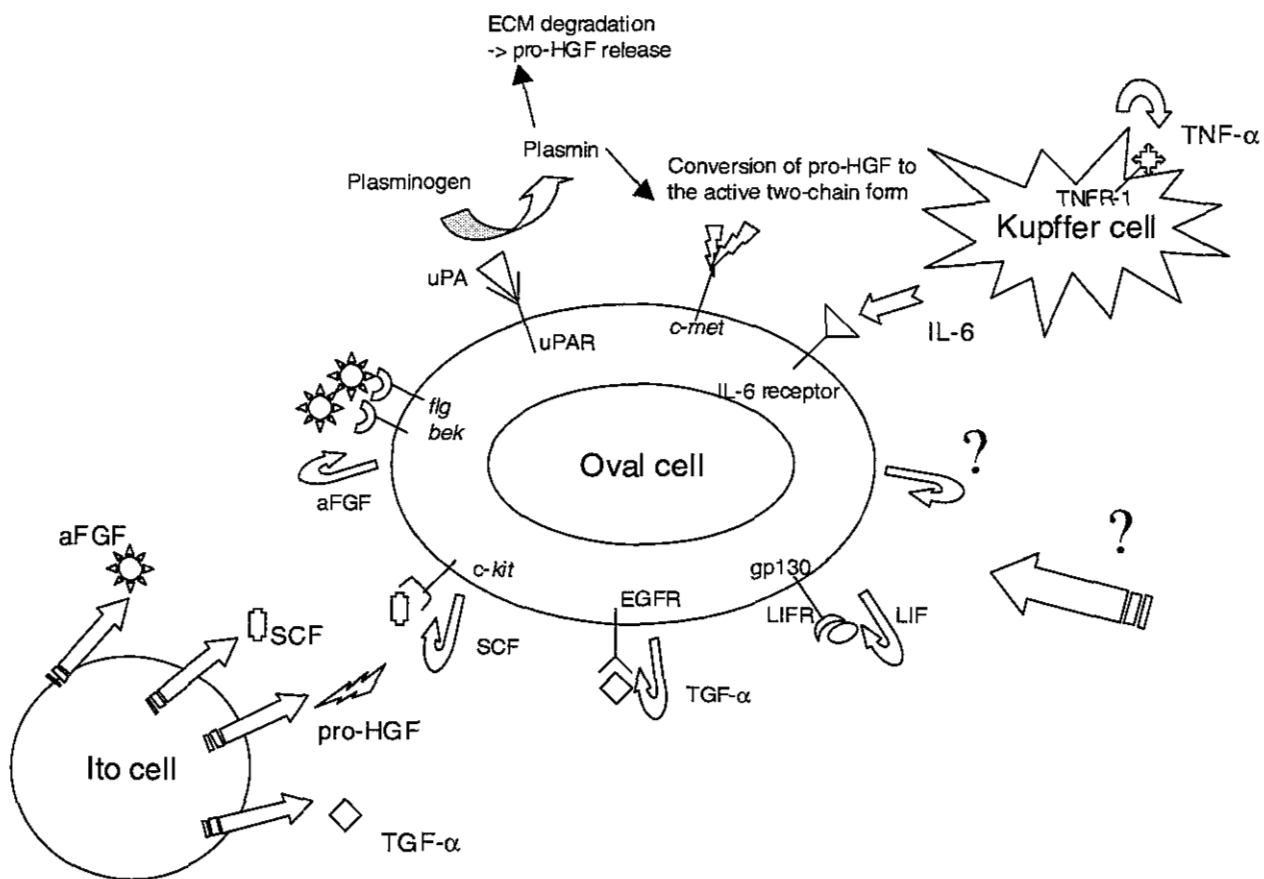


Figure 3. Schematic representation of positive regulators of oval cell proliferation operating through paracrine and/or autocrine loops. aFGF: acidic fibroblast growth factor; EGFR: epidermal growth factor receptor; HGF: hepatocyte growth factor; IL-6: interleukin 6; LIF: leukemia inhibitor factor; LIFR: leukemia inhibitor factor receptor; SCF: stem cell factor; TGF- α : transforming growth factor- α ; TNF- α : tumor necrosis factor- α ; TNFR-1: tumor necrosis factor receptor-1; uPA: urokinase-type plasminogen activator; uPAR: urokinase-type plasminogen activator receptor. Redrawn and modified from Alison (1998).

assist in the migration of oval cells into the parenchyma. Further, the matrix degradation may not only result in the release of pro-HGF, but also matrix bound TGF- β and possible EGF (Bisgaard *et al*, 1998; St Hilaire *et al*, 1983; Burwen *et al*, 1984). The injury caused by GalN, and the regenerative response caused by PHx, is diffuse throughout the entire hepatic lobule, whereas damage caused by CCl₄ and AA is limited to centrilobular and

periportal regions, respectively. To examine the relationship between the site of lobular damage and the induction of oval cell proliferation, Petersen *et al* (1998 b) compared the regenerative responses to CCl₄, AA or PHx when combined with 2-AAF. It was found that the oval cell response to 2-AAF/AA treatment was significantly smaller than the corresponding response to 2-AAF/PHx and 2-AAF/CCL₄ treatment. Conversely, the proliferative response of intraportal bile duct epithelia was most intense in the 2-AAF/AA model. As the AA induced death of hepatocytes is likely to have effects on the connective tissue matrix of the periportal sites, the results suggest that the periportal matrix contributes significantly to the stimulation of oval cell activities.

1.9. Aims of the present study

The studies of regeneration from oval cells performed so far illustrate several aspects in which the process differs from ordinary liver regeneration based on mature cell populations. These differences include alterations in the networks of autocrine and paracrine mechanisms which stimulate the involved cells, and the presence of factors associated with regeneration from oval cells only. The number of molecules identified as associated with the oval cell response, and consequently our understanding of the mechanisms involved, is, however, rather limited. In order to increase our understanding of the mechanisms which control the activation, proliferation, migration and differentiation of oval cells, the following aims were defined for the present study:

- To start a mapping of genes that are highly expressed when oval cells are activated and recruited for liver regeneration.

- Characterize the roles of the identified gene products by analyzing the expression of the genes themselves and possible associated molecules.

Two different experimental approaches were used to identify genes associated with regeneration from oval cells: i) Suppression subtractive hybridization (SSH) analysis and ii) Signal peptide selection (SPS) combined with Northern blot analysis. For both approaches, the 2-AAF/PHx protocol was used to induce a regenerative response from oval cells. SSH analysis selects for differentially expressed genes at the cDNA level, that is, irrespective to the functional properties of the corresponding products. Consequently, this approach identifies products of differentially expressed genes which are present at all the cellular levels by which the oval cell associated response is characterized. Contrary, the SPS method selects for genes encoding secreted and membrane-bound molecules. This subgroup of molecules includes the growth modulators and receptors which regulate the actions of individual cells with respect to a regenerative response. The SPS method was introduced in the present study with the aim to focus on the category of secreted regulating molecules.

2. Materials and methods

Besides the materials and methods described in Paper I & II, the following experimental procedures were used in this study:

Preparation of the λ -RK18 vector

The λ -RK18 phage was amplified in *E. coli* strain BB4 (LE392.23 [F'*LacI*^rZ Δ M15*proAB*Tn10(Tet^r)]) as described by Sambrook *et al.* (1989). λ -RK18 DNA was purified by use of the QIAGEN® Lambda Maxi kit according to the manufacturers instructions, except for the addition of a Proteinase K (50 μ g/ml – approx. 20 U/ μ g) (BOEHRINGER MANNHEIM, Germany) treatment step by incubation at 56°C for 1 hour in 10 mM Tris (pH 7.6), 5 mM EDTA, 0.5% SDS, prior the column purification step. To prepare the λ -RK18 vector for cloning of cDNA in the unique *Not* I site, lambda cohesive ends were ligated followed by *Not* I digestion and dephosphorylation, essentially as described by Sambrook *et al.* (1989). The effect of the individual treatments was verified by comparative examination of infection efficiency. Obtained reductions were 2×10^{-3} and 5.5×10^{-2} for ligated versus ligated/*Not* I treated and ligated/*Not* I treated versus ligated/*Not* I/dephosphorylated aliquots, respectively, and thereby within the described ranges for successful manipulations. Optimal λ -RK18:cDNA ratio was determined by trial ligations as described by Sambrook *et al.* (1989). Phage DNA was packed by use of GigaPack III Gold (Stratagene, La Jolla, CA) according to manufacturers instructions except for the termination volume, which was reduced to 1/10, in order to obtain a concentration of plaque forming units that made it possible to use the primary (non-amplified) phage library directly in phagemid excision reactions.

Construction of the λ RK18/PAI-1 positive control.

The 405 bp 5' PAI-1 sequence used to verify the functionality of the SPS method in our laboratory was prepared by PCR on full length PAI-1 in a pCR2.1 (Invitrogen Corp., Carlsbad, CA) vector background (from Dr. Hanne Cathrine Bisgaard). Primers for PCR amplification and introduction of *Not* I cloning sites were forward 5' – gcggccgcggcacttccgaaagctccag- 3' and reverse 5' –gcggccgcgtggcgtccgcagt-actgatctc- 3'. The PCR reaction mixture was composed as follows: 1x PCR buffer (Perkin Elmer Corp., Norwalk, Connecticut), 200 μ M each dNTP, 10 pmole each forward and reverse primers, 2.5 U Ampli Taq polymerase (Perkin Elmer Corp.) and amplified in 30 cycles of 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 30 seconds. The amplification product was subsequently ligated into pCR2.1 (Invitrogen) and plasmid DNA amplified in *E. coli*. The PAI-1 *Not* I fragment was obtained by *Not* I digestion and purification from agarose gel and ligated into λ -RK18. Correct orientation and in-frame cloning was verified by sequence determination of the cDNA insert from a sucrose positive YT455 PAI-1 yeast transformant as described in paper II.

3. Primary experimental approaches

3.1. Identification of genes associated with regeneration from oval cells by Suppression Subtractive Hybridization.

The Suppression Subtractive Hybridization method (SSH) used in this study (CLONTECH PCR-Select™ cDNA subtraction, Clontech Laboratories Inc., Palo Alto, CA) is based on the principles of traditional subtractive hybridization, in which two mRNA populations (converted to cDNA) are compared by the use of one of the populations (called driver cDNA) as a reference in hybridization reactions. This subtraction makes it possible to isolate sequences which are expressed in only one of the populations (referred to as the tester cDNA). In addition to this basic concept, the PCR-Select™ method uses a hybridization strategy which levels the concentration of high and low abundance sequences and a subsequent PCR amplification increases the sensitivity for detection of differentially expressed sequences.

In the present study, mRNA from the nonparenchymal cell (NPC) population at day 7 in the 2-AAF/PHx protocol was used to construct the tester cDNA population and mRNA from the NPC population of normal rats were used to construct the driver cDNA population. Although hepatocytes may be involved in generating the oval cell response, use of the NPC populations aimed the identification of differentially expressed genes towards the cell fraction which include the oval cell population. At day 7 in the 2-AAF/PHx protocol, no overt differentiation of oval cells is seen (see Paper I). Use of NPC populations from day 7 thereby selected for the identification of differentially expressed genes involved in the activation and proliferation of oval cells rather than differentiation.

3.1.1. The molecular basis of Suppression Subtractive Hybridization

In practice, the PCR Select™ procedure implicates the following events (depicted in Figure 4): The two mRNA populations are converted into cDNA and further modified into short blunt-end sequences by digestion with the four base cutter *RsaI*. The cDNA representing the material under examination (the tester cDNA) is then divided into two aliquots which are ligated with adaptor 1 or 2, respectively. These adaptors do not have a phosphate group and consequently only one strand of each adaptor attaches to the 5' end of the cDNA. The tester cDNA aliquots are then separately hybridized with the second cDNA population (the driver cDNA), giving rise to molecules of the following types (Figure 4):

- a; Single stranded cDNAs present in the tester population only. The concentration of low and high abundance sequences is leveled as high abundance molecules re-anneal and form type b hybrids.
- b; Double stranded cDNAs of high abundance sequences from the tester population.
- c; tester + driver hybrids of cDNAs present in both populations.
- d; single and double stranded cDNAs representing low and high abundance molecules present in the driver population only.
- The two hybridization reactions are subsequently mixed, without prior denaturation, resulting in the formation of type e hybrids, composed of double stranded subtracted cDNAs with different ends. Type e molecules are further enriched by addition of a new aliquot denatured driver cDNA.

The ends are then filled in by DNA polymerase, resulting in differentially expressed tester sequences (e type molecules) with different adaptor sequences in their 5' and 3' ends.

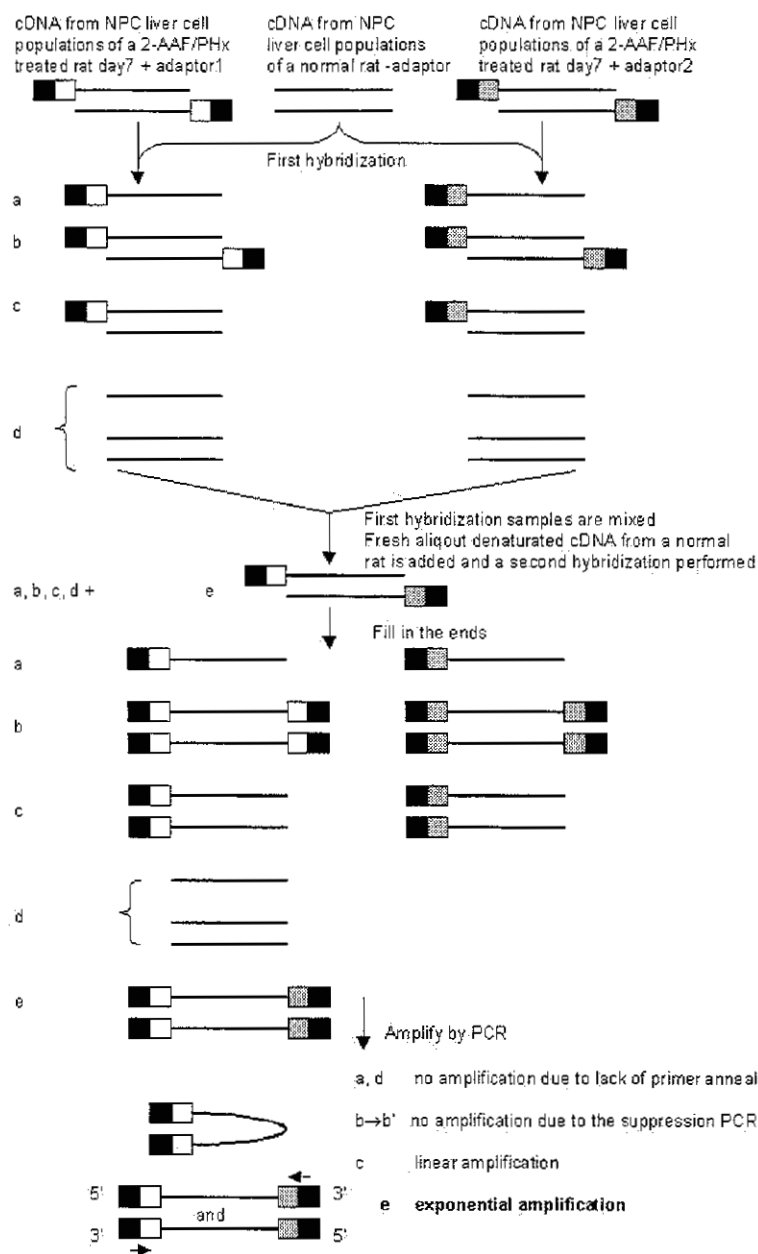


Figure 4. The molecular events of PCR-Select cDNA subtraction. Redrawn and modified from CLONTECH manual #PT1117-1.

Finally the entire reaction is subjected to PCR amplification. Only the differentially expressed tester cDNAs (type e) are exponentially amplified as type a and d molecules lack primer annealing sites and type c molecules are amplified in a linear fashion as primer

annealing sites are present in only one end. Type b molecules are potential targets for PCR amplification but this reaction is efficiently suppressed due to the adaptor design. Each of the two adaptor types are self-complimentary, resulting in the formation of pan-like secondary structures of type b molecules in favour of primer annealing during the PCR cycle annealing step.

3.1.2. Clones identified from the 2-AAF/PHx day 7 NPC population by use of SSH analysis

In the present study, the secondary SSH PCR reaction was used directly for cloning of differentially expressed sequences by use of the T/A vector pCR2.1 (Invitrogen Corp., Carlsbad, CA). The identity of the cDNA inserts from 119 clones was revealed by sequence analysis and comparison to known DNA or amino acid sequences or expressed sequence tags (ESTs) using the BLAST algorithm (<http://www.ncbi.nlm.nih.gov/BLAST/>). Twenty-nine known and five novel unique cDNA sequences in addition to four ESTs were identified as shown in Table 2.

As a SSH cDNA library is likely to contain false positives, the differential expression of identified genes must be confirmed by expression analysis. In the context of this thesis, eight of the known genes; Interleukin 1- β (IL-1 β), α -fetoprotein (AFP), ebnerin, eukaryotic initiation factor 67 kDa associated protein, interferon- γ receptor α (IFN- γ R α), gp91phox, lymphocyte function-associated molecule-1 α (LFA-1) and CXCR4 (Table 2, written in bold), were further analyzed by Northern blot analysis and all found to be differentially expressed. The calreticulin, PRxV and Hex sequences have subsequently been examined but differential expression was not detected for either of these genes (Bisgaard, unpublished results) As described in Paper II, the chemoattractant receptor CXCR4 is induced when oval progenitor cells are activated and might be a central

component in a pathway which brings about oval cell activity-associated T-lymphocyte recruitment. The remaining seven examined genes are all parts of the gene network connected to Interferon γ and are modulated in regeneration from oval progenitor cells, as described in Paper I. The possible implications of the eight differentially expressed genes in oval progenitor cell activity will not be paid any further attention in this chapter, but are thoroughly examined in chapter 4, Paper I and II.

Table 2. Unique clones identified from the suppression subtractive hybridization library using non-parenchymal cell populations from a 2-AAF/PHx treated rat day 7 as tester cDNA and nonparenchymal cell populations from a normal (control) rat as driver cDNA. Genes written in bold were further analyzed in Paper I or Paper II.

Gene name	Remarks	Acc no./References
<i>High mobility group protein 14 (Hmg14)(murine)</i>	non-histone chromosomal protein	NM_008251. Landsman and Bustin.(1990)
<i>YWK-II</i>	Rat sperm membrane protein related to A4 amyloid protein	M31322. Yan <i>et al.</i> (1990)
<i>Atp1a1</i>	ATPase, Na+K+ transporting, alpha 1 polypeptide	NM_012504. Shull <i>et al.</i> (1986)
<i>CIIDBP</i>	DNA-binding protein; high mobility group 1 protein homologue	L08814. Wang <i>et al.</i> (1993)
KIAA0235 protein (H. sapiens)	Novel gene isolated from male bone marrow myeloblast cell line KG-1	D87078. Nagase <i>et al.</i> (1996)
<i>Interleukin-1β (IL-1β)</i>	Inflammatory cytokine released from macrophages. IL-1 β binds to IL-1R1 and induces transcription and translation of IFN- γ in hepatocytes.	M98820. Unpublished; Schroeder <i>et al.</i> (1998)
<i>CXCR-4</i> (murine)	PBSF/SDF-1 receptor. Involved in liver regeneration from oval progenitor cells (Müller <i>et al.</i> submitted to AJP)	D87747. Nagasawa <i>et al.</i> (1996)
<i>Alpha-fetoprotein (AFP)</i>	Oval progenitor cell marker. Suppresses IFN- γ production (Yamashita <i>et al.</i> 1994)	NM_012493. Jagodzinski <i>et al.</i> (1981)
<i>Ebnerin</i>	Involved in liver regeneration from oval progenitor cells. (Bisgaard <i>et al.</i> in preparation)	U32681. Li and Snyder. (1995)
<i>Eukaryotic initiation factor (Eif-2) 67 kDa associated protein</i>	Protects eIF-2 from inhibitory phosphorylation by IFN- γ induced eIF-2 kinases	L10652. Wu <i>et al.</i> (1993); Kumar <i>et al.</i> (1997)
<i>Interferon-γ receptor α (IFN-γRα)</i>	Cell surface receptor which exhibits ligand binding properties towards IFN- γ (Arany <i>et al.</i> 1998)	AF201901. Direct submission 1999
<i>Conserved ATPase domain protein of 44kDa (CADp44) (S. tridecemlineatus)</i>	A regulatory subunit of the 26S Proteasome.	U36395. Bauer <i>et al.</i> (1996)
<i>sid23p</i> (murine)	Actin depolymerizing factor.	AB025406. Direct submission 1999

Gene name	Remarks	Acc no./References
54 kDa oligo-adenylate synthetase-like protein p540ASL (murine)	induced during maturation of murine dendritic cells.	AF068835. Tiefenthaler <i>et al.</i> (1999)
Calreticulin	Lectin-like chaperone which participates in the synthesis of several molecules including surface receptors and integrins (Michalak M <i>et al.</i> 1999). Not specifically associated with oval cell activity (Bisgaard unpublished result)	X53363. Murthy <i>et al.</i> (1990)
Mannosidase 2, alpha B2(Man2b2) (murine)	Mannose trimming	NM_008550. Hiramoto <i>et al.</i> (1997)
PRx IV	Peroxidins play a role in protecting protein free thiol groups against oxidative damage and thioredoxin-dependent peroxidase activity. PRxIV is a secretory form which functions within the extracellular space. Not specifically associated with oval cell activity (Bisgaard unpublished result)	AF106945. Matsumoto <i>et al.</i> (1999)
F-0-ATPase subunit b	Precursor of subunit b in H ⁺ -ATP synthase from mitochondria	M35052. Tsurumi <i>et al.</i> (1990)
F-box protein FBL4 (H. sapiens)	Member of the expanding F-box protein family. F-box proteins are one of the four subunits of ubiquitin protein ligases (Cenciarelli <i>et al.</i> 1999)	AF199420. Direct submission 1999
Rap1B	Ras homologous GTPase implicated in cell proliferation and differentiation (Posern <i>et al.</i> 1998)	U07795. Direct submission. 1994
Putative secreted protein ZSIG37 (murine)	Function unknown.	AF192499. Direct submission. 2000
gp91phox (Cybb) (murine)	Heavy chain of cytochrome b558. Induced as a primary response gene by IFN- γ (Eklund and Skalnik. (1995)	U43384. Bjorgvinsdottir H <i>et al.</i> (1996)
Hex	Homeobox gene. Transcriptional regulator. Not specifically associated with oval cell activity (Bisgaard unpublished result)	D86383. Tanaka T <i>et al.</i> (1999)
Lymphocyte function-associated molecule-1 α subunit (LFA-1)(murine)	Lymphocyte adhesion receptor. Regulated in response to IFN- γ . Binds ICAM-1 and ICAM-2, which are induced as secondary response genes by IFN- γ . (Barker <i>et al.</i> 1989).	M60778. Kaufmann <i>et al.</i> (1991)
Ribosomal proteins L3, L4, L6, L12 and S7.	Ribosome subunits	X62166, X82180, X87107, X53504, X53377, respectively.
SSH clone G6	EST292485 from normalized rat liver	AW142249
SSH clone 41	EST189325 from normalized rat heart	AA799828
SSH clone 85	EST va73d09.r1 Mouse cDNA	AA272079
SSH clone 112	EST294771 from normalized rat embryo	AW913993
Five novel unique cDNA sequences		

Table 2. continued.

3.2. Identification of genes encoding secreted and membrane-bound proteins associated with regeneration from oval progenitor cells. Combination of The Signal Peptide Selection (SPS) method and expression analysis.

Virtually all proteins which are secreted or bound in the cell membrane of prokaryotes as well as eukaryotes are characterized by the presence of a provisional N-terminal sequence, a signal peptide, which controls their entry to the secretory pathway (Gierasch, 1989; von Heijne, 1990; Rapoport, 1992). Common to the structure of signal peptides is a positively charged n-region, followed by a hydrophobic h-region and a c-region in which the residues at position -3 and -1 must be small and neutral for cleavage to occur correctly (von Heijne, 1985). These features can be identified in the signal peptide of most known secreted proteins but the level of degeneracy within any one of these structural elements is rather high. Therefore, it is not possible to identify novel extracellular proteins by use of standardized signal peptide encoding cDNA probes or DNA database searches using e.g. expressed sequence tags. Consequently, *in vivo* methods were developed to select for secreted proteins (Tashiro *et al*, 1993; Klein *et al*, 1996; and variations of these e.g. Lim and Garzino-Demo, 2000; Jacobs *et al*, 1997). These methods all identify signal peptide encoding cDNA's based on their ability to complement for a deleted signal peptide encoding sequence of a naturally secreted gene product. An *in vitro* method based on sedimentation velocity of mRNA species bound to membrane-associated polysomes and subsequent hybridization to DNA microarrays has just been introduced (Diehn *et al*. 2000)

When the present study was initiated the Signal Peptide Selection (SPS) method (Klein *et al.*, 1996) appeared as the most suitable, as it has integrated a selection step and allows simultaneous examination of millions of cDNA clones (see below). The

capacity of the method developed by Tashiro *et al.* (1993) is much smaller as it requires a subsequent antibody based screening step, which makes this method unsuitable for examination of large amounts of cDNA's.

3.2.1. The Signal Peptide Selection method

The method to isolate genes encoding secreted and membrane-bound proteins developed by Klein *et al.* (1996) is based on a single-step selection in yeast. It takes advantage of the fact that the ability of *Saccharomyces cerevisiae* to grow on a sucrose medium depends on the secretion of the *SUC2* encoded invertase enzyme, which catalyze the breakdown of sucrose into glucose and fructose (Figure 5, B). A library representing the cDNA of interest is established as *Not* I fragments in a λ -based shuttle vector, upstream a modified *suc2* gene, from which the sequence encoding the signal peptide and the initiator methionine is deleted (*suc2*^{ΔMSP}) (Figure 5, A). The cDNA/*suc2*^{ΔMSP} constructs are subsequently converted to an *E.coli*/yeast plasmid library and introduced into a *SUC2* deleted yeast strain. To select for signal peptide encoding cDNAs cloned in-frame with the *suc2*^{ΔMSP} gene, yeast transformants are finally replicated onto a sucrose medium.

Only 0.6% of the wild-type invertase activity is sufficient to allow growth on sucrose medium (Kaiser *et al.*, 1987), why even inefficient heterologous (e.g. mammalian) signal peptides are able to direct an invertase fusion protein to the secretory pathway. Klein *et al.* (1996) demonstrated the potential of the SPS method by examining five cDNA libraries representing human bone marrow, whole rat embryo, rat embryonic brain or specific parts of embryonic rat brain. From a total of \approx 20 million yeast transformants, 49 sucrose positive clones were found to contain unique cDNAs encoding known proteins localized

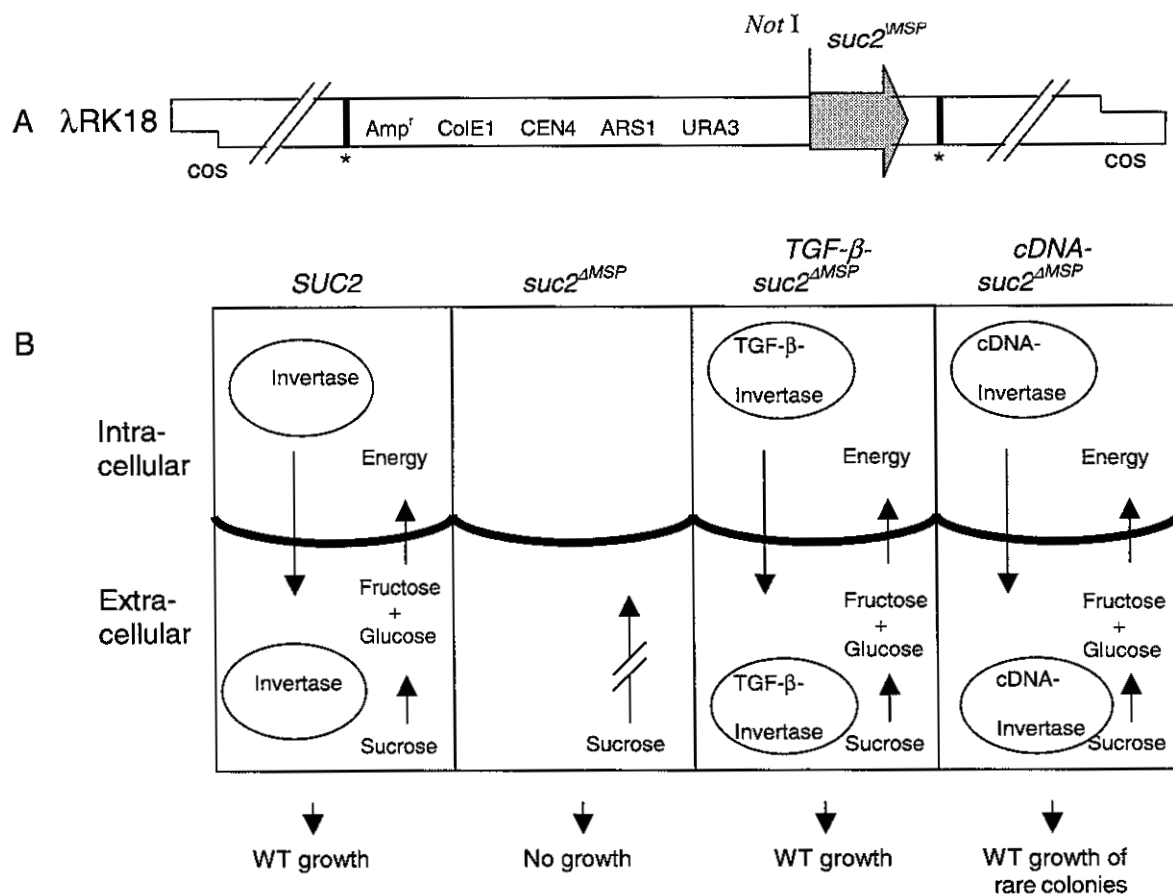


Figure 5. The Signal Peptide Selection method. **A)** Schematic representation of the λ -based *E. coli* /yeast shuttle-vector for cloning of cDNAs upstream the modified invertase gene lacking the initiator methionine and the signal peptide (*suc2*^{ΔMSP}). Asterisks represent the initiator and terminator sites for f1 DNA synthesis and define the boundaries of the sequence excised as phagemid. This sequence include genes for selection of transformants and replication in *E. coli* and yeast, respectively. **B)** Schematic representation of the SPS method. The panels indicate the ability of the yeast strain YT455 to grow on sucrose when the *SUC2* gene is present in pRK18 as a wild type copy, MSP truncated, includes the first 46 amino acids of the secreted transforming growth factor β in the MSP truncated pRK18 version or when fused to a random collection of cDNAs, respectively. Redrawn and modified from Klein et al. (1996).

along the secretory pathway or exported to the cell surface or the extracellular environment. Another 17 cDNAs were related to known proteins which belonged to the same categories of destinations. Finally, 74 unique clones representing unknown genes were by computer analysis found to contain possible signal peptide encoding sequences.

The known cDNAs included sequences encoding: members of the transforming growth factor and epidermal growth factor families, endocrine hormones, tyrosine- and serine/threonine kinase receptors, cell adhesion molecules, extracellular matrix proteins and plasma proteins.

3.2.2. Construction of a λ RK18/PAI-1 positive control

In order to verify the functionality of the SPS method in our laboratory, a positive control was constructed. A 405 bp fragment, encoding the 23 amino acid signal peptide sequence and the first 99 amino acids of the structural gene from the secreted rat serine protease inhibitor, Plasminogen activator inhibitor-1 (PAI-1) (Zeheb and Gelehrter, 1988), was cloned in-frame with the *suc2* ^{Δ MSP} gene. Replication of YT455 pRK18/PAI-1 transformants to sucrose plates resulted in positives after incubation for 2-3 days. Parallel examination of YT455 pRK18-insert transformants did not result in growth on sucrose plates even after 4 weeks of incubation.

3.2.3. Examination of a 2-AAF/PHx cDNA library by SPS analysis

Selection of cDNAs by SPS analysis is solely based on the functional properties of the examined sequences. As no subtraction or leveling steps are included in the method, highly expressed cDNAs which are able to complement for the truncated sucrose gene sequence will naturally constitute the preferential output. In order to increase the possibility of identifying molecules associated with regeneration from oval progenitor cells, cDNA from day 11 in the 2-AAF/PHx protocol was examined. At this time point the presence of progenitor cells was massive according to histological evaluation (See Figure 1 Paper II).

A total of 6×10^6 non-amplified λ RK18/2-AAF/PHx cDNA plaque forming units (pfu) were excised in pools of 10^5 pfu. To verify efficient ligation of cDNAs into the λ RK18 vector, phagemid DNA aliquots representing the excised pools were examined by restriction analysis. Representative examples of successful ligation reactions are shown in Figure 6 A, the excised pRK18 vector in 6 B. Note that the smear between 1.3 and 2.3 kb representing the 500-1500 bp cDNAs (present in the 0.8 kb *Hind* III/*Xba* I fragment) is dominated by specific fragment lengths which differ between the individual cDNA pools. The weak 0.8 kb band in lane 1 and 3 – 7 indicates the presence of re-ligated pRK18 without insert. This background appears small when the relative intensity between the 6.3, 2.2 and 0.8 kb *Hind* III/*Hind* III and *Hind* III/*Xba* I vector bands is compared to the relative intensity of the corresponding bands in the pRK18 x *Hind* III/*Xba* I control (lane 2).

The 60 phagemid DNA pools were realized in a total of 4×10^6 YT455 transformants and transferred to sucrose plates by replica plating. More than 500 sucrose positive transformants were preliminarily examined by PCR on yeast lysate and amplification products from the individual pools were subjected to insert size-alignment by gel electrophoresis, as a rough means to detect redundancy of positives. Accordingly, the 3 – 500 terminal bases of 110 inserts from sucrose positive transformants were sequence determined. By comparison to known DNA or amino acid sequences using the BLAST algorithm (<http://www.ncbi.nlm.nih.gov/BLAST/>) 18 inserts were identified as unique known genes, included a signal peptide encoding sequence and were found to be cloned in-frame with the signal peptide sequence deleted sucrose gene (Table 3). Two inserts (aocp1 and aocp3 (associated with oval cell proliferation)) showed no significant homology to known sequences at either DNA or amino acid level but were identified as expressed sequence tags (EST) (acc. no. A1233493 and BE106481, respectively), one (aocp4) showed 87%

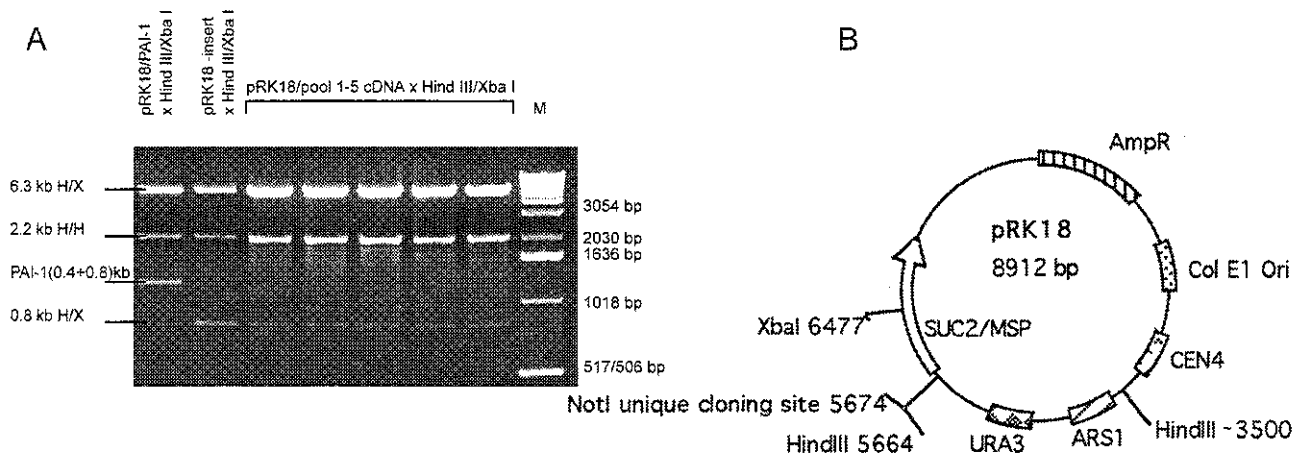


Figure 6. A) Analysis of λ RK18/insert ligation efficiency by *Hind* III/*Xba* I digestion of pRK18/insert DNA aliquots and electrophoresis on a 1% agarose gel. Lane 1 represents the ligation of the 405 PAI-1 positive control fragment, lane 3 – 7 represents the ligation of 500 – 1500 bp size fractionated cDNA from a 2-AAF/PHx treated rat 11 days post PHx. H/X = *Hind* III/*Xba* I vector fragment, H/H = *Hind* III/ *Hind* III fragment, M = DNA ladder. B) Schematic representation of the excised phagemid pRK18, showing the position of the *Not* I cloning site, *Hind* III and *Xba* I restriction sites and the relative positions of *E. coli* and yeast markers for selection of transformants and plasmid replication.

identity at the DNA level to human NY-REN-25 antigen (acc no. AF155103) and one (aocp2) showed 89% identity to a homo sapiens EST encoding a putative calcium-transporting ATPase (acc no. A1371849). The putative polypeptides generated from the preliminary sequence data of the inserts which showed no or limited homology to known sequences were analyzed by use of a computer program designed to search for signal peptides (Nielsen et al. 1997) and as the prognoses were positive all the sequences were further examined.

The remainder of the 110 examined inserts mainly consisted in varying 3'end truncations of the hemopexin, serum albumin, transferrin, ceruloplasmin or corticosteroid binding globulin cDNAs, but also in sequences which included a signal peptide cloned in the wrong orientation or sequences encoding internal regions of polypeptides localized to the secretory pathway (data not shown).

Table 3. cDNA clones isolated from a rat liver at the 11th day after the combined treatment of 2-AAF exposure and a 70 % hepatectomy in the 2-AAF/PHx protocol and cloned by use of the SPS method.

Clone	Name	Main features	Acc no./ References
1	<i>Leucocyte-common antigen; LCA-1; CD45</i>	Transmembrane receptor-linked protein tyrosine phosphatase.	Y00065. Barclay <i>et al</i> (1987)
2	<i>Ceruloplasmin</i>	Plasma copper dependent oxidase involved in the acute phase response and antioxidant systems.	L33869. Fleming and Gitlin (1990)
3	<i>Transferrin</i>	Major iron binding protein in vertebrate serum.	D38380. Escriva <i>et al</i> (1995)
4	<i>Complement factor I</i>	Regulatory plasma serine protease. (Schlaf <i>et al</i> , 1999)	Y18965. Direct submission (1999)
5	<i>Alpha-1-macroglobulin</i>	Plasma protease inhibitor.	M84000. Waermegard <i>et al</i> (1992)
6	<i>OX47 antigen; Basigin</i>	Plasma membrane glycoprotein of the immunoglobulin superfamily.	X54640. Fossum <i>et al</i> (1991)
7	<i>Hemopexin</i>	Plasma glycoprotein. Acute phase protein.	M62642. Nikkilae <i>et al</i> (1991)
8	<i>Alpha albumin</i>	A member of the serum albumin multigene family.	X76456. Belanger <i>et al</i> (1994)
9	<i>Vitamin D-binding protein</i>	A member of the serum albumin multigene family.	J05148. McLeod and Cooke (1989)
10	<i>Corticosteroid-binding Globulin</i>	Steroid-binding protein in plasma. Main carrier of glucocorticoids.	X70533. Scrocchi <i>et al</i> (1993)
11	<i>Complement component C6</i>	A late complement component of the membrane attack complex following complement activation. (Zhu <i>et al</i> , 2000)	AF184900. Direct submission (1999)
12	<i>Fc gamma receptor type II</i>	Receptor for the Fc domain of immunoglobulin G.	X73371. Bocek and Pecht (1993)
13	<i>Serum albumin</i>	Major plasma protein.	V01222. Sargent <i>et al</i> (1981)
14	<i>Plasma protein; Inter-alpha-inhibitor H4</i>	The single chain member of the plasma protein family inter-alpha-inhibitor.	Y11283. Soury <i>et al</i> (1998)
15	<i>LMW T-kininogen; major acute phase alpha-1 protein</i>	Plasma glycoprotein.	M11883. Furutokato <i>et al</i> (1985)
16	<i>Rat core protein</i>	Transmembrane glycoprotein.	M81687. Pierce <i>et al</i> (1991)
17	<i>Kidney injury molecule-1</i>	Type 1 membrane protein.	AF035963. Ichimura <i>et al</i> (1998)
18	<i>HMW K-kininogen</i>	Plasma glycoprotein; Splice variant of LMW T-kininogen.	L29428. Kitagawa <i>et al</i> (1987)
19	<i>aocp1</i>	Novel cDNA 1 (SP561); associated with oval cell proliferation (aocp); 99% identity to rattus norvegicus EST	A1233493
20	<i>aocp2</i>	Novel cDNA 2 (SP587); associated with oval cell proliferation; 89% homology to homo sapiens EST; putative calcium-transporting ATPase.	A1371849
21	<i>aocp3</i>	Novel cDNA 3 (SP611); associated with oval cell Proliferation; 99% identity to rattus norvegicus EST	BE106481
22	<i>aocp4</i>	Novel cDNA 4 (SP622); associated with oval cell proliferation; 87% identity to H. sapiens NY-REN-25 antigen.	AF155103

None of the potential candidates for identification by SPS analysis, encoding secreted or membrane-bound proteins which are known to respond in the 2-AAF/PHx protocol by increased transcriptional activity, e.g. transforming growth factor- α (TGF- α), acidic fibroblast growth factor (aFGF), hepatocyte growth factor (HGF) (Evarts *et al.* 1993), stem cell factor (SCF) and its receptor *c-kit* (Fujio *et al.* 1994), the fibroblast growth factor receptor-1 and -2 (Hu *et al.* 1995), leukemia inhibitory factor (LIF) and its receptor LIFR (Omori *et al.* 1996) or urokinase-type plasminogen activator (uPA), its receptor uPAR and plasminogen activator inhibitor-1 (PAI-1) (Bisgaard *et al.* 1998), were cloned in the present study. The absence of genes which are known to be induced in the 2-AAF/PHx model may reflect a relatively low abundance of the corresponding transcripts within the total RNA pool as compared to the identified transcripts e.g. albumin and transferrin (proteins normally produced and secreted by hepatocytes).

3.2.4. Northern blot analysis of cDNAs identified from the SPS library

To examine for a possible role of the identified genes (Table 3) during liver damage and regeneration, particularly in regeneration from oval progenitor cells, the cDNAs were analyzed by Northern blotting of RNA extracted from livers of rats exposed to experimental regimens inducing different regenerative responses (Figure 7). Three regimens represent responses which are known to induce either activation or activation, extensive proliferation, migration and differentiation of oval progenitor cells;

- 2-AAF treatment (Figure 7, lane B, H and K) inhibits mitotic activity in hepatocytes and activates oval progenitor cell proliferation in bile ducts and ductules, as shown by [^3H]-thymidine labeling and detection of the liver-specific transcription factors hepatocyte nuclear factor 1 β (HNF1 β), HNF3 γ and their target genes AFP and albumin, in the activated structures (Bisgaard *et al.* 1996). It was further shown that 2-AAF treatment

resulted in an increased number of apoptotic cells in portal areas, a process that contributed to the overall retention of liver morphology.

- When 2-AAF is combined with a 70% PHx (Figure 7 lane G and J), inhibition of mitotic activity in hepatocytes and activation of oval progenitor cells are followed by extensive proliferation, expansion into the liver parenchyma and differentiation of oval progenitor cells (Nagy et al. 1994, Evarts et al. 1996).
- D-galactosamine exposure (Figure 7 lane C) cause parenchymal necrosis and compromise mitotic activity in hepatocytes for two days whereas oval progenitor cells replicate at day 1 and subsequently differentiate into small hepatocytes (Lemire et al. 1991). Normal liver structure is restored within only 7 – 10 days after GalN injection (Lemire *et al*, 1991) as mature hepatocytes make up an important source of tissue renewal.

The remaining examined regimens are known to induce extensive regenerative responses but oval progenitor cell activity has not been reported in these models;

- Ligation of the common bile duct (BDL) (Figure 7 lane E) induces bile duct hyperplasia without penetration of the parenchyma (Lenzi et al. 1992) or expression of hepatocyte enriched transcription factors (Bisgaard et al. 1996).
- Removal of 90% of the liver mass (90% PHx) without toxic injury to the liver (Figure 7 lane M and N) stimulates all liver cell populations to proliferate (Tygstrup et al. 1996).
- Both acetaminophen intoxication (APAP) (Figure 7 lane D) and endotoxin stimulation (LPS) (Figure 7 lane O and P) induce hepatic dysfunction and hepatocyte death.

Injection of NaCl, sham operation with laparotomy only and late stages of regeneration from 70% PHx was used as controls for the above regimens (Figure 7 lane A, F, I and L, respectively).

To verify oval cell activity in animals exposed to GalN and the 2-AAF/PHx protocol (Figure 7 lane C, G and J), expression of the commonly used oval cell marker α -fetoprotein (AFP) was included in the Northern blot analysis. The observed signal intensity in the GalN and 2-AAF/PHx protocols is in accordance with the relative presence of oval cells at the examined time points. AFP expression could not be detected in the remaining examined regimens, reflecting the absence of significant oval cell response in these protocols.

In general the Northern blots show that among the known cDNAs, only CD45 expression is specifically associated with the regimens which extensively induce oval cell activity, whereas expression of all the novel cDNAs seems to be associated with oval cell activity. This pattern of differential expression is more clearly illustrated in Figure 8, in which the relative intensity of the Northern blot hybridization signals are quantified by densitometric scanning. The implications of the Northern blot analysis in the context of liver regeneration from oval progenitor cells are further analyzed in Paper II and will not be commented any further in this chapter. Only, it should be noted that the Northern blot analysis in the case of complement component C6 revealed an additional hybridization signal which the BLAST database search could not account for. Significant alignment values were produced for sequences from *M. musculus* and *H. sapiens* only. The mRNA from these species are stated as 2.8 kb and 3.5 kb, respectively. The rat complement component C6 cDNA cloned in this study hybridized to RNA of approximately 3 kb but also to RNA of approximately 0.8 kb. Further, the relative intensity of the 3 kb and 0.8 kb signals was not identical within the individual regimens (Figure 7). Northern blot analysis of the Fc gamma receptor cDNA did also produce hybridization signals in addition to the corresponding 1.5 kb mRNA, but in this case the BLAST search identified several

sequences from related Fc gamma receptor types (data not shown). As neither the complement component C6 nor the Fc gamma receptor was identified as specifically associated with regeneration in oval progenitor cell activating protocols, the Northern blot analysis results were not paid any further attention in the present study.

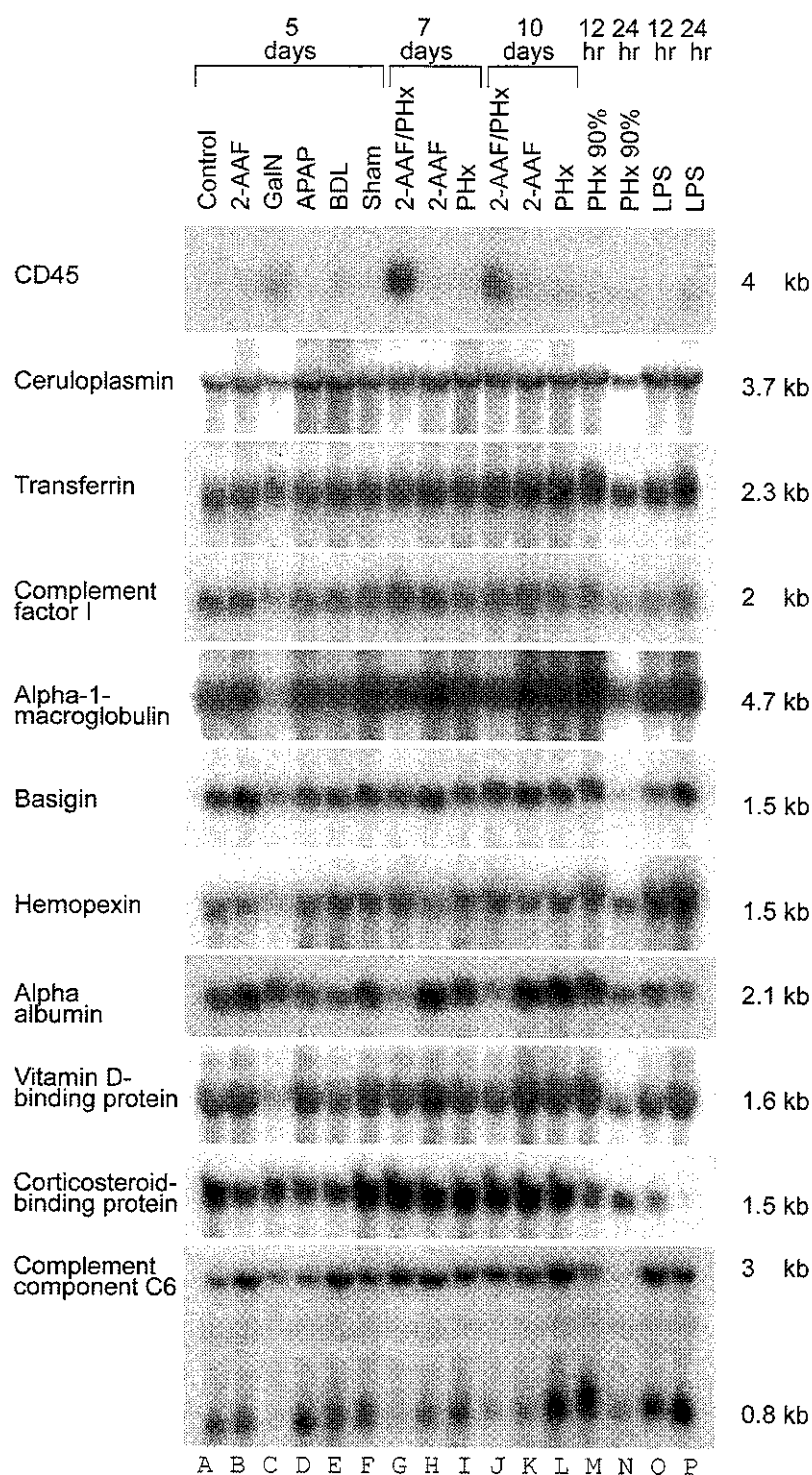


Figure 7. Northern blot analysis of cDNAs identified by use of the Signal Peptide Selection method applied on a cDNA library from the 11th day of a rat liver regenerating in the 2-AAF/PHx regimen. Hybridization of α -fetoprotein (AFP) (Figure 7 continued) is shown to verify oval cell activity. Each lane was loaded with 10 μ g of total liver RNA isolated from male Wistar rats exposed to the regimens indicated and sacrificed at the time points stated. Control liver, (A); 2-acetylaminofluorene (2-AAF), (B, H, K); D-galactosamine (GalN), (C); acetaminophen (APAP), (D); bile duct ligation (BDL), (E); sham operation with laparotomy only (Sham), (F); 2-acetylaminofluorene and 70% partial hepatectomy (2-AAF/PHx), (G, J); 70% partial hepatectomy (PHx), (I, L); 90% partial hepatectomy (PHx 90%), (M, N); lipopolysaccharide (LPS), (O, P). PCR generated copies of the cloned cDNAs and AFP were ³²P-labeled and used for hybridization.

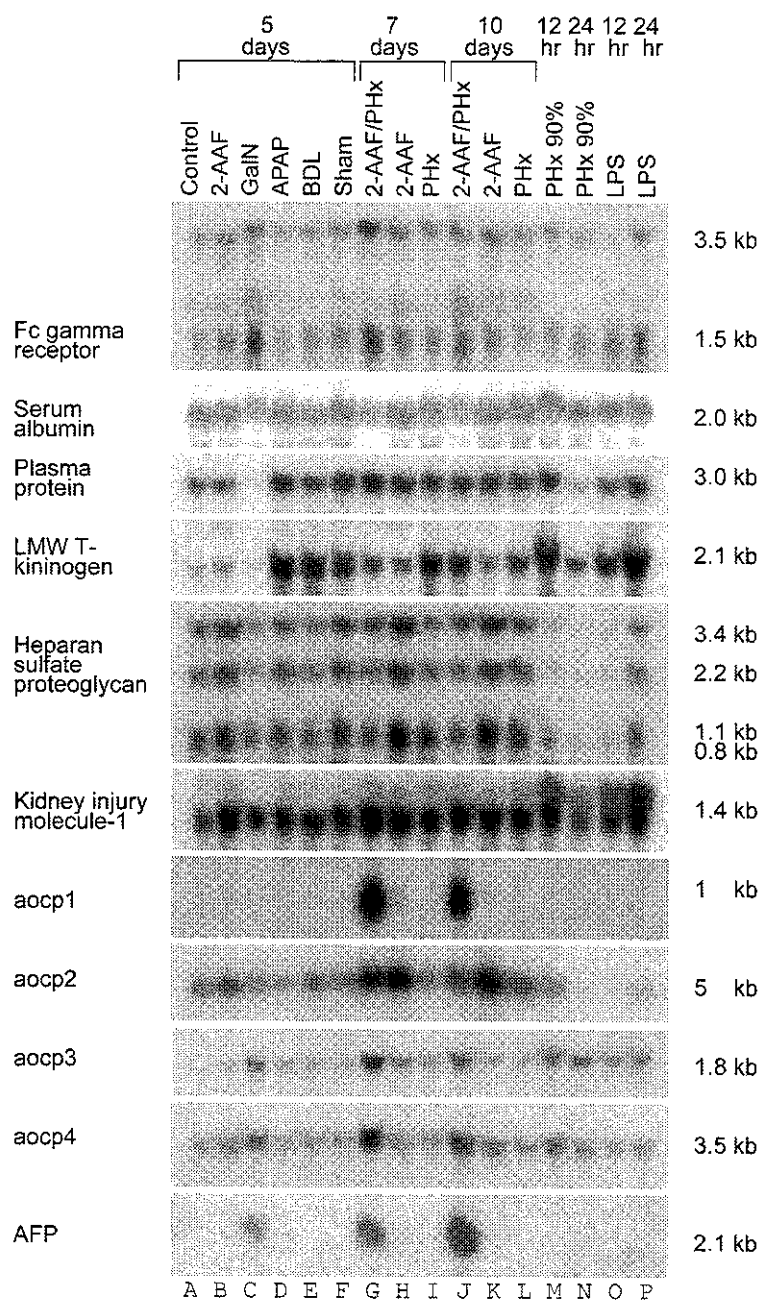


Figure 7. Continued

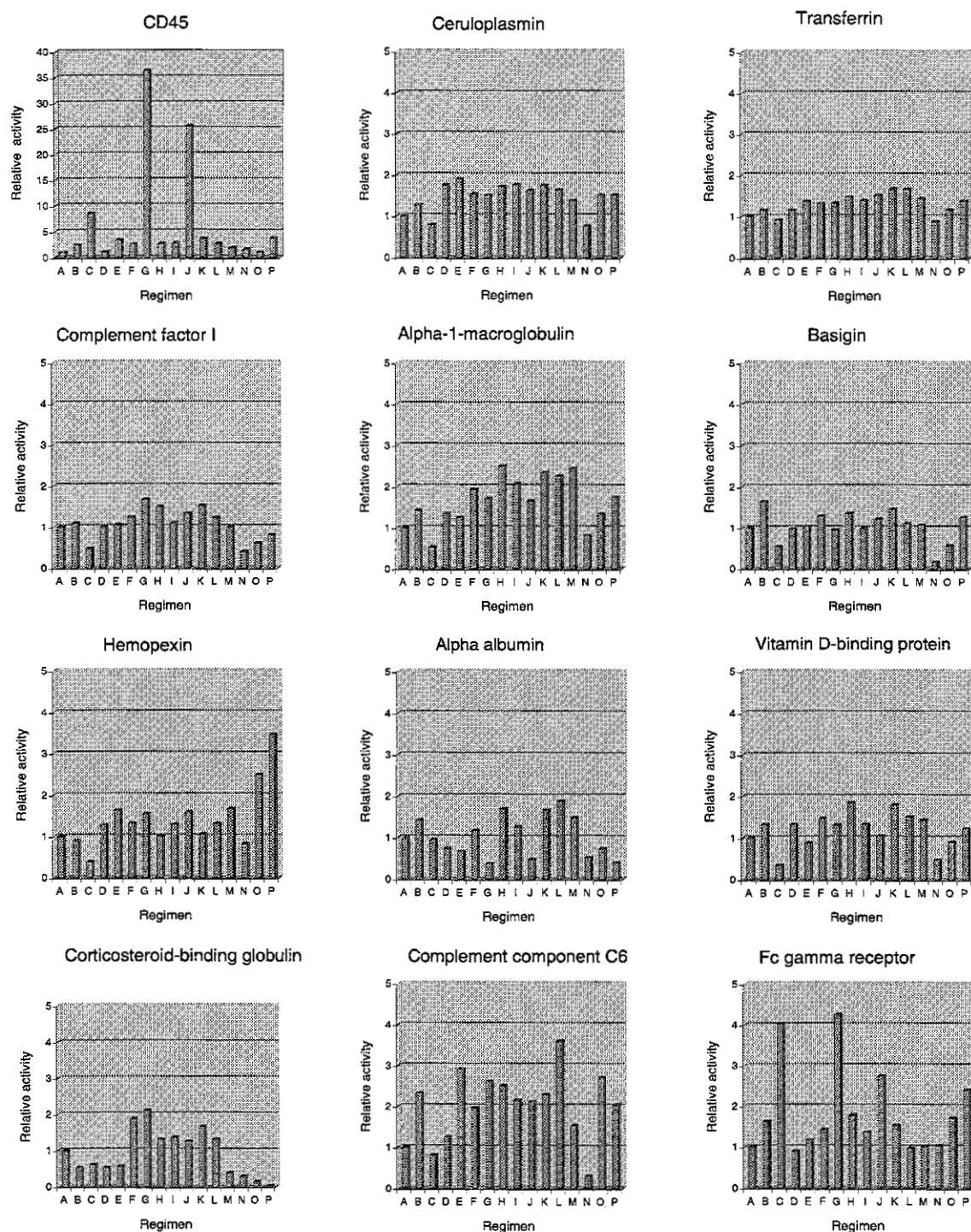


Figure 8. Quantification of the relative expression levels by densitometric scanning of the Northern blot analysis (Figure 7). Expression values of the individual experimental models were quantified relative to the control liver in which the density value was set to one. Note that the scale of the Y-axis differs between the diagrams. Capitals (X-axis) refer to the individual regimens as in Figure 7.

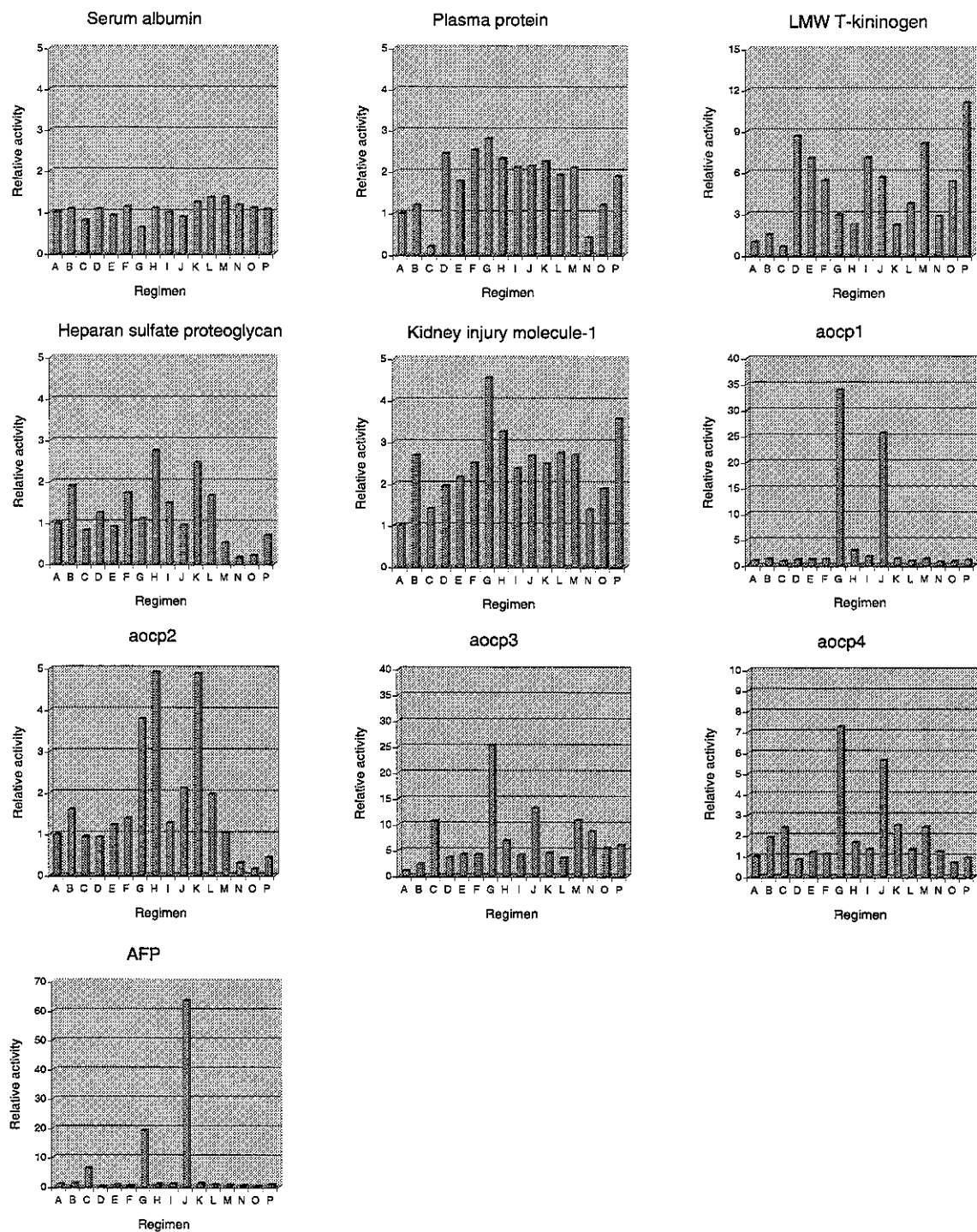


Figure 8. Continued

4. Discussion

Numerous studies have described the functional and physical properties of oval cells but our knowledge concerning the molecular mechanisms which regulate regeneration from this cell type is quite limited. A mechanism for priming of hepatocytes through the cascade initiated by TNF- α and IL-6 can, to a great extent, explain the efficient response after PHx treatment, but the mechanism by which oval cells are primed, does still appear rather unclarified. The GalN protocol demonstrates that the oval cell response can be quite fast, but oval cells are not significantly involved in the response to mass reduction by PHx. The ability of oval cells to compensate for a reduced proliferative activity of hepatocytes in IL-6 knockout mice (Rosenberg *et al*, 2000) further indicates that different mechanisms are responsible for the priming of hepatocytes and oval cells. The majority of growth modulators known to be involved in regeneration after PHx treatment are also present in regeneration from oval cells. The prolonged expression and individual characters of many of these growth modulators indicate their pleiotrophic functions as stimulators of migration and differentiation in addition to the proliferative effect (see chapter 1). However, the expression of other factors such as SCF (Fujio *et al*, 1994), IL-1 β , IL-18 and IFN- γ (Paper I), indicates, that regulatory mechanisms exist, which are involved only when liver regeneration is effectuated by the activation, proliferation and differentiation of oval cells.

Considering the present status of oval cell research, a mapping of differentially expressed genes seemed as an appropriate means to enhance our understanding of the molecular mechanisms involved in regeneration from oval cells. Experimental data provided in this work, concerning genes associated with oval cell activity, are summarized in Table 4. A complete mapping is, however, an extremely resource demanding task.

Table 4. Summary of genes examined in the present study which were found to be associated with oval cell activity.

Gene products identified by SSH or SPS combined with Northern blot analysis	
Gene name	Remarks
<i>IFN-γRα</i>	Cell surface receptor which exhibits ligand binding properties towards IFN- γ (Arany <i>et al.</i> 1998). Constitutively expressed by NPC populations in normal liver but the expression increases in response to 2-AAF/PHx treatment when the relative abundance of specific cell types in the NPC populations increases. Protein detected in ductular structures of oval cells as well as in cells located in the sinusoids.
<i>gp91phox</i>	Heavy chain of cytochrome b558. Induced as a primary response gene by IFN- γ (Eklund and Skalnik. (1995). Expressed at low levels in normal liver. Induced in response to 2-AAF.
<i>IL-1β</i>	IL-1 β binds to IL-1R1 and induces transcription and translation of IFN- γ in hepatocytes (Schroeder <i>et al.</i> 1998). Expressed at low levels in normal liver. Induced in response to 2-AAF but expression peaks at 12 hours and 36 hours in the PHx protocol too.
<i>LFA-1</i>	Lymphocyte adhesion receptor. Regulated in response to IFN- γ . Binds ICAM-1 and ICAM-2, which are induced as secondary response genes by IFN- γ . (Barker <i>et al.</i> 1989). Expressed at low levels in normal liver. Transcript level increases in the 2-AAF and 2-AAF/PHx protocols.
<i>eIF-2-associated 67-kd protein</i>	Protects eIF-2 from inhibitory phosphorylation by IFN- γ induced eIF-2 kinases (Wu <i>et al.</i> 1993; Kumar <i>et al.</i> 1997). Constitutively expressed by NPC populations in normal liver but the expression increases in response to 2-AAF/PHx treatment when the relative abundance of specific cell types in the NPC populations increases.
<i>α-fetoprotein</i>	Oval cell marker which suppresses IFN- γ production (Yamashita <i>et al.</i> 1994). Constitutively expressed by NPC populations in normal liver. Induced in response to 2-AAF.
<i>Ebnerin</i>	Homologous gene expressed in the bile ducts of the adult mouse liver (Cheng <i>et al.</i> 1996). Constitutively expressed by NPC populations in normal liver. Induced in response to 2-AAF.
<i>CXCR4</i>	PBSF/SDF-1 receptor (Nagasawa <i>et al.</i> 1996). Transcript not detectable in normal liver. Expressed in response to 2-AAF and 2-AAF/PHx treatment.
<i>CD45RO</i>	Transmembrane receptor-linked protein tyrosine phosphatase predominately expressed by memory T lymphocytes (Altin and Sloan, 1997). Constitutively expressed at low levels in normal liver. Expression of transcript increases in response to 2-AAF and 2-AAF/PHx treatment.
<i>aocp1</i>	Novel cDNA cloned in the present study. 99% identity to <i>rattus norvegicus</i> EST (acc. no. A1233493). Transcript not detectable in normal liver. Expressed in response to 2-AAF and 2-AAF/PHx treatment. In a multiple tissue Northern blot analysis detected in testis only.
<i>aocp2</i>	Novel cDNA cloned in the present study. 89% identity to <i>homo sapiens</i> EST (acc. no. A1371849) encoding a putative calcium-transporting ATPase. Expression of transcript increases in response to 2-AAF and 2-AAF/PHx treatment. Detected in several tissues of normal rat.
<i>aocp3</i>	Novel cDNA cloned in the present study. 99% identity to <i>rattus norvegicus</i> EST (acc. no. BE106481) Expression of transcript increases in response to 2-AAF and 2-AAF/PHx treatment. Detected in several tissues of normal rat.

Gene name	Remarks
<i>aocp4</i>	Novel cDNA cloned in the present study. 87% identity to homo sapiens NY-REN-25 antigen (acc. no. AF15503) Expression of transcript increases in response to 2-AAF and 2-AAF/PHx treatment. Detected in several tissues of normal rat.

Additional genes studied by Northern blot analysis and/or immunohistochemistry

<i>IFN-γ</i>	High affinity ligand of IFN-γRα (Boehm <i>et al</i> , 1997). mRNA levels not detectable in normal liver but increases to low levels in 2-AAF and 2-AAF/PHx treated animals.
<i>IL-18</i>	Induces IFN-γ production in T-lymphocytes (Okamura <i>et al</i> , 1995). Transcript levels increases in response to 2-AAF and increases further when combined with PHx. IL-18 protein highly expressed in sinusoidal lining cells as well as in the ductular structures of oval cells.
<i>ICE</i>	Induced as a secondary response gene by IFN-γ, involved in the activation of IL-1β and IL-18 (Gu <i>et al</i> , 1997; Tamura <i>et al</i> , 1995). Transcript levels increases in response to 2-AAF and increases further when combined with PHx. Protein detected over the ductular structures of oval cells.
<i>IFN-γRβ</i>	β-chain of the IFN-γ receptor complex, involved primarily in signaling (Boehm <i>et al</i> , 1997). Among NPC populations only oval cell structures stained for IFN-γRβ. Mature hepatocytes also stained (weakly) positive.
<i>uPAR</i>	Induced by IFN-γ as a secondary response gene (Sitrin <i>et al</i> , 1994). Transcript levels increases in response to 2-AAF and 2-AAF/PHx treatment. Protein present in NPC populations of bile duct and/or oval cells.
<i>uPA</i>	Induced by IFN-γ (Sitrin <i>et al</i> , 1994). Transcript levels increases in response to 2-AAF and 2-AAF/PHx treatment. Protein present in NPC populations of bile duct and/or oval cells.
<i>ICAM-1</i>	Induced as a secondary response gene by IFN-γ, binds to LFA-1 (Boehm <i>et al</i> , 1997). Protein present in NPC populations of bile duct and/or oval cells.
<i>Thy-1</i>	Membrane-anchored molecule which can interact with CD45 (Volarevic <i>et al</i> , 1990; Altin and Pagler, 1995). Transcript not detectable in normal liver. Transcript expressed in response to 2-AAF treatment, expression level increases further when combined with PHx.
<i>SDF-1</i>	Sole ligand for CXCR4 (Zou <i>et al</i> , 1998). High steady state transcript level in normal liver, decreases in response 2-AAF treatment and decreases further when combined with PHx.

Table 4. Continued.

In a recent study, Phillips *et al* (2000) applied an experimental approach based on subtracted cDNA libraries from highly purified murine fetal liver hematopoietic stem cells (FLHSC) in order to map the genetic program on which stem cell regulation is based. 5' end sequencing of almost 6000 subtracted clones identified at least 161 transcription

factors, 174 cell-surface or membrane-associated molecules, 28 secreted proteins and 147 signaling molecules. Many of the identified molecules were novel or found in stem cells for the first time. The efficacy of the subtraction was verified by the absence of β -actin and the retention or enrichment of gene products such as the marker of hematopoietic stem- and progenitor cells, CD34 (Krause *et al*, 1996). The database representing the sequences of the FLHSC subtraction libraries (<http://stemcell.princeton.edu>) includes several of the molecules (or associated molecules) known to be expressed by oval cells, e.g. uPAR, ICAM-1, IL-6 receptor and IL-18 receptor associated protein (Chapter 1 and Paper I). This coincidence of molecules associated with hematopoietic stem cell and oval cell activities is not surprising, considering the stem cell-like properties of oval cells and the ability of bone marrow stem cells to substitute hepatocytes in the presence of 2-AAF (Petersen *et al*, 1999). A Blast search with aocp1-4 against the hematopoietic stem cell database did not result in significant hits. In addition to the incompleteness of the database, several circumstances can account for this lack of hits, including the possibility aocp1-4 genes are not expressed by oval cells themselves. Of the unknown cDNAs from the SSH library (Table 2) only #SSH clone 112 (EST294771 from normalized rat embryo), was identical to an EST sequence in the stem cell database (SC LL2 in 14518T7119945).

In the present study, an approach based on SPS analysis was introduced in addition to the SSH analysis, in order to focus on the subgroup of oval cell associated molecules, which may be involved in the regulation of oval cell activities through an extracellular destination or presence on cell surfaces. The identified genes from the SSH and SPS libraries need to be analyzed further, but the genes that were examined more extensively indicated a quite interesting aspect of oval cell regulation: A part of the genes identified from the SSH library (Paper I); IFN- γ R α , gp91phox, IL-1 β , LFA-1, eIF-2-

associated 67-kd protein and AFP, are all implicated in the gene network connected to IFN- γ . Their expression in the 2-AAF/PHx protocol was analyzed by Northern blotting and immunohistochemistry and additional components of the gene network; IFN- γ itself, the β -subunit of the IFN- γ receptor, the IFN- γ induced uPA, three secondary response genes induced by IFN- γ (IL-1 β -converting enzyme (ICE), intercellular adhesion molecule-1 (ICAM-1) and uPAR), and IL-18, were included in the expression analysis. All the examined IFN- γ -connected molecules turned out to be modulated in regeneration from oval cells. It was found that the IFN- γ receptor α and β subunits and ICAM-1 all were expressed on oval cells and Northern blot analysis revealed an increased expression of LFA-1. It was also shown that IL-18, a strong inducer of IFN- γ production in T-cells (Okamura *et al*, 1995), is expressed by oval cells. Further, the ICE protein, a protease that converts IL-18 precursors to an active form (Gu *et al*, 1997), was detected over the ductular structures of oval cells, indicating that oval cells not only can produce IL-18 but also process it to an active form.

Of the known proteins identified from the SPS library, only CD45 turned out as specifically associated with oval cell activity (Chapter 3; Paper II). This observation is, however, quite interesting, as CD45 may be a central factor in the microenvironmental changes orchestrating oval cell activity and which include the induction of the gene network connected to IFN- γ . The CD45 isoform cloned from the SPS library corresponds to the low molecular form (CD45RO-positive) predominantly expressed by memory T-cells (Altin and Sloan, 1997). CD45 can associate with the membrane-anchored oval cell marker Thy-1 (Volarevic *et al*, 1990; Altin and Pagler, 1995; Petersen *et al*, 1998 a). Thy-1 might be involved in cellular recognition, adhesion and T-cell activation (Williams, 1985; Huber *et al*, 1992; Hozumi *et al*, 1994). The increased expression of CD45 and Thy-1

which associates oval cell recruitment (Paper II) thereby may reflect that CD45 mediates a contact between IFN- γ producing T-lymphocytes and oval cells, through Thy-1. This hypothesis is supported by the observation that several molecules which can reinforce the contact and mutual stimulation between T-lymphocytes and oval cells are induced when oval cells are activated (Paper I; Paper II); The membrane-bound molecule ICAM-1 was found to be extensively expressed by oval cells and the expression of its adhesion-partner, LFA-1, has been found to be highly increased on the surface of memory T-cells (Sanders *et al*, 1988). ICAM-1 and LFA-1 are both induced by IFN- γ . Further, oval cells provide components for the regulation of IFN- γ expression by the secretion of AFP and IL-18, which inhibits or stimulates IFN- γ production, respectively (Yamashita *et al*, 1994; Okamura *et al*, 1995).

The chemokine receptor CXCR4 was among the genes identified from the SSH library (Chapter 3, Table 2). CXCR4 mRNA is expressed by lymphocytes (Loetscher *et al*, 1994) and is the sole receptor for the chemoattractant stromal cell derived factor-1 (SDF-1) (Zou *et al*, 1998). SDF-1 has been recognized as a highly efficient inducer of lymphocyte migration. (Bleul *et al*, 1996). Both CXCR4 and SDF-1 expression is modulated when oval cells are activated by 2-AAF (Paper II, Figure 5) and thereby appears to be central elements in the pathway by which T-lymphocytes can be guided to the localization of oval cells. The SDF-1 mRNA steady state level is high in normal liver, but no expression of CXCR4 can be detected (Paper II, Figure 5), indicating that SDF-1 is either modified, inhibitors are removed, or that SDF-1 interacts with co-factors, in order to elicit its function as a chemoattractant. The possible scenario for an interaction between T-lymphocytes and oval cells is illustrated in Figure 9.

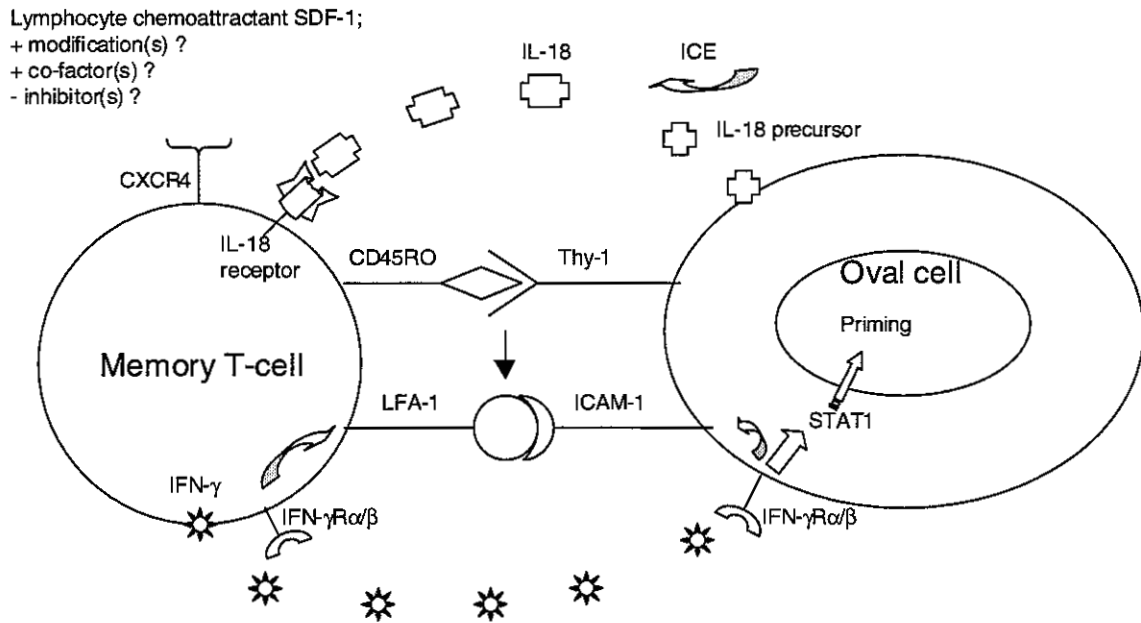


Figure 9. Model representing the interactions between IFN- γ producing T-lymphocytes and oval cells. CXCR4 and SDF-1 are central factors in the pathway involved in T-lymphocyte recruitment and the initial contact between the two cell types is established by CD45 and Thy-1. Paracrine and autocrine loops reinforce the contact and the exposure of oval cells to IFN- γ .

The functional implication(s) of a recruitment of IFN- γ producing T-lymphocytes when oval cells are activated is not known. However, for all examined components of the gene network connected to IFN- γ , IFN- γ itself, SDF-1 and CXCR4, expression is modulated in response to 2-AAF alone, indicating a role for this system in an early phase of oval cell activation. The properties of IFN- γ include orchestration of lymphocyte-endothelium interactions and effects on cell proliferation and apoptosis, but more than 200 genes are known to be regulated by IFN- γ , reflecting an extraordinary complexity of the IFN- γ response (Boehm *et al*, 1997). In mice with a demonstrable lack of IFN- γ mediated cellular responses, due to targeted mutations in the IFN- γ genes, in the gene of the only known receptor for IFN- γ , or the main transcription factor which mediates IFN- γ responses, STAT-1, the only phenotypic abnormalities are seen in the lymphoid system and

consequently heightened susceptibility to a variety of pathogens (Dalton *et al*, 1993; Huang *et al*, 1993; Meraz *et al*, 1996). *In vitro* studies by Marra *et al* (1996) showed that DNA synthesis rates were only slightly influenced by IFN- γ itself but increased significantly when pre-treatment with IFN- γ was combined with exposure to growth factors like EGF. It therefore seems possible that IFN- γ , through STAT-1, primes oval cells *in vivo* for the mitogenic effect of growth factors.

The indications that IFN- γ is a key molecule involved in an early phase of oval cell activation, eventually as a priming factor, need to be examined more thoroughly. In case that the role of IFN- γ , in protocols which induce an oval cell response, is parallel to the role of TNF- α in simple regeneration, new treatment strategies can be implemented in the clinic, e.g. in the context of pathological conditions which are characterized by uncontrolled growth of oval cells. A more thorough examination of the role of IFN- γ in the context of the data presented here, might include examination of CD45/CXCR4 co-expression from T-lymphocytes by immunohistochemical analysis or *in situ* hybridization. Ultimately, exposure of an IFN- γ deleted rat to the 2-AAF/PHx protocol would enlighten the importance of this cytokine.

Recent results have demonstrated that cells of bone marrow origin can function as a source of several liver cell types (see chapter 1.4). As an alternative to the described hypothesis of T-lymphocyte recruitment in the 2-AAF and 2-AAF/PHx protocols, the modulation of CD45, CXCR4 and LFA-1 expression observed in the present study (Paper I and Paper II) might reflect a recruitment of hematopoietic progenitor cells. Bone marrow derived CD34⁺ subpopulations, defined by CD45 isoform expression, have been studied by flow cytometry and it was shown that the most primitive hematopoietic CD34⁺ cells are CD45RO positive (Craig *et al*, 1994). SDF-1 is a highly efficacious lymphocyte

chemoattractant which mediates its effect through CXCR4 (Zou *et al*, 1998; Bleul *et al*, 1996), but CXCR4 is expressed by primitive hematopoietic CD34⁺/Thy-1⁺ cells also (Möhle *et al*, 1998). Human CD34⁺ hematopoietic progenitor cells are attracted by bone marrow derived stromal cell SDF-1 (Aiuti *et al*, 1997) why liver derived SDF-1 may bring about a similar effect on this cell type. Further, LFA-1 and ICAM-1, which were found to be associated with regeneration from oval cells (Paper I), seems important in the process of hematopoietic stem cell homing too (Peled *et al*, 2000). As both T-lymphocytes and hematopoietic progenitor cells can express molecules such as CXCR4, CD45RO and LFA-1, Northern blot analysis alone does not account for the recruitment of a specific cell type when the liver regenerates in the 2-AAF/PHx protocol. Immunohistochemical analyses will be more suitable to address this question. Recruitment of hematopoietic progenitor cells, rather than T-lymphocytes, in the 2-AAF and 2-AAF/PHx protocols involve the existence of an alternative to T-lymphocytes as a source of IFN- γ production. IL-1 β has been shown to induce IFN- γ production in hepatocytes (Schroeder *et al*, 1998) and the induction of IL-1 β in response to 2-AAF and 2-AAF/PHx (but also PHx alone) (Paper I) thereby makes hepatocytes to a possible source of IFN- γ in the 2-AAF and 2-AAF/PHx protocols. However, in the BMx and WLTx experiments by Petersen *et al* (1999), which included the 2-AAF/CCl₄ protocol, less than 0.2% of the hepatocytes were shown to originate from donor bone marrow at day 13 after hepatic injury. The proportion of Thy-1⁺ cells of donor origin was about 0.1% and the biological importance of cells originating from bone marrow, when the liver regenerates in the 2-AAF/CCL₄ protocol, therefore seems questionable.

By use of the SPS approach (Chapter 3; Paper II), four novel genes were identified as associated with oval cell proliferation. One of these, *aocp1*, appeared

especially interesting, as its expression was exclusively restricted to the 2-AAF and 2-AAF/PHx regimens. Further, in tissues of normal rats, the *aocp1* transcript was detected in testis only. The latter finding might reflect that *aocp1*, like SCF-1, is involved in spermatogenesis as well as the stimulation of oval cells (Fujio *et al*, 1994; Loveland and Schlatt, 1997). Identification of *aocp1* producing cells, e.g. by immunohistochemical analysis or *in situ* hybridization and identification of interacting molecules, e.g. by a yeast two hybrid screening of a testis library, are obvious future steps in the functional characterization of *aocp1*.

Experimental situations have been described in which renewal of liver cells, as well as fully differentiated cells of other tissues, involve tissue-specific adult stem cells (Petersen *et al*, 1999; Theise *et al*, 2000 a; Theise *et al*, 2000 b; Kooy and Weiss, 2000). These recent observations does not only indicate an extraordinary plasticity of adult stem cells but also that the process of stem cell differentiation into mature cell types is influenced by the tissue-specific microenvironment. The integrity of this microenvironment is adequately conserved in the 2-AAF/PHx and GalN protocols to allow complete regeneration from oval cells, and apparently destroyed in some chronically liver diseases or poisoning in which the mechanisms controlling oval cell differentiation are absent. A complete characterization of the liver microenvironment seems as a quite distant prospect. However, future mapping strategies based on subtraction cloning could be directed towards the individual pathological situations and thereby identify relevant key molecules, e.g. by use of 2-AAF/PHx cDNAs as the tester population and cDNAs from an APAP poisoned liver as the driver population.

5. Concluding remarks

Despite considerable research efforts during the past years, surprisingly little is known about the molecular mechanisms which control the actions of oval cells in liver regeneration. The majority of studies conducted so far, has focused on the role of factors known to be involved in regeneration from mature cell types.

The experimental approaches, based on Suppression Subtractive Hybridization and Signal Peptide Selection, applied in the present study, have demonstrated that regeneration from oval cells differ from simple regeneration by the specific expression of several known and novel molecules. These findings include, that genes of the network connected to IFN- γ are specifically induced when oval cells are recruited for regeneration. Some members of this network, e.g. the receptor for IFN- γ , ICAM-1 and IL-18, are expressed by oval cells. Others, like IFN- γ and LFA-1, appears to be characteristics of the changes in the microenvironment which associate regeneration from oval cells. Additional microenvironmental alterations was demonstrated by the modulation of CD45, CXCR4 and SDF-1 expression. The increased expression of CD45, LFA-1 and CXCR4 may reflect that cells of the hematopoietic system are recruited when liver regeneration is accomplished by oval cells. Whether such hematopoietic cells are represented by oval cell stimulating IFN- γ producing T lymphocytes, or more primitive progenitor cells, which themselves can differentiate into liver cells, needs to be examined further.

Finally, the detection of *aocp1* expression as highly restricted to the 2-AAF and 2-AAF/PHx protocols, as well as the restricted association with testis among multiple adult tissues, indicates a central role of *aocp1* in the pathways associated with recruitment of oval cells for liver regeneration.

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Modulation of the Gene Network Connected to Interferon- γ in Liver Regeneration from Oval Cells

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Suppression subtractive hybridization was used to clone genes associated with proliferation of oval cells in rat liver regenerating after a 70% partial hepatectomy combined with the feeding of 2-acetylaminofluorene. A subset of the identified genes comprised interferon- γ receptor α subunit (IFN- γ R α), gp91phox, interleukin-1 β (IL-1 β), lymphocyte function-associated molecule-1 α (LFA-1), eukaryotic initiation factor-2-associated 67-kd protein (eIF-2-associated 67-kd protein), and α -fetoprotein, which constitute part of the cellular program modulated by IFN- γ . Therefore, expression analysis performed by Northern blotting and immunohistochemistry were extended to include IFN- γ , the IFN- γ receptor β subunit (IFN- γ R β), three secondary response genes induced by interaction of IFN- γ with IFN- γ receptor complexes, ie, IL-1 β -converting enzyme (ICE), intercellular adhesion molecule-1 (ICAM-1), and urokinase-type plasminogen activator receptor (uPAR), and a cytokine inducing IFN- γ expression, ie, interleukin-18 (IL-18). The Northern blot analysis showed that all examined genes were modulated when progenitor-like oval cells were activated and recruited for liver regeneration. Immunohistochemistry localized the subunits of the IFN- γ receptor complex, IFN- γ R α and IFN- γ R β , the secondary response genes uPAR and ICAM-1, the IFN- γ -inducing factor IL-18, and ICE to the ductular structures of oval cells. In contrast, during liver regeneration after a 70% partial hepatectomy, only modulation of IL-1 β and ICE was observed. Our results, therefore, indicate that IFN- γ -mediated events may be particularly important when cells in the bile ductules must respond to liver damage by production of ductular oval cells. (*Am J Pathol* 1999, 155:1075–1085)

Selective Cloning of Genes Encoding Secreted and Membrane-Bound Proteins with Putative Roles in Liver Regeneration from Oval Cells

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aocp1, was exclusively expressed in the testis indicating its putative role in both spermatogenesis and liver regeneration accomplished by recruitment of multi-potent oval cells.

Regeneration of the adult rat liver in response to severe damage can be accomplished through the activation and differentiation of multi-potent oval cells. This process appears to be modulated by altered expression of secreted and membrane-bound growth modulators. To acquire further knowledge of the molecules involved, we have attempted to systematically identify genes encoding secreted and membrane-bound proteins, the expression of which is specifically associated with progenitor cell activity of the oval cell phenotype. Since secreted and membrane-bound proteins must pass through a secretory pathway involving a signal peptide sequence, we have used a single-step, signal peptide selection method in yeast to clone cDNA sequences from a rat liver containing numerous proliferating oval cells. Twenty two unique cDNAs encoding eighteen known membrane-bound or secreted proteins, and four novel proteins were identified. Northern blot analysis in various experimental models of liver damage and regeneration showed that increased expression of one transcript encoding CD45 and of the four novel transcripts was associated with recruitment of oval cells. Additional expression analysis of genes linked to the function of CD45, (i.e. Thy-1, CXCR4, and SDF-1) further indicated a role for CD45 in oval cell-based liver regeneration. Finally, Northern blot analysis of multiple adult rat tissues revealed that one novel cDNA,