Serial Analysis of Gene Expression of Rat Liver

Regeneration by Oval Hepatic Stem Cells

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List of Abbreviation

2-AAF/PH: model of 2-Acetoamidofluorene administration and 70% partial hepatectomy operation

2-AAF: 2-Acetoamidofluorene

AFP: Alpha foeto-protein

Alb: Albumin

APS: Ammonium Persulfate

ATPase: Adenosine Tri-Phosphate Hydrolase

BF: Basophilic Foci of Hepatocytes

BP: Base Pairs

BSA: Bovine Serum Albumin

CD: Choline Deficient Diet

CDC42: Cell Division Cycle 42

cDNA: complementary Deoxyribonucleic Acid

CK: Cytokeratin

CoA: Coenzyme A

Conc: Concameter

Cpm: Counts per minute

CV: Central Vein

DDC: 3,5-diethoxycarbonyl-1,4-dihydrocollidine

DNA: Deoxyribonucleic Acid

EF2: Elongation Factor 2

EGF: Epidermal Growth Factor

EST: Expressed Sequence Tag

FGF: Fibroblast Growth Factor

GC content: Guanosine-Cytosine content

GGT: Gamma Glutamyl Transferase (also designed Gamma Glutamyl Transpeptidase)

GTPase: Guanosine Tri-Phosphate Hydrolase

HGF: Hepatocyte Growth Factor

kb: kilo base (pairs)

kDA: kilo Dalton

M: DNA markers

mRNA: messenger Ribonucleic Acid

NL: Normal Liver

OC: Oval Cell Liver

PCR: Polymerase Chain Reaction

PETA-3: Platelet-endothelial cell tetraspan antigen 3

PF: portal field

PH: Partial Hepatectomy

P$_i$: inorganic Phosphate

PKC: Protein Kinase C

Real Time PCR: Real Time Polymerase Chain Reaction

RNA: Ribonucleic Acid

SAGE: Serial Analysis Gene Expression

SL: Sham Liver

TNFα: Tumour Necrosis Factor alpha

UV: Ultra Violet

1. Summary of the Work

The liver has the unique capability to regenerate by itself after an injury. Normally adult hepatocytes maintain a perfect balance between cell gain and cell loss and they have the ability to proliferate and regenerate the organ when the hepatic tissue is damaged.

Under certain circumstances like in hepatocarcinogenesis and chronic liver injury caused by drugs, viruses and toxins, when the hepatocytes proliferation is impaired, facultative hepatic stem cells called "oval cells" are recruited to generate the hepatic lineages of the hepatocytes and biliary cells. Hence, it was proposed that oval cells could represent a second compartment involved in the liver regeneration when the insult of the organ is too massive and proliferation of hepatocytes is suppressed.

We performed the model of rat liver regeneration *via* oval cells using the protocol of 70% partial hepatectomy (PH) plus 2-Acetoamidofluorene (2-AAF) treatment. By histological techniques we have characterized the oval cell development, proliferation and differentiation *in vivo*. Oval cells are induced between 1 and 3 days after PH inside the portal field and they express the typical onco-foetal marker alpha foeto-protein (AFP), at mRNA and protein levels. Oval cells proliferate and differentiate in hepatocytes expressing albumin, and they differentiate in biliary cells expressing cytokeratin 7 (CK 7).

Serial Analysis of Gene Expression (SAGE) was applied to the hepatic stem cell model of rat liver regeneration for studying gene expression pattern of liver regeneration during early stage of oval cell proliferation and differentiation. A total of 153,057 tags were analysed from normal liver (52,343 tags), from sham control treated with 2-AAF (50,502 tags), and from an early stage of the oval cell proliferation (50,212 tags). Comparative analysis of the transcriptomes from the 3 different conditions identifies 45 differentially expressed genes during oval cell regeneration, 27 up-regulated and 18 down-regulated. Temporal regulation of these genes in the context of the oval cell regeneration was studied by Real Time

PCR. Up-regulated genes include: cell cycle genes like CDC42 and cyclin D1; cytoskeleton associated proteins like stathmin 1, and E-tropomodulin; signal transduction triggering genes like CDC151 and lipopolysaccharide binding protein; transporter genes like Na^+/P_i co-transporter 4, phosphatidylcholine transfer protein, and ATPase H^+ 34 kDa lysosomial transporter; anti-apoptotic enzyme gene like thioredoxin like 2. Kinetically down-regulated genes involved in the lipid metabolism are: hydroxyacid oxidase 3, fatty acid CoA ligase long chain 2, and steroyl CoA desaturase 1. The regulation of protein expression of the cell cycle genes CDC42 and cyclin D1 was studied by Western Blot technique, in the oval cell liver regeneration. Interestingly, cyclin D1 and CDC42 proteins are co-expressed temporally with the oval cell protein marker AFP. From the data of the present study we conclude that the cell cycle genes CDC42 and cyclin D1 could be involved in the mechanism of proliferation of the oval cell in the liver regeneration process.

2. Introduction

2.1. Anatomy and function of the adult mammalian liver

2.2. Embryonic liver development

2.3. Liver regeneration by hepatic oval stem cells

2.4. Serial Analysis of Gene Expression (SAGE)

2.1. Anatomy and function of the adult mammalian liver

The *liver* is not only the largest gland but also the biggest organ in mammalians. Indeed, in the adult human the liver reaches a weight of 1.5 kilograms, and about 2% of the total body weight. The liver is located in the upper part of the abdominal cavity, just below the diaphragm, and on top of the stomach (Figure 1).
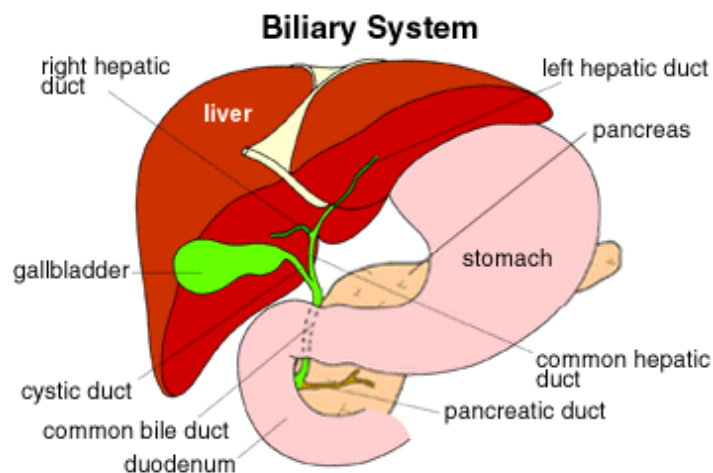
Figure 1. Anatomy of the human liver organ.
(Source: http://www.umm.edu/liver/liver.htm)

The microanatomical and functional unit of the liver is the *hepatic lobule*. The hepatic lobule has polygonal shape with a diameter of 1-2 mm and high of 2 mm. The human liver is constituted by approximately 1 million of hepatic lobules. Each lobule is constituted by *hepatic cords* irradiating from the *central vein* or centrolobular vein. In the pig liver it is possible to observe easily the lobular structure which is delimited by connective tissue septa (Figure 2). The *portal triad space* is situated between the edges of the lobules and contains a *portal vein branch*, a *hepatic artery branch*, a *bile duct* and finally a *lymphatic vessel*.
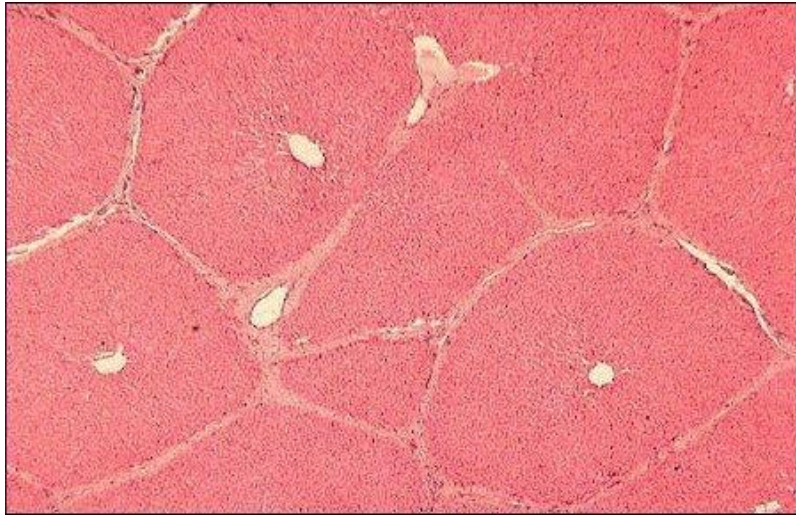
Figure 2. In the pig liver it is possible to observe the morphology of the liver lobules.
(Source http://arbl.cvmbs.colostate.edu/hbooks/pathphys/digestion/index.html).

The liver has two separate afferent systems of blood supplies: the *portal vein* and the *hepatic artery*. The portal vein receives the blood from the intestine, stomach, spleen and pancreas. The portal vein system ramifies inside the liver in portal vein *branches* located between the interlobular spaces and the portal triad spaces. From the portal veins branches are generated the *hepatic sinusoids* which are confluent in the central vein. The central veins from the liver lobules are connected with the *hepatic veins*, which are flowing into the *vena cava inferior*. The hepatic artery branches are also confluent in the sinusoids, thus the arterial blood and the portal venous blood flowing through the sinusoids to the central vein is mixed inside the lobule. Hence the lobules receive oxygenated blood from the hepatic artery and blood rich of nutrients from the hepatic vein.

The *hepatocytes* are the main liver cell population, and they are separated from the fenestrated sinusoid wall by the *space of Disse*. The space of Disse is important for the exchange of metabolites between the hepatocytes and the blood flow.
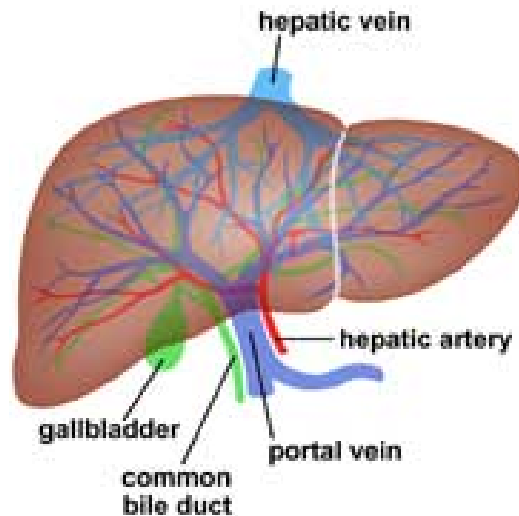
Figure 3. Liver blood supplies and bile duct system of the human liver. (source: http://www.cincinnatichildrens.org/svc/prog/liver/liver-anatomy.htm).

On the opposite side of the sinusoids, the hepatocytes form the *bile canaliculi* which are connected to the *canals of Hering* and the *interlobular bile ducts*. The bile is drained from the bile canaliculi to the interlobular bile ducts in the opposite direction respect the blood stream. The bile ducts are connected to the *right hepatic duct* and *left hepatic duct*, which are confluent in the *common hepatic duct*. From the common hepatic duct the *cystic duct* and the *gallbladder* originate (surprisingly none of them are present in the rat). The common hepatic duct continues in the *common bile duct*, from which the bile is secreted in the duodenum. The bile produced by the liver is important for the digestion, the absorption of lipids and fat soluble *vitamins* from the intestine (vitamin A, D, E and K), and for excretion of end products of the metabolism in the intestine.

The main cell type populations of the liver are the hepatocytes, the *biliary cells*, the *stellate cells* or *Ito cells*, the *fibroblasts*, the *Kupffer cells*, the vascular *endothelial cells*, and the *leucocytes*. The hepatocytes and the biliary cells have common embryonic origin from epithelial precursor cells, while the others cell types are mesenchymal derived.

The hepatocytes represent circa 80 to 90% of the liver weight and they are responsible for the exocrine liver function. The hepatocytes are important for many metabolic functions: 1) synthesis of plasma proteins like *albumin*, *fibrinogen*,

apolipoproteins, alpha globulins and *gamma globulins*, 2) metabolism of amino-acids (comprehending the urea metabolism), carbohydrates and lipids, 3) storage of many compounds like carbohydrates, iron and copper, 4) detoxification of drugs and toxic metabolites, 5) hormonal catabolism.

The biliary cells form the biliary ducts and ductules inside the liver. In particular, the biliary cells located in the ductules of Hering are believed to be the progenitors of the *oval hepatic stem cell* which will be discussed in this manuscript.

The Ito cells are located outside the sinusoids in the space of Disse, surrounding the hepatocytes with long processes, and they store almost 75% of the total body vitamin A as lipid droplets inside the cytoplasm. In addition, the Ito cells are involved in the regulation of hepatic growth and regeneration by secretion of *cytokines* and growth factors. Upon an injury, the Ito cells are activated, change their morphology from star-shaped to that typical of fibroblast or myofibroblast, loosing vitamin A. The strong phenotypic similarity between the fibroblasts and the activated Ito cells has led to confusion regarding the function and the characterisation of these two cell populations in the process of liver fibrosis. Although authors are convinced that activated Ito cells can synthesize extra-cellular matrix proteins and trigger liver fibrosis (Friedman, 1997), we believe that the myofibroblasts are the most likely responsible for the generation of fibrotic tissue in the liver (Novosyadlyy et al., 2004;Ramadori and Saile, 2004).

The sinusoidal endothelial cells form the fenestrated sinusoids and are important for the blood supply from the hepatic arterial and venous system inside the lobules. The Kupffer cells are immunitary cells with phagocytic activities. They represent the largest population of resident tissue macrophages in the animal body and are important for demolition of dead erythrocytes, and for the protection from micro-organism which penetrates from the colon organ to the portal blood flow. Finally, the leucocytes are immunitary cells important for the protection of the organ from viral and bacterial infections.

2.2. Embryonic liver development

The hepatic oval stem cell is generally considered to be phenotypically corresponding to the foetal epithelial liver cell, or *hepatoblast* (Zheng and Taniguchi, 2003) (Table 1). Oval cells and hepatoblast not only share many phenotypical markers but are also able to differentiate in adult hepatocytes and biliary cells and they possess the self-renewal capability to proliferate and propagate (Alison et al., 2002). Thus, oval cell differentiation and characters can be compared with the hepatoblasts in the foetal liver development.

The liver organ develops from two anlages: from the *ventral foregut endoderm* and the *septum transversum* (Zaret, 2002). The ventral foregut endoderm buds off and invades the mesenchyme of the septum transversum containing the vitelline veins (Zaret, 2001;Zaret, 2000). The endodermal cells forms sheets and cords of hepatoblast arrayed along the sinusoidal vascular channels deriving from the vitellin veins of the yolk sac. The mesenchymal cells of the septum transversum are involved in the generation of the sinusoidal endothelial cells (Wilson et al., 1963), the cells of the Glisson's capsule and the haematopoietic cells (Houssaint, 1981). The interaction between the endodermal cells and the mesenchymal components is critical for the differentiation and lineage development of endodermal cells in parenchymal cells and biliary epithelium (Houssaint, 1981;Cascio and Zaret, 1991). The hepatoblasts, immediately adjacent to the mesenchymal channels of the portal tracts, differentiate into a *ductal plate*, a circumferential layer of biliary epithelial cells, while the remaining hepatoblast differentiate in hepatocytes arranged in the hepatic cords.

The hepatic differentiation is characterised by the expression of the foetal marker a*lpha foeto-protein* (AFP) and the albumin gene (Alpini et al., 1992;Gualdi et al., 1996) (Figure 4). The cardiac mesoderm plays an important role in induction of AFP and albumin gene expression in the endodermal precursors of the liver bud. The *Fibroblast Growth Factor* (FGF) produced by the cardiac mesoderm is an important specific signal for the induction of the liver marker gene expression and the specification of the hepatoblasts located in the foregut endoderm (Jung et al.,

1999). Rat hepatoblasts express the hepatic markers (AFP and albumin) and as soon as they migrate inside in the mesenchymal stroma, they start to express also the g*amma glutamyl transferase* (GGT) gene, a bile ducts specific enzyme (Holic et al., 2000). The intrahepatic bile ducts express different *cytokeratin* filaments (CK 7, CK 8, CK 18 and CK 19) during the ductular morphogenesis. In addition, the intrahepatic bile ducts still express the hepatic markers AFP and Albumin in the first 7 to 14 days after the birth (Shiojiri et al., 1991). Thus hepatoblasts, adjacent to the portal mesenchymal channels, which differentiate in intrahepatic bile ducts, transiently express both hepatocytes and ductular biliary markers can be considered equivalents to the oval cells. Hepatoblasts that are not in contact with the portal mesenchyma, are differentiate into hepatocytes, and continue to express the GGT until birth while their expression of cytokeratins is restricted to the type 8 and 18 (Shiojiri et al., 1991).
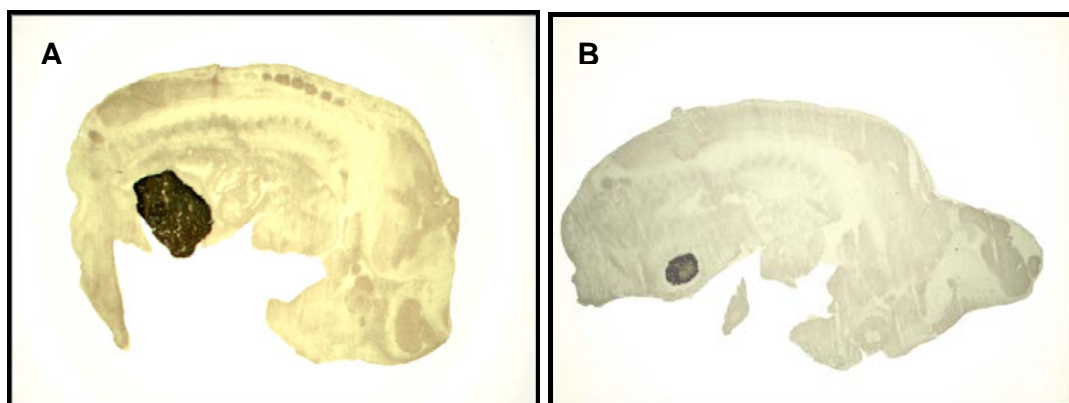
Figure 4. *In situ* hybridisation experiments in rat embryo at 14 days *post coitum* stage. A) Expression of the AFP gene inside the liver. B) Expression of the Albumin gene inside the liver.

2.3. Liver regeneration by hepatic oval stem cells

The liver is one of the few organs in the body, like the skin, gut mucosa and bone marrow, able to regenerate. In the normal condition, the proliferation from quiescent hepatocytes can maintain a perfect balance between cell gain and cell loss, by the "wear and tear" renewal (Alison et al., 1998). The knowledge that the liver can regenerate after injury, dates back to Greek mythology in the myth of *Prometheus* (Ankoma-Sey, 1999). Because Prometheus was stealing the secret of the fire from Olympus, he was condemned to have a portion of his liver eaten daily by an eagle. The ability of the liver to regenerate made his punishment and torture eternal, because the eagle had a perpetual feeding from a constantly replenished source. An important breakthrough in the transformation of a myth in an experimental research model was established by Higgins and Anderson (Higgins and Anderson, 1931) by the method of two thirds *partial hepatectomy* (PH) in rats (Figure 5). The operation consists in the removal of the intact *left lateral lobe* and the *median lobe*, without damaging the lobes left behind (Forbes et al., 2002). The residual lobes are able to enlarge and replenish the original liver mass, while the resected lobes with their structure never grow back. For this reason, many authors defined such process more specifically like a *compensatory hyperplasia* or growth, although in practice the regeneration term is used more frequently (Alison et al., 1998). The whole process of liver growth after the operation is very fast (7days) and well orchestrated (see Results: Figure 9). In the normal PH, the liver regeneration is carried out by the proliferation of adult liver cells: the hepatocytes start to replicate first 24 hours after the PH operation, followed 1 day later by the replication of biliary and non-epithelial cell types (Michalopoulos and DeFrances, 1997). Thus, the proliferating hepatocytes express growth factors responsible for the replication of the other cell types. It has been definitely demonstrated that in the model of PH, only the hepatocytes undergo significant proliferation and no stem cells are required for the regeneration and growth process.
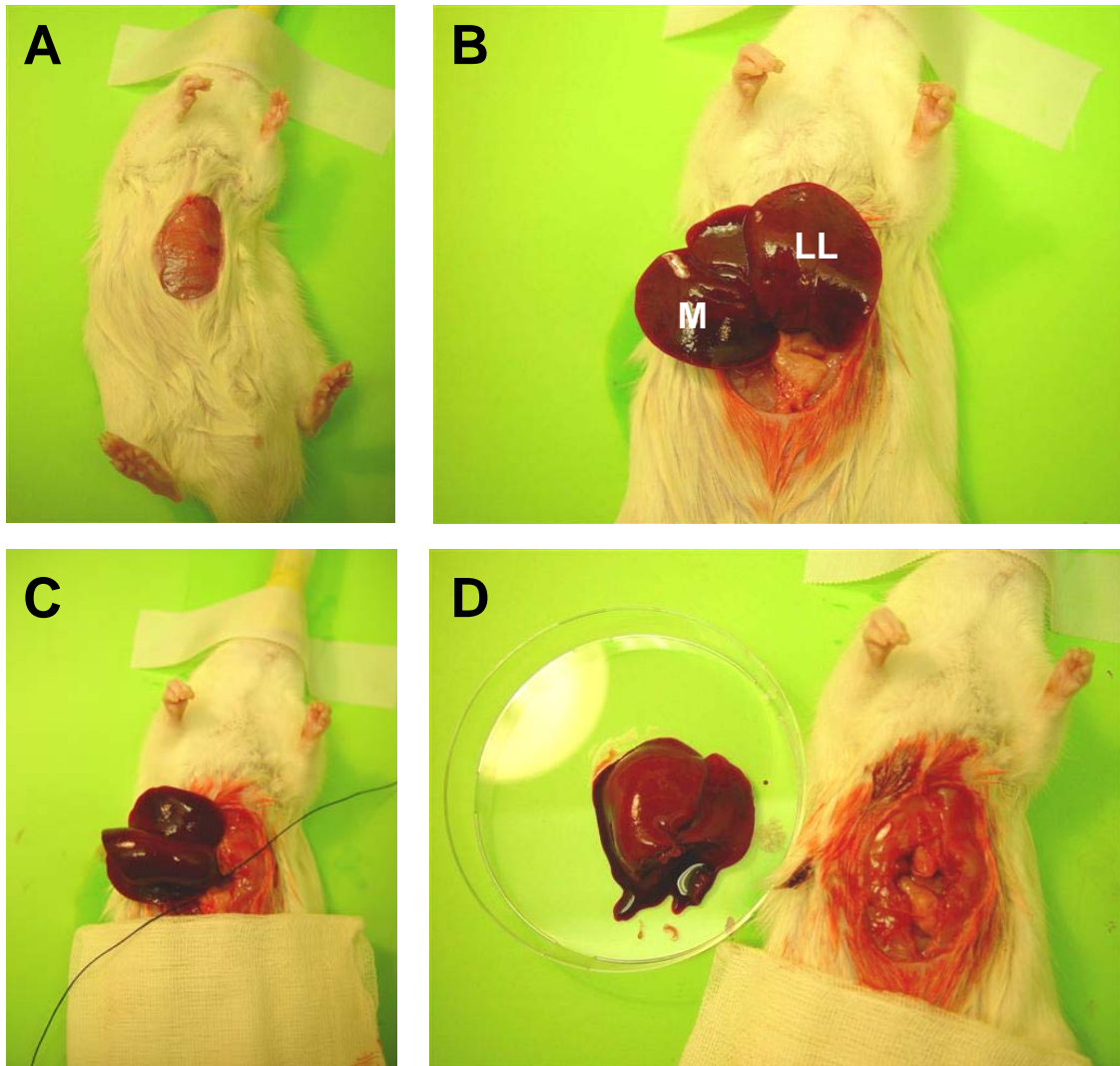
Figure 5. The main phases of the PH operation performed in our department.
A) The abdomen is opened below the thorax cavity. B) The main bigger liver lobes are brought outside the abdominal cavity (M, median lobe; LL, left lateral lobe). C) The lobes are ligated distally to block any arterial and venous blood outflow. D) The lobes are resected leaving the minor lobes intact in the animal.

Anyway, if the proliferation of the hepatocytes is prevented by toxic injury, massive necrosis or carcinogenesis, poorly differentiated adult stem cells, called "*oval cells*", can generate hepatic lineages. The oval cell is an epithelial cell population, originated from the biliary cells of the ductules of Hering and activated only when the insult of the organ is too massive and the regeneration from pre-existing mature hepatocytes is impaired. For this reason, the oval cell is considered to be a

second compartment for the liver regeneration and a facultative stem cell. This hepatic stem cell population was termed "oval" for its particular morphology and characterised for the first time in studies of carcinogenesis and other toxic hepatic agents (Farber , 1956;Wilson and Leduc , 1958).

Different protocols were established for triggering the oval cell proliferation in the rat liver, basically consisting of a combination of a stimulus for the liver growth together an inhibition of the hepatocyte proliferation. The most frequently used experimental model for oval cell proliferation, also used in our studies, is a variation of the Solt-Farber protocol consisting in 2-Acetoamidofluorene (2-AAF) treatment combined with the PH operation (2-AAF/PH) (Evarts et al., 1987;Sarraf et al., 1994). Other common alternative protocols are choline-devoid ethionine diet (Lenzi et al., 1992), galactosamine treatment (Dabeva and Shafritz, 1993), 2-AAF and CCl_4 treatment (Petersen et al., 1998), retrosine and PH operation (Laconi et al., 1998), diethylnitrosamine treatment (Schwarze et al., 1984).

The oval cells are morphologically characterised by small cellular size (10 µm), high nuclear/cytoplasm volume ratio and oval shape. The oval cells emerge from the portal fields, proliferate extensively and migrate from the portal area towards the central vein (see Results: Figure 11 and 12) through the bile canaliculi between the hepatic cords. Study of thymidine incorporation demonstrates *in vivo* the potential of the oval cells to differentiate in *foci of basophilic hepatocytes* which are reorganizing the liver lobules (Evarts et al., 1989). The oval cells form irregular duct-like structures (*ductular reaction*) connected with pre-existing bile ducts (see Results: Figure 12C and D) (Theise et al., 1999). Thus, the oval cell is considered bi-potential progenitor stem cell able to generate mature hepatocytes and biliary cells (see Results: Figure 12). Interestingly, the oval cells are able to differentiate in particular conditions in intestinal epithelium or pancreatic acinar cells (Alison et al., 1996;Yang et al., 2002). The oval cells are heterogeneous cell population expressing a combination of several markers (Table1): biliary ductular cell markers (GGT, CK 7 and CK 19), foetal hepatoblast markers (Albumin and AFP), and surprisingly also haematopoietic markers (Thy1, CD34, c-kit, flt-3) (Petersen et al., 1998;Omori et al., 1997;Fujio et al., 1994). The particular expression of the

haematopoietic markers in the oval cell compartment was considered no more coincidental, but it inspired the idea that bone marrow cells could transdifferentiate in hepatic cells (Petersen et al., 1999;Petersen, 2001). Indeed, Petersen was the first to describe that oval cells derived hepatocytes can originate from bone marrow cells. Lethally irradiated female rat animals were transplanted with bone marrow cells from male donor after a liver injury caused by CCl_4 and 2-AAF treatment. "Y" chromosome positive cells were engrafted inside the liver and showed hepatocytes phenotype. Other authors described generation of adult hepatocytes from bone marrow cells in humans, studying the liver of female patients who had previously received bone marrow transplant from a male donor (Theise et al., 2000). On the other hand, later reports shows that hepatocytes derived from bone marrow cells are extremely rare in the regenerative liver (Wagers et al., 2002;Theise and Krause, 2002). In addition, it was proposed that hepatocytes derived bone marrow cells are originated by *in vivo* cells fusion forming hepatocytes-bone marrow hybrid cells (Grompe, 2003;Wang et al., 2003;Fausto, 2004). Hence, we believe that the capability of the liver to regenerate from bone marrow cells must be still properly demonstrated.

	Oval Cell	Hepatoblast	Bile Duct Cell	Hepatocytes
AFP	+	+	-	-
Alb	+	+	-	+
CK7, CK19	+	+	+	-
CK8, CK18	+	+	+	+
GGT	+	+	+	-
Thy 1	+	+	-	-
c-kit	+	+	+	-
CD34	+	+	+	-
Flt-3	+	nd	+	-

Table 1. The most common markers of the oval cells population. AFP is the best marker for identifying the oval cells in the adult liver (Alb, albumin; CK, cytokeratin; GGT, gamma-glutamyl transpeptidase; nd, not detected) (Zheng and Taniguchi, 2003).

2.4. Serial Analysis of Gene Expression (SAGE)

Liver regeneration by hepatic oval stem cells is a well orchestrated and complex process which includes origin, proliferation and differentiation. The critical research issues studied in the stem cells regenerative biology are: 1) characterisation of regeneration competent cells by marker genes expressed specifically; 2) finding how to direct the differentiation of stem or progenitor cells in specific directions; 3) identification of factors that are involved in stimulating the stem cell regeneration process; 4) finding of signal molecules which are involved in the localisation and tropism of transfused stem cell; 5) developing a procedures to avoid immuno-rejection of transfused stem cells, 6) finding the molecular mechanism involved in stem cell origin, proliferation and differentiation. To elucidate the molecular mechanism of oval cells regeneration, accurate quantification of gene transcripts process must be achieved.

Serial Analysis of Gene Expression (SAGE) is a powerful tool that allows a quantitative digital analysis of overall gene expression patterns. SAGE is a high throughput technique, established by Velculescu (Velculescu et al., 1995), which is based on three experimental confirmed principles. First, a short sequence called *tag*, consisting of 10 base pairs (bp), is extracted from each transcript from a defined position adjacent to the restriction enzyme recognition site closest to the 3' end of the transcript (Figure 6). Each tag sequence can ideally identify uniquely one gene. This is statistically reasoned by the fact that the maximum number of possible tag sequences (4^{10}= 1,048,576 possible tags) is far greater than the total number of transcripts expressed in an organism (in human about 120,000) (Velculescu et al., 1995). Second, a concatenation of several tags into a single molecule allows efficient cloning, sequencing and data acquisition. And third, the frequency of a tag inside a SAGE library is directly proportional to the level of expression of its transcript (Velculescu et al., 1999). The SAGE technique generates immortalised data which can be statistically compared for the identification of repressed or induced genes between different samples. For this reason SAGE is a convenient technique when identification of gene expression

profiles should be determined in a particular physiopathological situation by comparison of different samples.

The SAGE method is applied in many fields and different organism, from bacteria to mammalian and plant, leading to relevant discoveries. In human and in the mammalian models SAGE is providing information for the identification of genes involved in pathophysiological processes like cancer and other diseases (Argani et al., 2001;Patino et al., 2002;Wu et al., 2002;Cornelissen et al., 2003). SAGE has had also application in embryology for the study of the genes involved in development, (Jasper et al., 2001), and in biotechnology for the study of the metabolism of plants and microorganism (Velculescu et al., 1997). The SAGE data set are also available in public repository (http://cgap.nci.nih.gov/SAGE) for everyone is interested in performing analysis of transcriptomes from different sources (Lash et al., 2000).

A brief and schematic description of the method is following in this section (Figure 7). The RNA sample is converted in a double stranded cDNA covalently linked to magnetic beads which are allowing easy separation and purification of the products. The cDNA is digested with a restriction enzyme defined as "anchoring enzyme": the most common enzymes used for the SAGE protocols are NlaII or Sua3. Linker cassette of 40 bp is ligated to the digested extremity of each digested cDNA. The linker cassette contains a recognition site for the "tagging enzyme" (BsmFI) which will bind a linker sequence and cleave 12 to 16 bp away from its binding site. The digestion with the tagging enzyme produces a construct containing a tag linked with the linker cassette. Two linker-tag molecules can be joined tail to tail to generate a 108 bp construct called "*ditag*". The ditag population can be amplified by PCR using primer set which anneals the linker cassettes. Purified ditags can be digested with the restriction anchoring enzyme releasing the ditags molecules of 24-26 bp from the linkers. The ditags can be polymerised in a long molecule called "*concatemer*" by ligation reaction. The concatenation of several ditags in the concatemer allows efficient cloning in a plasmid vector for subsequent sequencing. Each ditag is punctuated by the restriction recognition anchoring enzyme, and is possible to identify inside the sequenced vector (see

Results: Figure 18). The sequences data must be analysed by bioinformatics tools for the counting and the identification of the tags in the SAGE library. The tag abundances of different SAGE libraries can be compared statistically for identification of up-regulated and down-regulated genes (Figure 8). For further detailed information about the method: see section 4.4. Serial Analysis of Gene Expression method.
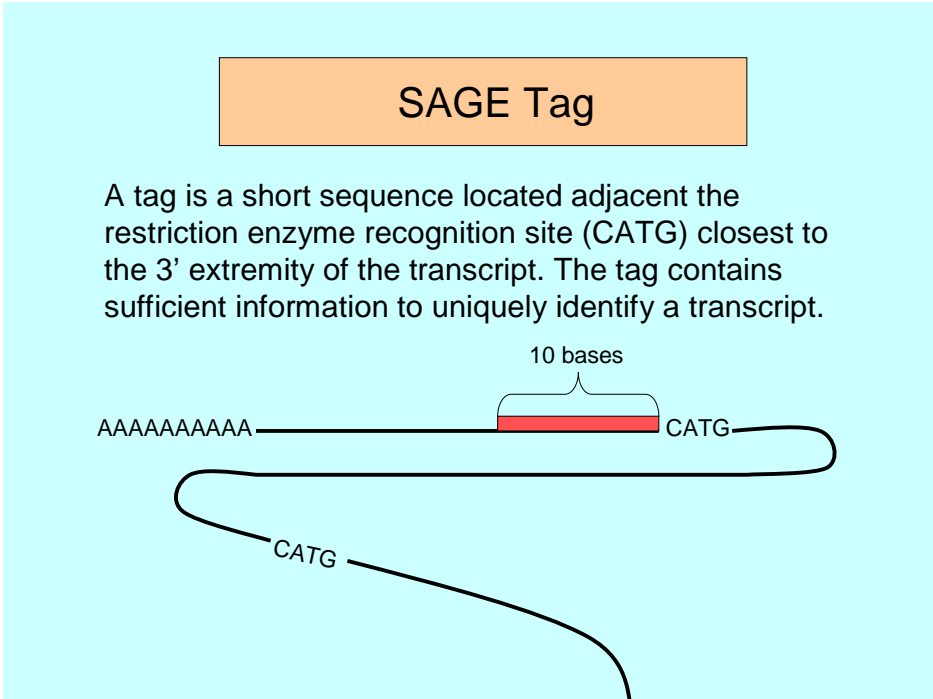
Figure 6. Definition of the SAGE tag.
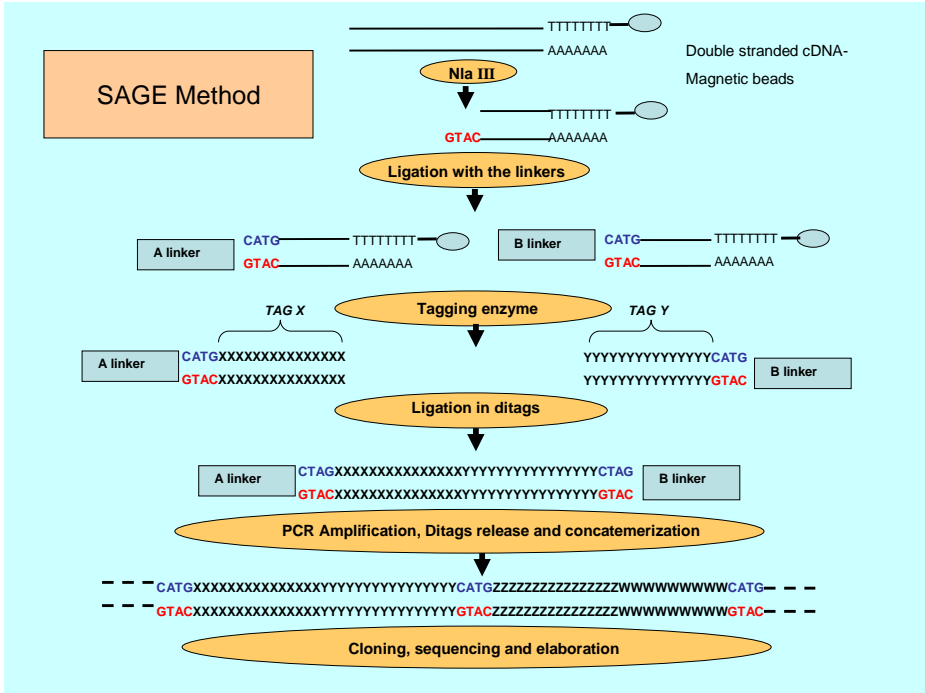
Figure 7. The scheme of the SAGE method.
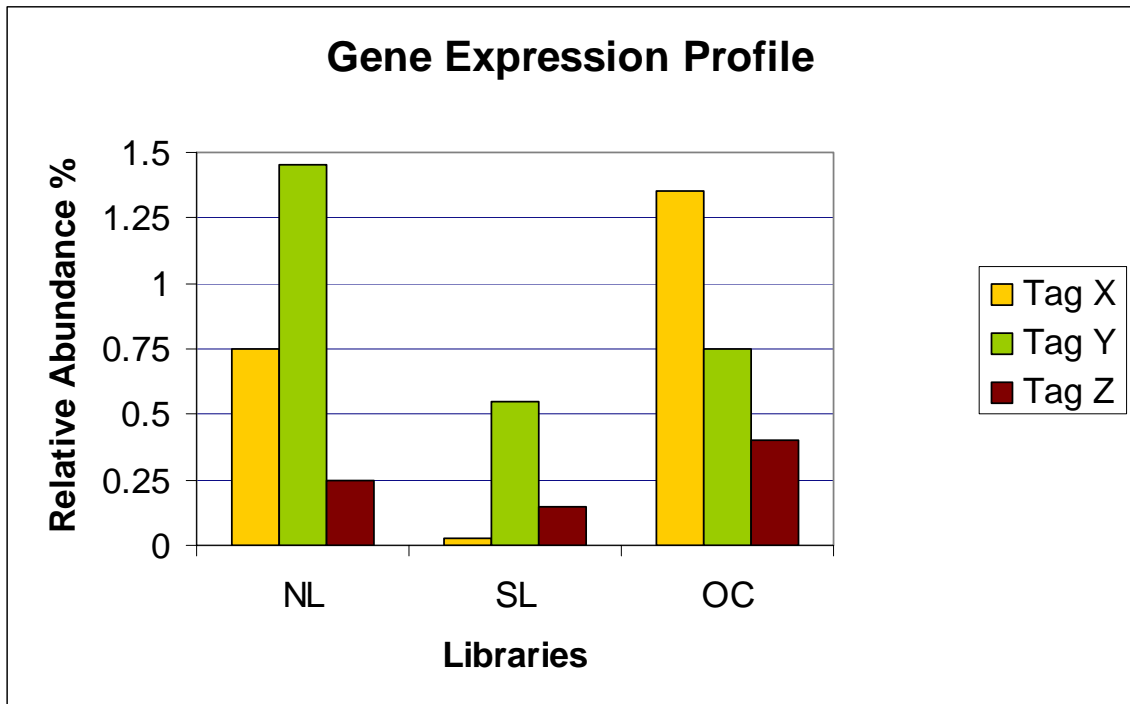
Figure 8. Expression profile of three tags (X, Y, and Z) in three different SAGE libraries (NL, normal control liver; SL, sham liver; OC, oval cell liver). By the comparison between different SAGE libraries is possible to characterise the regulation of gene expression in a particular process.

3. Materials

3.1. Animal model materials

3.2. Northern Blot of AFP transcript materials

3.3. Histological techniques materials

3.4. Serial Analysis of Gene Expression materials

3.5. Real Time PCR materials

3.6. Western Blot materials

3.1. Animal model materials

Animals

Fisher F-334 rats (180 to 220 g) were provided from the Winkelmann company.

Solution and reagents

2-AAF solution: 1mg/ml 2-acetoamidofluorene (SIGMA-Aldrich, Cat. No. A7015) in 1:1 of PBS (Biochrom, Istamed PBS Dulbecco Cat. No. L182-10) and polyethylenglycol 600 (Merck Cat. No. 8.17004.1000), ether (SIGMA-Aldrich, Cat. No. 44,354-9).

Saline irrigation solution (B. Braun, NaCl 0.9%).

Surgical tools

Suture (Perma-Hand Seide 2/0, Cat. No. K833H), surgical tape (Durapore 3M, Cat. No. 1538-1), scissors, forceps, clamp forceps, scalpels (Martin and Aesculap).

3.2. Northern Blot of AFP transcript materials

Solutions and reagents

RNAse free H_2O: to distillate H_2O is added DEPC to final concentration 0.1% (SIGMA-Aldrich, Cat. No.D5758), after mixed the solution is autoclaved the following day.

Guanidine-isothiocyanate solution: 4M guanidine-isothiocyanate (Invitrogen, Cat. No.15535-016), 25mM Na-citrate buffer pH 7.0, (SIGMA-Aldrich, Cat. No. C8532 and S4641), 0.5% N-lauroylsarcosine (SIGMA-Aldrich, Cat. No. L-5125), 0.1 M 2-mercaptoethanol (Merck, Cat. No. 12006.0100).

$CsCl_2$ solution: 5.7M $CsCl_2$ (Invitrogen, Cat. No 15507-023), 25mM Na-citrate buffer pH 7.0 (SIGMA-Aldrich, Cat. No. C-8532 and S-4641), 0.5M EDTA (SIGMA-Aldrich, Cat. No. ED-255).

10X running buffer: 200mM MOPS (SIGMA-Aldrich, Cat. No. 8899), 50mM sodium acetate (SIGMA-Aldrich, Cat. No. S-2889), 10mM EDTA (SIGMA-Aldrich, Cat. No. ED-255), pH 7.0.

RNA loading buffer: 0.25% bromophenol blue (SIGMA-Aldrich, Cat. No. B-8026), 4mM EDTA, 0.9M formaldehyde (SIGMA-Aldrich, Cat No. F-1635), 20% glycerol (Merck, Cat. No. 1.12011.0100), 30.1% deionised formamide (Merck, Cat. No. 1.12027.1000), 4X running buffer.

TE buffer: 1M Tris-Cl (Merck, Cat. No.1.08219.1000), 0.5M EDTA (SIGMA-Aldrich, Cat. No. ED-255).

20X SSC: 3M sodium chloride (Merck, Cat. No. 1.06404.1000), 0.3M sodium citrate-2-H_2O (SIGMA-Aldrich, Cat. No. S4641), pH 7.0.

Ethanol (Merck, Cat. No. 8.19760.1000), agarose (Agarose 1000, Invitrogen-GibcoBRL, Cat. No. 10975-035), ethidium bromide (SIGMA-Aldrich, Cat. No. E7637), DNA markers and gel loading buffer (25bp DNA Step Ladder, Promega, Cat. No. G4511, 100bp DNA Step Ladder, Promega, G6951), MinElute Gel Extraction Kit (Qiagen, Cat. No. 28604), Random Primer DNA Labelling System (Invitrogen, Cat. No. 18187-013), $dCTP^{32}$ (Amersham Biosciences, Cat. No.

RT3002-250µCurie), DNA from fish sperm (Roche, Cat. No. 1 467 140), Quick Hyb hybridization solution (Stratagene, Cat. No. 201220), SDS (SIGMA-Aldrich, Cat. No. L-4509), liquid scintillation solution (Wallac OptiPhase 'HiSafe', Cat. No. 1200-473), DNA-oligo primers were ordered from the MWG biotech, 0.01µmol quantity for each DNA-oligo.

Consumables

Beckman tubes (Beckman, Cat. No. 326819), Membrane Hybond-N (Amersham Biosciences, Cat. No. RPN 203N), Nick Columns (Amersham Biosciences, Cat. No. 170855-02).

Equipment

Ultracentrifuge (Kontron Instruments, Centrikon T2070), PCR thermal cycler (Perkin Elmer, Gene Amp PCR System 9600), UV Crosslinker (Stratagene, UV Stratalinker 1800), Wallac liquid scintillation counter (Wallac 1409), auto-radiographic film (Hyperfilm Amersham Biosciece, Cat. No. RPN 1675K).

3.3. Histological techniques materials

Solution and reagents

Maleic acid buffer: 0.1M maleic acid (SIGMA-Aldrich, Cat. No. M-0375), 0.15M NaCl (Merck, Cat. No. 1.06404.1000), pH 7.5 using NaOH pellets (Merck, Cat. No. 6498.1000).

Washing buffer: maleic acid buffer, 0.3% Tween (SIGMA-Aldrich, Cat. No. P-1379).

Detection buffer: 0.1M Tris-HCl (Merck, Cat. No.1.08219.1000), 0.1M NaCl (Merck, Cat. No. 1.06404.1000), pH 9.5 using NaOH pellets.

Blocking solution: 1% blocking reagent (Roche) in maleic acid buffer.

Entellan (Merck, Cat. No. 1.07961.0100), Kayser's glycerol gelatine (Merk, Cat. No. 1.09242.0100), Meyer hemalum (Merck, Cat. No. 1.09249.0500), eosyn Y (Merck, Cat. No. 15953), methanol (Merck, Cat. No. 1.13351.2500), ethanol (Merck, Cat. No. 8.19760.1000), aceton (Merck, Cat. No. 8.22251.1000), xylol (Merck, Cat. No. 108681.1000), glucose oxidase (SIGMA-Aldrich, Cat. No. G6641), sodium azide (Merck, Cat. No. 822335), glucose (Merck, Cat. No. 8342), DAB (3,3'-Diaminobenzidine, SIGMA-Aldrich, D8001), H_2O_2 30% solution (Merk, Cat. No. 1.07209.0250), PBS (Biochrom, Istamed PBS Dulbecco Cat. No. L182-10), foetal bovine serum (Invitrogen-Gibco, Cat. No. 16250-078), rat serum (Dako, Cat. No. X0912), DNA-oligo primers were ordered from the MWG biotech, 0.01μmol quantity for each DNA-oligo.

Antibodies

Anti-mouse Alpha-1-fetoprotein (Dako, Cat. No. A00008), anti-mouse Cytokeratin 7 (Dako, Cat. No. M7018), anti-rabbit Albumin (Cat. No.A0001), polyclonal swine anti-rabbit immunoglobulins/HRP (Dako, Cat. No. P0217), polyclonal rabbit anti-mouse immunoglobulins/HRP (Dako, Cat. No. P0161)

Kits

DIG Nucleic Acid Detection Kit (Roche, Cat. No. 1 175 041), RNAse A (Roche, Cat. No. 0109142), DIG RNA Labeling Kit (SP6/T7) (Roche, Cat. No. 1 175 025).

Consumables

Microscope slides (Menzel-Gläser, Cat. No. 021102), coverslip 25 X40 (Menzel-Gläser, Cat. No. BB02504A01), folded filters (Schleicher & Schuell Cat. No. 311 647), OCT compound (Sakura, TissueTek).

Equipment

Cryostat (Reicher-Jung, 2800 Frigocut E), Microscopes (Zeiss, Axioskop and Standard 25), digital camera (Canon EOS D60).

3.4. Serial Analysis of Gene Expression materials

For materials required the RNA purification: see also the section 3.2. Northern Blot of AFP transcript materials.

Solutions and reagents

1X binding buffer: 10mM Tris-HCl, 0.5M NaCl, 1mM EDTA, 0.5% SDS, pH 7.5.

Elution buffer: 10mM Tris-HCl, 1 mM EDTA, 0.05% SDS, pH 7.5.

TBE buffer (SIGMA-Aldrich, Cat. No. T4415).

2X B+W Buffer: 10mM Tris-HCl (pH 7.5), 1mM EDTA, 2.0 M NaCl.

Phenol-chloroform solution: 480ml phenol, 320ml 0.5M Tris-HCl (pH 8.0), 640ml chloroform, stored at -20°C.

10X PCR buffer: 166mM $(NH_4)_2SO_4$, 670mM Tris pH 8.8, 67mM MgCl2, 100mM beta-mercaptoethanol, aliquots of 0.5ml are stored at -20°C.

Agarose (Agarose 1000, Invitrogen-GibcoBRL, Cat. No. 10975-035), Ethidium Bromide (SIGMA-Aldrich, Cat. No. E7637), DNA markers and gel loading buffer (25bp DNA Step Ladder, Promega, Cat. No. G4511, 100bp DNA Step Ladder, Promega, G6951), glycogen (Roche, Cat. No. 901-393), acrylamide (Acrylamide/Bis-acrylamide 29:1, SIGMA-Aldrich, Cat. No. A2792), ammounium persulfate (SIGMA-Aldrich, Cat. No. A3678), TEMED (SIGMA-Aldrich, Cat. No. 87688), Sybr Green Nucleic Acid Gel Staining (SIGMA-Aldrich, Cat. No. S9430), 10mM dNTP mix (Invitrogen, Cat. No. 18427-013), DMSO (SIGMA-Aldrich, Cat. No. D2650), 7.5 M ammonium acetate (SIGMA-Aldrich, Cat. No. A2706), LB agar (SIGMA-Aldrich, Cat. No. L-2897).

Kits and enzymes

Superscript Choice System cDNA Synthesis Kit (InvitrogenCat. No. 18090-019), electro-competent cells (Invitrogen, DH10B Electromax, Cat. No. 18290-015), pZERO-1 plasmid (Invitrogen, Cat No. K2500-01). BsmFI (NEB Cat. No. 572S), NlaII (NEB Cat. No. 125S), Sph 1 (NEB Cat. No. 182S), Klenow (Pharmacia/USB Cat. No. 27-0929-01), T4 ligase high concentration (5U/µl) (Invitrogen Cat. No.

15224-041), T4 ligase regular concentration (1U/µl) (Invitrogen Cat. No. 15224-017), Platinum Taq (Invitrogen Cat. No.10966-034)

Consumables

Oligo(dT)cellulose (Roche, Cat. No. 808229), Glass Wool Silanized (Serva, Cat. No. 22367), magnet (Dynal Cat No. 120.04), SpinX tubes (Costar, Cat.No. 8160), electroporation cuvettes (BioRad), magnetic beads (Dynabeads M-280 Streptavidin Slurry Dynal, Cat. No. 112.05).

SAGE primers sequences and linker preparation

We order the following oligo-DNA from Integrated DNA Technologies (USA).

Primer 1: 5' GGA TTT GCT GGT GCA GTA CA 3'.

Primer 2: 5' CTG CTC GAA TTC AAG CTT CT 3'.

Biotinylated oligo dT (obtain gel-purified) 5' [biotin]T18.

M13 Forward: 5' GTA AAA CGA CGG CCA GT 3'.

M13 Reverse: 5' GGA AAC AGC TAT GAC CAT G 3'.

Linker 1 A (obtain gel-purified):

5' TTT GGA TTT GCT GGT GCA GTA CAA CTA GGC TTA ATA GGG ACA TG 3'.

Linker 2 A (obtain gel-purified):

5' TCC CTA TTA AGC CTA GTT GTA CTG CAC CAG CAA ATC C[amino mod. C7] 3'.

Linker 1 B (obtain gel-purified):

5' TTT CTG CTC GAA TTC AAG CTT CTA ACG ATG TAC GGG GAC ATG 3'.

Linker 2 B (obtain gel-purified):

5' TCC CCG TAC ATC GTT AGA AGC TTG AAT TCG AGC AG[amino mod. C7] 3'.

Kinasing reaction for linkers is performed using the following ingredients and protocol. Each linker is diluted to 350ng/µl. 2 kinasing reactions are prepared for each linker 2A and 2B respectively. The following ingredients are mixed for the kinasing reaction: Linker 2A or 2B (350ng/µl) 9µl, LoTE 6µl, 10X kinase buffer 2µl, 10mM ATP 2µl, and T4 Polynucleotide Kinase (10U/µl) 1µl.

The reaction is incubated 37℃ for 30 minutes.

The enzyme is heat inactivate at 65°C for 10 minutes.

The annealing of the linkers is performed by the following procedure.

9μl Linker 1A are mixed with 20μl kinased Linker 2A (final conc 200 ng/μl)

9μl Linker 1B are mixed with 20μl kinased Linker 2B (final conc 200 ng/μl)

To anneal linkers the reaction is heated to 95°C for 2 min, then placed at 65°C for 10 min, 37°C for 10 min and room temp for 20 minutes. The annealed linkers are stored at -20°C.

Kinasing should be tested by self ligating about 200ng of each linker pair and running on 12% polyacrilamyde gel (section 4.4.12. Polyacrylamide gel purification of 108 bp ditags). Kinased linkers should allow linker-linker dimers (80-100 bp) to form after ligation, while unkinased linkers will prevent self-ligation. Only linker pairs that self-ligate more than 70% should be used in further steps.

3.5. Real Time PCR materials

For materials required for the RNA purification and cDNA synthesis and RNA quality control: see the section 3.2. Northern Blot of AFP transcript.

Primers

DNA-oligo primers were ordered from the MWG biotech, 0.01µmol quantity for each DNA-oligo (Table 3).

Enzymes

Platinum SYBR Green qPCR SuperMIx UDG (Invitrogen, Cat. No. 11733-038), RNAfree DNAase (Promega RQ1 RNAse free DNAse, Cat. No. M610A).

Equipment

ABI prism 7000 thermal cycler and SYBR Green Master Mix Taq Polymerase (Applied biosystem).

Software

Primer sets were designed using the "Primer Express" software (Applied biosystem).

3.6. Western Blot materials

Solutions and reagents

Lysis buffer: 20mM Tris-HCL (Merck, Cat. No.1.08219.1000), 5mM EDTA (SIGMA-Aldrich, Cat. No. ED-255), 3 mM EGTA (SIGMA-Aldrich, Cat. No. E-4378), 1mM DTT (SIGMA-Aldrich, Cat. No. D-9779), 1% SDS (SIGMA-Aldrich, Cat. No. L-4509), 1mM PMSF (SIGMA-Aldrich, Cat. No. P7626) protease inhibitors cocktail (SIGMA-Aldrich, Cat. No. P9599), pH 8.0.

TBS: 10mM Tris-HCl, 150 mM NaCl. TrisGly SDS running buffer (Invitrogen, Cat. No. LC2675).

Blocking solution: 5% milk powder (AppliChem, Cat. No. 0830.0500) in TBS.

TrisGly transfer buffer (Invitrogen, Cat. No. LC3675), TrisGly SDS sample buffer (Invitrogen, Cat. No. LC2676). Ponceau S solution (Serva, Cat. No. 33427), MultiMark Multi-Colored Standard (Invitrogen, Cat. No. LC5725).

Antibodies

Primary antibodies used were: CDC42 1:100 diluted (Santa Cruz Biotech sc-8401 clone B2), Cyclin D1 1:100 diluted (DAKO M7155, clone DCS-6), Alpha-1-fetoprotein 1:100 diluted (DAKO, A0008). Depending from the primary antibody, the secondary antibody used were: anti mouse immunoglobulins peroxidase 1: 1000 diluted (DAKO, P0161), or anti rabbit immunoglobulins peroxidase 1:1000 diluted (DAKO, P0217).

Kits

ECL detection system (Amersham Biosciences, Cat. No. RPN2106V).

Consumables

Hyperfilm ECL (Amersham Biosciences, Cat. No. RPN2103K), nitrocellulose membrane (Serva, Cat. No. 71208), ProGel-Tris-Glycin-Gel 8-16% (anamed, Cat No. TG81612).

Equipment

Electrophoresis and Protein transfer apparatus: XCell SureLock Mini-Cell & XCell II Blot Module Invitrogen, Cat. No. EI0002).

4. Methods

4.1. Animal model

4.2. Northern Blot of AFP transcript

4.3. Histology Techniques

4.4. Serial Analysis of Gene Expression method

4.5. Real Time PCR

4.6. Western Blot

4.1. Animal model

4.1.1. 2-AAF/PH protocol

Male young Fisher F-344 rats, from 180 to 220 g of weight, are used for all the experiments. Rats are kept under standard conditions of temperature and daylight cycle and they are maintained with rodent chow and water *ad libitum*. We used a model of oval cell proliferation in principle as described previously, using a low daily dose of 2-AAF (7.5 mg/kg) (Evarts et al., 1989). The rats received by oral administration 7.5 mg/kg of 2-AAF suspended in a solution of 1:1 PBS:polyethylenglycol 600 for 5 days (Alison et al., 1996). On day six, during the morning, rats are subjected to 70% PH or to sham laparotomy under light ether anaesthesia during the morning and in semi-sterile conditions. The PH is performed in accordance to the standard protocol (Higgins and Anderson, 1931). The abdomen is open under the *Processus Xiphoideus* by a median longitudinal incision. The main liver lobes, the left lateral lobe and the median lobe, are brought outside from the abdominal cavity by gentle finger manipulation and pressure of the abdomen around the incision. The medial and left lateral lobes are excised after placement of a suture ligature around the distal part of them. The abdominal cavity is rinsed with saline sterile solution and is closed first by suture of the abdominal wall, followed by suture of the skin. See in the Introduction section for the pictures description of the PH operation performed in our department (Figure 5). Laparotomy or sham operation was performed by a median longitudinal incision of the abdomen. After the incision, the liver parenchyma was exposed to gentle fingertips manipulation. The wound was rinsed with sterile saline solution and double suture was performed to close the abdomen. After the operation, rats are kept in warmed cages using an infrared lamp until they don't recover completely. The following day a second 2-AAF administration was continued for a period of 4 days, using the same daily dosage. On postoperation days 1, 3, 7, 11 and 16, rats of each group (PH and Sham) are sacrificed by exsanguination through the vena cava inferior and livers are removed and immediately weighed. The liver samples

are rinsed in cooled 4°C sterile saline solution, cut in 1cm^3 pieces and snap-frozen in liquid nitrogen.

4.1.2. Calculation of the regenerative liver

We used the following empirical formulas for calculating the percent of resected material:

1) Percent of resected material = Weight resected of material X 100 / Weight total liver;

2) Weight total liver (without exsanguinations) = 0.054 X Animal Weight.

We used the following empirical formulas for calculating the percent of regenerative liver:

3) Percent of regenerative liver = Weight resected of material X 100 / Weight total liver;

4) Weight total liver (after exsanguinations) = 0.036 X Animal Weight.

4.2. Northern Blot of AFP transcript

4.2.1. Sample RNA preparation by ultracentrifuge method

All the material and reagents are kept under RNAse free condition. Frozen liver tissue of about $1cm^3$ of volume is homogenised on ice in 3 ml of guanidine-isothiocyanate solution using an Ultra-Turrax electric homogenizer. Cushion of 2ml of $CsCl_2$ solution is poured in a 5ml Beckman centrifuge tube. A layer of homogenised liver sample in guanidine-isothiocyanate solution is added carefully over the $CsCl_2$ solution cushion. Each sample tube is inserted in a bucket. The buckets are balanced by using an analytic scale and adding guanidine-isothiocianate solution: the weight of a bucket should not be minor than 0.01 g from the heaviest bucket. The buckets are placed in a swinging rotor, before the rotor is transferred in the ultracentrifuge. The centrifugation is run at 18.000 rpm, for approximately 12 to 16 hours at 20°C. After the centrifugation, the guanidine-isothiocyanate and $CsCl_2$ solutions are decanted and the RNA pellet attached to the bottom of the tube is washed twice with 200µl of 70% ethanol cold solution. RNA pellet is re-suspended in 100µl of RNAse free water. Concentration and purity of total RNA is measured by spectrophotometer at 260nm and 280nm wavelength. An aliquot of 2µl of RNA sample is diluted 1:100 in H_2O for the spectrophotometric measurement and the H_2O is used as a blank. RNA concentration and purity is obtained by using the following formulas: RNA conc. (ng/µl) = Abs_{260} X 40 X dilution factor; RNA Purity= Abs_{260}/Abs_{280} (1.7 to 2.0 are considered the optimal range of nucleic acid purity).

4.2.2. Northern Blot membrane preparation

RNA sample are run in formaldehyde denaturing 1% agarose gel. Agarose gel is prepared by dissolving 1.5g of agarose in 72ml of H_2O boiling in a microvawe oven. After, to the solution are added: 10X running buffer 10ml, 0.9M formaldehyde 18ml and ethidium bromide 5µl of 1 mg/ml concentrated solution. Quickly the gel solution is casted in the electrophoretic chamber containing combs for sample loading. When the gel is solidified (after circa 1 hour) is inserted in the

electrophoretic apparatus containing the 1X running buffer and the combs are removed. RNA sample 5µg to 20µg is diluted in RNAse free H_2O 10µl, and RNA loading buffer 2µl. The RNA samples are denatured 5 min at 65°C, placed on ice for approximately 2 min, and loaded onto the gel wells. The gel is run at 100 Volts until bromophenol blue is 5cm far from the gel wells (after about 1 hour). The RNA samples are visualized by UV transilluminator and picture are played by digital or Polaroid Camera. A good RNA sample should show two clear and sharp ribosomal bands, the major ribosomal band (28S, 4.7 kilobase) should have double intensity of the minor ribosomal band (18S, 1.9 kilobase) (See Results: Figure 14A). Smear of the ribosomal bands is indication of possible RNA degradation. 20X SSC solution is poured in the disc container below a glass support of the blotting apparatus. A rectangular Whatman paper layer, wider than the gel, is placed over the glass support dipping in the solution from two opposite sides. The Whatman paper is also made wet with 20X SSC solution. The gel is positioned upside-down over the paper layer. A saran wrap sheet is placed around the gel covering the paper layer. A nylon membrane with the dimension of the gel is pre-soak in 20XSSC solution for 1 min and placed over the gel carefully. Once the membrane is covering the gel is not moved anymore. The air bubbles are removed gently by rolling with a plastic pipet back and fourth over the surface. Two Whatman paper layers are placed over the nylon membrane and 20cm stack of dry paper towels are placed over them. A glass is placed on the top of the paper towels. A weight consisting in a bottle of water of about 1kg is placed over the glass plate to press the blotting apparatus. After a night, the nylon membrane is taken from the apparatus and the RNA is cross-linked over the nylon membrane by UV radiation using UV cross-linker apparatus. The membrane is washed with H_2O and is ready for hybridisation or can be stored at -20 °C wet or dry.

4.2.3. Probe synthesis and labelling
4.2.3.1. cDNA synthesis from RNA liver samples
The AFP cDNA probe was obtained by a standard PCR protocol using a cDNA template from rat oval cell regenerating liver. cDNA is synthesised from a RNA

sample from rat liver containing oval cells, using the first strand synthesis reaction protocol. A quantity of 5µg of RNA sample in 5µl of RNAse free H_2O, is incubated with 1µl of oligo-dT (500 ng/µl), at 70°C for 10min and placed on ice for RNA denaturation.

The tube is centrifuge for a quick run. To the reaction tube is added:

5X first strand buffer 4µl, 0.1M DTT 2µl, 10mM dNTPs 1µl, DEPC treated H_2O 1µl, and Superscript II RT 1µl. The reaction is incubated for 1 hour at 37°C, and then incubated 10 min at 70°C for inactivating the endonuclease activity of the enzyme. The reaction is diluted adding 230µl of RNAse free H_2O.

4.2.3.2. Cloning of the AFP gene

cDNA sample is used as a template for PCR cloning of the AFP transcript using the following primers: AFPfor 5'-GCCCAGCATACGAAG-3', AFPrev 5'-ATGTAAATGTCGGCCAGTCC-3. The PCR reaction is achieved with the following ingredients: cDNA sample 2µl, 10X PCR Buffer 5µl, DMSO 3µl, 10 mM dNTPs 2µl, Primer Forward (20 pmol/µl) 1µl, Primer Reverse (20 pmol/µl) 1µl, H_2O 35µl, and Platinum Taq (5U/µl) 1µl. 3 to 5 PCR reactions are required for purify enough PCR products (500ng-1µg) for the following steps. One reaction is performed with H_2O instead the cDNA template as a negative control.

The thermal cycler protocol is performed with the following parameters.

Steps	Temp / time
1 step	94°C 1 min
40 cycles	94°C 30"; 55°C 1 min; 72°C 1 min;
1 step	72°C 5 min

4.2.3.3. Agarose gel analysis of the PCR reaction

Analysis of the PCR product is done by 1.5% agarose gel electrophoresis and ethidium bromide staining. An agarose gel is prepared dissolving 1.5g of agarose in 100ml of TBE buffer by heating with a microwave oven. When the agarose is

dissolved, is added 10µl of 1mg/ml of ethidium bromide solution, and mixed gently. The gel solution is casted in a horizontal chamber provided of combs for wells loading. After the gel is solidified (after circa 1 hour), the gel with its chamber is inserted in the electrophoresis apparatus, and TBE buffer is added to the apparatus until gel is covered. Each PCR sample of 10µl is mixed with 2µl of 6X DNA loading buffer. After removal of the gel comb the samples are loaded in the gel well by pipetting carefully. Size of the PCR products is determined by using 1µg of 100 bp ladder DNA marker running together the samples with the same loading condition. The gel is run at 100 Volts until the bromophenol blue dye font has reached the distance of 1 circa 10cm from the loading well (after circa 30 min). The gel is analysed over a short wave UV transilluminator for visualize the DNA products. Documentation pictures of the gel are made by digital or Polaroid camera. The AFP specific PCR product must be 750bp and no other products must be seen in the PCR samples. The negative control must show no any PCR products.

4.2.3.4. Purification of the AFP cDNA PCR product
PCR products are purified by using MinElute Gel Extraction Kit (all the components are provided in the kit). To 100µl of the PCR reaction is added 500µl of PB buffer. The sample is applied to a MinElute column inserted in microcentrifuge tube. The tube is centrifuged 1 min at max speed and the flow-through is discarded from the collection tube. 750µl of PE buffer is applied to the column inserted in microcentrifuge tube. The tube is centrifuged 1 min at max speed and the flow-through is discarded from the collection tube. The column is placed in a new collection tube and 15µl of EB buffer is applied to elute the DNA from the column. After 1 min of incubation, the tube is centrifuged for 1 min at max speed. The purified eluted DNA is collected in the tube and the column is discarded. Concentration of the DNA is measured by spectrophotometer at 260nm and 280nm of wavelength. An aliquot of 2µl of the sample is diluted 1:100 for the measurement and the H_2O is used as a blank. Concentration and purity of the DNA sample is obtained by using the following formulas: DNA conc. (ng/µl) = Abs_{260} X 50 X

dilution factor; DNA purity= Abs_{260}/Abs_{280} (1.7 to 2.0 are considered the optimal range of nucleic acid purity).

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4.2.3.5. Labelling of the AFP cDNA

A sample of 100ng of purified cDNA is labelled by Random Primer DNA Labelling System. All the reagents, excluded the radioactive $dCTP^{32}$, are included in the kit. The cDNA is diluted in 10µl final volume of H_2O and denatured at 95 °C for 5min. The tube is centrifuged at 10,000 rpm and then placed on ice. To the cDNA is added the following reagents: dGTP solution 2µl, dATP solution 2µl, dTTP solution 2µl, random primer buffer solution 15µl, $dCTP^{32}$ solution (50 µCurie) 5µl, H_2O 14µl. After mixed the components is added Klenow enzyme 1µl.

The reaction tube is mixed and place at 25°C for 1 hour. The reaction is stopped adding 5µl of stop buffer. The radioactive labelled probe is purified by columns chromatography. A chromatography column is washed twice with 2ml of TE buffer before. The entire volume of the reaction is placed on the top of the column after. When the reaction volume is entered in the column (after 5 min approximately), 2 volumes of 400µl of TE buffer is applied and the 2 eluted fractions of 400µl each one are collected. Beta radiation is measured using 5µl from the first and the second fractions by liquid scintillation system. The counts per minute (cpm) of the first fraction are representing the not incorporated radioactivity, while the cpm of the second fraction is representing the specific incorporated radioactivity of the probe. Specific activity of at least 50.000 cpm/µl should be required for proceeding to the hybridization. Radioactive probes are active for a maximum period of two weeks and must be store at -70°C.

4.2.4. Northern Blot hybridisation

The membrane is inserted in the hybridisation roller bottle avoiding air bubbles between the filter and the glass bottle. Pre-hybridisation is performed in a rolling oven for 1 hour with 10ml of Quick-Hyb hybridization solution at 68°C. 1 million cpm of labelled probe is added every 1 ml of hybridization solution. The probe is denatured with 1 ml salmon sperm DNA 10mg/ml concentrated, for 10 min at 95°C,

then placed on ice for 2 min. The probe is added directly to the hybridisation solution and mixed. The membrane is hybridised for 5 hours. The radioactivity of the membrane is monitored by Geiger counter during the washing steps, and depending on the radioactive value of the membrane the washing time and condition are determined. Generally, the membrane is washed with

2X SSC-0.1% SDS solution for 15 min, 0.1X SSC-0.1% SDS solution for 15 min, and finally with 2X SSC-0.1% SDS solution for 15 min at room temperature. The membrane is exposed to auto-radiographic film for a period of hours to days depending on the specific signal intensity.

4.3. Histological Techniques

4.3.1. Hematoxylin-eosin staining

Frozen liver pieces are cut in section of 4 to 8µm using cryostat at the temperature of -25°C. In each glass slides, they are fixed sections from livers from different time points of oval cell regeneration, from liver normal control, and from livers from sham operated animals as additional controls. The sections are dried 1 hour at room temperature, fixed in cold methanol at -20°C for 10 min, fixed in cold acetone at -20°C for 10 sec, and finally dried for 1 hour to overnight at room temperature. Sections can be store at -20°C for at least one year.

Glass slides of fixed sections are first immersed in Meyer hemalum solution for 1 min, washed in H_2O 10 min, immersed in eosin 0.5% solution for 5 min, and washed again in H_2O for 10 min. After the staining, the sections are dehydrated in serially concentrated solutions of ethanol (70%, 80%, and 96%) immersing the slides 1 min, in each solution. After all, the slides are immersed in xylol overnight. The day after, the slides are mounted with coveslip using the Entellan media.

4.3.2. Immunostaining

Liver tissue are cut and fixed on glass slides as already described in the previous section 4.3.1. Hematoxylin-eosin staining. The endogenous peroxidase is blocked by incubating the sections 30 min, at 37°C in 100 ml PBS solution containing glucose oxidase 5 mg, sodium azide 6.5mg, and glucose 180 mg. The sections are washed for 10 min in PBS solution. The blocking step is performed incubating the sections with foetal bovine serum for 30 min. Then a washing step with PBS is performed for 10 min. Each slide is incubated with 200µl of primary antibody diluted in PBS solution at different concentration (e.g. 1:50, 1:100. 1:200) and for different time of incubation and temperature; from 30 min to 5 hours at room temperature or over night at 4°C. Negative control, fundamental for the staining interpretation, is consisting in PBS with immunoglobulins from the same species of the primary antibody.

Sections are washed in PBS for 10 min. For each slide is prepared 25µl of secondary antibody and 5µl of rat serum which are incubated for 60 min at 37°C. The secondary antibody is centrifuged for 10 min at 13,000 rpm. To 25µl of the supernatant, is added PBS 175µl and immunoglobulin inactivated serum or foetal calf serum 300µl.

The slides are incubated with the prepared secondary antibody solution for 50 min. A washing step is achieved with PBS for 15 min. The staining solution is prepared by stirring 1 hour 50mg of DAB in 100ml of PBS. The staining solution is filtrated with paper filter and 33µl of 30% hydrogen peroxide solution is added next before the slides are ready for the staining. Slides are immersed in the staining solution until the desired stain intensity is developed (circa 10 min). The slides are washed by tap water and counterstained with Meyer hemalum solution for 30 sec for nuclear staining. The slides are washed for 10 min with running tap water and the coverslips are mounted with 100µl of pre-warmed at 50°C Kaiser's glycerol gelatin.

4.3.3. In situ hybridization

Riboprobes are generated by in vitro transcription of a cDNA construct containing the cloned gene and the T7 promoter. The cDNA construct is synthesized by PCR using as template a cDNA from rat liver and using primers pairs with T7 promoters in two different configurations (Table 2). When a T7 promoter is upstream of the reverse primer we obtain a cDNA construct for the synthesis of an antisense riboprobe useful for detecting the transcript. Instead, when a T7 promoter is upstream of the forward primer, we generate a cDNA construct for the synthesis of sense probe useful as a negative control (Figure 9).

Cloning by the PCR protocol is achieved using the protocol described above in the section 4.2. Northern Blot of AFP transcript method. In the table 2 are shown primer sets for the synthesis of AFP cDNA constructs.
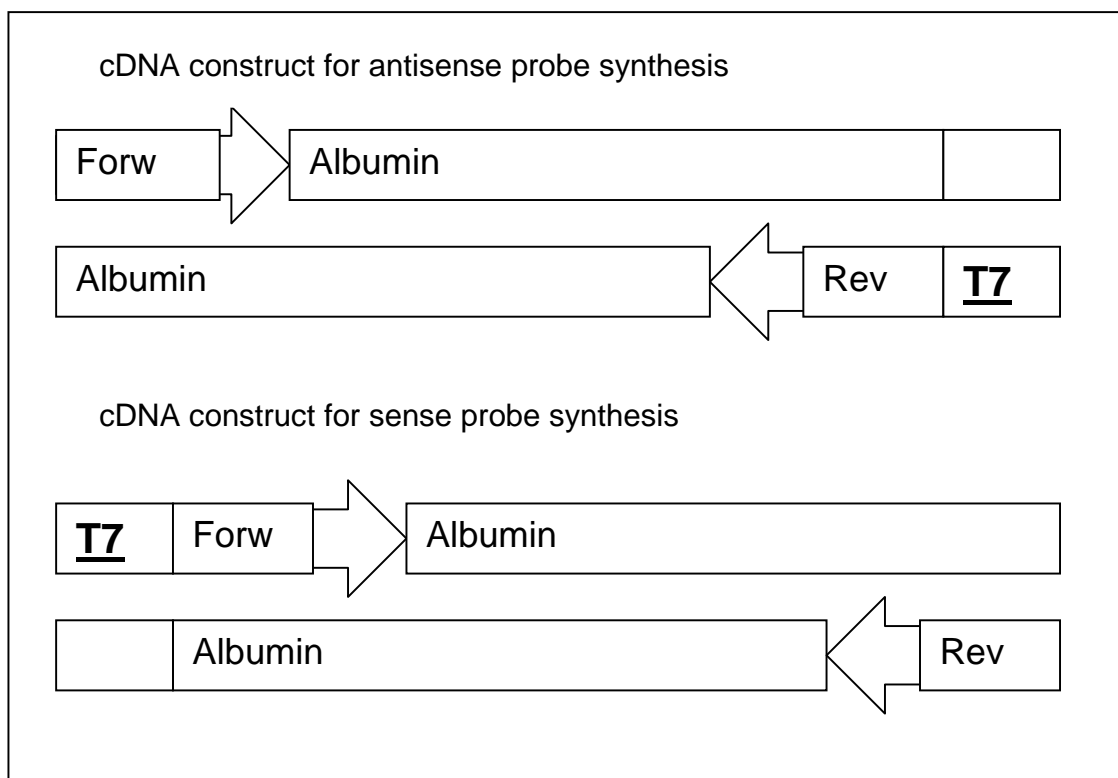
Figure 9. cDNA constructs of the albumin gene for the synthesis of antisense probe and the sense probe

Primer	Sequence
AntisenseAFPfor	GGCGATGTCCATAAACACGTTC
AntisenseAFPT7rev	TAATACGACTCACTATAGGGCCGGTTTGTCGCCATTTTC
SenseAFPT7for	TAATACGACTCACTATAGGGGGCGATGTCCATAAACACGTTC
SenseAFPrev	CGGTTTGTCGCCATTTTC
AntisenseAlbfor	GGATTCCAAAACGCCGTTCT
AntisenseAlbT7rev	TAATACGACTCACTATAGGGCCTCAGTGGCGAAGCAGTTATC
SenseAlbT7for	TAATACGACTCACTATAGGGGGATTCCAAAACGCCGTTCT
SenseAlbrev	CCTCAGTGGCGAAGCAGTTATC

Table 2. Primer sets for the PCR synthesis of the cDNA constructs.

In vitro transcription is performed using the DIG RNA Labeling Kit of Roche and all the components are provided in the kit.

To 1μg of purified cDNA in 26μl of H_2O is added: 10X NTP labelling mixture 4μl, 10X transcription buffer 4μl, RNase inhibitor 2μl, and RNA Polymerase T7 4μl.

The reaction is mixed gently and incubated for 2 hours at 37°C.

The enzyme activity is stopped by adding 0.2M EDTA (pH 8.0) 4μl.

An aliquot of 1μl of the reaction is analysed by agarose gel electrophoresis (see section 4.2.3.3 Agarose analysis of the PCR reaction) and RNA product should be seen as a band or smear with the half size of the PCR product. For each section is used 1μl of the reaction that should contain about 500ng of DIG-labelled RNA.

Sections of 10μm are cut from frozen liver pieces or frozen rat embryos using a cryostat. Liver sections are immediately fixated after cutting, while rat embrios sections are dried 30 min at room temperature before fixation. The sections are fixed 2 hours over the glass slides using 4% parafolmaldeyde solution in DEPC treated PBS. The sections are washed two times for 15 minutes in PBS solution containing 0.1% active DEPC. 50μl of hybridisation solution is poured over each slide, and a clean coverslip is used for keeping the hybridisation solution covering the sections. Pre-hybridisation is achieved at 50°C for 1 hour in a sealed glass slide chamber. Antisense or sense riboprobe is added to each slide to the final concentration of 50ng/μl and pre-hybridization is achieved at 50°C for 12-16 hours. After hybridisation the slides are washed 15 min at 50°C in 2X SSC solution, 15 min at 50°C in 0.1X SSC solution. RNAse treatment of the slides is achieved 30 min at 37°C with 10μg/ml of RNAse A in 2X SSC solution. A final washing step is achieved for 15 min at 50°C with 0.1X SSC solution. The following steps are done using the DIG Detection Kit. Blocking step is achieved for 30 min with blocking solution. Slides are incubated for 1-2 hours with antibody anti-digoxygenine, alkaline phosphatase-coupled diluted 1:500 in blocking solution. The slides are washed two times 15 min in washing buffer and incubated 5 min with detection buffer. The staining is achieved from 2 hours to overnight depending by the speed of the development reaction and it is monitored by optic microscopy. After the staining the slides are washed 10 min in H_2O, counterstained 10 second with 0.1%

methyl-green solution, washed again for 5 min in H_2O and finally a coverslip is mounted with pre-warmed at 50°C liquid Kaiser gelatin.

4.4. Serial Analysis of Gene Expression method

4.4.1. Sample RNA preparation

RNA samples are obtained by ultracentrifuge method: see section 4.2.1 Northern Blot of AFP transcript.

4.4.2 Preparation of poly(A)$^+$ RNA from total RNA by Oligo(dT)cellulose chromatography

All the material and reagents are kept under RNAse free condition. Chromatography column is prepared using 1 ml syringe, with a filter made with glass wool silanized in the bottom of the syringe. A quantity of 0.1g of oligo(dT)cellulose is inserted in the column by first suspending the powder in 5 ml of 1X elution buffer and then flowing the solution inside the column. The column is washed 10 times with 1ml of binding buffer. The total RNA sample of 1mg is diluted in 0.5ml of RNAse free H_2O. RNA denaturation is achieved by heating at 65°C 10 min, and chilling quickly to room temperature. A volume of 2X Binding Buffer (0.5ml) is added to RNA sample. The sample is applied to the column, followed by 1ml of 1X binding buffer, and the eluted fraction of 2ml is collected. To the eluted fraction is repeated the denaturation steps and it is reapplied to the column. The column is washed with 10ml of 1X binding buffer and eluted fractions of 1ml each ones are collected separately. The Abs$_{260}$ of each eluted fraction is measured spectrophotometrically at 260nm wavelength and using 1X binding buffer as blank. When the Abs$_{260}$ value is below 0.01, the poly(A)$^+$ sample is eluted with 2.1ml of Elution Buffer. Six eluted fractions of 350µl containing the poly(A)$^+$ sample are collected. Poly(A)$^+$ sample contained in each fraction is precipitated by adding 40µl of 2M ammonium acetate and 1050µl of ethanol. The eluted fractions are kept at -20°C overnight and centrifuged 30min at 14.000 rpm and 4°C. The pellets are washed with cold 70% ethanol twice and dried in vacuum centrifuge. Each pellet is resuspended in RNAase free H_2O 15µl, the reaction tubes are warmed at 60°C for 1 min, and the resuspended poly(A)$^+$ samples are pooled. An aliquot of 4µl of the poly(A)$^+$ sample is diluted 1:25 in H_2O for the spectrophotometric measurement.

For the spectrophotometric measurement see the procedure described in section 4.2.1. Sample RNA preparation by ultracentrifuge method. Poly(A)$^+$ sample of 1 µg is used for Northern Blot hybridization for checking the quality of the sample following the procedure described in the section 4.2. Northern Blot of AFP transcript section.

4.4.3. cDNA synthesis by biotylinated oligo(dT) primers

4.4.3.1. First strand synthesis reaction

A sample of 1µg/µl of poly(A)$^+$ RNA in 5µl of RNAse free H$_2$O, is incubated with 2.5µl biotin-oligo-(dT) (1 µg/µl), at 70°C for 10min and then the reaction tube is placed on ice. Then the tube is centrifuge for a quick run.

To the reaction tube is added:

5X first strand buffer 4µl, 0.1M DTT 2µl, 10mM dNTPs 1µl, DEPC treated H$_2$O 1µl, and Superscript II RT 5µl.

Reaction is incubated for 1 hour at 37°C, and then place on ice

4.4.3.2. Second strand synthesis

To 20µl of the first strand reaction products is added:

DEPC treated H$_2$O 94µl, 5X second strand buffer 30µl, 10mM dNTPs 3µl, E. coli DNA ligase 1µl, E. coli DNA Polymerase I 4µl, and E. coli RNase H 1µl.

The reaction tube is mixed gently, incubated for 2 hours at 16°C, and then placed on ice. To the reaction tube is added 2µl of T4 DNA Polymerase (10units) the incubation is continued at 16°C for 5 minutes

The reaction is stopped by adding 10µl of 0.5M EDTA (pH 7.5), and heating to 70°C for 10 minutes.

4.4.3.3. cDNA phenol-chloroform extraction and ethanol precipitation

To the reaction tube is added 50µl of H$_2$O to a final volume of 200µl. Than it is added 200µl of phenol-chloroform solution, the tube is mixed briefly, centrifuged for 10 min at 10.000 rpm. The top aqueous layer is collected in a new tube while the phenol-chloroform solution is discarded.

The cDNA is precipitated by ethanol.

To circa 200μl of the sample is added:

7.5M ammonium acetate 133μl, glycogen 3μl, and ethanol 777μl.

The tube is kept 10 min in -70°C refrigerator and then it is centrifuged for 45 min at 4°C at 14.000 rpm. Supernatant is decanted and the pellet is washed twice with cold 70% ethanol. The pellet is dried briefly in a fume hood and re-suspended in 20μl of LoTE buffer.

4.4.4. Cleavage of cDNA with anchoring enzyme (Nla III).

To the sample of 20μl is added:

LoTE buffer 153μl, 100X bovine serum albumin (BSA) 2μl, 10X buffer 4 20μl, and NlaIII (10 U/μl) 5μl.

The reaction is incubated at 37°C for 1 hour.

cDNA is purified by phenol-chloroform extraction and ethanol precipitation like described above (section 4.3.3).

4.4.5. Binding of biotinylated cDNA to magnetic beads

Two tubes containing 100μl Dynabead M-280 Streptavidin slurry are first washed using the magnet rack to immobilize beads and remove the supernatant.

The beads are washed as follows: 200μl of 1X B+W buffer is added to the tubes containing the magnetic beads, the tube is mixed briefly, a magnetic separation is performed by the magnetic rack, and the supernatant is removed.

To each of the 2 tubes containing the washed magnetic beads is added:

2X B+W buffer 100μl, H_2O 90μl, and cDNA restriction products 10μl. Thus the restriction product sample is split in 2 fractions which will be ligated alternatively with linker adapter A and linker adapter B. The tubes are incubated 15 minutes at room temperature and mixed intermittently. The beads binding the biotilynated cDNA are washed as above three times with 200μl of 1X B+W buffer, and then once with 200μl of LoTE buffer, removing the supernatant washing buffer each time.

4.4.6. Ligating linkers to the cDNA

One of the two tubes is ligated with the linker A, the other with linker B.

To each tube is added 25µl of LoTE buffer and 8µl of 5X ligase buffer.

In one tube is added 5µl of linker A (200ng/µl) and to the second tube is added 5µl of linker B (200 ng/µl). The beads are re-suspended by mixing the tubes gently. The tubes are incubated at 50°C for 2 minutes, then let sit at room temperature for 15 minutes. Tubes are put on ice and 2µl of T4 ligase is added to each tube. The ligation reaction is incubated for over night at 16°C, gently mixing every 15min for the first two hours. After the ligation, each tube is washed 3 times with 200µl of 1X B+W buffer using the magnetic separation method as described in the section 4.4.5. The beads contents are transferred to new tubes.

Again the tubes are washed once with 200µl of 1X B+W buffer and twice with 200µl of 1X buffer 4.

4.4.7. Release of cDNA tags using Tagging Enzyme (BsmFI)

To each microcentrifuge tube is added:

LoTE 86µl, 10X buffer 4 10µl, 100X BSA 2µl, and BsmFI (2U/µl) 2µl.

The cDNA samples are incubate at 65°C for 1 hour, gently mixing intermittently.

The supernatants containing the released cDNA are collected from each tube by magnetic separation and they are put in two separated new tubes. To each tube is added 100µl of LoTE buffer. The samples are purified by phenol-chloroform extraction and ethanol precipitation like described in the section 4.4.3.3. cDNA phenol-chloroform extraction and ethanol precipitation. Each pellet is resuspended in 10µl of LoTE buffer.

4.4.8. Blunt ending of released cDNA tags

To each tube containing 10 µl of the tagging enzyme-derived cDNA fragments is added:

5X second strand buffer (Invitrogen cDNA synthesis kit) 10µl, 100X BSA 1µl,

dNTPs (10mM) 2.5µl, H_2O 23.5µl, and Klenow enzyme (1U/µl) 3µl.

The tubes are incubated 30 minutes at 37°C.

The sample size of each tube is increased with LoTE buffer to 200µl. The samples are purified by phenol-chloroform extraction and ethanol precipitation like described in the section 4.4.3.3. Each pellet is re-suspended in 6µl of LoTE buffer.

4.4.9. Ligating tags to form ditags

Blunt-ended sample 1 and 2 are ligated to each other in the following way: the first tube is the ligation reaction (ditag reaction); the second serves as a control for contamination control in later PCR steps (Negative Control).

Contents	Ditag Reaction	Negative Control
Blunt-ended sample 1	2 µl	2 µl
Blunt-ended sample 2	2 µl	2 µl
5X ligase buffer	1.2 µl	1.2 µl
T4 ligase (high conc 5U/µl)	0.8 µl	0 µl
H_2O	0 µl	0.8 µl

The ditag reaction and the negative control are incubated overnight 16°C.

To each to ligation mixture is added 14µl of LoTE buffer to proceed to PCR amplification of the ditags or storing at -20°C.

4.4.10. PCR amplification of 108 bp ditags

We amplify ditags using primers 1 and 2 (see section 3.4.2. SAGE primer sequences and linkers preparation section) which anneal to linkers A and B respectively.

The amplification is optimized by using 1µl of ditag reaction from different dilutions solutions: 1:50, 1:100 and 1:200. The negative control is important to test eventual contamination and consist in a PCR reaction with 1µl of undiluted negative control as template.

A PCR reaction of 50µl is prepared using the following ingredients:

10X PCR buffer 5µl, DMSO 3µl, 10 mM dNTPs 7.5 µl, Primer 1 (350 ng/µl) 1µl, Primer 2 (350 ng/µl) 1µl, H_2O 30.5 µl, and Platinum Taq (5U/µl) 1µl.

. The PCR program of the thermal cycler is the following:

Step	Temp / time
1 step	94°C 1 min
26-30 cycles	94°C 30"; 55°C 1 min; 70°C 1 min;
1 step	70°C 5 min at end.

The appropriate cycle number is critical for isolating an adequate amount of DNA for SAGE. Too few cycles will result in a low yield and may cause problems with subsequent steps. Too many cycles will give erratic results of incorporation of wrong nucleotides in the polymerisation reaction, and can also result in low yields. Therefore, we find the optimal conditions trying various cycle numbers (e.g. 26, 28, and 30). Analysis of the PCR product is done by 4% agarose gel electrophoresis: the method for casting, running and analysing the electrophoretic agarose gel is described in the section 4.2.3.3. Agarose gel analysis of the PCR reaction.

Amplified ditags product should have 108 base pairs size, while the background linker-linker product should have 80 base pairs size (see the Results: Figure 14). We found 30 cycles of PCR amplification and 1/100 dilution of the ditag reaction template the optimal conditions for amplifying the ditags.

4.4.11. Scale up PCR of 108 bp ditag

96 PCR reaction of 50µl volume each one is prepared with the same ingredients like above, using optimized conditions of template concentration and number of PCR cycles. PCR reactions are collected in a 15 ml conical tube and purified by phenol-chloroform extraction and ethanol precipitation. An equal volume of phenol-chloroform is added to the PCR scale up sample (5ml). The tube is briefly and gently mixed and centrifuge at 2,400 x g for 10 minutes at room temperature, after the aqueous top phase is split in two other 15ml tubes.

To circa 2.5ml of the each sample is added: 7.5M ammonium acetate 0.64ml, mussel glycogen 15µl, 100% ethanol 5ml. The solution is mixed vigorously and the tube is placed 10-20 minutes at -70°C before is centrifuged at 12,000 rpm for 30

minutes at +4°C. The supernatant is carefully removed and the pellet is washed 2-3 times with 25ml of cold 70% ethanol. The pellet is air dried for 15 to 20 minutes. The pellet is re-suspended in 300µl of LoTE buffer and incubated at 37°C for 5 to 10 minutes to aid the solubilisation. Before the gel electrophoresis 60µl of 6X DNA sample buffer is added to the sample.

4.4.12. Polyacrylamide gel purification of 108 bp ditags

4.4.12.1. Polyacrylamide gel electrophoresis purification
12% Acrilamide gel is prepared for isolating and purify ditags from the PCR products. The gel is prepared with the following ingredients:
40% Polyacrylamide 10.5ml, H_2O 21ml, 10X TBE Buffer 3.5ml, 10% Ammonium persulfate (APS) 350µl, and TEMED 30µl.
The solution is mixed and immediately poured in a vertical gel chamber 15 cm high, with 1.5mm spacers. The chamber is sealed below and laterally using a rubber gasket. A comb with 11 gel wells is inserted from the top and the gel is let at least 30 min to polymerize. The gasket is removed after the polymerization and the vertical gel chamber is installed to the electrophoresis apparatus and fixed by using clamps. TBE buffer is poured on the top and the bottom of the electrode containers bathing the gel. Air bubbles are removed using a syringe on the bottom of the gel and the gel wells are rinsed using a Hamilton syringe. DNA marker is loaded in the two lateral wells 1µg each ones. The samples are loaded 30µl for each of the 9 wells. The apparatus is connected to the power supply and a constant current of 160 Volts for 2 to 2.5 hours is applied. When the xylene cyanol reaches 1 cm distance from the bottom of the chamber, the chamber is disassembled and the gel is carefully removed for the staining with SYBR Green fluorescence dye. The staining solution is made using TBE buffer 100ml and 10,000 time concentrated SYBR Green dye solution 10µl. The gel is poured in the staining solution for 10 min and a long wave UV transilluminator is used for the analysis of the DNA electrophoresis (see Results: section 6.4. Construction of SAGE libraries, Figure 15).

4.4.12.2. Crush and soak elution purification

Bands of 108 nucleotides is excised from the gel using a clean scalpel and poured in 0.5ml micro-centrifuge tube which was previously perforated with a syringe needle on the bottom. The 0.5ml sample tubes are inserted in 2ml tubes and centrifugation is achieved for 15 min at 12,000 rpm. The gel is crashing in small pieces from the upper tube and they are collected in the lower tube. The 0.5ml tubes are discarded and a 300µl of elution solution (250µl TE and 50µl 7.5M ammonium acetate) is added to the tubes containing the crashed gel pieces. Tubes are incubated over night at 65°C. After the incubation time, the sample tubes are centrifuged and each supernatant is transferred to a SpinX micro-centrifuge tube containing a filter to separate the eluted solution from the polyacrylamide gel pieces. The tubes are centrifuged for 5 min at max speed, and the filters are discarded from each tube. To each sample solution is added:

glycogen 5µl, ammonium acetate 133µl, and ethanol 1000µl.

Precipitation of the DNA is achieved by incubating 10 min the at -70°C and by centrifugation for 30 min at 14,000 rpm. Pellets are washed twice with 1ml of cold 70% ethanol solution and they are air dried. Each pellet is re-suspended in 10µl LoTE buffer and the samples are pulled for a total final volume of 90µl. Concentration of the purified DNA is achieved by spectrophotometer measurement as described in the section 4.2.3.4. Purification of the AFP cDNA PCR product.

At this point are needed 10 to 20µg of ditags to continue the protocol, otherwise steps of PCR reaction and polyacrylamide gel electrophoresis purification of 108 bp ditags must be repeated.

4.4.13. NlaIII digestion of the 108bp ditags

PCR products are digested with NlaIII by adding the following ingredients to the sample tube: LoTE buffer 226µl, 10X buffer 4 40µl, 100X BSA 4µl, and NlaIII (10U/µl) 40µl.

The tube is incubated 1 hour 37°C.

The digested reaction is extracted with equal volume of phenol-chloroform and the aqueous phases are transferred into 5 tubes and then ethanol precipitate as

follows: sample 200µl, 7.5 M ammonium acetate 66µl, glycogen 5µl, and ethanol 825µl.

The tubes are incubated for 10 min at -70°C and then are centrifuged at 4°C for 30 min. Pellets are washed with cold 70% ethanol solution and air dried on ice. The pellets are re-suspended and pooled in a total volume of 30µl of TE buffer (not in LoTE) into a new tube. A volume of 6µl of 6X sample buffer is added to the digested ditags and mixed (36µl final volume).

4.4.14. Polyacrylamide gel electrophoresis purification of 26 bp ditags

12% polyacrylamide gel is prepared using the protocol described in the section 4.4.12.1. Polyacrylamide gel electrophoresis purification. The sample is loaded into 4 lanes of a 12% polyacrylamide. The 2 lateral lines of the gel are loaded with 0.5µg of DNA standard markers ranging from 25bp to 300bp. The gel is run at 160 Volts for 2.5 hours when the bromophenol blue line is 2 cm from the bottom of the gel. The line of the xylene cyanol dye migrates approximately in the position of the 26 bp ditags.

The gel is stained using SYBR Green, 1:10,000 diluted in TBE buffer. A strong band of 24-26 bp of the digested ditags must be visible. In the gel will be also present side products of the digestion: a 40 bp band representing the linker A and linker B. Sometimes when the digestion is not complete is visible a 66 bp band and a 108 bp band which are representing respectively the partially digested material and the starting material not digested (see Results: section 6.4. Construction of SAGE libraries, Figure 16).

The 24-26bp bands are cut out from the 4 lanes, and placed each in a 0.5ml microcentrifuge tube (4 tubes total).

The crush and soak method to elute the purified DNA from the gel is performed as described in the section 4.4.12.2. Anyway, it is extreme important to incubate the elution solution containing the polyacrylamide pieces at 37°C, instead 65°C for avoiding that ditags are melting (Margulies et al., 2001b). Two hours of incubation is performed and longer incubations (even overnight) do not appear to result in

significantly higher yields. SpinX tubes are used to isolate elute solutions as described in the section 4.4.12.2.

Ethanol precipitation is achieved in 3 tubes:
sample 200µl, 7.5M ammonium acetate 66µl, glycogen 5µl, and ethanol 825µl.
The tubes are incubated 10 min at -70°C and centrifuged for 30 min at 4 °C and at 14,000 rpm. Pellets are washed twice with 70% cold ethanol solution and air dried on ice. Each DNA sample is re-suspended in 2.5µl of TE (not LoTE). The samples are pooled in a new tube (7.5µl total).

4.4.15. Ligation of ditags to form concatemers

4.4.15.1. Concameter reaction
Length of ligation time depends on quantity and purity of ditags.
Typically, several hundred nanograms of ditags are isolated and produce large concatemers. We have carried out normally the ligation reaction 3 hours at 16°C but lower quantities or less pure ditags will require longer ligations reaction.
The concameter reaction is carried out with the following ingredients:
Pooled purified ditags 7.5µl, 5X ligation buffer 2µl, T4 ligase (high concentration 5 U / µl) 1µl.
The reaction is incubated for 1 to 3 hours at 16°C depending from the quantity of the starting material.
Afterwards, 2µl of 6X Sample Buffer is added to the ligation reaction.
The sample is heated to 65°C 5 min then placed on ice next before the gel electrophoresis purification (Powell, 1998).

4.4.15.2. Polyacrylamide purification of concatemers
8% polyacrylamide gel is prepared as described above in the section 4.4.12.1. Polyacrylamide gel electrophoresis purification.
The gel is prepared with the following ingredients:

40% Polyacrylamide 7ml, H_2O 24.5ml, 10X TBE Buffer 3.5ml, 10% APS 350µl, and TEMED 30µl.

In the first lane of an 8% polyacrylamide gel is loaded 250ng of 1 kb ladder marker. The entire concatemer sample is loaded in the third well. The gel is run at 130 Volts for 3 hours until the bromophenol blue dye front reaches approximately 10cm of distance from the gel wells. The gel is stained with SYBR Green 1:10,000 diluted in TBE solution. Gel is visualized on UV box using long wave UV radiations. Concatemers will form a smear on gel with a range from about one hundred base pairs to several kilo bases (see Results: section 6.4. Construction of SAGE libraries, Figure 17). We usually isolate region 600 to 1200bp and 1200 to 2500bp cutting the gel pieces with a clean scalpel. The DNA concameter is isolated with the crush and soak method already described in the section 4.4.12.2. Crush and soak elution purification. Briefly each gel pieces from the two regions are collected into a 0.5ml microcentrifuge tube pierced in the bottom with a 21 gauge needle (2 tubes total). The 0.5ml tube are placed in a 2.0ml microcentrifuge tube and centrifuged at full speed for 2 min. The 0.5ml tubes are discarded, and 300µl of elution solution (250µl TE and 50µl 7.5M ammonium acetate) is added to the crashed gel pieces on the bottom of the 2.0ml tubes. The tubes are mixed and place at 65°C for 2 hours. If necessary this incubation can be extended to overnight but yields are not significantly increased. The content from each tube is transferred to a SpinX microcentrifuge tubes (2 SpinX tubes total) and a centrifugation is carried out for 5 minutes at full speed to separate the eluted samples from the polyacrylamide gel pieces.

Each eluted fraction is precipitated as follows:

eluted fraction 300µl, glycogen 3µl, 7.5M ammonium acetate 133µl, ethanol 1000µl.

The tubes is mixed and incubated for 10 min at -70°C before centrifugation is achieved at full speed for 30 min. The pellets are washed twice with 70% ethanol cold solution, and air dried. Pellets are resuspended in 6µl of LoTE.

4.4.16. Cloning concatemers and sequencing

4.4.16.1. Cloning in a vector.

Concatemers can be cloned and sequenced in a vector of choice. We currently clone concatemers into a SphI cleaved pZero.

Contents	Library	Vector Alone Control	No Ligase Control
Purified concatemer	6µl	0	0
H$_2$O	0µl	6µl	7µl
pZero (25ng/µl)	1µl	1µl	1µl
5X ligase buffer	2µl	2µl	2µl
T4 ligase enzyme (1U/µl)	1µl	1µl	0

The ligase reaction is incubated overnight at 16°C.

To the sample is added volume to 190µl of LoTE buffer.

The sample is purified by equal volume of phenol-chloroform as described in the section 4.4.3.3. cDNA phenol-chloroform extraction and ethanol precipitation. Ethanol precipitation is performed with the following ingredients:

sample 200µl, 7.5M ammonium acetate 133µl, glycogen 5µl, and ethanol 777µl.

The sample is incubated 10 min at -70°C and centrifuged for 30 min at 4°C. The pellet is washed four times with 70% ethanol cold solution, centrifuged to remove ethanol, air dried and resuspended in 10µl of LoTE buffer.

4.4.16.2. Bacterial transformation

A volume of 1µl from ligase reaction from the library sample and from the controls (vector alone control, no ligase control) is transformed into ElectroMAX DH10B by electroporation. We use electroporation cuvettes with 1 mm of size capacitor chamber. The cuvettes are cooled on ice before starting. Aliquot of 50µl electro-competent bacteria are thaw on ice. To 1µl of each ligase reaction is added the aliquot of cells and the mixing is performed by gently moving the pipette tip inside

the tube. The cells are transferred immediately inside the electroporation cuvette chamber avoiding bubbles between the capacitor walls. The electroporetor is set to 1,800 Volts and the cuvette is inserted inside the machine. A pulse of electric current is discharged and 1ml of room temperature SOC medium is added immediately to suspend the bacteria. The bacteria in SOC medium are incubated shaking for 30 min at 37°C. The transformed bacteria are plated in the following dilution in SOC medium (1/10, 1/100, 1/1000) in 10 cm zeocin containing plates pre-warmed at 37°C. The colonies are incubated 16 to 24 hours at 37°C.

Insert containing plates should have hundreds to thousands of colonies while control plates should have 0 to tens of colonies

Save plates for each concatemer ligation reaction since, if insert size appears appropriate these may be used for sequencing as described below.

4.4.16.3. Check insert sizes by PCR

Each 25µl of PCR reaction is set using the following ingredients:

10X PCR buffer 2.5µl, DMSO 1.25µl, 10 mM dNTPs 1.25µl, M13F (350 ng/µl) 0.5µl, M13R (350 ng/µl) 0.5µl, H_2O 19µl, and Platinum Taq (5U/µl) 0.2µl.

Each PCR mix is added to a well of a 96-well PCR plate

With a sterile tip (autoclaved toothpick) is gently touched a colony and then dip tip into PCR mix.

PCR is performed with the following steps and conditions:

Step	Temp / time
1 step	94°C 1 min
30 cycles	94°C 30"; 56°C 1 min; 72°C 1 min;
1 step	72°C 5 min at end.

To each PCR products is added 5µl of 6X loading buffer and 10µl of each PCR sample is run in a 1.5% agarose gel. Preparation of the electrophoretic agarose gel is described in the section Northern Blot of AFP transcript 2.3.4.

Determination of the size and the percent of inserts present in the SAGE library are crucial. Most of the clones must contain an insert of >500 bp (a plasmid without insert gives a 200 bp PCR product). The quality of a SAGE library is determined by the average of tags per colony. Average of number of tags per colony should be more than 20 for an efficient sequencing, while 25 tags per colonies is the ideal maximum number for a SAGE library.

When the library is fulfilling the criteria of quality, automated sequencing is strongly recommended for fast acquisition of the SAGE data.

The Doctor Hanke Hanne in the department of microbiology of Göttingen has arranged the sequencing of the libraries. Briefly the colonies were picked manually and growth in LB medium containing zeocin. The plasmids were purified by automated robot and the sequencing reaction was performed over the plasmids. The analysis of the sequence reactions was done using capillary sequencer.

4.4.17. SAGE data elaboration

Library analysis was performed using the SAGE2000V45 software provided from Invitrogen and the eSAGE software kindly provided by Margulies E. H (Margulies and Innis, 2000). The sequencing data were analyzed using the Phred algorithm for the control of the quality of the sequencing trace data and for the exclusion of the poor quality data from the SAGE analysis.

4.5. Real Time PCR

4.5.1. Real Time PCR experiments by standard curve method

RNA samples are obtained by ultra-centrifugation and their quality is tested by Northern Blot hybridisation. For the RNA purification and Northern Blot hybridisation see the section 4.2. Northern Blot of AFP transcript method.

The RNA sample is treated by RNAse free DNAse for the digestion of genomic DNA using the following ingredients: RNA sample 5µg in 8µl of RNAse free H_2O, 10 X reaction buffer 1 µl, RNAse free DNAse 1µl (1 enzymatic unit).

The reaction is incubated 30 min ant 37 °C and then 1 µl of stop solution is added. The reaction is incubated at 65°C for 10 min for inactivate the DNAse enzyme. The A quantity of 5µg of the RNA sample is retro-transcribed using the protocol described in the section 4.2.3.1. cDNA synthesis from RNA liver samples.

Primers sets are elaborated using the program "Primer Express" (Abi Sistem) and the gene bank data (http://www.ncbi.nlm.nih.gov). In the table 2 are showed the primers set used for the Real Time PCR experiments.

The cDNA samples are analyzed by the Real Time PCR using the following ingredients for each PCR reaction:

"X" primer-forward (5mM) 1.5µl, "X" primer-reverse (5mM) 1.5µl, H_2O 8µl, SYBR Green Master Mix Taq Polimerase 13.5 µl.

To each PCR reaction is added 2µl of the cDNA sample or 2µl of H_2O for the negative control. Each sample is analysed by Real Time PCR in duplicates reaction.

The thermal-cycler is programmed with the following parameters:

Steps	Temp / time
1 step	95°C 2 min
40 cycles	94°C 15"; 60°C 30";

The PCR program is followed by dissociation curve protocol for controlling the specificity of the PCR products. Specific temperature of dissociation of the PCR

product is calculated by the Primer Express program. Curves of amplification are analyzed to measure the Ct value in the linear range of the amplification. The values of gene regulation are calculated using the standard curve method. PCR products are purified with the MinElute system already described in the section 4.2.3.4. Purification of the AFP cDNA PCR product. The concentration of the PCR products are measured by spectrophotometer as described in the section 4.2.3.4. Purification of the AFP cDNA PCR product. Standards are made from $1e^9$ to $1e^4$ copies/µl by dilution of the purified PCR product and using the following formula:

Copies per µl = conc. PCR product (µg/µl) * 9.1×10^{11}/ size of the PCR product (Kb). Serially diluted PCR products, of the gene of interest are amplified by Real Time PCR and Ct values are calculated. The standard curves are obtained graphically by using the following parameters: Ct values and the logarithm of the number of copies. Standard curve are calculated for the gene of interest and the housekeeping gene ubiquitin C. Number of copies of gene in the sample is obtained by extrapolation of the measured Ct value to the relative standard curve. Normalization of gene expression in each sample is performed by calculating the ratio of number of copies of the gene of interest in respect the number of copies of the housekeeping gene. The regulation of gene expression is calculated by the ratio of gene expression of the sample respect the gene expression of the calibrator that in our case is the normal liver control. Kinetic of gene expression regulation during the liver oval cell regeneration is obtained over the time points 1, 3, 7, 11, and 16 days after PH and Sham operation.

GENE	Primer Forward	Primer Reverse
Housekeeping gene		
Ubiquitin C	CAC CAA GAA GGT CAA ACA GGA A	AAG ACA CCT CCC CAT CAA ACC
Early up-regulated genes		
Cyclin D1	GCC ATC CAT GCG GAA AAT C	AGA GAC AAG AAC CGG TCC AGG T
Stathmin 1	GAA GTG CTC CAG AAA GCC ATT G	GCC TCC CGG TTC TCT TTG TTA G
CD151	CAC CAT AGG AGT GGC AAG ATC C	TTG AAT CCC CCA TCC TGC G
Lipolysaccaride binding protein	AAA GCA AAA TTG TAG CCC CCC	CCG ATG AGC GTT TGA TTT GG
Na$^+$/P$_i$ cotransporter 4	GTA TGG CAT GAT GCT GAT GCA G	AGT GAG CAG TGA CGA TCC CAA C
Phosphatidylcholine transfer protein	GAT TAT GTC TAC ACC CGC CAG C	TAC CCC AGA CTT CTC CGG AAA
ATPase H$^+$ 34KDa lysosomial transpoter	ACG AGG ACC TGC TGT TCG AGT A	CTG CCA AAA TAA ATA CCC GCA C
Thioredoxin Like 2	AAG CCG AAG CTG TTC CTG AAG T	AAA ACC ACA GCG TGG TTC CTG
Alpha foeto-protein co-expressed genes		
E-Tropomodulin	GCC TCC CAC AAT GTC CTA CAG A	TCG TTT TCC AGT GTC CTG AGC T
Cell division cycle 42 (CDC42)	GGA GAG GCT GAG GTC AAC ATC A	ACA GCA CCA TCA CCA ACA ACA A
Alpha foeto-protein (AFP)	GCC CAG CAT ACG AAG AAA ACA	TCT CTT TGT CTG GAA GCA TTC CT
Down-regulated genes		
Hydroxyacid oxidase 3	AGC AAT GAA GCA CAA CGT CCA	AGC CAC CAC TTC TCT CAG AGC A
Fatty acid CoA ligase long chain 2	GCC CAT ATG TTT GAG ACC GTT G	TTG AGG TCA TCC ATA AGC AGC C
Stearoyl-coenzyme A desaturase 1	ATG ACC CAG CCA AAG TGC AAG	TCA CAA ATA ACC GCC CCA CA

Table 3. Primer sets used for the Real Time experiments.

4.6. Western Blot

4.6.1. Protein sample preparation from liver tissue

Proteins lysates are prepared by ultrasound homogenization on ice of frozen liver tissue pieces and using lysis buffer containing inhibitor of proteases. Samples are cleared by centrifugation at 15,000 rpm for 15 min at 4°C. Protein concentrations of supernatant samples are measured by BCA assay using BSA as a standard. BSA standard solutions are prepared using the dilution scheme in the product datasheet and the components provided in the kit. The volume of working reagent is calculated depending on the number of samples and standards and it is prepared using the reagent A and reagent B in 50:1 proportion. Standard or sample aliquot is mixed with 1 ml of working solution in a plastic spectrophotometric cuvette. The prepared standard solutions and the sample solutions are incubated for 30 min at 37 °C and cooled at room temperature. Spectrophotometer measurements are performed at 562 nm wavelength using the H_2O as a blank. Standard curve is prepared plotting the standard solution measurement. The concentrations of the samples are obtained by extrapolation from the standard curve.

4.6.2. Polyacrylamide Gel Electrophoresis (PAGE) of liver protein

The pre-cast gel cassette is washed with H_2O, and the tape is peeled off from the bottom. The gel cassette is inserted in the electrophoretic apparatus following the manufacture instructions. The chamber is filled with 1X running buffer. The comb is removed from the gel cassette and the gel wells are rinsed with 1X running buffer using a Hamilton syringe. The protein sample of 25 to 50µg is diluted in 10 µl of H_2O and 10µl of 2X loading buffer containing 50mM DTT. The samples are incubated 2 min at 85°C. Denatured protein samples are loaded in the gel wells by using a Hamilton syringe. Protein marker is loaded in one side, and the gel is run at constant 125 Volts for circa 1 hour, until the blue dye font has reached the bottom of the gel cassette.

4.6.3. Protein transfer in nitrocellulose membrane

Two layer of Wathman filter paper and a nitrocellulose membrane are cut with the size of the gel and pre-soak in transfer buffer for 30 min before the gel transfer procedure. The gel cassette is removed from the electrophoretic apparatus and opened with the gel knife. The gel is placed carefully onto a pre-soak paper filter soaked with the transfer buffer. The gel is wet with additional transfer buffer and a pre-soak nitrocellulose membrane is placed over. Air bubbles are removed by rolling with a plastic pipet back and fourth over the surface. Another pre-soak filter paper layer is placed over the membrane and air bubbles are removed. The gel-membrane "sandwich" is placed between two sponge pads pre-soaked with transfer buffer, and inserted inside the blotting apparatus. The apparatus is filled with transfer buffer solution and constant current of 25 Volts is applied to the system for 3 hours. The blotting apparatus is kept on ice. After the blotting, the apparatus is disassembled and the membrane is stained 5 min with Pounceau staining solution for visualize proteins transferring and separation.

4.6.4. Protein immunodetection

After the blotting procedure, the nitrocellulose membrane is subjected to the following steps: incubation in blocking solution for 1 hour, incubation with primary antibody diluted in blocking solution for 1 hour, washing 3 times for 10 min in TBS buffer containing 0.1% Tween, incubation with secondary antibody diluted in blocking solution for 1 hour, washing 3 times for 10 min in TBS buffer containing 0.1% Tween, and finally development using the ECL system following manufacture instructions.

6. Results

6.1. The 2-AFF treatment blocks the hepatocyte proliferation

6.2. Oval cell proliferation and differentiation

6.3. Study of the oval cell induction

6.4. Construction of SAGE libraries

6.5. Transcriptome analysis of early stage of the oval cell regeneration by SAGE

6.6. Differentially expressed genes during the oval cell regeneration

6.7. Kinetic of differentially regulated genes during the oval cell regeneration

6.8. Regulation of protein expression of the cell cycle genes: CDC42 and cyclin D1

6.1. The 2-AFF treatment blocks the hepatocyte proliferation

We have studied the regenerating liver mass after PH operation in the model of oval cell regeneration in comparison with the normal regeneration where no 2-AAF treatment is achieved. In the oval cell model the liver mass did not raise significantly until 11 days after PH (from 33% to 57 +/- 11%), while after 16 days the liver mass was completely recovered (102 +/- 1 %). In the normal regeneration model, consisting in PH operation only, the liver mass increased sooner: at 2 days after PH the liver mass was almost duplicated (from 33% to 61%), and 7 days after PH the liver mass was nearly completely recovered (90%) (Figure 9). We conclude that the 2-AAF treatment impairs effectively the liver regeneration blocking the hepatocyte proliferation (Farber , 1956) .

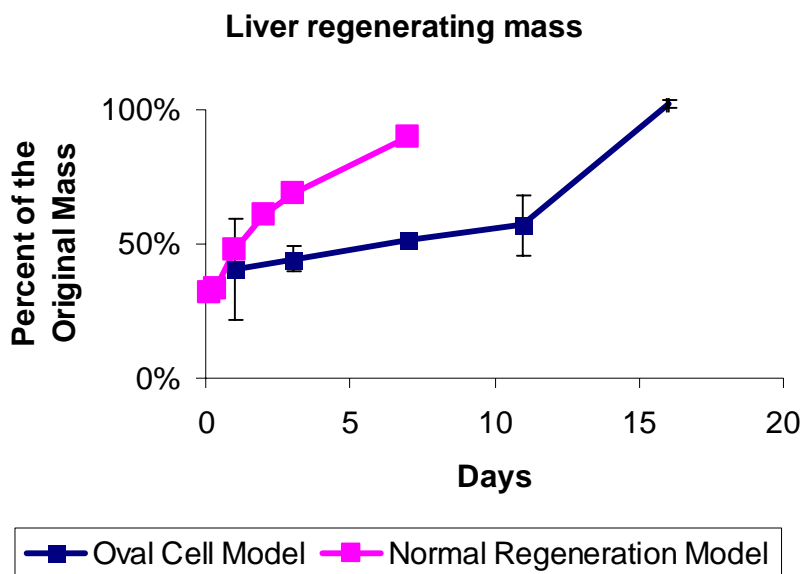**Liver regenerating mass**

Figure 10. Liver mass during the regeneration process: the liver mass in the model of oval cell regeneration (2-AAF/PH) was recovering slowly compared to the model of normal regeneration.

6.2. Oval cell proliferation and differentiation

We performed histological studies of the regenerating liver by Hematoxylin-eosin staining. Oval cells can be recognised in the liver tissue parenchyma by their characteristic morphology: they have scant cytoplasm, the most part of their volume consists of the nucleus, and the cell size (10 μm) is comparable to precursor biliary cells (Sell, 1997). Oval cells appeared inside the portal fields around the triads at 3 days after PH, they proliferated in the portal fields at 7 days after PH, and infiltrated the liver lobules at 11 days after PH. In particular at 11 days after PH basophilic foci of hepatocytes appeared in proximity of proliferating oval cells (Figure 11).

To study the proliferation of the oval cells we performed immunohistochemistry with the onco-foetal marker AFP of undifferentiated oval cells (Alpini et al., 1992). At 7 days after PH, we have stained undifferentiated positive AFP oval cells diffused in the portal field (Figure 12A). For characterising the differentiation of oval cells into hepatocytes and biliary cells we have studied respectively the expression of the hepatocyte marker Albumin and the biliary marker CK 7. At 11 days after PH, we have characterised Albumin protein expression of oval cells differentiating in hepatocytes inside basophilic foci (Figure 12B) (Shiojiri et al., 1991). At the later time point 16 days after PH, we have observed CK 7 positive oval cells differentiating in biliary cells and forming new bile ductules (ductular reaction) (Theise et al., 1999) (Figure 12C and 12D). Taken together the histological results, we can conclude that oval cells appeared inside the portal field at 3 days after PH, they strongly proliferated at 7 days after PH. Oval cell differentiated in hepatocytes at 11 days after PH and biliary cells at 16 days after PH (Figure 13)(Paku et al., 2001).
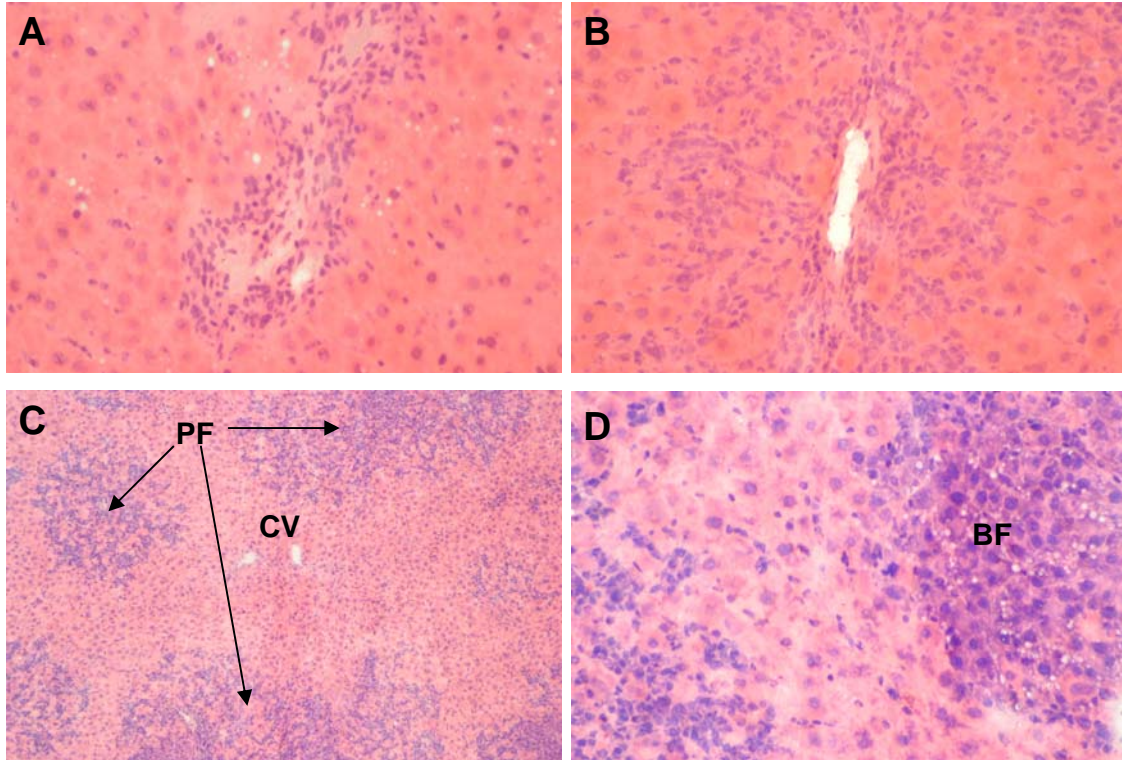
Figure 11. Hematoxylin-eosin staining of the liver tissue from different time points of oval cell regeneration: A) 3 days after PH, the oval cells appeared from the triads; B) 7 days after PH, the oval cells proliferated and migrated in the portal field area; C) 11 days after PH, the liver lobules are infiltrated by oval cells (PF, portal field; CV, central vein); D) 11 days after PH, basophilic foci (BF) of newly formed hepatocytes develops in the liver lobule from differentiating oval cells.
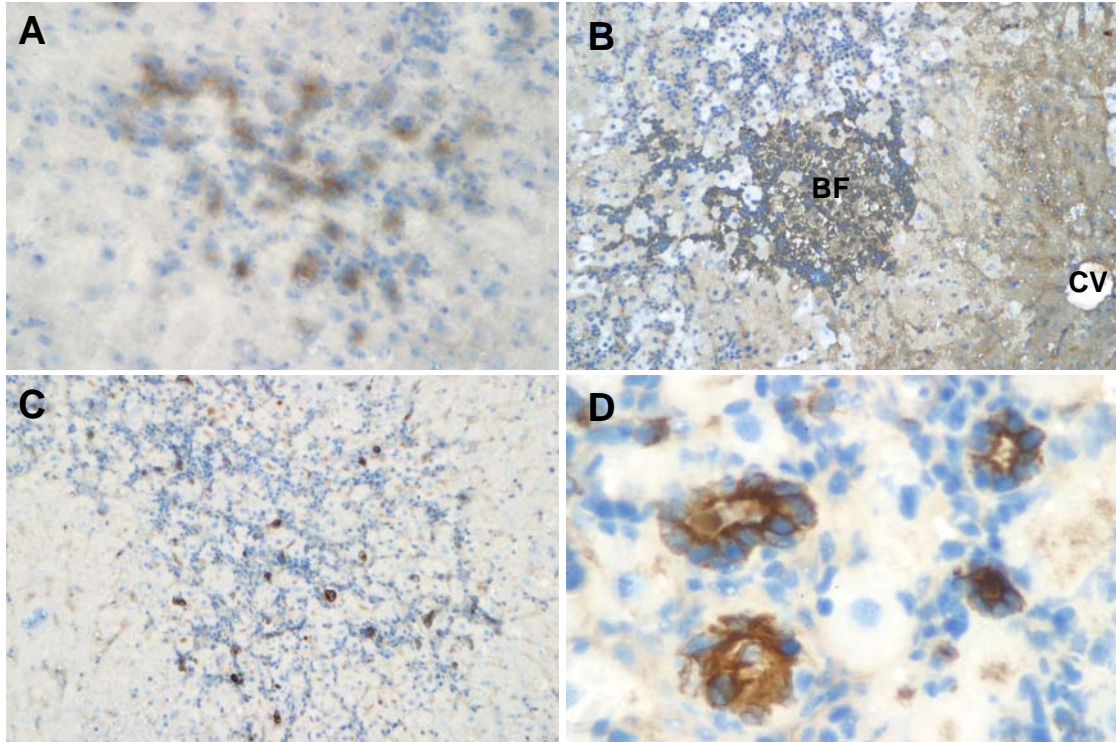
Figure 12. Immunohistochemistry study of oval cell proliferation and differentiation: A) 7 days after PH, oval cells were undifferentiated and AFP positive; B) 11 days after PH, albumin positive oval cells differentiated in hepatocytes inside the basophilic foci (BF, basophilic foci; CV, Central Vein); C) 16 days after PH, CK 7 positive oval cells differentiated in biliary cells; D) 16 days after PH, higher magnification of CK 7 positive oval cells formed ductular structures (ductular reaction).
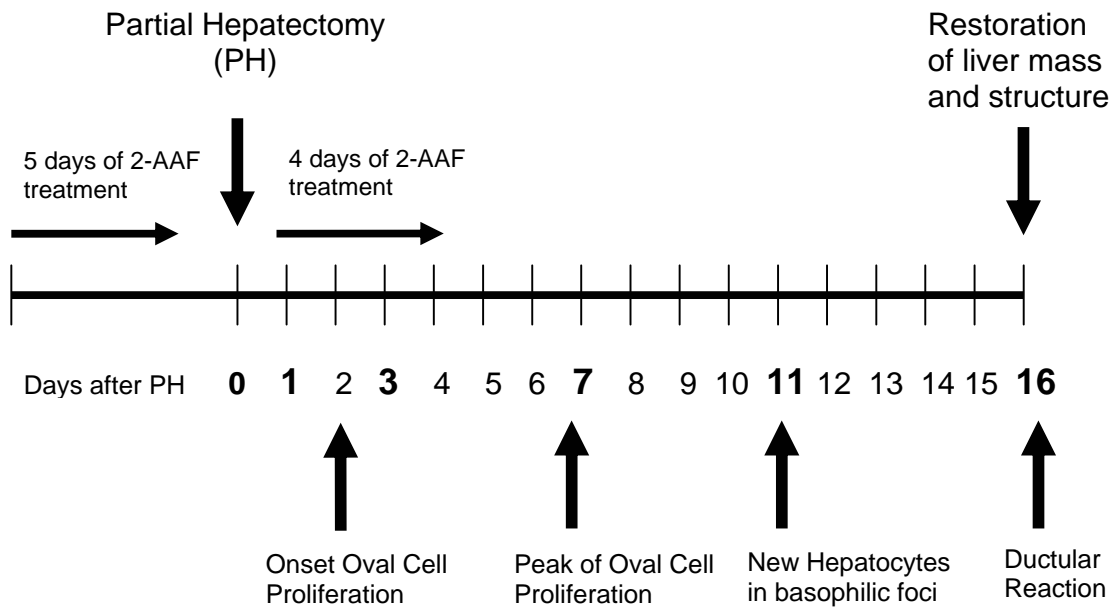
Figure 13. Description of the 2-AAF/PH model of oval cell regeneration. Oval cells originated at 3 days after PH in the portal field. Undifferentiated oval cells reached a peak of proliferation at 7 days after PH. Newly formed hepatocytes appeared in basophilic foci at 11 days after PH. New bile duct structures and ductular reactions were formed at 16 days after PH when the liver mass is restored.

6.3. Study of the oval cell induction

To determine the oval cell response on the transcriptional level in the regenerating liver samples, we analysed by Northern Blot hybridization the expression of AFP as a typical marker of the oval cell population (Figure 14A) (Lemire and Fausto, 1991). The AFP transcript became detectable at 3 days after the PH operation as reported earlier (Paku et al., 2001). AFP gene expression peaked at 7 days, and declined at 11 days after the PH and was hardly detectable in the later stages after PH. No induction of AFP transcript was observed in the corresponding sham operated control livers. In addition no AFP transcript was detected by Northern Blot in regenerating livers after PH only (data not shown).

By *in situ* hybridisation we characterised the spatial distribution of the AFP transcript in liver tissue (Figure 14B, 14C and14D). AFP positive oval cells were detected from 3 to 7 days after the PH operation; they seem to proliferate from the portal fields and to migrate inside the liver lobules, infiltrating the liver parenchyma toward the central veins. Based on the AFP expression kinetic, we decided to analyse the transcriptome in the early time point of oval cell regeneration at 3 days after PH operation.
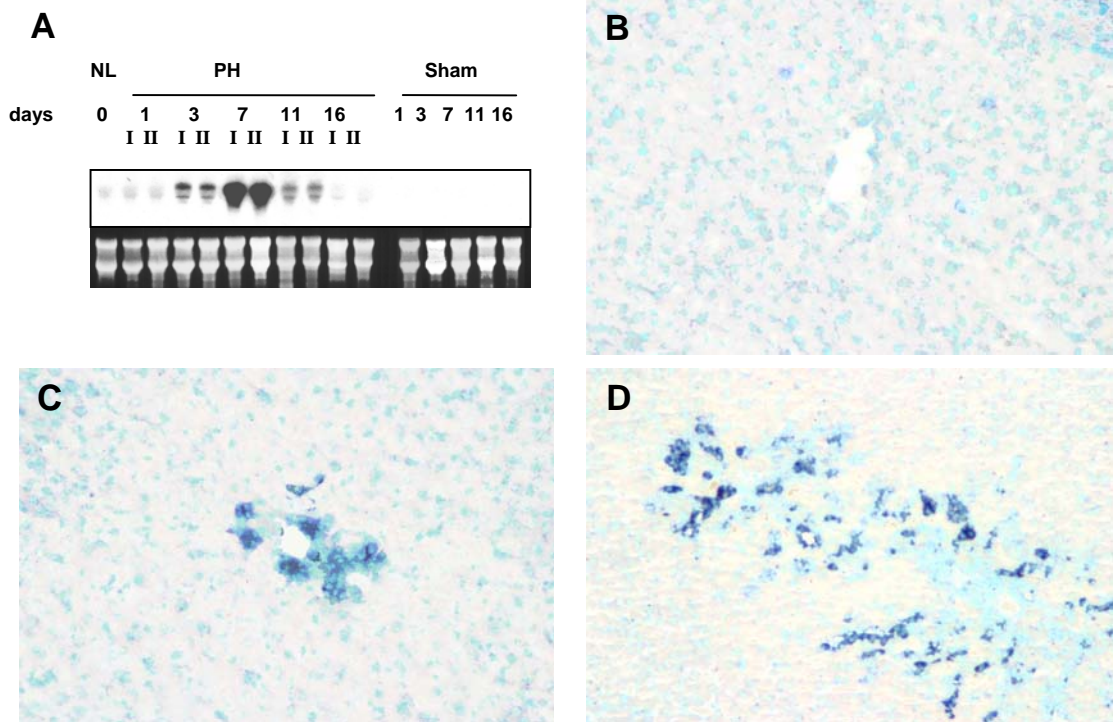
Figure 14. A) Northern blot analysis of the AFP expression during the oval cell regeneration process. The upper panel shows the hybridisation picture of the AFP gene in different RNA liver samples; in the PH series we analysed two different animals for each time point (I and II). The lower panel shows the ethidium bromide staining of the RNA electrophoretic gel (NL, Normal liver control; PH, partial hepatectomy operated; Sham, sham operated).

In situ hybridization with AFP probe in oval cell regenerating livers: the oval cells are expressing the AFP transcripts, appeared in the portal field area and proliferated inside the liver lobule: B) Normal Liver; C) 3 days after PH; D) 7 days after PH.

6.4. Construction of SAGE libraries

SAGE is a complex technique consisting in several steps (see Introduction: Figure 6, 7 and 8). Here we show the most relevant methodological steps of the SAGE technique (Munasinghe et al., 2001).

Initially, a SAGE library construction consists in converting the RNA sample in a double stranded cDNA. From each transcript a sequence of 10 bp called tag is extracted joined to an adapter linker cassette. The linker-tags construct are ligated tail to tail to generate the 108 bp ditag molecule. The ditags are PCR amplified and purified by polyacrylamide gel electrophoresis (Figure 15). The purified 108 bp ditags are digested with the "tagging enzyme" for the releasing of the linkers and generation of the "26 bp ditags" (Figure 16). The 26 bp ditags are gel purified and polymerized in long molecules called "concatemers" which can be cloned and sequenced efficiently (Figure 17). The insert from each bacterial colony can contains several tags (2 to 100 circa) which are punctuated by the restriction enzyme site "CATG" (Figure 18). The sequences were elaborated by bioinformatics tools for the tags identification and counting.
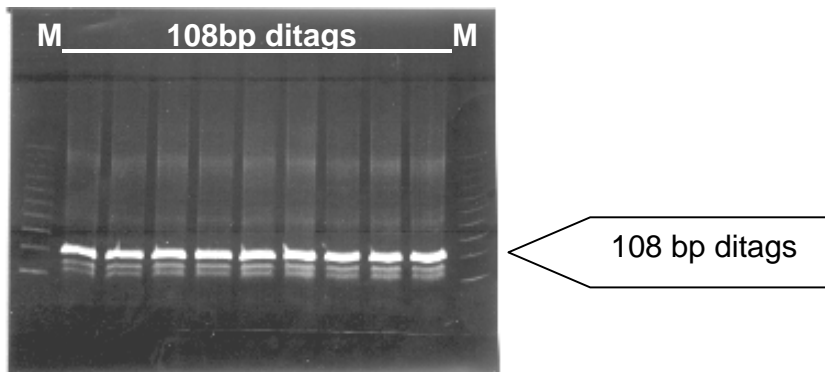
Figure 15. The arrow indicates the 108 bp ditags PCR product generated from a normal control rat liver sample (M, DNA markers). Additional band of 80 bp represent the PCR amplification of linker-linker side product of tag ligation to form ditag.
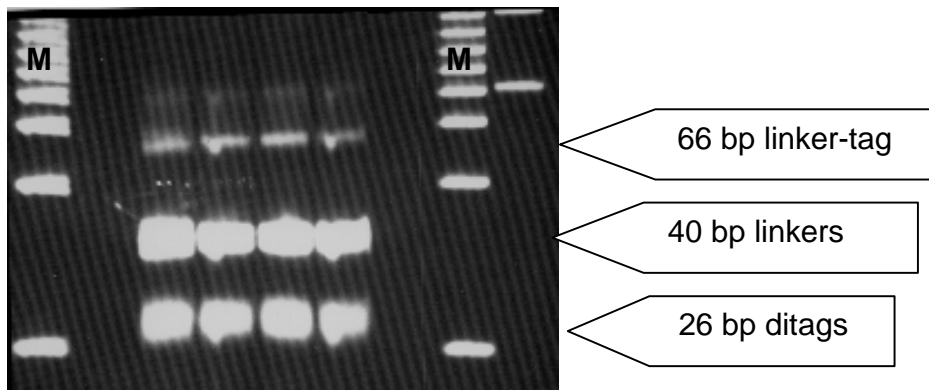
Figure 16. The arrow indicates the 26 bp ditags. In the gel there are also present bands representing: the 40 bp band of the linkers, the 66 bp band of partially digested ditags (M, DNA markers).
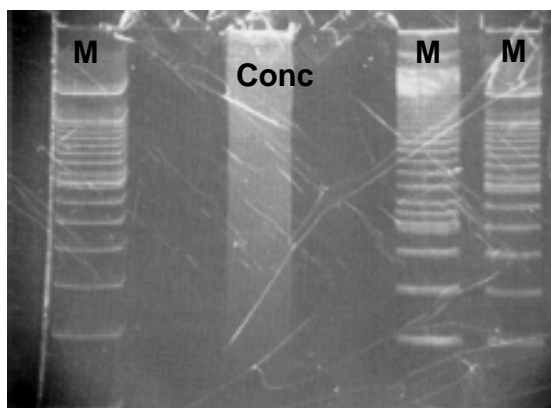
Figure 17. The 26 bp ditags are joined in a long concatemer (Conc, Concatemer; M, DNA markers).

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>grig0209f sequence exported from chromatogram file
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DITAG

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GCATGATACATCACTATAGGCGAATTGGGCCTCTAATGCATGGTGGGTCTG

GGGTCCTGAGTCTTCATGACACTGGGTGACTTCAATATGCCATGAAGACTC

AGGACTTACACTTGGCCATGATCATTCCGCACCCCACAGGTGCCATGATCA

TTCCGCACGTGGGGGGCATCCATGAGTATGATCGAGTAAGTGGGCATGCCT

GTACACCATCCTGATCTTCATGACCTTTGTATTTTCATGACC
```

Figure 18. DNA sequence from one of our clone from a SAGE library. Ditag can be identified by the restriction enzyme recognition site (CATG).

Three SAGE libraries were generated: from a normal rat liver control (NL library; 52,343 tags), from a sham rat liver control 2-AAF treated (SL library; 50,502 tags), and finally from a regenerating liver from an early stage of oval cell proliferation (OC library; 50,212 tags), for a total of 153,057 tags. We applied the Phred algorithm to the sequence chromatogram data for the control of the quality of the sequencing trace data, and in order to exclude of the poor quality data from the SAGE analysis and for reducing the generation of erroneous sequenced tags (Ewing et al., 1998;Ewing and Green, 1998;Margulies and Innis, 2000). Table 3 summarizes the SAGE statistics and the quality parameters. The number of tags accumulated indicates the size of the library. The number of unique tags is a theoretical parameter to estimate the total number of transcripts included in the library. The number of clones sequenced shows the amount of data must be elaborated. In particular we have found that the number of common tags in the different libraries is quite limited with a total of 3,514 tags; the number of unique tags in each library is 3-4 folds higher. Concerning the quality parameters, the number of tags sequenced from each clone indicates the efficiency of data

acquisition; the frequency of duplicated tags indicates if PCR amplification bias occurred; the linker contamination is a parameter indicating the degree of purity of the ditags from their linker adapter. The Guanidine-Cytosine content (GC content) is a parameter important for controlling if GC content bias occurred. GC content bias consist in an increase of GC content in the tags population sequences generated by melting of 26 bp tags during purification steps (Margulies et al., 2001b). In conclusion the data set of our SAGE libraries responds to the quality criteria defined by the different parameters. Gene expression profile of our libraries was compared with SAGE libraries from human and mouse liver previously described finding a good agreement (Yamashita et al., 2001;Yamashita et al., 2000;Kurachi et al., 2002).

Statistic Parameters	NL	SL	OC	Total
Accumulated tags	52343	50502	50212	153057
Number of unique tags	12291	12987	14110	39388
Number of clones sequenced	2094	2020	2092	6206
Number of common tags[a]				3514

Quality Parameters	Average
Tags per clone	25
Frequency of duplicated ditags[b]	8.90%
Linker contamination	0.37%
%GC content[c]	44%

[a]Tags observed in common between the three libraries.

[b]Examination of the most of the duplicated ditags reveals that are composed by the most abundant tags, hence they are not generated by PCR ditag amplification bias.

[c]See references (Margulies et al., 2001b).

Table 3. SAGE parameters: statistical and quality parameters are showing the good reliability of our SAGE data set.

6.5. Transcriptome analysis of early stage of the oval cell regeneration by SAGE

The transcriptome of a rat liver from an early time point of the oval cell regeneration (3 days after PH) was analysed by SAGE for studying the gene expression profile of early stage of proliferation and differentiation of the oval cells. By SAGE, we analysed also the gene expression profile of a liver from a normal control rat and a liver from a sham operated rat which was treated with 2-AAF. SAGE data demonstrates that each gene can be identified uniquely by a short sequence called tag and the frequency of the tag inside the library, is proportional to the expression or abundance of the relative gene (Velculescu et al., 1995;Velculescu et al., 2000).

The analysis of the global gene expression in the rat liver by SAGE showed that the transcriptome consists in a large number of low abundant genes represented by a low frequency number of tags while the very abundant genes are numerically few (Figure 19 and Table 4). Indeed, we have found that the genes represented by more than 10 tags are only 4.4% of the all transcriptome.
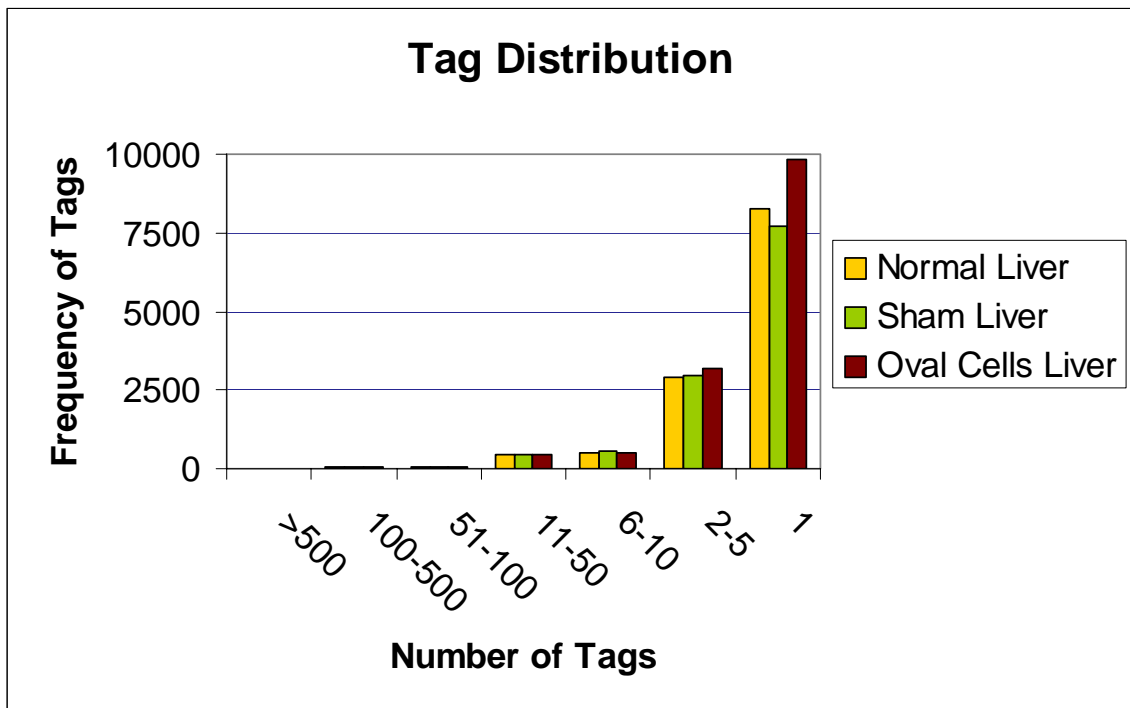
Figure 19. The histogram represents the gene expression profile of the liver transcriptome.

Tag Frequency	>500	100-500	51-100	11-50	6-10	2-5	1
% of Total, StDev	0.07+/- 0.01	0.33+/- 0.04	0.39+/- 0.08	3.61+/- 0.30	4.07+/- 0.43	23.87+/- 1.33	67.70+/- 2.06

Table 4.The table shows the percentage of each class of tag frequency respect the liver transcriptome (StDev, Standard Deviation).

The elaboration of the SAGE data demonstrated that the plasma protein mRNAs are the most abundant transcripts in the liver and they are constitutively expressed. Table 5 shows the most abundant transcripts represented in our libraries. Tags highly abundant match many plasma secreted proteins like albumin, fibrinogen and apolipoproteins. Previous SAGE library generated in human and mouse liver showed the high abundance of the plasma protein transcripts in the liver organ (Yamashita et al., 2001;Yamashita et al., 2000;Kurachi et al., 2002). The abundant genes were expressed without significant differences in respect to the number of tags in a comparison of the 3 libraries. Three abundant tags don't match any known gene (No match), and this result suggests the presence of potentially novel genes in the rat liver, still not characterised (Green et al., 2001). Considering the small size of each individual tag (10 bp), it seems possible that a tag is matching more than one gene. Indeed, in many previous studies such situation was already discussed (Margulies et al., 2001a;Pleasance et al., 2003). For example, the second abundant tag sequence matches two different genes belonging to the alpha-2U globulin family. Indeed, we found 4 abundant tags lad matching two different genes (data not shown). To overcome this problem, we analysed the 11th base pair of the tag consensus sequence for the determination of the right match like it was already described before (Wahl et al., 2004).

Description and Function	Unigene	TAG	NL	SL	OC
Albumin	34353	AAGACTCAGG	2691	2238	2059
Alpha-2u globulin PGCL1	86422	ATCATTCCGC	1617	1164	955
Alpha-2u globulin PGCL4	86413	ATCATTCCGC	1617	1164	955
Similar to Urinary protein 3 precursor	102085	TATATCATTA	1277	1278	1196
Protein tyrosine phosphatase, non-receptor type 1	11317	ATAACACATA	1140	680	645
ATPase inhibitor	108208	TATATCATAT	751	649	541
ATPase inhibitor	120356	TATATCATAT	751	649	541
No match		GATGCCCCCC	699	796	758
No match		ATACTGACAC	636	661	613
Transferrin	91296	CCCTGAGCGG	588	608	595
Apolipoprotein C-III	36813	CTGTCCCTAA	582	454	370
Betaine-homocysteine methyltransferase	11406	CCGGTTGAAA	564	172	134
No match		AAAAATCATC	467	406	327
Transthyretin	1404	AATACGCAGA	374	354	270
Apolipoprotein E	32351	ACCAGCCAGG	358	298	231
Glycerol-3-phosphate dehydrogenase 2	89705	AATCAATAAA	336	416	361
Haptoglobin	10950	ACACGGAGGA	331	375	341
Serine (or cysteine) proteinase inhibitor	1419	ATAGTTGAAT	305	336	294
Fibrinogen, alpha polypeptide	98846	AGCCATCCCT	285	270	294
Alpha-2u globulin PGCL1	86422	GAGCATCCTG	247	211	194
Alpha-2u globulin PGCL2	103016	GAGCATCCTG	247	211	194
Alpha-2u globulin PGCL3	86412	GAGCATCCTG	247	211	194
Alpha-2u globulin PGCL4	86413	GAGCATCCTG	247	211	194
Fibrinogen, gamma polypeptide	1702	CATTTCAATA	228	222	200
Retinol binding protein 4	108214	GGAATGCCTG	210	241	189
Cytochrome P450, subfamily IIC	10870	TGCTAAATAA	195	114	75
Fatty acid binding protein 1	36412	ACACTGGGTG	190	175	203
Carbonic anhydrase 3	1647	CCTATTAAAA	183	137	91
Alpha-2-HS-glycoprotein	32083	CCTTCTCTCC	182	147	186
Apolipoprotein C-I	8887	GCTTTGGAAG	179	163	140
Group specific component	1437	GTGGCCAAGC	177	194	134
Similar to Ac2-248 (LOC304917)	106537	GGGAGAGTGT	176	176	168
Serine protease inhibitor	97597	TTTCTGAACC	176	210	162

Table 5. The most abundant genes expressed in the rat liver identified in our 3 libraries (NL, normal liver; SL, sham liver; OC, oval cell liver).

6.6. Differentially expressed genes during the oval cell regeneration

We compared the tags by the statistical model of Audic and Claverie (Audic and Claverie, 1997), considering differentially regulated genes with p value of 0.1 in comparison of the oval cell liver (OC) library, the normal control (NL) library and the sham control (SL) library. In addition, we applied a second criterion; we considered differentially regulated tags from the OC library if up-regulated or down-regulated equal to or more than two fold. The statistical analysis of the liver libraries allowed the identification of 107 over-expressed and 54 down-regulated tags.

The most part of these differentially regulated tags could not be identified because they are probably belonging to novel genes. Only 27 induced genes and 18 repressed genes could be unambiguously identified by the SAGE map repository (Lash et al., 2000) (Table 6 and 7). Genes were classified by function in accordance with the OMIM repository (http://www.ncbi.nlm.nih.gov/), and Gene Ontology repository (http://www.geneontology.org/). Particularly we identified by SAGE up-regulated genes functionally classified in cell cycle genes (cyclin D1, and CDC42) and signal transduction genes (calmodulin 2, CD151, nicastrin, lipopolysaccharide binding protein, and asialoglycoprotein receptor 2). However, we have not found the AFP transcripts differentially regulated between the libraries and the number the AFP tag was quite low in each library (Table 6). The most of the down-regulated identified genes were metabolic enzymes (Table 7). Interestingly two of the down-regulated genes are involved in the vitamin K metabolism and synthesis (diaphorase 1, vitamin K epoxide reductase complex subunit 1) while other 8 genes are important for the steroid and fatty acid metabolism [hydroxysteroid (17-beta) dehydrogenase 10, electron-transferring-flavoprotein dehydrogenase, cytochrome P450 8b1, sulfotransferase hydroxysteroid gene 2, steroyl-coenzyme A desaturase 1, hydroxyacid oxidase 3, fatty acid coenzyme A ligase long chain 2, lisophospholipase 1].

Description and Function	Unigene	TAG	NL	SL	OC
Apoptosis and Cell Cycle					
Outer mitochondrial membrane receptor rTOM20	2143	CAGTTCAATG	1	0	4
Cyclin D1	22279	GTCCAGGAAA	1	1	5
Cell division cycle 42 homolog (CDC42)	60067	TGAGACAAGG	4	4	11
Signal Transduction					
Calmodulin 2	5968	TTGCTGTTGA	3	1	8
CD151 antigen	1465	GCTGTTTTCA	4	1	8
Nicastrin	51975	CTGTTAGGTG	1	1	11
Lipopolysaccharide binding protein	48863	TCTGACGGAG	6	9	17
Asialoglycoprotein receptor 2	9834	AGGGGGGTTA	7	9	18
Cytoskeleton Associated Protein					
Stathmin 1	555	AAAAAGAAGT	1	0	4
Microtubule-associated protein tau	2455	GAGAGAAGAG	0	0	4
Tropomyosin isoform 6	37575	AAAGTCATCT	1	1	5
E-Tropomodulin	1646	AAGTTGGCAT	0	0	6
Enzyme					
Carnitine palmitoyltransferase 2	11389	TAGCTGGAAG	1	3	8
Thioredoxin-like 2	3578	AAGCATAGAA	5	7	18
Protein Metabolism					
Eukaryotic translation elongation factor 2	55145	AACTTCAGGA	0	0	6
Ribosomal protein L27	1254	CCCACAAGGT	10	12	24
Transcription Regulator					
Nuclear receptor subfamily 1, group H member 2	786	CACCTTGAGT	2	2	8
Transporter and Vesicle Trafficking					
Ruby-eye-like protein	69203	TTTCGGGCAC	0	0	4
Adaptor-related protein complex 2, beta 1 subunit	56138	CTGTACCAGT	0	0	4
Na/P_i cotransporter 4	16357	AAGGTGTGTT	0	1	7
Adaptor protein complex AP-2, alpha 2 subunit	34928	ACTACCGGGC	2	3	10
Multidrug resistance 1	82691	GCACGTAACC	0	2	11
Phosphatidylcholine transfer protein	9487	AATAAACACA	5	3	11
ATPase H$^+$ 34Kda lysosomial transporter	106041	AACAGAGTAA	1	8	16
Solute carrier family 28, member 2	10140	GAAGTCGGAA	2	13	93
Plasma Protein					
Complement component factor H	101777	AACTAGAAAG	1	1	5
Alpha-2-HS-glycoprotein variant	32083	CGGTGGAGGG	5	21	46
Alpha foeto-protein (AFP)	9174	TGGGATTTCT	4	1	3

Table 6. Up-regulated genes in the oval cell liver regeneration and AFP gene (NL, normal liver; SL, sham liver; OC, oval cell liver).

Description and Function	Unigene	TAG	NL	SL	OC
Enzymes					
Diaphorase 1	35994	ATGCCTTGAG	4	5	0
Hydroxysteroid (17-beta) dehydrogenase 10	2700	ACACTGCCCA	5	5	0
Dipeptidylpeptidase III	10902	TGACAGAGCC	4	5	0
Vitamin K epoxide reductase complex subunit 1	97942	AGCCTTGCCC	8	7	1
Hydroxyacid oxidase (glycolate oxidase) 3	10417	CCAATTACCT	10	9	1
Electron-transferring-flavoprotein dehydrogenase	37277	AAATATTTGT	9	8	2
Fatty acid Coenzyme A ligase, long chain 2	6215	TCTTCAGAAG	11	7	2
Lysophospholipase 1	3594	TTTTTCACAG	17	10	2
Liver glycogen phosphorylase	21399	GAGCCTTCCG	10	8	3
Selenium binding protein 2	16617	ATGAGACCAT	10	10	4
Histidine ammonia lyase	10037	GCAGCAATAC	14	12	5
Cytochrome P450, 8b1	23013	TGTGCAATTG	31	20	6
Sulfotransferase hydroxysteroid 2	91378	GGAATAAAAT	21	16	6
Steroyl-coenzyme A desaturase 1	1023	GCAGAGGTTT	33	20	11
Transporter and Vesicle Trafficking					
ATP-binding cassette, subfamily D, member 3	7024	TCTCCTTAGC	7	5	1
Solute carrier family 10, member 1	9913	TTACTCAAAA	51	41	18
Plasma Protein					
Apolipoprotein A-II	89304	AAGCTGCTTG	29	21	5
Clusterin, apolipoprotein J	1780	TCTCCAGCGC	25	24	11

Table 7. Down-regulated genes in the oval cell liver regeneration (NL, normal liver; SL, sham liver; OC, oval cell liver).

Considering the number of genes differentially regulated in each functional group, it is possible to characterize which metabolic pathway is regulated. The histogram shows how apoptosis-cell cycle, signal transduction, cytoskeleton associated proteins and transporter-vesicle trafficking genes were particularly up-regulated while enzyme genes were repressed during the early stage of oval cell regeneration (Figure 20).
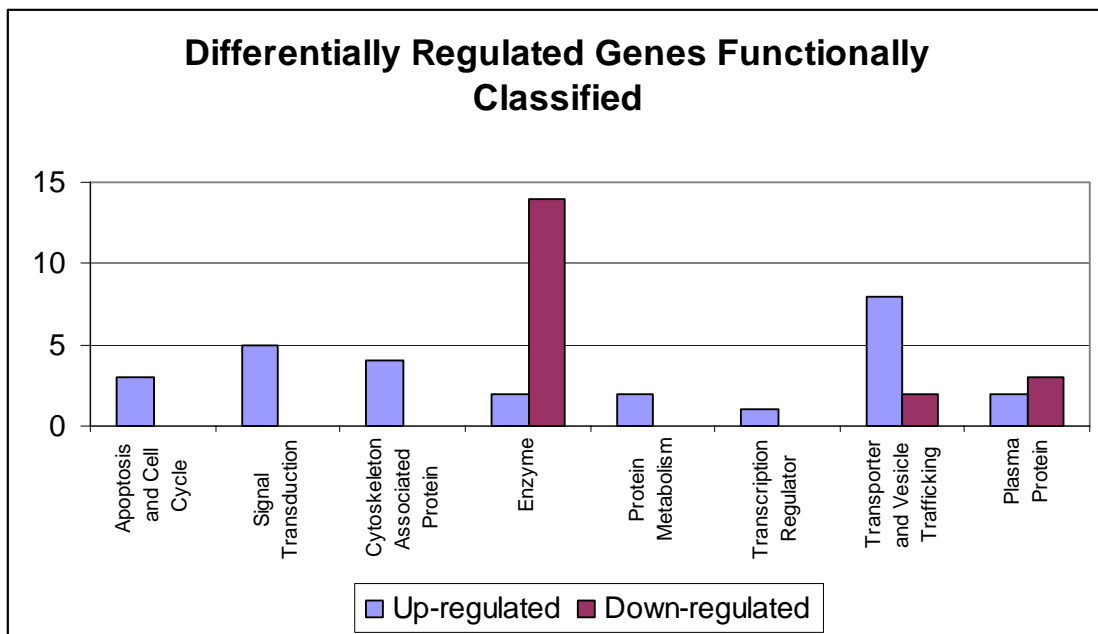

Figure 20. Differentially regulated genes were classified in accordance with their function. The oval cell regeneration was characterized by the up-regulation of apoptosis-cell cycle, signal transduction, cytoskeleton associated protein, and transporter-vesicle trafficking genes, and by the down-regulation of enzyme genes.
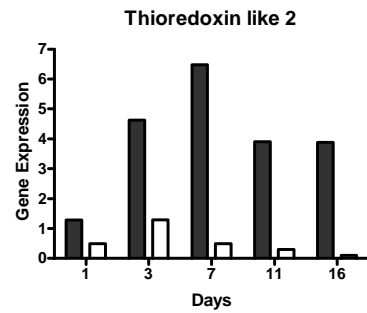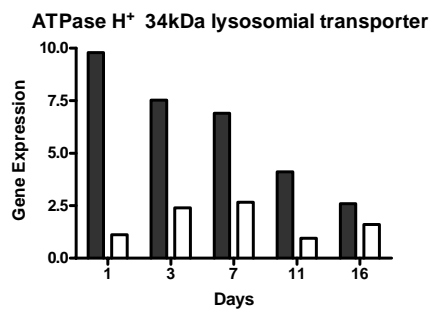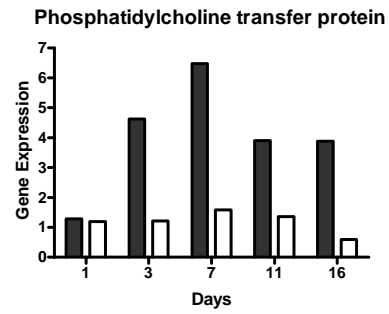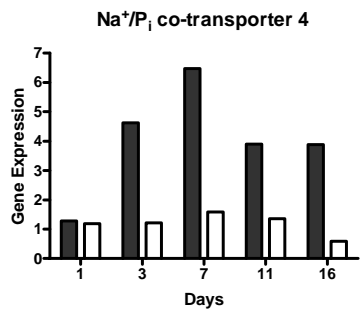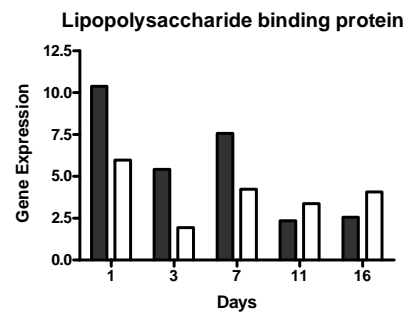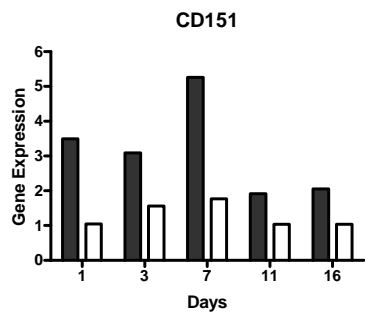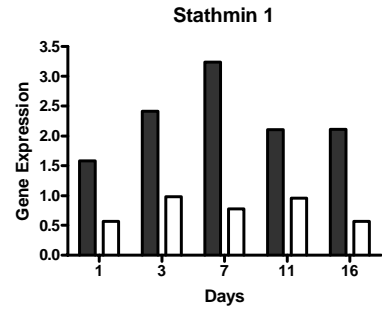
6.7. Kinetic of differentially regulated genes during the oval cell regeneration

Real Time PCR experiments are strongly affected by the normalisation in reference to a housekeeping gene; hence a proper reference gene is a pivotal factor for the analysis of relative gene expression levels (Dheda et al., 2004;Vandesompele et al., 2002). By SAGE, it was possible not only to identify the differentially regulated genes, but also to identify the most reliable and stable housekeeping gene in our model. The classical housekeeping gene ubiquitin C had very similar number of tags among the 3 libraries, while beta-actin transcripts showed a variable number of tags between them (Table 8). Hence we performed normalisation in our Real Time PCR experiments using the ubiquitin C gene. Up-regulated and down-regulated genes were analysed by Real Time PCR. We analyzed the kinetics of gene expression in regenerating and sham control livers, at the time points of 1, 3, 7, 11, and 16 postoperative days, in respect to the normal liver control (Figure 21). Differentially regulated genes were classified as: 1) "Early up-regulated", when a gene was immediately induced after 1 day PH; 2) "Alpha foeto-protein co-expressed genes" when their kinetic of induction was similar to AFP; 3) "Down-regulated genes" (Figure 20, Table 9). We found as early up-regulated genes: cyclin D1, stathmin 1, CDC151, lipopolysaccharide binding protein, thioredoxin-like 2, Na^+/P_i cotransporter 4, phosphatidylcholine transfer protein, and ATPase H^+ 34 kDA lysosomial transporter (Figure 21A). The AFP co-expressed genes were: CDC42, and E-tropomodulin, which shows to be strongly induced at 7 days after PH (Figure 21B). The down-regulated genes included: hydroxyacid oxidase 3 and fatty acid CoA ligase long chain 2 which started to be down-regulated immediately after PH, while steroyl CoA desaturase 1 gene was strongly down-regulated at 7 days after PH (Figure 21C).

Description and Function	Unigene	TAG	NL	SL	OC
Ubiquitin C	3761	CAGATCTTTG	33	38	38
Beta Actin variant I	94978	CCCTGAGTCC	17	7	9
Beta Actin variant II	94978	GCTTTATTGT	15	8	4
Beta Actin variant III	94978	GATGCAGCCA	12	6	10
Beta Actin variant IV	94978	AAAATCAAAA	3	0	0
Beta Actin variant V	94978	AAGATCAAGA	1	6	7

Table 8. Ubiquitin C housekeeping gene was stable and more reliable than the beta actin gene in our model of oval cell liver regeneration (NL, normal liver; SL, sham liver; OC, oval cell liver).

A

Early up-regulated genes

Figure showing bar charts of gene expression over days (1, 3, 7, 11, 16) for: Cyclin D1, Stathmin 1, CD151, Lipopolysaccharide binding protein, Na⁺/Pᵢ co-transporter 4, Phosphatidylcholine transfer protein, ATPase H⁺ 34kDa lysosomial transporter, and Thioredoxin like 2.

B

Alpha foeto-protein co-expressed genes

Alpha foeto-protein

CDC42

E-tropomodulin

C

Down-regulated genes

Hydroxyacid oxidase 3

Fatty acid CoA ligase long chain 2

Stearoyl CoA desaturase 1
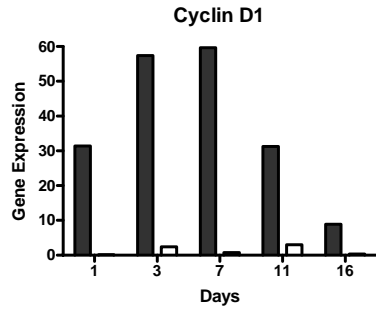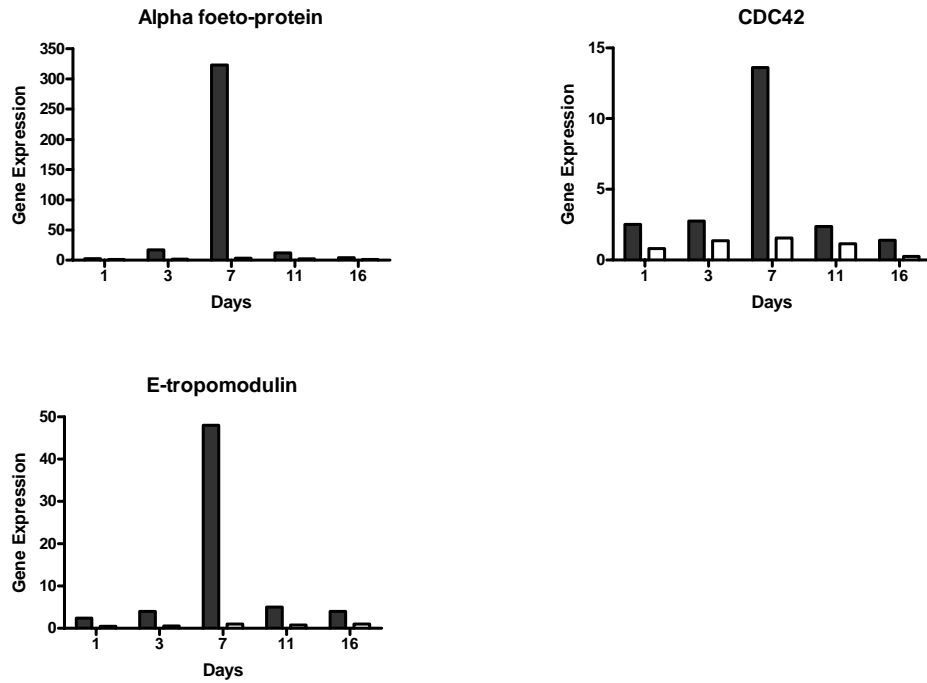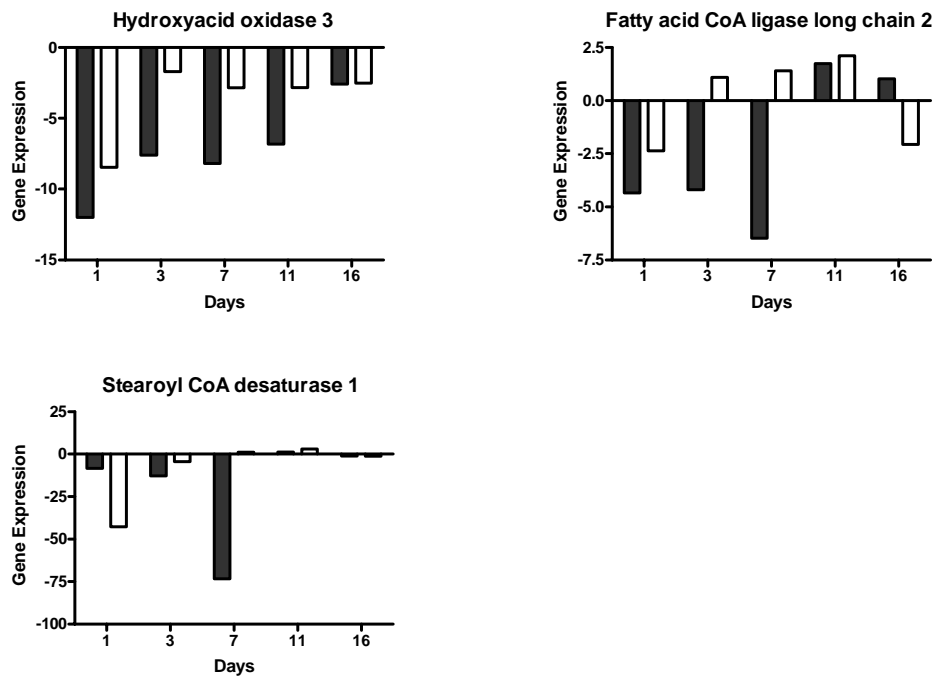
Figure 21. Real Time PCR of kinetically differentially regulated genes during the oval cell regeneration (black bars, PH livers; white bars, sham livers). We have classified the kinetically up-regulated genes according to their temporal expression: A) early up-regulated genes were induced after 1 to 3 days after PH, B) alpha foeto-protein co-expressed genes had kinetic of expression similar to the AFP gene which was strongly expressed at 7 days after PH; C) down-regulated genes.

Early up-regulated genes	Alpha foeto-protein co-expressed genes
- Cyclin D1 - Stathmin 1 - CD151 - Lipopolysaccharide binding protein - Na$^+$/P$_i$ co-transporter 4 - Phosphatidylcholine transfer protein - ATPase H$^+$ 34Kda lysosomial transporter - Thioredoxin like 2	- Cell division cycle 42 (CDC42) - E-tropomodulin
	Down-regulated genes
	- Hydroxyacid oxidase 3 - Fatty acid CoA ligase long chain 2 - Steroyl CoA desaturase 1

Table 9. Genes kinetically regulated during the oval cell regeneration characterised by the Real Time PCR method.

6.8. Regulation of protein expression of the cell cycle genes: CDC42 and cyclin D1

We studied by Western Blot the regulation of protein expression of the two cell cycle genes CDC42 and cyclin D1 compared to the oval cells marker AFP during the regeneration process. Interestingly, the cell cycle proteins CDC42 and cyclin D1 were induced when also the AFP was detectable, at 7 and 11 days after PH (Figure 22). We believe that such temporal co-expression of CDC42 and cyclin D1 with the AFP could be related to the expression of those cell cycle genes in the proliferating oval cells during the liver regeneration.
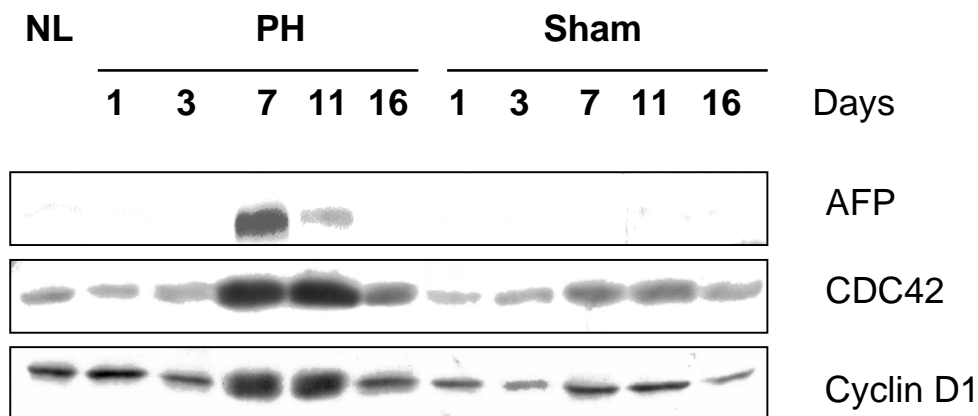
Figure 22. Western Blot analysis of AFP and the cell cycle genes CDC42 and cyclin D1 in oval cell regenerating livers. CDC42 and cyclin D1 were over-expressed at 7 and 11 days after the PH and co-expressed with the oval cell marker AFP.

7. Discussion

7.1. The oval cells are facultative stem cells able to generate hepatic lineages

7.2. Transcriptome profile of early stage of oval cell regeneration by SAGE

7.3. Comparison between SAGE and Microarray results in the study of oval cell liver regeneration

7.4. Study by Real Time PCR and Western Blot of the differentially regulated genes

7.5. Possible role of the kinetically regulated genes in the oval cell regeneration

7.6. Final Remarks

7.1. The oval cells are facultative stem cells able to generate hepatic lineages

The liver has the potential to regenerate by itself after an injury (e.g. toxic injury or viral infection). In normal condition the hepatocytes are the cell population responsible for the liver growth and for the maintaining of the liver mass (Michalopoulos and DeFrances, 1997). It is particular remarkable that in normal state the hepatocytes are quiescent and rarely divide (Zimmermann, 2002). But when an alteration of hepatic tissue takes place like cell loss or surgical tissue resection, the hepatocytes can proliferate until the organ has not reached the original mass and function (Fausto, 2004). By transplantation experiments in mouse models was demonstrated that the hepatocytes can undergo clonogenic expansion and restore the liver mass (Overturf et al., 1996;Rhim et al., 1994). In clinical studies it was described that under particular circumstances in which the proliferation of pre-existing hepatocytes is impaired (e.g. carcinogenesis, massive necrosis and toxic injury), hepatic stem cells can be activated and originate hepatic lineages. It is believed that hepatic stem cells, called "oval cells" for their particular shape, are derived from biliary cells located in ductules of Hering, and they are considered as a second facultative compartment for the liver regeneration (Alison, 1998).

In the rat it is possible to trigger oval cell proliferation and liver regeneration, combining a regenerative stimulus of PH operation with an inhibition of the hepatocytes proliferation by 2-AAF treatment (2-AAF/PH). Indeed 2-AAF is metabolised to its cytotoxic and mitoinhibitory N-hydroxy derivative molecule by the phase I metabolic enzymes which are strongly expressed specifically in adult hepatocytes but relatively low expressed in biliary cells and oval cells (Alison et al., 1998). In the present study, by measuring the regenerative liver mass in the model of 2-AAF/PH in comparison with the model of PH alone, we have observed how the recovery of the liver mass was strongly impaired by the 2-AAF treatment. Indeed, the liver mass in the model of 2-AAF/PH was not increased significantly until 11 days after the PH (from 33% to 57% +/- 11%) and it was recovered completely only at 16 days after the PH. In the model of normal regeneration by PH only the liver

growth was much faster and at 7 days after PH the liver mass was nearly completely restored (90%) (see Results: Figure 9). Taking into consideration the studies of the liver growth in the two models of regeneration (PH and 2-AAF/PH), we can conclude that the hepatocyte proliferation is strongly inhibited by the 2-AAF treatment after the PH operation. The suppression of the hepatocyte proliferation by 2-AAF after the PH is a necessary condition for the oval cell regeneration of the liver.

We have characterised the oval cells development and differentiation in the regenerative liver by the histological techniques of hematoxylin-eosin and immunohistochemistry staining. Oval cells appeared at 3 days after PH inside the portal field. Afterwards, oval cells reached a peak of proliferation at 7 days after PH and infiltrated the liver lobule while they were still undifferentiated and expressing the AFP marker. Following proliferation and migration, oval cells were able to differentiate in hepatic lineages. Basophilic foci of newly formed hepatocytes expressing the albumin protein emerged at 11 days after PH. New CK 7 positive ductular biliary structures were formed at 16 days after PH by oval cell ductular reaction (see Results: Figure 10, 11 and 12).

To study the oval cell induction at transcriptional level, we have analysed the AFP transcript expression by Northern Blot and Real Time PCR. We have temporally characterised the AFP transcript expression during the regeneration process. AFP started to be detectable at 3 days after PH, was strongly induced at 7 days after PH, declined at 11 days after PH, and returned to its basal level at 16 days after PH (see Results: Figure 13A, Figure 20B). Such a kinetic of AFP regulation was already described previously in the same model (Lemire and Fausto, 1991). To characterise the spatial distribution of the AFP transcript, we have performed non-radioactive *in situ* hybridisation (see Results: Figure 13B, 13C, and 13D). AFP positive oval cells were individuated inside the portal field at 3 days after PH and they diffused in the liver parenchyma at 7 days after PH. Based on the histological analysis and the AFP transcript expression kinetic we decided to apply SAGE at 3 days after the PH operation in order to analyse the early expressed genes responsible for the oval cell development in the regenerative liver.

7.2. Transcriptome profile of early stage of the oval cell regeneration by SAGE

SAGE offers a digital analysis of gene expression: ideally each gene is represented uniquely by a SAGE tag and the frequency of the tag found in the library is proportional to its level of expression (see Results: Table 5) (Velculescu et al., 1995). The SAGE method allows the quantitative and simultaneous analysis of a large number of transcripts and the identification of novel genes (Green et al., 2001). SAGE is a convenient technique which allows to circumvent the unwanted unspecific cross-hybridisation typical of the hybridisation methods like the Microarray technique (Lee et al., 2004). Three libraries for a total of 153,057 tags were generated using the liver samples from a normal rat control (library NL, 52,343 tags), from a sham control treated with 2-AAF (library SL, 50,502 tags), and from an early stage of the oval cell proliferation at 3 days after PH (library OC, 50,212 tags). The SAGE data were evaluated by different statistic and quality parameters; in the Table 3 we have reported the good reliability of our SAGE data in agreement with the literature (Margulies et al., 2001b;Wahl et al., 2004). Analysing our SAGE data set we have found that the global gene expression of the rat liver is characterized by the expression of a large number of low abundant genes represented by less than 10 tags of frequency (95,6%) and few genes represented by more than 10 tags of frequency (4,4%) (see Results: Figure 18, Table 4). Such a situation was previously described not only in human and mouse liver, but more generally in every cell and tissue by the SAGE method (Yamashita et al., 2001;Kurachi et al., 2002;Blackshaw et al., 2003). We believe that the transcriptome consists of a few very abundant genes, while the most part of the genes is expressed at a low level and they could be switched alternatively on or off. Our SAGE data from the 3 libraries showed that the plasma protein mRNAs are the most abundant transcripts in the liver, which we found to be in agreement with the already described SAGE libraries from human and mouse livers (Yamashita et al., 2001;Yamashita et al., 2000;Kurachi et al., 2002). The number of tags of the most abundant genes between the libraries shows that they were stably expressed

during the regeneration process (see Results: Table 5). By statistic analysis we identified 107 over-expressed and 54 repressed tags. Most of the tags differentially regulated were not matching any known genes and for this reason we can consider them probably novel. We identified by reliable match 45 differentially expressed genes in the oval cell model of rat liver regeneration from which 27 were up-regulated and 18 down regulated (see Results: Table 6 and 7). Although we detected over-expression of AFP by Northern Blot hybridisation and Real Time PCR at 3 days after PH, however, on our SAGE data set, the AFP tag (TGGGATTTCT) was represented by a very low number of tags in the different libraries (library NL, 4; Library SL, 1; Library OC, 3) and was not found induced in the OC library as expected (see Results: Table 6). In the literature it was already described the limitation of the SAGE method in representing the real level of expression of the very low abundant marker genes (Wahl et al., 2004;Cornelissen et al., 2003). Indeed, we have seen by Northern Blot and Real Time PCR that the AFP transcript has a robust expression only at 7 days after PH, while at 3 days after PH (analyzed by SAGE) its level of expression is 20 times minor. In addition we have found a limited number of tags between the different libraries 3-4 fold times minor in comparison to the number of unique tags in each library (see Results: Table 3), which cannot be explained by the different patterns of gene expression. Rather, we believe that such a situation is reflecting the limitation of the SAGE method in representing the entire transcriptome including the very low abundant genes (Anisimov et al., 2002;Stern et al., 2003). SAGE is a sampling method based on information data from a limited number of accumulated tags, and indeed we have calculated that for achieve a fully comprehensive SAGE library from the rat liver are required at least 200,000 accumulated tags (data not shown). We have functionally classified the differentially regulated genes and we have found that during the early stage of oval cell regeneration the up-regulated genes belong to the functional groups of: cell cycle-apoptosis, signal transduction, cytoskeleton associated proteins and transporter-vesicle trafficking. While, we have found 14 enzyme genes are down-regulated (see Results: Figure 19). We believe that induced genes functionally classified in cell cycle, signal transduction and

cytoskeleton associated protein could be directly involved in the proliferation and migration of the oval cells during the regeneration process, while the role of the induced transporter-vesicle trafficking genes needs further examination. The repression of such large number of enzymes could be the result of the strong injury of the PH operation combined with the 2-AAF treatment which could affect the normal liver metabolic functions.

7.3. Comparison between SAGE and Microarray results in the study of oval cell liver regeneration

Recently gene expression profiles in two different models of oval cells regeneration in mice, by the Microarray technique has been described (Arai et al., 2004). In their first approach, a group of mice (5) were subjected to the 2-AAF/PH protocol and the regenerating liver were analysed 48 hours after the PH operation. In the second approach, another group of mice (5) was feed with a choline deficient diet (CD) and then sacrificed 3 weeks later. They analysed the pattern of the gene expression profile in the oval cells regeneration using as controls two PH operated mice without 2-AAF treatment and sacrificed 48 hours later. Excluding the EST sequences, they described 22 genes up-regulated in the 2-AAF/PH model and 24 genes up-regulated in the CD model. Only 6 genes were found up-regulated in the 2 models demonstrating how the gene expression profile is strongly depending by the protocol used for inducing the regeneration. We performed a comparison of their described results with our data and we have found no any relevant overlap; indeed only a variant of the Fetuin/Alpha-2-HS-glycoprotein gene was found up-regulated in our and their results. We conclude that the molecular mechanism of liver regeneration by induction of oval cells is strongly depending by the protocol and the animal model used. We believe that the use of a proper control is a pivotal factor to determine the genes involved in the liver regeneration. While they analysed the oval cell regeneration gene expression profile using as a control livers from PH animals without any chemical treatment, we used as controls not only a normal liver but also a sham animal which received the chemical treatment (2-AAF). Indeed, we believe that such control is important for identify the genes induced or repressed by the acute phase response and the chemical intoxication. While 2-AAF/PH and CD diet are protocols quite effective in rat for triggering oval cell in the liver, in mice animals such protocols have not been applied and characterised. Indeed, the most common protocols for activate murine oval cells are the Dipin/PH model (Engelhardt et al., 1990;Factor and Radaeva, 1993) and the cocaine treatment model (Rosenberg et al., 2000). However many authors

lately use an alternative protocol consisting in a treatment with the chemical 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) in a standard chow at a concentration of 0.1% for 4 to 6 weeks (Preisegger et al., 1999). DCC protocol is supposed to be more effective and suitable also for murine oval cell isolation (Preisegger et al., 1999;Petersen et al., 2003;Wang et al., 2003). We conclude that Microarray is an alternative technique for the study of gene expression profile which can be conveniently applied for the study of oval cell regeneration with the appropriated controls and a reliable model.

7.4. Study by Real Time PCR and Western Blot of the differentially regulated genes

Differentially regulated genes were analysed by Real Time PCR and their kinetic of expression was characterised during the regeneration process.

The normalisation in the Real Time PCR experiment is critical to measure the regulation of the gene expression (Dheda et al., 2004;Vandesompele et al., 2002). The beta-actin gene was shown by our SAGE data to be not enough stable and reliable housekeeping gene for this model (see Results: Table 6). By our SAGE data, we have seen that ubiquitin C expression is not affected by the different physiopathological situations of our liver samples, hence we have considered ubiquitin C as an alternative housekeeping gene (see Results: Table 6).

We classified the differentially regulated genes by their kinetic of regulation: 1) "Early up-regulated genes" which are induced after 1 to 3 days after PH (cyclin D1, stathmin 1, CDC151, lipopolysaccharide binding protein, thioredoxin-like 2, Na^+/P_i cotransporter 4, phosphatidylcholine transfer protein, and ATPase H^+ 34 kDA lysosomial transporter) , 2) "Alpha foeto-protein co-expressed genes" when the kinetic of regulation is similar to the AFP (CDC42 and E-tropomodulin), 3) "Down-regulated genes" (hydroxyacid oxidase 3 fatty acid CoA ligase long chain 2 starting, and steroyl CoA desaturase 1) (see Results: Figure 20A, 20B, 20C and Table 7).

Two cell cycle genes are kinetically regulated during the regeneration process: CDC42 and cyclin D1. By Real Time PCR we have seen that the CDC42 transcript is co-expressed with AFP having the same kinetic of regulation, while cyclin D1 is earlier induced at 1 day after PH declining later at 11 days after PH.

By Western Blot we studied the protein expression of CDC42 and cyclin D1 in comparison with AFP (see Results: Figure 21). Interestingly, the proteins of the two cell cycle genes were induced concomitantly with the expression of the AFP protein at 7 and 11 days after PH. We believe that CDC42 and cyclin D1 proteins could be expressed specifically in the oval cells population and important for the molecular mechanism of oval cell proliferation.

7.5. Possible role of the kinetically regulated genes in the oval cell regeneration

We have analysed by SAGE the liver transcriptome in an early proliferation of the oval cell compartment and studied by Real Time PCR the kinetic of expression of the differentially regulated genes induced or repressed in the liver during the oval cell regeneration. We have found 10 up-regulated genes and 3 down-regulated genes kinetically regulated during the oval cell regeneration never characterised before. The up-regulated genes functionally characterised are: cell cycle genes like CDC42 and cyclin D1; cytoskeleton associated proteins like stathmin 1 and E-tropomodulin, signal transduction genes like CD151 and lipopolysaccharide binding protein; transporter genes like Na^+/P_i Co-transporter 4, phosphatidylcholine transfer protein, and ATPase H^+ 34 kDa lysosomial transporter; and finally anti-apoptotic enzyme gene like thioredoxin like 2. We have found 3 kinetically down-regulated genes involved in the lipid metabolism: hydroxyacid oxidase 3, fatty acid CoA ligase long chain 2, and steroyl CoA desaturase 1.

In particular we have studied the protein expression of the identified up-regulated cell cycle genes CDC42 and cyclin D1, finding them co-expressed temporally with the AFP protein concomitant with the proliferation of the oval cells inside the liver. We believe that CDC42 and cyclin D1 proteins could be important for the oval cell proliferation during the liver regeneration and CDC42 could be a molecular key signal cascade regulator for triggering the oval cell proliferation by induction and activation of the cyclin D1 protein (Gjoerup et al., 1998;Roovers and Assoian, 2003). CDC42 is a guanosine tri-Phosphate hydrolases (GTPases) belonging to the Rho family and it's a signal molecule involved in several pathways activated by the Ras effectors (Macaluso et al., 2002). The cyclin D1 expression and activity is regulated by the small GTPase, such as Ras, Rho, Rac and finally CDC42. Indeed in a later report in the fibroblast cell line NIH 3T3, was shown that Rac/CDC42 pathway induces the accumulation of cyclin D1 with subsequent retinoblastoma hyperphosphorilation and elongation factor 2 (EF2) mediated transcription (Gjoerup et al., 1998). CDC42 is not only involved in the cell cycle control, but is also a key

molecular signal for cytoskeleton actin reorganisation (Shigeta et al., 2003), in the cell-cell adhesion and in the migration (Kurokawa et al., 2004). Hence, we don't exclude a possible role of CDC42 in the oval cell migration and invasion inside the liver lobule during the regeneration process.

We identified two cytoskeleton associated protein which are up-regulated during the oval cells regeneration: stathmin 1 and E-tropomodulin. Stathmin (also designed as oncoprotein 18) is a 19 kDa cytoplasmatic phosphoprotein, ubiquitous expressed, which its phosphorylation and expression is tightly correlated with the cell growth and differentiation (Mistry et al., 1998). The phosphorylation of stathmin is important in the dynamics of the microtubule polymerisation and assembly (Daub et al., 2001;Jourdain et al., 2004). Indeed the phosphorylation of stathmin deactivates the protein leading the assembly of the microtubules and the formation of the mitotic spindle during the cell division (Niethammer et al., 2004). Interestingly the phosphorylation of stathmin is controlled by the Rac/CDC42 signalling pathway and can be triggered by growth factor like Epidermal Growth Factor (EGF) (Daub et al., 2001;Ji et al., 1993). In addition, stathmin 1 is described to be transiently expressed in embryonal rat liver and it is induced during the liver regeneration by proliferating hepatocytes, in the model of normal PH (Okazaki et al., 1993). Hence it was proposed that stathmin over-expression correlates with the proliferation and differentiation of hepatocytes in the adult liver. E-tropomodulin (homolog of human Tropomodulin 1) belongs to the family of tropomodulins. It's a cytoskeleton tropomyosin binding protein important in controlling the growing of the slow ends of the actin filaments and the cell structure and motility (Ito et al., 1995;Sung et al., 1996;Sung et al., 1992). Same experimental evidences are suggesting that E-tropomodulin gene could be expressed specifically in the oval cells. Indeed we have found in our SAGE data that E-tropomodulin is expressed specifically in the oval cell liver (6 tags) but is neither detectable in normal rat liver and nor in sham control liver (0 tags) (see Results: Table 4). In addition we found by Real Time PCR that E-tropomodulin gene is co-expressed with the AFP gene. We believe that E-tropomodulin could be important for the motility and migration of the oval cells inside the liver.

Two signal transduction triggering genes are early up-regulated: CDC151 and lipopolysaccharide binding protein. CD151 (also designed as PETA-3) is a transmembrane protein belonging to the tetraspanins protein family ubiquitously expressed and implicated in the regulation of cell development, proliferation, differentiation, motility and tumour cell invasion (Maecker et al., 1997;Fitter et al., 1999;Lammerding et al., 2003). The tetraspanins are associated with the beta and alpha integrin proteins and they are important in extracellular matrix adhesion (Lammerding et al., 2003). Later reports indicate that CD151 is implicated in cell-cell adhesion and filopodium formation in epithelial cells (Shigeta et al., 2003). Indeed, over-expression of CD151 enhances cell-cell adhesion and filopodium formation by modulating the cytoskeletal reorganisation through Protein Kinase C (PKC) and CDC42 induction. In the contest of the liver organ, CD151 was demonstrated to be expressed in the hepatic stellate cells (Ito cells) and it is implicated in their motility and migration in vitro (Mazzocca et al., 2002). Thus it was proposed that CD151 could be a regulatory protein involved in the wound healing migration of stellate cells when an injury in the liver occurs.

Lipopolysaccharide binding protein is believed to be mainly an acute phase response protein involved in the inflammation process of the liver and could play a protecting role after the injury of the operation in the early stages of liver regeneration (Immenschuh et al., 1999;Ramadori et al., 1979).

We have found three kinetically up-regulated transporter genes: Na^+/P_i co-transporter 4, phosphatidylcholine transfer protein, and ATPase H^+ 34 kDa lysosomial transporter. Na^+/P_i co-transporter 4 is a transporter transmembrane protein involved in the sodium dependent up-take of inorganic phosphate (P_i), and it was lately cloned in rat (Ishibashi et al., 2003). The Na^+/P_i Co-transporter 4 is specifically expressed in the rat liver more than in the kidney, suggesting an important role of Na^+/P_i co-transporter 4 in the cytosolic P_i regulation and homeostasis in the liver. Unfortunately, very little is known about the activity and expression of Na^+/P_i co-transporter 4 in rat and human liver. Phosphatidylcholine transfer protein is highly specific transporter energy dependent for phosphatidylcholine (van Helvoort A. et al., 1999). It's was shown that

phosphatidylcholine transfer protein is strongly and transiently expressed in the liver of mice pups and decreasing two weeks after the birth. Although the phosphatidylcholine transfer protein was believed to be important in the liver for the supply of phosphatidylcholine secreted in the bile, the knock-out mice show no any defect concerning bile secretion. In addition the knock-out mice do not show any visible defects in the foetal liver development where the phosphatidylcholine transfer protein expression is more pronounced. In conclusion the general physiological function of phosphatidylcholine transfer protein remains unknown like its role in the oval cells liver regeneration and further and more detailed studies are needed.

ATPase H^+ 34 kDa lysosomial transporter is a vacuolar ATPase responsible for acidifying a variety of intracellular compartments in the cell, thus providing most of the energy required for transport process in the vacuolar system. Despite ATPase H^+ 34 kDa lysosomial transporter was cloned in the rat brain, still its function in the liver is not yet characterised (Strausberg et al., 2002).

Thioredoxin like 2 is an anti-apoptotic enzyme, a mitochondrial specific member of the thioredoxin family (Tanaka et al., 2002;Jurado et al., 2003;Kwon et al., 2003;Spyrou et al., 1997), found kinetically over-expressed during the oval cell regeneration. The anti-apoptotic activity of thioredoxin was demonstrated in the liver; indeed, over-expression of thioredoxin prevents acute hepatitis by thiocetamide or lipopolysaccharide administration (Okuyama et al., 2004). We could consider the anti-apoptotic activity of thioredoxin like 2 as important factor for maintaining the liver cells viable, after a chemical and operation injury (2-AAF/PH), which is a prerequisite for the regeneration process. We have identified by SAGE 14 down-regulated enzyme genes in the oval cells. We have identified 3 enzymes involved in the lipid metabolism kinetically down-regulated. Hydroxyacid oxidase 3, and fatty acid CoA ligase long chain 2, genes starts to be down-regulated in the beginning of the regeneration process, while steroyl CoA desaturase 1 is down-regulated especially at 7 day after PH when the peak of the oval cell proliferation is reached (see Results: Figure 20 C). The regeneration process and the hepatocarcinogenesis are characterised by strong changes in the bile composition

(Abel et al., 2001). We think that the down-regulation of gene expression of enzymes involved in the lipid metabolism and transport could affect the bile composition and production.

7.6. Final Remarks

This is the first report about a transcriptome analysis by SAGE performed on liver regeneration *via* oval hepatic stem cells. We have identified novel candidate genes that are differentially regulated during the regeneration process.

We identified CDC42 and cyclin D1 cell cycle genes which are over-expressed kinetically during the regeneration process at RNA level and protein level. By Western Blot we have seen that the proteins of the two cell cycle genes are co-expressed with AFP. CDC42 is an important molecular key regulator involved in many pathways (Bishop and Hall, 2000). CDC42 induces cyclin D1 and cell cycle progression (Gjoerup et al., 1998;Roovers and Assoian, 2003), it regulates the stathmin activity important in the control of the mitotic spindle assembly and cell division (Daub et al., 2001), and finally it is involved in the cell-cell adhesion CD151 dependent (Shigeta et al., 2003). We think that CDC42 could play an important role in proliferation, migration and differentiation of the oval cell. A report about *in vitro* studies of isolated hepatocytes shows that CDC42 can be induced by the growth factors TNFα and HGF or glucose (Auer et al., 1998). In addition the authors found that CDC42 is implicated in the stimulation of DNA replication. Indeed, a dominant negative CDC42 construct blocks the DNA synthesis after growth stimulation.

We believe that it would be important to study the role of CDC42 in the proliferation of isolated oval cells (Pack et al., 1993) and find a possible mechanism of cyclin D1 induction and stathmin 1 phosphorylation by CDC42.

The cell cycle and proliferation is a well regulated process by multiple environmental influences; a mitogenic response can be triggered through the activation of the signal transduction cascade intracellular pathways, by synergic action of growth soluble factors, and by extracellular matrix and cell-cell adhesion (Coleman and Marshall, 2001). Mitogens, like EGF, stimulate oval cell proliferation *in vivo* and *in vitro* (Nagy et al., 1996;Isfort et al., 1997). In addition, EGF promotes the activation of CDC42, as well as its phosphorylation in cells (Tu et al., 2003). It would be interesting to determine if the EGF induction of oval cells is CDC42 dependent.

Finally, it is still significantly important to determine the role of the other candidate genes we have identified by SAGE and Real Time PCR in the contest of liver regeneration by oval hepatic stem cells. Particularly we would be interested in characterising the antiapoptotic gene thioredoxin 2 (Tanaka et al., 2002) and the cytoskeleton associated protein gene E-tropomodulin (Sung et al., 1992) expression and regulation in the oval cells (see Results: Table 6, 7 and 8, Figure 21; see Discussion).

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