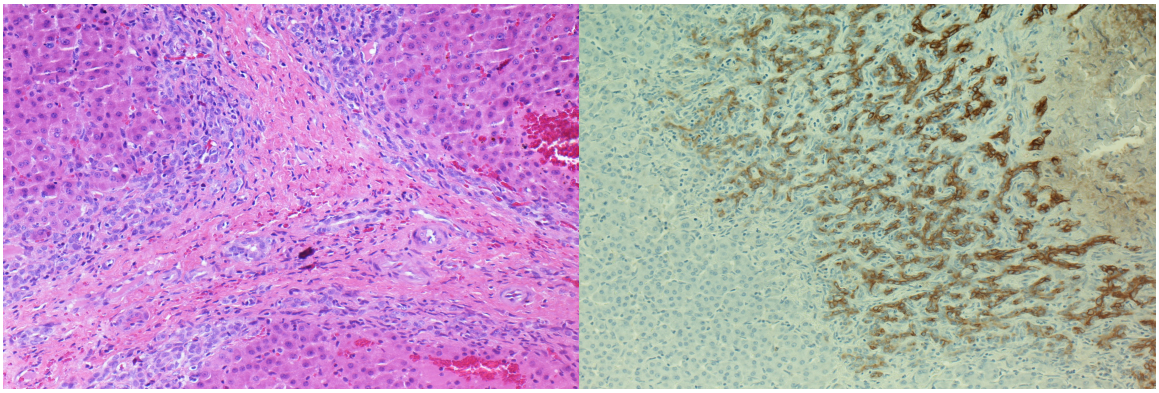
UNIVERSITY OF TROMSØ UIT

FACULTY OF HEALTH SCIENCES
DEPARTMENT OF CLINICAL MEDICINE

The terminal phase of liver regeneration

-differential gene expression, tissue remodelling and growth arrest of regenerating porcine liver



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Cover page: Liver tissue sections at six weeks after a partial hepatectomy stained with HE (left) and pan-CK (right). Note the broadening of the connective tissue (left) and the heavily towards lightly stained cells in the adjacent hepatocyte parenchymal tissue (right).

Scientific environment

This study was performed between 2005 and 2012 at 1) the Department of Digestive Surgery, University Hospital of Northern Norway, Tromsø; 2) at the Surgical Research Laboratory, Institute of Clinical Medicine, Faculty of Medicine, University of Tromsø; and 3) at the Institute of Medical Biology, Faculty of Medicine, University of Tromsø. During this period I was a medical student at the University of Tromsø, doing my internship at Vestfold Hospital, Tønsberg, while working as a residence doctor at the Department of Digestive Surgery, University Hospital of Northern Norway, Tromsø. This work was supported by research grants from both the Northern Norway Regional Health Authority and the University of Tromsø.

Contents

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List of papers

Paper I

Nygård IE, Lassen K, Kjæve J, Revhaug A. Mortality and survival rates after elective hepatic surgery in a low volume centre are comparable to those of high volume centres. *ISRN Surg. 2012; 2012: 783932. doi: 10.5402/2012/783932.*

Paper II

Nygård IE, Mortensen KE, Hedegaard J, Conley LN, Kalstad T, Bendixen C, Revhaug A. The genetic regulation of the terminating phase of liver regeneration. *Comp Hepatol. 2012 Nov 20; 11(1): 3. doi: 10.1186/1476-5926-11-3.*

Paper III

Nygård IE, Mortensen KE, Hedegaard J, Conley LN, Bendixen C, Sveinbjørnsson B, Revhaug A. Tissue remodelling in the terminal phase of porcine liver regeneration. *Submitted Liver Int. 2013.*

Abbreviations

AHNAK - AHNAK nucleoprotein, desmoyokin
α-SMA - Alpha smooth muscle actin
BMP-1 - Bone morphogenetic protein-1
BMP-2 - Bone morphogenetic protein-2
CARD-11 - Caspase recruitment domain-containing protein-11
CCl_4 - Carbon tetrachloride
COL1A1 - Collagen type 1, alpha 1
COL1A2 - Collagen type 1, alpha 2
DSP - Desmoplakin
ECM - Extracellular matrix
ERAS - Enhanced recovery after surgery
ERBB2 - Erytroblastic leukemia viral oncogene homolog 2
ERBB3 - Erytroblastic leukemia viral oncogene homolog 3
ESR1 - Estrogen receptor 1
F11R - F11 receptor
GNG11 - Guanine nucleotide binding protein (G protein), gamma 11
GPC3 - Glypican 3
HE - Haematoxylin-Eosin
HGF - Hepatocyte growth factor
HRP - Horse radish peroxidase
HSC - Hepatic stellate cell
IL - Interleukin
ILK - Integrin-linked kinase
ITGAV - Integrin, alpha V
Log FC - Log fold change
LOS - Length of stay
LPC - Liver progenitor cell
MMPs - Matrix metalloproteinases
OCLN - Occludin
PAI - Plasminogen activator inhibitor
Pan-CK - Pan-cytokeratin
PBS - Phosphate buffered saline
PCNA - Proliferating cell nuclear antigen
PEG - Percutaneous endoscopic gastrostomy
PHx - Partial hepatectomy
PPAR - Peroxisome proliferator-activated receptor
PTK2B - Protein tyrosine kinase 2 beta
RT-PCR - Real-time PCR
SEC - Sinusoidal endothelial cell
SKI - Sloan-Kettering viral gene oncolog
SOCS3 - Suppressor of cytokine signalling 3
SPARC - Secreted protein, acidic and rich in cysteine (osteonectin)
SPSS - Statistical Package for the Social Sciences
STAT - Signal Transducer and Activator of Transcription
STEAP1 - Six transmembrane epithelial antigen of the prostate 1
TCF4 - Transcription factor 4
TFPI2 - Tissue factor pathway inhibitor 2
TGF- β - Transforming growth factor beta
TIMP1 - Tissue inhibitor of metalloproteinase 1
TNF-α - Tumor necrosis factor-alpha
TOB1 - Transducer of ERBB2
TSP-1 - Thrombospondin-1
uPA - Urokinase plasminogen activator
VASH2 - Vasohibin 2
ZNF490 - Zinc finger protein 490

1. Introduction

1.1 Rationale for the study

The need for research on the terminal phase of liver regeneration is evident considering the lack of scientific articles focusing on this specific event. Many studies have been conducted to investigate the initiating phase of liver regeneration, however, the terminating phase persists in both being deficiently investigated and described.

On an annual basis, an approximate number of 28 hepatic resections are performed at the University Hospital of North Norway with acceptable morbidity and mortality. To strengthen the theoretical basis upon which this small volume practice is maintained, parallel research programs of experimental surgery related to liver failure and liver regeneration are conducted in porcine models in the surgical research facility at the University of Tromsø. The purpose of this activity is not only to improve the technical skills amongst the involved personnel, but also to contribute in the maintenance of updated knowledge on liver pathophysiology.

In a wider perspective, liver cancer has the fifth highest cancer incidence in the world, and is the third highest cause of cancer related deaths [1], with resection of the liver remaining the only curative option [2]. Hepatic resections are both associated with, and dependent on a rapid proliferation and regeneration of the remnant liver. However, liver failure following partial hepatectomy still occurs, primarily due to a massive resection, to a pre-existing liver disease (neoadjuvant chemotherapy) or to advancing age [3]. New scientific discoveries governing liver regeneration could contribute to enlightening our understanding of the mechanisms behind this process. This could offer new treatment strategies, not only to patients with advanced liver cancer, but also to patients suffering from acute and chronic liver failure, as the liver is the major detoxifying organ of the body and is likely to be injured by ingested toxins.

After a liver transplantation, the donor grafts may not be of an optimal size for adequate function in the recipient. According to Kawasaki et al. [4], the size of the transplanted liver converged to the standard liver volume over time, regardless of whether the initial liver-graft volume was smaller or larger than the standard liver volume. In order to develop improved treatment strategies to patients eligible for liver transplantation,

another fundamental basis for research on this topic is to understand the mechanisms behind the up- and down-regulation of the transplanted graft volume in the recipient after a liver transplantation.

Over the past decades, much effort has been devoted to furthering a better understanding of the molecular and cellular mechanisms underlying liver regeneration. Not only is this knowledge crucial for clinical medicine, but also for the use of stem cells for cell therapy and liver regeneration. Since the liver is the only internal mammalian organ that can fully regenerate after injury, it is likely to believe that studies on liver regeneration will contribute to our understanding of pathways governing mammalian organogenesis.

1.2 Historical perspectives of liver regeneration

The term partial hepatectomy (PHx) means a surgical resection of one or more liver segments. The word is derived from the Ancient Greek, *hepat*: "liver" and *ectomy*: "to cut out". The fact that the liver regenerates after parenchymal loss was alluded to in ancient Greek mythology, when the Greek God Prometheus stole fire from heaven and delivered it to mankind. As punishment for this act, Zeus arrested Prometheus and bound him to a rock on Mount Caucasus where an eagle was set to feed upon his liver every day. However, his liver grew back the next day, allowing the eagle to feed upon it for many years until the Greek hero Heracles (Hercules) slew the eagle and freed Prometheus from his chains (Fig. 1).

Figure 1. The Greek God Prometheus bound to a rock on Mount Caucasus.

In the 19th century the Russian surgeon Nikolai Eck presented the Eck fistula in dogs, thus initiating modern studies on liver regeneration in 1877. The Eck fistula is an artificial passage between the portal vein and the inferior vena cava in order to direct the portal blood flow away from the liver and directly to the heart. The study suggested that the function of the liver was not dependent upon the portal blood perfusion [5]. However, the theory was challenged in 1893 when Hahn et al. [6] reported a study of the Eck fistula in dogs which resulted in liver atrophy, weight loss and encephalopathy. Rous and Larimore [7] indicated in 1920 the possibility that the portal venous blood contained a hepatotrophic factor necessary for liver regeneration. In 1952 Grindlay and Bollman [8] demonstrated liver regeneration in a dog model after a 70% PHx combined with an increased hepatic pressure by constricting the vena cava above the liver, and in 1953 Child et al. [9] conducted a study on liver regeneration following portocaval transposition in dogs. The work of Child et al. did not support the hepatotrophic theory of Rous and Larimore in that liver regeneration was dependant on the quality of portal venous inflow; instead they established the quantity of the total hepatic blood flow to be the main determinant for regeneration [10]. Since Rous and Larimore hypothesised the theory that the portal venous blood contained a hepatotrophic factor, 40 years passed before new investigations challenged the theory of that liver regeneration was dependant on the delivery of certain substances from the portal venous blood. During the 1960s and -70s, the research evolved to screen for hormonal and nutritional hepatic growth factors. In 1976 Starzl et al. [11] demonstrated that insulin infusion into the left portal vein after portocaval shunting in dogs reduced liver atrophy, preserved hepatocyte ultrastructure and trebled cell renewal, and in 1991 Francavilla et al. [12] demonstrated how the Eck fistula could be used to screen for hepatic growth factors. During the past 20 years, the research of liver regeneration has evolved in several directions, from the extrinsic hepatic growth factors, to the intrinsic changes in the extracellular matrix, the intracellular signal transduction mechanisms and the genetic response in the liver [13].

1.3 Models of liver regeneration

Since Higgins and Anderson pioneered the experimental model for the study of liver regeneration in 1931 [14], multiple studies have been conducted to study the liver

regeneration in rodents. However, compared to rodents, a pig bears a closer anatomic, genetic and physiological resemblance to a human being. Furthermore, their comparable size to the human anatomy allows for a greater clinical application of surgical procedures than what is the case for many small animal models. Another advantage with the partial hepatectomy is that compared to other methods, e.g. the use of hepatic toxins such as carbon tetrachloride (CCl_4), the model of partial hepatectomy is not associated with tissue injury and inflammation, since the removal of liver segments is precisely defined [15]. Due to the lack of a current adequate model to study liver regeneration in humans, the partial hepatectomy in a porcine model presents a simple and reproducible model in which the process of liver regeneration can be studied.

1.4 Liver anatomy and physiology

The liver is the largest internal organ in mammals, weighs an average of 2.5% of total body weight and receives its blood supply from the portal vein and the hepatic artery. The portal vein carries largely deoxygenated blood from the spleen, pancreas, stomach, small intestine and large intestine, and supplies approximately 75% of the hepatic blood flow. The hepatic artery carries oxygenated blood from the aorta and supplies about 25% of the hepatic blood flow. Unlike the human liver with two lobes, the porcine liver is divided into three main lobes, the left lateral, the median and the right lateral lobes respectively. The porcine liver lobes can again be divided into eight segments (I-VIII), and each segment has its own arterial supply, venous and biliary drainage [16].

The functional microscopic unit of the liver is referred to as the liver lobule, and the hepatic parenchyma is divided into these small units, with each consisting of a hexagonal arrangement of hepatocytes with a hepatic vein in the centre. The portal triads are located at the verticals of the lobules, with each containing a bile duct, a hepatic artery and a portal vein. The blood flows directly from the portal triad towards the central vein, while in between the portal triads and the central vein, rows of hepatocytes are arranged in single lines with a sinusoid on one side, and a bile canaliculi on the other (Fig. 2). Bile is emptied from the hepatocytes into the bile canaliculi and flows in the opposite direction of the blood, i.e. towards the portal bile duct for drainage [17]. In between the lobules, the hexagons are divided by a fine, delicate lining of connective tissue.

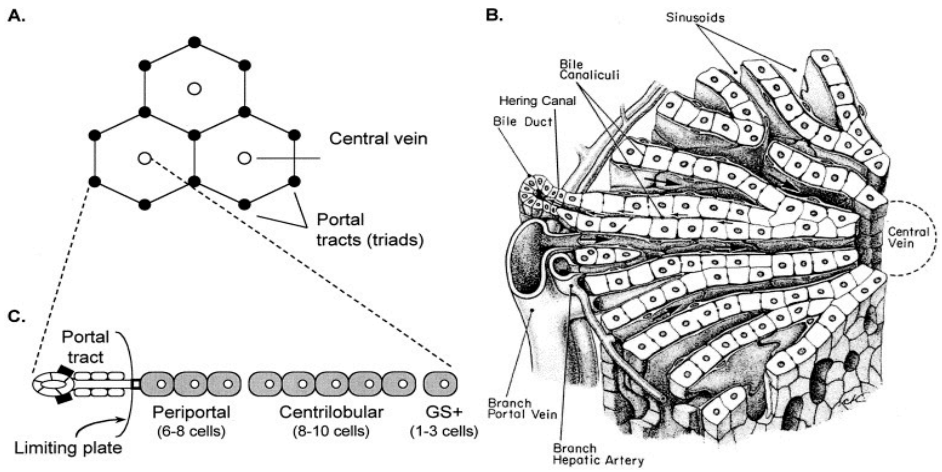

Figure 2. Lobular structure of the liver, demonstrating the portal triad, central vein and hepatocyte lining, from: From Fausto et al. 2003 [17], used with permission.

The fluid-filled space of Disse separates the hepatocytes from the sinusoidal endothelial cells (SECs), which are special because of their lack of intercellular junctions, creating fenestrae in between the endothelial cells, thereby allowing contact between the circulating blood and hepatocytes [18]. The hepatic stellate cells (HSCs) are located within the space of Disse, and their main functions are the production of extracellular matrix and the storage of vitamin A. The activation of HSCs is a cellular event in liver regeneration, as well as in the development and progression of liver fibrosis [19].

1.5 Hepatocyte proliferation

The proliferative capacity of the hepatocyte has been shown to be almost unlimited. A study in a mouse model using the serial transplantation of hepatocytes have shown that small numbers of hepatocytes can replicate more than 100 times, and are capable of fully reconstituting diseased livers multiple times [20]. Previous studies in rats have suggested the "streaming liver hypothesis", thus implying that new hepatocytes arise in the periportal area, and then gradually migrate towards the pericentral area [21]. During normal liver turnover or in response to mild injury, the maintenance of the liver mass is

achieved by mature self-duplicating hepatocytes. However, when an injury is severe or the proliferative capability of hepatocytes is impaired, liver progenitor cells (LPCs) located in or around bile ducts in the periportal areas provide a backup system for mouse liver regeneration [22]. During liver regeneration, hepatocyte clusters containing 10-14 hepatocytes are formed, which is followed by an invasion of HSCs and fenestrated SECs, thereby re-establishing the normal hepatocyte vascular relationship. During the development of hepatic cirrhosis, the vascularization of the regenerative clusters differs in that basement membranes and non-permeable continuous capillaries are formed [23]. More research on the mechanisms governing hepatocyte clustering and fenestrated vascularization may hopefully result in new treatment options for patients suffering from liver cirrhosis.

1.6 The terminal phase of liver regeneration

The process of liver regeneration is divided into three phases, namely that of initiation, proliferation and termination. Although great advances have been made over the past few years in understanding the phenomenon of liver regeneration, most have focused on the initiating phase. Interestingly, the terminating phase of liver regeneration is a field just entering a phase of rapid discovery. During liver regeneration two distinct pathways are activated, the growth factor and the cytokine-regulated pathway. These pathways have checkpoints that could be feedback-inhibited, thus regulating liver growth and size [24]. Among the cytokine pathway, several negative regulators (suppressors of cytokine signalling (SOCS), plasminogen activating inhibitor (PAI), interleukin-6 (IL-6)) and positive regulators (signal transducer and activator of transcription protein-3 (STAT-3), hepatocyte growth factor (HGF)) are reported to regulate cell growth [25-27]. Transforming growth factor-β (TGF-β) is produced by HSCs and is a well-known inhibitor of hepatocyte proliferation, with the up-regulation of TGF-β leading to both liver fibrosis and apoptosis [28]. MicroRNA genes are a class of posttranscriptional regulators, and the up-regulation of miR-23b is reported to promote BRL-3A cell proliferation, thereby inhibiting TGF-β induced apoptosis. The down-regulation of the gene may therefore contribute to the activation of the TGF-β signalling pathway at the terminal phase of liver regeneration [29]. Additionally, the up-regulation of another

microRNA, the miR-34a, is reported to contribute to the suppression of hepatocyte proliferation [30]. A recent report identified thrombospondin-1 (TSP-1), an inhibitor of liver regeneration by activating TGF-β [31]. In 2009, Apte et al. reported that signalling by integrin-linked kinase (ILK) was essential in the termination of liver regeneration [32], which was corroborated in 2010 when Donthamsetty et al. reported that a lack of ILK enhanced the prolonged proliferative response in hepatocytes [33]. Glypican 3 (GPC3) is reported to play an important role in terminating hepatocyte proliferation and regulating liver size in mice [34], and lipid metabolism and peroxisome proliferator-activated receptor (PPAR) signalling pathways are reported to participate in late-phase liver regeneration [35]. An illustration of previously described factors regulating the terminal phase of liver regeneration is presented in Fig. 3.

Although some mechanisms regulating hepatocyte proliferation and apoptosis have been revealed, mechanisms' switching "off" the regenerative response still remains unknown. As the liver to body weight ratio is relatively standardized in mammals (i.e. 2.5%), there seems to be a regulator of liver growth, a "hepatostat" that is able to both terminate liver growth and down-regulate the liver size in a liver transplanted recipient whose liver-graft was larger than the standard liver volume. Are the above-mentioned mechanisms all taking part in a negative feedback system that eventually results in growth arrest? Is the "hepatostat" a specific checkpoint in the regenerative pathway simply activated by an irregular liver to body weight ratio, or is based upon the functional or metabolic needs of the organism? We know that the liver regeneration process terminates when the standard liver volume is re-established, however, a precise definition of the "hepatostat" is yet to be defined.

More research is needed to understand the precise mechanisms governing the terminal phase of liver regeneration.

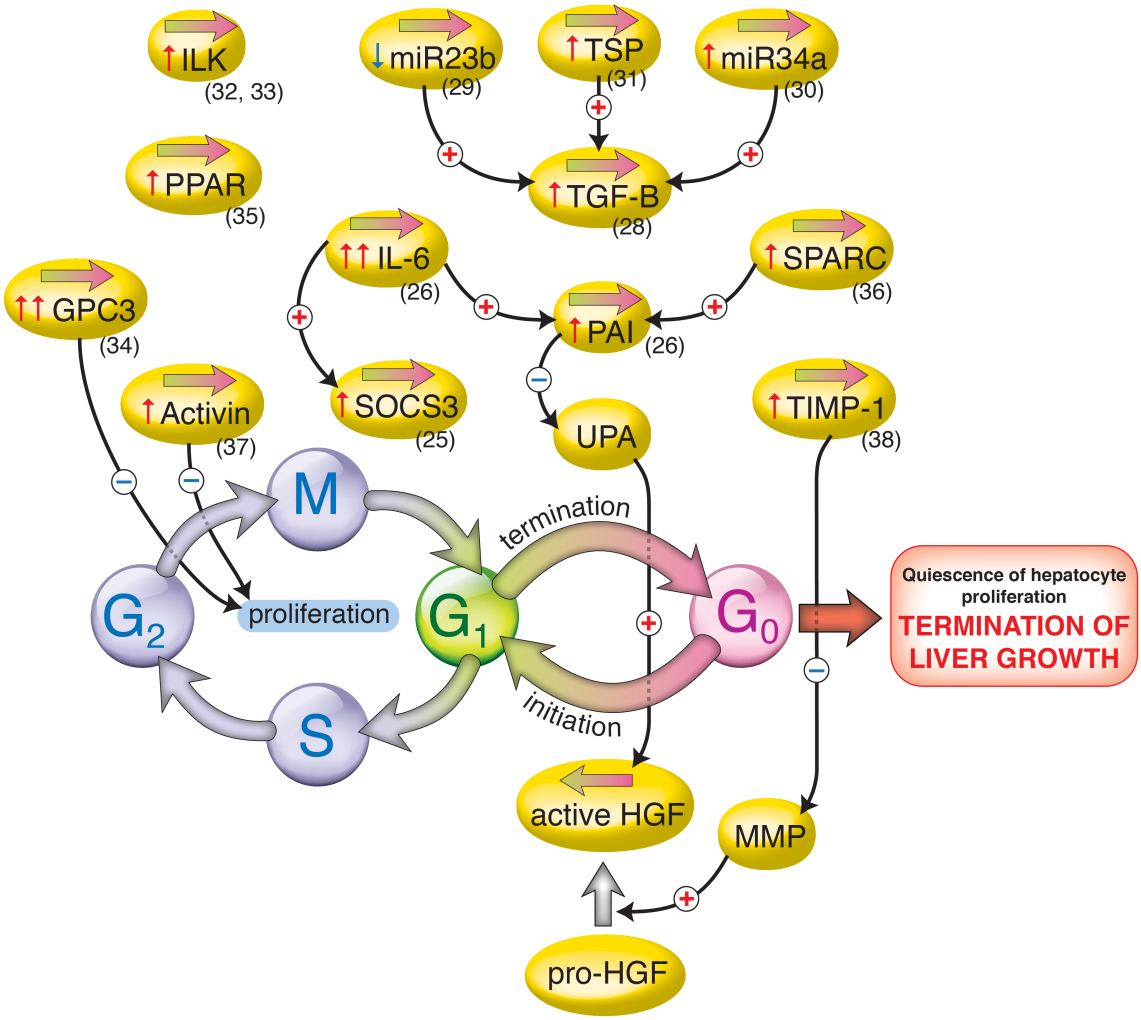

Figure 3. Previously reported regulators in the terminal phase of liver regeneration. Most regulators illustrated above are reported to participate in late phase liver regeneration/termination of liver regeneration. The rightward arrows indicate a participation in the terminal phase, and the leftward arrow indicates a participation in the initiation of liver regeneration. References are denoted in parentheses.

2. Aims

The aims of the present studies were:

I. To ascertain that the results of all elective hepatic resections performed at a low volume centre (UNN) since 1979 are comparable to the results reported internationally with regard to mortality and five-year survival rates *(Paper I)*.

II. To investigate differentially expressed genes in the liver remnant after a 60% hepatectomy in a porcine model, particularly for genes controlling cell cycle, apoptosis and angiogenesis in the termination of liver regeneration *(Paper II)*.

III. To study the architectural remodelling of the liver parenchyma, and to reveal genes regulating the extracellular matrix in the terminal phase of liver regeneration *(Paper III)*.

3. Methodological considerations

3.1 Paper I

Study design

Patients identified from the Hospital's patient administrative databases were studied in this paper and together with information from the Norwegian Central Population Register, the medical records of all patients who underwent liver resection from January 1979 to December 2011 were reviewed. A spreadsheet was created for registration of different variables registered, including death in hospital within 30 days and months survival.

Data collection

Medical records from an earlier time period (before 2003) were not included in the Hospital's patient administrative database, so the paper version had to be collected from the Hospital's archive. In order to find all patients who underwent liver resection in the early period, we went through the remittances of liver resected patients at the Department of Clinical Pathology.

Statistical analysis

All statistical analyses were conducted using the Statistical Package for Social Sciences (SPSS) 19.0.

3.2 Papers II-III

No experimental animal model will ever perfectly resemble a human being: however, porcine models are increasingly being used in experimental surgery due to their anatomic, genetic and physiologic resemblance to humans. A porcine model for the study of the terminal phase of liver regeneration after a partial hepatectomy constitutes the fundamental basis of Papers II and III.

3.2.1 The chronic porcine model

Ethics

All experiments were conducted in compliance with the Norwegian Animal Welfare Act § 21 and The Norwegian Regulation on Animal Experimentation §§ 7, 8 and 13. Moreover, our department is run in agreement with the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes, and all researchers and technicians contributing to both conducting the experiments and the postoperative care of the animals were certified by a course in animal science approved by the Norwegian Ministry of Agriculture.

Study design

The time points for biopsy sampling were: before PHx (t=0); three weeks post-PHx (t=1); and upon termination on the sixth week post-PHx (t=2). The first time point (t=0) was set before PHx to secure a sample of the normal non-resected liver. Based on the report from Court et al. [39], demonstrating that liver regeneration in the pig after a 70% PHx was completed within three weeks, the second time point (t=1) was set to three weeks post-PHx. At the time when we started this study in 2006, there were no previous reports on the terminal phase of porcine liver regeneration. However, previous studies reported the duration of rat liver regeneration to last from 5-7 days [15], and the human liver regeneration to last from 3-6 months [40]. The time point at six weeks post-PHx (t=2) was based on both an average duration of liver regeneration in rats and humans, and the report from Court et al. [39]; hence, the third time point may be a subject of debate. However, since the main aim of our study was to investigate the terminal phase of liver regeneration, and the reformation of a normal liver architecture only occurs after restoration of the original liver mass [28], we conclude that the chosen time point at six weeks was appropriate for our study.

To secure the homogeneity of animals, age and weight standardized (average 3 months and 32 kg, SD 5 kg) Norwegian landrace pigs from a single commercial farm were used in all experiments. We used only females, as intact male pigs are not readily available from commercial farms. We chose not to use castrated males as these have been

subjected to surgery at the farm, which potentially could inflict on the experiment. To reduce the incidence of aspiration and postoperative pneumonia, the animals were preoperatively fasted overnight with free access to water. During surgery, the anaesthesia was maintained with Isoflurane, because it is reported to provide the least cardio depressant effect while at the same time securing an acceptable depth of anaesthesia [41]. Although their pens were cleaned daily, the abdominally located postoperative wound inevitably made contact with the bedding on the floor increasing the risk for postoperative infections. Consequently, the animals received a peroperative intramuscular injection of antibiotic prophylaxis with Enrofloxacine 2.5 mg/kg.

Surgical procedures

To perform the PHx, a midline laparotomy was used for access to the hepatic hilus. Blood was extracted via a Hickman catheter (BARD Access Systems) placed in the Jugular vein. This access was also used for blood sampling and postoperative administration of intravenous fluids and medication. A Freka PEG (Fresenius Kabi AG) was placed in the stomach to prevent gastric retention, observed in pilot experiments. The hepatic artery supplying segments II and III together with these segments' portal branch were ligated using an absorbable polyfilament suture on a large needle. Thereafter the lobe was strangulated with a 0.5 cm wide cotton ribbon and then removed and weighed. Segments IV, V and VIII were removed in a similar manner leaving segments VI, VII and I in place, corresponding to an approximate 60% PHx.

Pigs subjected to sham also underwent a midline laparotomy; a biopsy was taken from segment IV, a Hickman catheter was placed in the Jugular vein and a Freka PEG was placed in the stomach.

Control animals underwent a minimal laparotomy for biopsy sampling from segment IV. Blood was sampled from the jugular vein. No catheters were used.

Postoperative treatment

Following major surgery in pigs, transdermal fentanyl in appropriate doses has been shown to be an effective means of delivering basal analgesia to farm pigs [42]. The pigs were monitored closely post-operatively with regard to pain assessment and well-being.

We also secured the patches using adhesive surgical drape (Ioban) to ensure proper drug delivery as well as to prevent ingestion and overdose. Compared to injections, the transdermal patches are also less stressful as there is no need for restraint and handling and no pain associated with its administration. As the liver regenerates and the pigs grow it is of utmost importance that the animals are in an adequate nutritional status. However, some of the animals in the resection group suffered from anorexia the first postoperative days. In order to maintain their appetite after surgery all pigs were already from the third preoperative day given a standardised amount of liquid dietary supplement (milk replacement for piglets). Postoperatively the pigs willingly consumed the dietary supplement, even those who rejected the pelleted food. Gastric ulceration is not uncommon in fattening pigs in commercial farms and mortality due to bleeding gastric ulcers has been reported [43]. It is known that the pig reacts on unfamiliar environments and overnight fasting with oesophago-gastric lesions [44]. Friendship et al. [45] reported that feed withdrawal 24 to 48 hours prior to slaughter could increase the incidence and severity of gastric ulcers in swine, however the report demonstrated that the proton pump inhibitor Omeprazole was effective in preventing some of the tissue damage due to fasting. Additionally, Baustad et al. [46] reported that including straw to 5-10% of the porcine diet provided almost full protection against gastric ulceration. Based on these studies, we tried to reduce the overall stress level by keeping the pigs in-house for approximately one week prior to surgery, feeding them with standardized amounts of solid pig feed and liquid dietary supplements and including hay as feed and bedding in their pens. Due to animal welfare reasons and to reduce stress, we housed the pigs together in groups of four, so that they could see, hear and smell another familiar pig. However, as pigs are curious and explorative by nature, and have a tendency to bite or ‘taste’ new things, each pig was separated in an individual pen in order to secure the catheters, surgical drape (Ioban) and postoperative wounds. The preoperative feed withdrawal was minimized since the pigs only fasted from midnight the day before surgery. Postoperatively they received a total of 40 mg of Esomeprazole daily in combination with Erythromycin in order to prevent gastric ulceration and to continue the antibiotic prophylaxis, respectively.

Porcine mortality

Out of 21 pigs, 12 survived the six-week experiment and a total of nine pigs were either terminated based on the clinical condition of the animals or died due to the extensive surgery. Chronic porcine models are challenging, and several considerations must be taken into account before initiating a chronic study. Firstly, in order to survive a chronic experiment with several surgical interventions, it is of utmost importance that the pig is in excellent health status prior to surgery. Secondly, the feasibility of the chronic model depends on strict animal husbandry in addition to a daily administration of intravenous fluids and medication. Thirdly, pigs are known for their vulnerability insofar as they easily achieve ischemia-induced ventricular dysrhythmias [47]. In our study, one pig subjected to sham surgery operation died during anaesthesia due to acute perioperative heart failure 24 days after primary surgery, whereas another pig subjected to PHx died due to ulcerative gastritis despite the prophylactic treatment with Esomeprazole, 19 days after primary surgery. Since the porcine liver anatomy exhibited some variation among different animals, none of the hepatic resections were completely identical. One pig suffered from postoperative bleeding, and had to be terminated, while another pig suffered from bile leakage and also had to be terminated, six and seven days after primary surgery, respectively.

Irrespective of any methodological drawbacks, the chronic porcine model presents a unique possibility to perform long-term studies in animals with a close resemblance to human.

Blood sampling (Paper II)

As a reference value, blood was sampled from the jugular vein at the time of laparotomy. After surgery, we sampled regularly from the jugular vein for analysis of cytokines: *IL-1, IL-6, IL-10* (Multiple cytokine analyses (Multiplex®, Tromsø, Norway), and humoral growth regulating factors: *TNF-α* (Multiple cytokine analyses (Multiplex®, Tromsø, Norway), *TGF-β* (MILLIPLEX MAP TGF ß1 (Transforming Growth Factor Beta) - Single Plex, Tromsø, Norway).

An advantage of the use of multiple cytokine analyses (Multiplex®, Tromsø, Norway) is the specific detection of *porcine* cytokines, as IL-1, IL-6, and IL-10 were all porcine-

specific, in addition to TNF-α. In contrast, the analysis of TGF-β was multi-species (MILLIPLEX MAP TGF ß1 - Single Plex, Tromsø, Norway), with the latter presenting a drawback in that it is not porcine-specific. Therefore, we find the paucity of assays and reagents for porcine cytokine detection to be a major handicap for studies in pigs.

3.2.2 Biostatistical considerations

Microarray methods

Two-colour microarray experiments were conducted to identify genes being significantly differentially expressed due to resection over time adjusting for effects by using the expression profiles obtained from the control animals and the sham operated animals. The microarray experiment was conducted as a common reference design using a reference consisting of equal amounts of total-RNA from all samples. Total-RNA was extracted from each sample and DNase treated using RNeasy Maxi Kit (Qiagen). The reference samples were labelled with Alexa-555 and the individual samples were labelled with Alexa-647. Following hybridization, washing and drying, the slides were scanned in a ScanArray Express HT system (version 3.0, Perkin Elmer, Hvidovre, Denmark), and the resulting images were analyzed using GenePix Pro (version 6.1.0.4, Molecular Devices). Statistical analysis was carried out in the R computing environment using the package Linear Models for Microarray Analysis (Limma, version 2.12.0, [48]). Time contrasts were formed referring to the time points for biopsy sampling, that is 3-0 weeks, 6-0 weeks and 6-3 weeks. Fig. 4 demonstrates the steps of a two-colour cDNA microarray analysis.

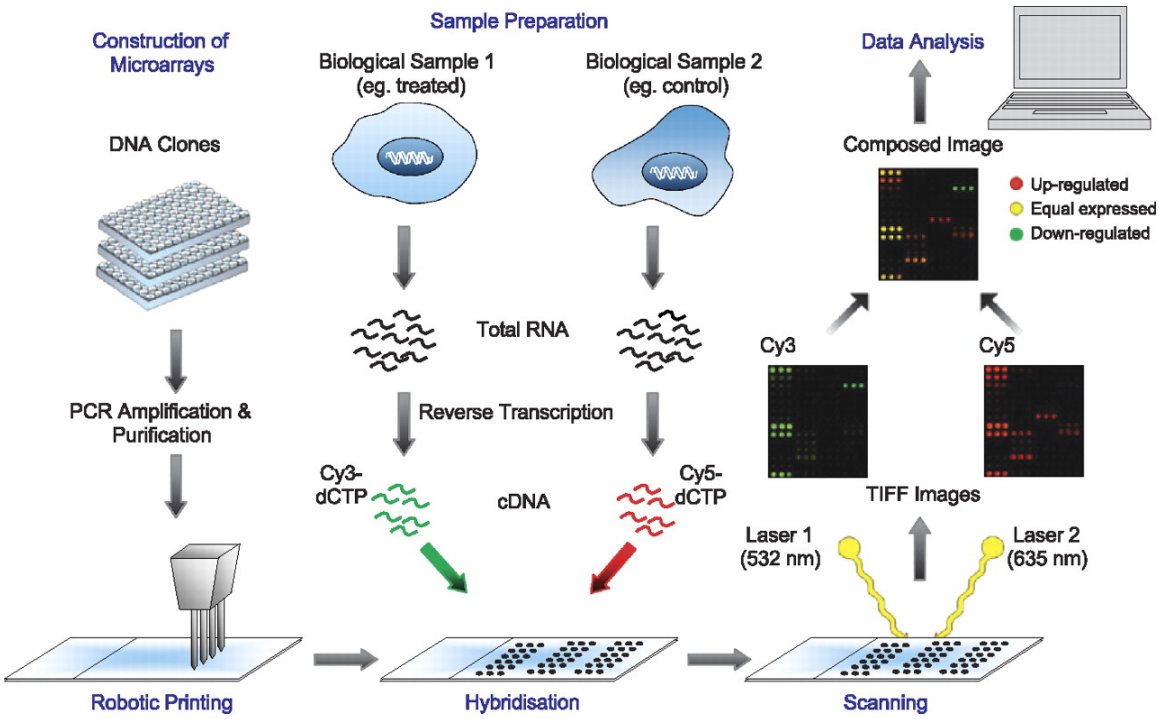

Figure 4. Steps of a two-colour cDNA microarray analysis. From White et al. 2005 [49].

Over the past decade, microarray analysis has gained acceptance as a standard tool for studies in molecular biology, though it is subject to several considerations. A microarray experiment cannot verify or falsify a null hypothesis, since it is, primarily a screening technique. Additionally, the use of microarray and fold change has the disadvantage that it needs cut-off values; consequently, it does not include all biologically present genes at any time, as some might have a very small fold-change, while some mRNA have a short half-life due to rapid degradation. On the other hand, the microarray analysis allows for massive data acquisition in parallel, which increases the experimental efficacy, and again allows for meaningful comparisons [50]. The ability to study the behaviour of many genes simultaneously is perhaps the greatest advantage of its use. Additionally, today's DNA microarray technology is convenient, and since the first reported use of this technology in 1982 [51], the method has improved, and the technique is now considered to provide reliable results. Because of the fact that the microarray experiment itself is a procedure involving multiple steps, the design needs to be strictly standardized. It is also of utmost importance that the starting material is of highest quality, and in order to secure

the quality of our biopsies, we immediately immersed them in RNAlater (Ambion) after sampling.

The standardized method to validate microarray results is by conducting real-time PCR (RT-PCR, also known as qPCR), though there are many potential pitfalls of microarray analysis validation. RT-PCR can be labour intensive, time consuming and expensive, and unfortunately with only a small fraction of the microarray results being validated [52]. RT-PCR is considered the "gold standard" for validating microarray analysis, but according to Smith [52], this is however debatable. As RT-PCR measures the abundance of a single transcript or transcript segment, it differs from microarray, in that microarray data may result from the hybridization of multiple transcript isoforms to a probe on an array. An alternative method that is more likely to be relevant to the biology of a cell or tissue of interest is to evaluate the protein expression levels [53]. Since immunohistochemistry is widely used as an alternative validation tool to validate microarray experiments [53], we utilized this method to validate our microarray analysis by assessing the presence of specific proteins in our samples.

Immunohistochemical validation (paper III)

Formalin-fixed and paraffin-embedded tissue sections were deparaffinised in xylene and graded alcohols, hydrated and washed in phosphate buffered saline (PBS). After an antigen retrieval in a sodium citrate buffer (pH 6) in a microwave oven, the endogenous peroxidase was blocked by 0.3% H_2O_2 for 15 min. Sections were incubated overnight at 4°C with primary antibody, and as a secondary antibody, a horseradish peroxidase (HRP) SuperPicTure Polymer detection kit was used (Invitrogen). As a chromogen substrate for HRP, either DAB or ACE (Dako) was used. A matched isotype control was used as a control for non-specific background staining, and a routine standard staining showing a normal histology of liver sections from respective groups was performed with hematoxylin/eosin (HE) and Masson's Trichrome, respectively.

Most antibodies for immunohistochemical analysis for diagnostics or research are made for humans, mice or rats. The reactivity and affinity to porcine epitopes remain uncertain, and species' cross-reactivity is often empirical, as both the time until fixation and the

cross-ligation of proteins after prolonged fixation can reduce the access to the epitope. In our study, we attempted to improve the antigen retrieval by using citrate buffer and microwaving. Since the pig compared to rodents bears a closer genetic and physiological resemblance to human, an obvious need to develop antibodies with a more specific affinity to porcine epitopes was presented.

4. Summary of results

4.1 Paper I

A total of 290 liver resections were electively performed between January 1979 and December 2011, with the most common indication for hepatic resections being colorectal metastasis (n=174, 60%). Fig. 5 shows the development of annual resections, while Table 1 shows the frequencies of indications. The five-year survival rate for patients who underwent liver resection for colorectal metastases was 35.8% (34/95), and of the 290 patients operated on at UNN, 109 were transferred to their local hospital for part of the postoperative care. The remaining 181 who were discharged directly from our hospital had a mean length-of-stay (LOS) of 11.1 days.

Postoperative mortality

Four out of 290 patients (1.4%) died while still in the hospital, all within nine days after surgery, whereas three patients died before 1996, all from serious cardiovascular complications. A fourth patient was operated on in 2011 for hepatocellular carcinoma, lost 250 ml blood during surgery and died five days postoperatively due to severe pneumonia.

Postoperative complications

Out of 290 patients, 65 (22.4%) developed complications, with six of these patients developing more than one complication. All complications were classified according to the modified Clavien classification system [54], and the most common complication was pneumonia, which occurred in 17 patients (5.9%).

Postoperative survival

Of the 290 patients who underwent liver resection over the past 33 years, 134 (46.2%) were still alive as of December 2011. The actual five-year survival rate was 45.5% (81 of 178) for all patients operated on > five years ago. By dividing time into early and late periods, we found a five-year survival of 42.3% (11/26) from 1979-1993, while the late period from 1994-2007 had a five-year survival of 33.3% (23/69).

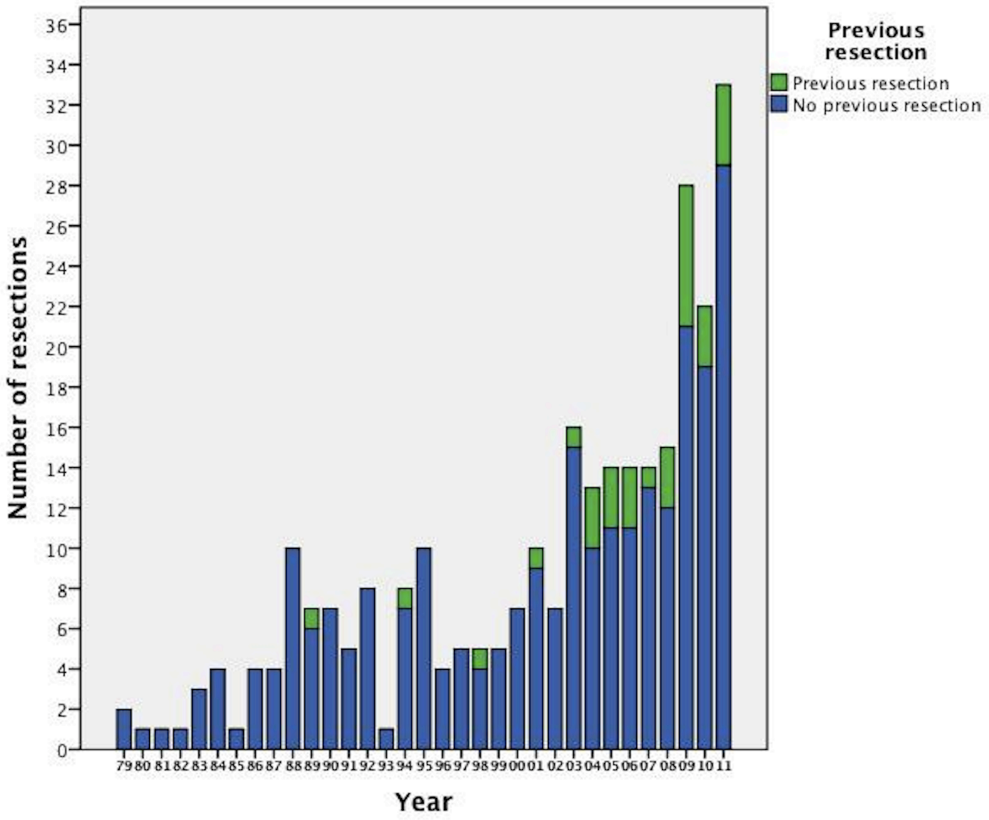

Figure 5. Number of patients who underwent liver resections from 1979-2011. From Nygård et al. 2012 [55].

	Frequency	Percent
Liver metastasis from colorectal cancer	174	60.0
Neuroendocrine carcinoid	16	5.5
Hepatocellular carcinoma	20	6.9
Other metastasis/cancer	50	17.2
Benign tumours	26	9.0
Trauma	4	1.4
Total	290	100.0

Table 1. Frequencies of indications. From Nygård et al. 2012 [55].

4.2 Paper II

Liver weight

A total of 12 pigs survived the six-week experiment, four PHx, four sham operated and four control animals. By the end of the sixth week, the liver had fully regenerated in all PHx pigs. In control animals, the liver constituted 2.33 % of the total body mass, in sham animals the liver constituted 2.48 % and in resected animals the liver constituted 2.78 % of the total body mass (Fig. 6).

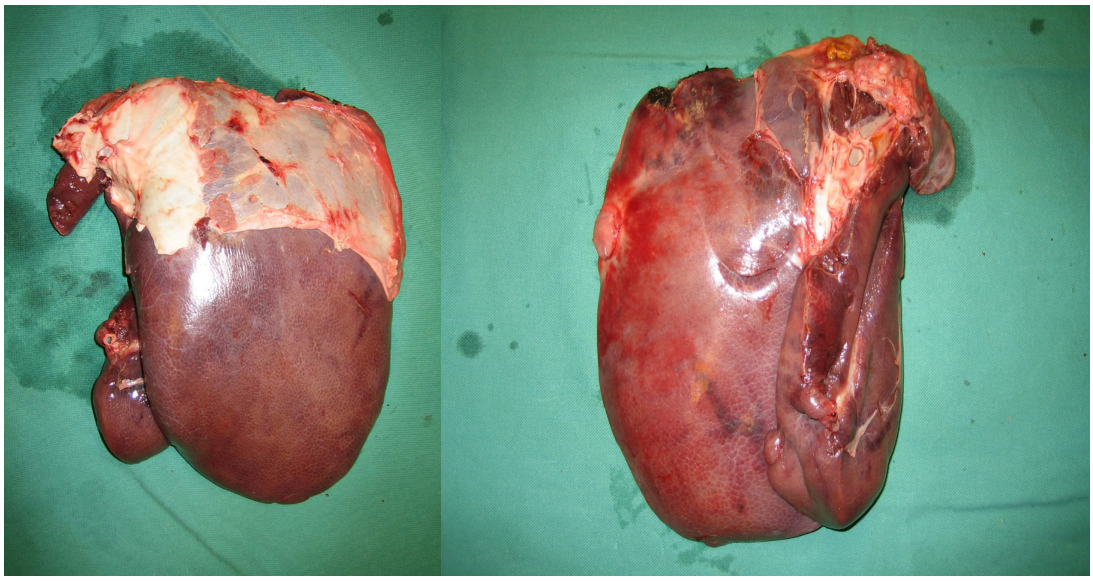

Figure 6. Macroscopic aspect of a regenerated liver six weeks post-PHx. Left; frontal, and right; dorsal view.

Microarray analysis

When comparing gene expressions at the different time contrasts, 3-0, 6-0 and 6-3 weeks, we found the resection group increasingly different over time from both the sham and control group. When comparing the three groups, seven genes were regulating apoptosis in the resection group, whereas only three and two in the sham and control group, respectively. Towards the end of regeneration, one gene found differentially expressed in both time contrasts 6-0 and 6-3 had a potential negative effect on cell cycle progression and promoted apoptosis, zinc finger protein 490 (ZNF490) [56]. By comparing the log fold change for genes in the resection group, this gene had the highest rate of 2.0 at t=1, and 2.4 at t=2. Another gene, caspase recruitment domain-containing protein 11

(CARD11) was up-regulated (log FC 0.4), and CARD domains of proteins have been shown to interact with B-cell lymphoma/leukemia 10 (BCL-10), a positive regulator of cell apoptosis [57]. Both ZNF490 and CARD11 are presented in Fig. 7.

Functional analysis

Through the classification of genes in functional groups, we were able to illustrate the differentially expressed genes in the top-tables (Fig. 8). Towards the end of the experiment (from t=1 until t=2), we found an increase in up-regulation for genes controlling lipid, hormone, amine, alcohol metabolism and transport. Amongst down-regulated genes in the resection group, there was an increase in the number of genes controlling cell cycle and transcription towards the end of the experiment (from t=1 until t=2). We also found a predominance of down-regulated genes regulating intracellular and cell-cell signalling towards the end of liver regeneration.

Angiogenesis

With regard to genes regulating angiogenesis, seven genes were differentially expressed in the resection group, three of these towards the end of regeneration. One gene was solely expressed in the resection group, vasohibin 2 (VASH2), which positively regulates both angiogenesis and the proliferation of endothelial cells in mice [58]. Moreover, VASH2 was down-regulated, both in the middle and towards the end of regeneration.

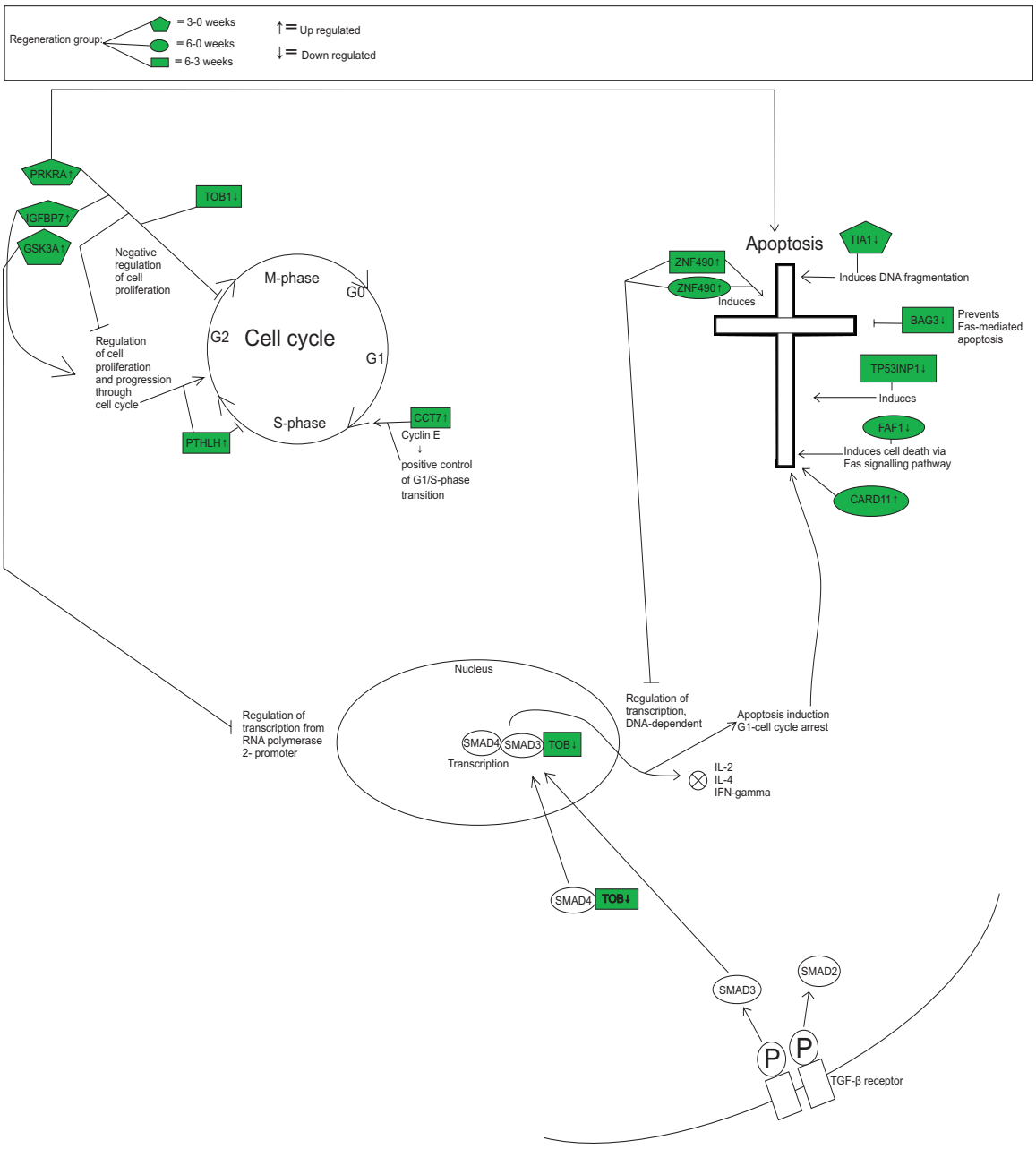

Figure 7. Differentially expressed genes at all time points after PHx. From Nygård et al. 2012 [59].

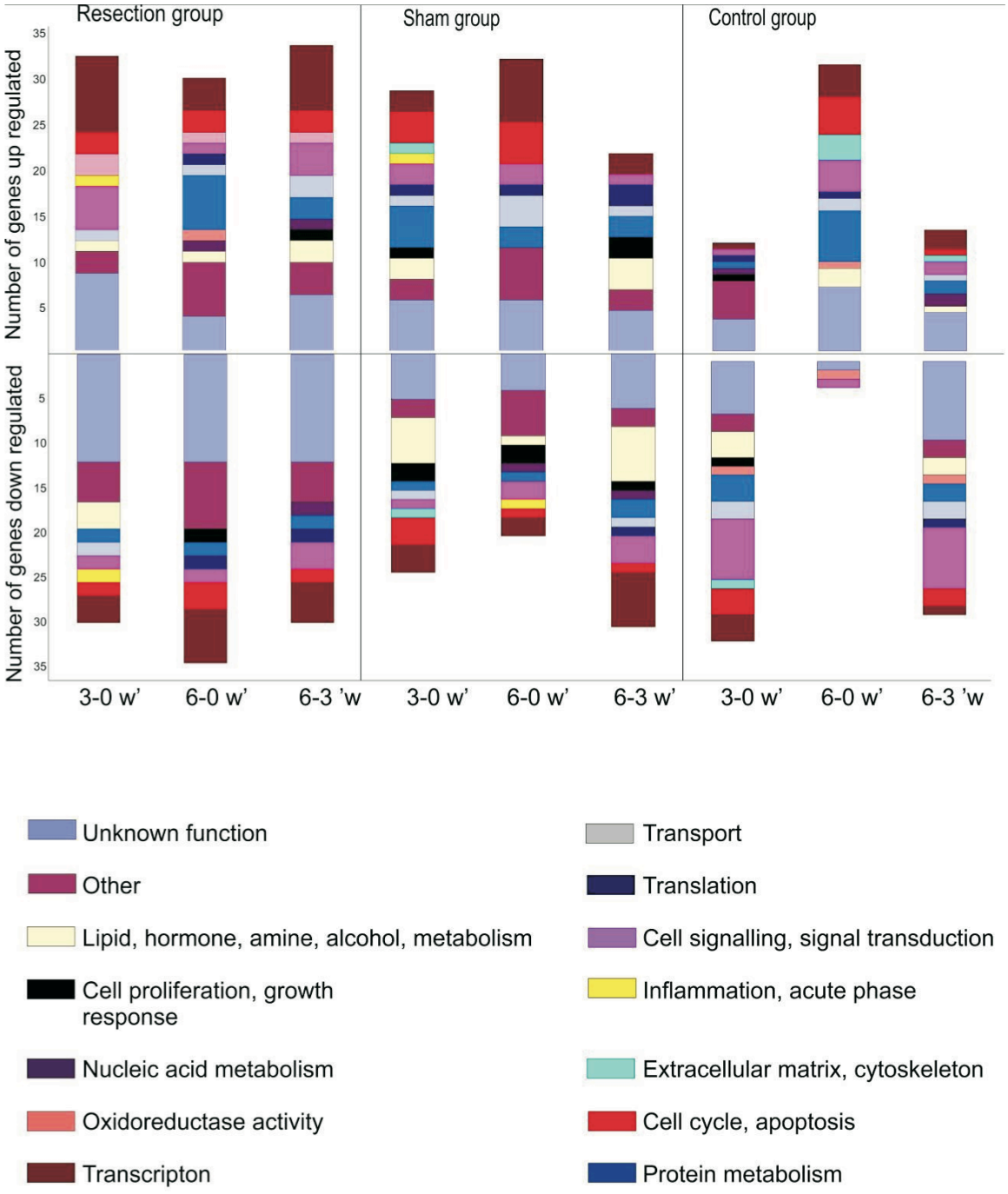

Figure 8. Functional classification of differentially expressed genes in all groups. From Nygård et al. 2012 [59].

4.3 Paper III

Microarray analysis

In the resection group, nine genes regulating the extracellular matrix were differentially expressed at the time contrast of 3-0 weeks, and two of these genes were repeatedly expressed at the time contrast of 6-3 weeks. One of these genes, secreted protein acidic and rich in cysteine (SPARC), is a matrix-associated protein that contributes to changes in cell shape, inhibits cell-cycle progression and influences the synthesis of extracellular matrix (ECM) in mice [60]. This gene was up-regulated at the beginning of regeneration (with a log FC of 0.78), and down-regulated towards the end of regeneration (with a log FC of -0.7).

Collagen type 1, alpha 2 (COL1A2) acts as a structural component of the ECM, and together with collagen type 1, alpha 1 (COL1A1), COL1A2 encodes the fibrillar collagen type I, while accounting for 36% of the total collagens in the ECM of healthy liver in rats [61]. COL1A2 was up-regulated at the beginning of regeneration with a log FC of 0.84, but not differentially expressed towards the end of regeneration.

In the sham group at the time contrast of 6-3 weeks, two genes were down-regulated, desmoplakin (DSP) and integrin, alpha V (ITGAV). Desmoplakin is a cell surface adhesion protein, and during the regeneration of rat hepatocytes, desmosomes between neighbouring cells remains constant [62]. ITGAV is a member of the integrin family, with the integrin-signalling pathway participating in regulating hepatocyte proliferation during rat liver regeneration [63].

In the control group towards the end of the experiment, one gene was down-regulated, matrix metalloproteinase 2 (MMP2). Proteins of the MMP family are involved in the breakdown of extracellular matrix in liver repair reactions [64]. All differentially expressed genes regulating the ECM are presented in Table 2.

Resection Group		
3-0 weeks (FC)	6-0 weeks (FC)	6-3 weeks (FC)
COL1A2 (0.84) *SPARC* (0.78) *TCF4* (0.33) *PTK2B* (0.27) *ERBB3* (0.26) *TFPI2* (0.26) *ESR1* (0.23) *TIMP1* (0.14) *ESR1* (-0.23)		*ESR1* (-0.38) *SPARC* (-0.7)
Sham Group		
3-0 weeks (FC)	6-0 weeks (FC)	6-3 weeks (FC)
BMP1 (0.22)	*GNG11* (0.35) *BMP1* (0.23)	*STEAP1* (0.29) *ITGAV* (-0.15) *DSP* (-0.47)
Control Group		
3-0 weeks (FC)	6-0 weeks (FC)	6-3 weeks (FC)
STEAP 1 (0.48) *AHNAK* (-0.6)	*STEAP1* (0.65) *F11R* (0.54) *OCLN* (0.4) *TFPI2* (0.38) *TCF4* (-0.28)	*MMP2* (-0.44)

Table 2: Differentially expressed genes regulating ECM in all groups over time. From Nygård et al. submitted manuscript 2013 [65].

Immunohistochemical analysis

As seen in Fig. 9, the HE staining of normal liver demonstrated the hepatic parenchyma being divided into lobules consisting of a hexagonal arrangement of hepatocytes. At six weeks post-PHx, islands of hepatocyte-like cells were presented in between the broadened connective tissue, thereby making the structural units of liver lobules less organized. Masson's Trichrome staining in normal liver demonstrated hexagons divided by a fine, delicate lining of connective tissue. At six weeks post PHx, the same staining revealed a broadened and re-established connective tissue capsule, thus dividing the parenchyma into separated nodular compartments. In normal liver, alpha smooth muscle actin (α-SMA), which is expressed in activated HSCs and smooth muscle cells, was positive in a linear pattern, both along the connective tissue and within the vascular walls. Towards the end of regeneration, α-SMA positive cells were identified in both the connective tissue and in between the hepatocytes. Pan-cytokeratin (Pan-CK) staining in normal liver revealed positive bile duct cells located within the connective tissue, while at six weeks post-PHx the same staining demonstrated a distinct pattern of hepatic

compartmentalization. At an original magnification x200, the pan-CK staining differed, from heavily stained cholangiocytes in the periportal region, towards lightly stained cholangiocytes in the pericentral region. In normal liver, the matrix-associated protein SPARC was positively stained in endothelial cells of vascular walls within the connective tissue, and COL1A2 revealed heavily positive cells all over the connective tissue. At six weeks post-PHx, SPARC-positive cells were located all over the unorganized and broadened connective tissue, and COL1A2 staining was also positive in the broadened connective tissue. Proliferating cell nuclear antigen (PCNA) positive cells exhibited a scattered distribution throughout the liver tissue during normal liver growth, whereas towards the end of regeneration, PCNA revealed a pattern of multiple heavily stained nuclei along the connective tissue compared with the staining of nuclei in the pericentral region. At high magnification, a significant number of cells immunopositive for pan-CK and PCNA were detected in the connective tissue layer and also in the adjacent hepatocyte parenchymal tissue.

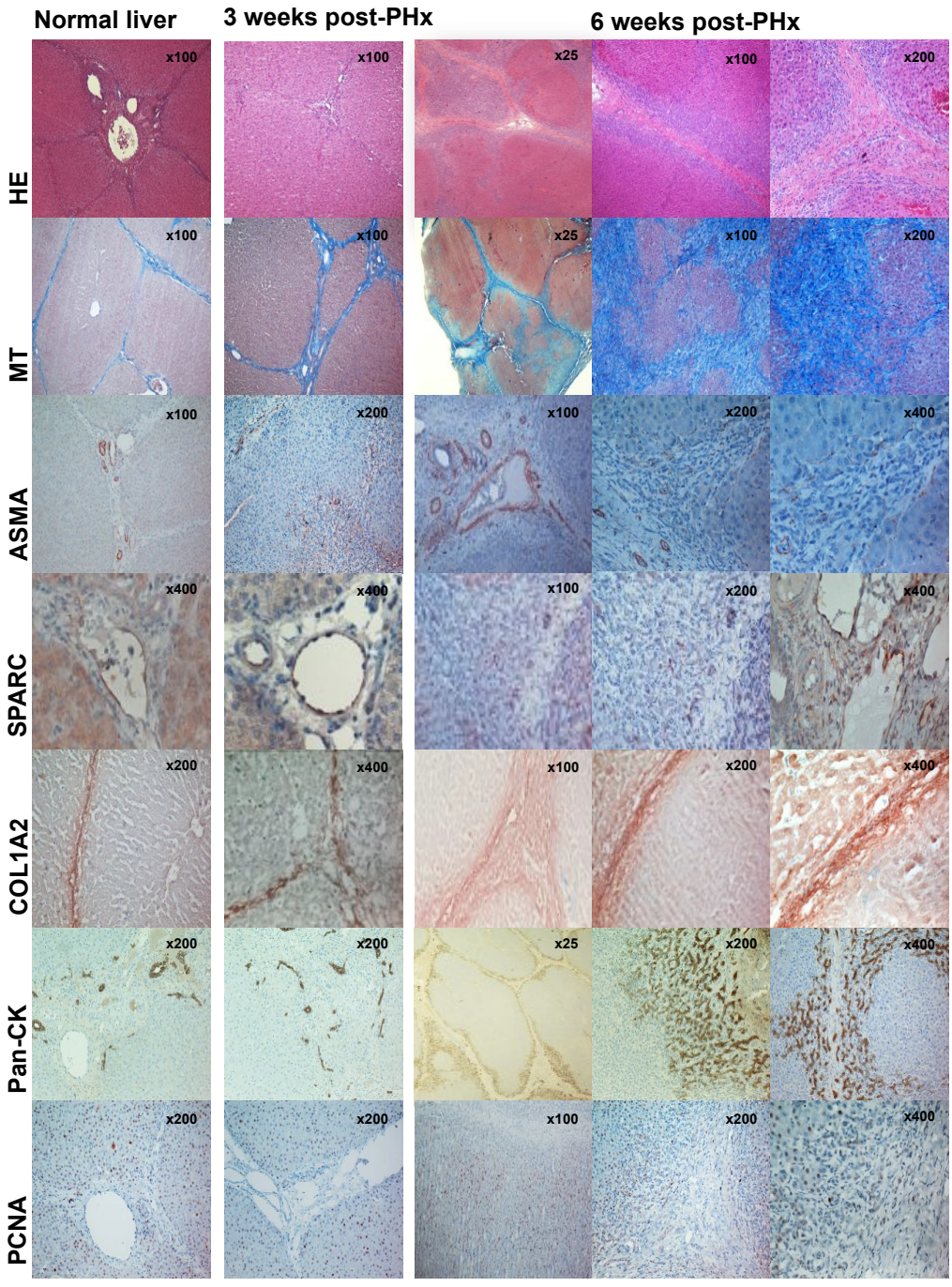

Figure 9. Overview of immunohistochemically stained normal and regenerated liver, three and six weeks post-PHx. From Nygård et al. submitted manuscript 2013 [65].

5. Discussion of papers

5.1 Paper I

It has been reported that 60-70% of recurrent colorectal cancer involves the liver, and that the liver is the only involved organ in 35% of cases [66]. Over the past few decades, liver resection has become a frequently performed procedure because of its acceptance as the most effective treatment for patients with selected cases of metastatic tumours. In particular, liver resections for colorectal metastasis have seen many refinements; improvements in anaesthesia and postoperative care have reduced both the morbidity and the mortality with a subsequently more aggressive surgical approach. Amongst these strategies are: portal vein embolization, staged liver resection, neoadjuvant chemotherapy, ablative procedures and locoregional chemotherapy [67]. The work presented in *Paper I* indicates that the results after elective hepatic surgery in a low volume centre are comparable to those of larger, high volume centres. The paper included all liver resections performed since 1979, totalling 290 resections over a 33-year period. The perioperative mortality rate after all resections was 1.4%, which is comparable to the results of this surgery reported internationally, with in-hospital mortality rates of less than 5% [68-70]. Despite the low volume in our hospital, our liver resection mortality has followed the international development for hepatic surgery.

Liver metastasis from colorectal cancer was the most common pathology (n=174), and the five-year survival rate for patients who underwent liver resection for colorectal metastases was 35.8 % (34/95) in the late period, which is comparable to recent studies from high volume centres reporting five-year survival rates of 29-37% [69-70]. In the early time period hepatic resections were only performed in a selected group of patients with a curative intention, following a five-year survival rate of 42.3% compared to the five-year survival rate of 35.8% in the late period. Resections were performed in 1.9 per 100,000 inhabitants per year, which is also in accordance with the prevalence in similar populations in Western countries. There has been a significant reduction in operating time, total amount of blood loss, hospital stay and 30-day mortality after surgery, which may be a result of gradually implementing an enhanced recovery after surgery protocol (ERAS) for our liver resected patients [71].

5.2 Paper II

The termination phase of liver regeneration is a field that has traditionally received relatively little attention, though it is now entering a phase of rapid discovery. In *Paper II,* we aimed to investigate genes regulating the terminal phase of liver regeneration, to illuminate the genetic interactions between genes controlling cell cycle, apoptosis and angiogenesis, and to clarify the role of TGF-β signalling in the termination of liver regeneration.

The study showed several trends governing the termination of the regeneration process in the liver, and as expected, more genes were found to be associated with the regulation of the cell cycle and apoptosis when comparing gene expression in the biopsies from the regenerating livers to the liver biopsies from the control animals. We also observed genes regulating angiogenesis after PHx, which might give us an indication of how the vascularisation process is controlled towards the end of liver regeneration.

How can we explain our observation that several other genes regulating cell cycle, apoptosis and angiogenesis are differentially expressed in the sham and control groups? It may be an indication of the fact that a normally growing, non-resected liver is under constant control by the opposing actions of pro-mitotic and pro-apoptotic genes and their protein products, hence maintaining a constant liver to body weight ratio. Furthermore, more genes were differentially expressed towards the end of the experiment in the resection group compared with the sham and control group. This is probably a reflection of the fact that the regenerating liver is genetically more active not only after a resection as compared to sham and control livers, but it also indicates that the regenerative response continues for many weeks.

Since the liver to body weight ratio is relatively well conserved in mammals (i.e. 2.5%), there seems to be a "hepatostat" regulating liver size. With regard to established "stop" signals of hepatocyte proliferation and liver regeneration, this paper could only partially corroborate the conclusions of most previous studies. However, we can report the "finding" of genes associated with genes known to interact with cell cycle propagation and apoptosis. For instance, TGF-β was not found in our material, although transducer of ERBB2, 1 (TOB1), a down-regulated gene in regenerating livers, has been reported to repress liver regeneration in mice [72]. This gene occurred in the resection

group at time-contrast 6-0, thus indicating a down-regulation of its antiproliferative property. Additionally, we found Sloan-Kettering viral gene oncolog (SKI) down-regulated in the early phase of the sham group, and bone morphogenetic protein 2 (BMP2), a member of the TGF-β-superfamily, down regulated in the control group during the early time period. In our opinion, the findings of TOB1, SKI and BMP2 add credibility to our study at the same time that the lack of TGF-β supports the finding from Oe et al. [73].

Interestingly, we observed in the resection group that genes promoting apoptosis and inhibiting cell cycle, such as ZNF490 and CARD11, were up-regulated towards the end of the experiment, suggesting a crucial role of these genes at this time. In addition, genes regulating apoptosis in the middle of the experiment were both down- and up-regulated, indicating a complex process before the termination of regeneration. Within the sham and control group at the end of the experiment, three and four genes regulated apoptosis, respectively. From these results, it seems as if the gene expression in the resection group was more focused towards apoptotic function compared to the sham and control groups.

5.3 Paper III

Since the reformation of normal liver architecture occurs only after restoration of the original liver mass, we aimed in *Paper III* to study the differentially expressed genes regulating the extracellular matrix, together with the tissue remodelling of normal liver microarchitecture at the termination of liver regeneration.

The study demonstrated several observations corroborating previous studies in rodents. As described by Steer [74], the extracellular matrix is of critical importance in both maintaining growth arrest in the adult liver and regulating liver regeneration. Interestingly, our study demonstrated more differentially expressed genes regulating the extracellular matrix in the resection group, when comparing gene expression in the regenerating livers to the gene expression in sham and control livers. The up-regulation of genes regulating the extracellular matrix was followed by a broadened connective tissue, which was validated by histochemical analysis six weeks post-PHx. Since the extracellular matrix is suggested to play a role in growth arrest of the regenerating liver, and our study exhibits an increased deposition of extracellular matrix in the connective tissue, these findings seem to be part of the above mentioned "hepatostat" regulating liver size.

Our study demonstrated an early genetic up-regulation of SPARC at the initiation of liver regeneration. Interestingly, SPARC is known to influence the synthesis of the extracellular matrix [60, 75], with immunopositive SPARC cells presented in the broadened connective tissue at six weeks post-PHx validating the early genetic up-regulation. The collagen forming gene COL1A2 was also up-regulated in the early phases of liver regeneration, and the immunohistochemical staining of COL1A2 revealed heavily positive collagen cells within the broadened connective tissue six weeks post-PHx. The demonstration of both SPARC- and COL1A2-positive cells within the broadened connective tissue at the termination of regeneration suggests that the early genetic up-regulation plays a role in growth arrest at the end of regeneration. Additionally, α-SMA, a marker of HSC's and smooth muscle cells, displayed positive cells along the connective tissue, in addition to α-SMA-positive cells in between the hepatocytes at six weeks post-PHx. This implicates not only the presence of activated

HSC's, but could also explain the broadening of the connective tissue as part of a liver injury-induced production of ECM.

The question "where do hepatocytes come from?" during liver regeneration still remains controversial. However, hepatocytes are suggested to derive form Sox9-postive cells that were cholangiocytes in an initial morphology [76]. Hepatocyte progenitor cells and normal biliary epithelial cells are strongly immunopositive for cytokeratins [77], and our study shows the presence of pan-CK-positive cells six weeks after injury (PHx) to be in a migrating pattern from heavily stained cells in the portal region, fading towards lightly stained cells in the central area. Our study also exhibits multiple PCNA-positive nuclei along the parallel periportal parenchyma, and from these results it seems as if the periportal parenchyma is the zone of growth during regeneration. These data corroborate with "the streaming hypothesis", which suggests an ongoing process of hepatocyte proliferation and migration that starts in the periportal and "streams" towards the pericentral region.

Compared to rodents, the pig bears a closer genetic and physiological resemblance to humans, and *Paper III* is the first to account for the histological changes in a porcine model during the terminal phase of liver regeneration. In addition, our data support previous reports in rodent models, not only as it pertains to the "streaming" hypothesis that suggests a pattern of hepatocyte migration, but also with regard to the cholangiocyte origin of hepatocytes in the termination of liver regeneration.

6. Conclusions

I. By following international guidelines, hepatic resections are safely performed at a low volume centre, with regard to perioperative- and in-house mortality, as well as five-year survival rates.

II. The terminal phase of liver regeneration in a porcine model reveals more impact on genes regulating cell cycle, apoptosis and angiogenesis when comparing gene expression in the liver remnants to the liver biopsies from the sham and control animals.

III. The architectural remodelling in the terminal phase of liver regeneration in a porcine model corroborates previous reports in rodents with regard to the deposition of ECM proteins, hepatocyte migration and cholangiocyte origin of hepatocytes.

7. Further studies

Since the liver itself is a complex organ conducting over 5,000 different functions [78], it is unlikely to believe that the terminal phase of liver regeneration is controlled by a few differentially expressed genes. This thesis contributes with the identification of differentially expressed genes at three time periods, firstly, in between three and zero weeks of liver regeneration, second, in between six and zero weeks, and third, in between six and three weeks of liver regeneration. Even though the liver, according to the liver to body weight ratio, had fully regenerated at six weeks, we do not know if the reorganisation of the liver parenchyma was completed at the same time. As liver regeneration is a highly orchestrated phenomenon, it is likely to believe that the terminal phase is regulated over some time. This presents a need for a protocol with smaller sample intervals in order to study the terminal phase of liver regeneration.

Traditionally, most studies on liver regeneration have been conducted in rodents and from *in vitro* hepatocyte cultures. This is not without difficulties, as rodents significantly differ from humans in both physiology and overall time for the liver to regenerate. Groenen et al. reported in late 2012 the fully genome sequence of a female domestic Duroc pig (Sus Scrofa) [79], thus extending the valuable resource of the pig in biomedical research by enabling more accurate genetic analysis.

Many aspects of the terminal phase of liver regeneration are unanswered, and unfortunately very few studies have been conducted on large animal models. Information from such models would probably better reflect the human situation, and further *in vivo* studies are certainly required to better understand the molecular biology and genetic regulation throughout this process.

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Paper I

Paper II

Paper III