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# Gene Expression in the Liver Remnant Is Significantly Affected by the Size of Partial Hepatectomy: An Experimental Rat Study

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Extended hepatectomies may result in posthepatectomy liver failure, a condition with a high mortality. The main purpose of the present study was to investigate and compare the gene expression profiles in rats subjected to increasing size of partial hepatectomy (PH). Thirty Wistar rats were subjected to 30%, 70%, or 90% PH, sham operation, or no operation. Twenty-four hours following resection, liver tissue was harvested and genome-wide expression analysis was performed. Cluster analysis revealed two main groupings, one containing the PH(90%) and one containing the remaining groups [baseline, sham, PH(30%), and PH(70%)]. Categorization of specific affected molecular pathways in the PH(90%) group revealed a downregulation of cellular homeostatic function degradation and biosynthesis, whereas proliferation, cell growth, and cellular stress and injury were upregulated in the PH(90%) group. After PH(90%), the main upregulated pathways were mTOR and ILK. The main activated upstream regulators were hepatocyte growth factor and transforming growth factor. With decreasing size of the future liver remnant, the liver tended to prioritize expression of genes involved in cell proliferation and differentiation at the expense of genes involved in metabolism and body homeostasis. This prioritizing may be an essential molecular explanation for posthepatectomy liver failure.

**Key words: Hepatectomy; Regeneration; Liver failure; Gene expression; Microarray analysis**

## INTRODUCTION

Partial hepatectomy (PH) remains the standard treatment with a curative intend for both primary and secondary liver malignancies, both of which are on the rise worldwide[1–3]. Because of the unique regenerative ability of the liver, PH of the healthy human liver can be performed, as long as the volume of the future liver remnant (FLR) is 20%–30% of the total initial liver volume[4]. Larger PHs may result in posthepatectomy liver failure (PHLF), which is associated with a high morbidity and mortality[5]. The corresponding minimal size of the FLR (MSFLR) in healthy rats is approximately 10%[6–8].

The healthy liver's ability to recover lost mass, without jeopardizing the viability of the entire organism, is unique. Liver regeneration is the result of hepatocyte hypertrophy and hyperplasia[9]. Regeneration terminates rather precisely when the normal liver-to-body weight ratio (LBWR) is restored. Initiation, progression, and termination of liver regeneration are very complex processes involving a large number of pathways[10]. The overall regulator of these complex processes is often termed the hepatostat[11–13]. The most widely used model to examine liver regeneration is the 70% PH[14,15], in which the regenerative process is described from the early moments until termination of the process. The kinetics of liver regeneration seem to be different in the MSFLR model. In a recent study, we found a delay in hepatocyte proliferation, when comparing 90% PH to 70% PH[16]. This finding is also in agreement with other previous studies[17,18]. This suppression or delay of hepatocyte proliferation in the early phase of MSFLR regeneration could be crucial in understanding PHLF.

Genome-wide expression analysis can be used to uncover molecules and pathways affected by different sizes of the PH. The main purpose of the present study was to investigate and compare the gene expression profiles in rats subjected to the increasing size of the PH [PH(30%), PH(70%), and PH(90%)]. In addition, we aimed to determine which genes and gene networks are regulated in

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order to identify which pathways the liver prioritizes depending on the extent of PH and consequently the size of the FLR.

## MATERIALS AND METHODS

### *Experimental Design*

In total, 30 healthy, 10-week-old, male Wistar rats (M&B Taconic, Eiby, Denmark) with a mean weight of 206 g (range: 188–227) were randomized into blocks of 5 according to the size of the PH: PH(30%) ($n=6$), PH(70%) ($n=6$), PH(90%) ($n=6$), sham (laparotomy without PH) ($n=6$), and baseline (no surgery) ($n=6$). All rats (except baseline) were euthanized 24 h postoperatively [postoperative day (POD) 1].

The experimental protocol was approved by the Danish Animal Research Committee, Copenhagen, Denmark (License No. 2012-15-2934-00591). Animals received care in accordance with the *Guide for Care and Use of Laboratory Animals* published by the US National Institutes of Health[19]. The animals were housed in standard animal laboratories with the temperature maintained at 23°C, with an artificial 12-h light–dark cycle, and with free access to food and water. The rats were kept in the animal facilities until the end of the experiment.

### *Surgical Procedure*

The surgical procedure was performed as described in a previous publication by this study group[16]. In brief, performing PH(30%), the left lateral lobe (LLL) was resected (Fig. 1). In PH(70%), the LLL and the median lobe (ML) were resected. In PH(90%), the right lobe (RL), ML, and LLL were all resected, leaving only the two caudate lobes [posterior caudate lobe (PCL) and anterior caudate lobe (ACL)] intact[6].

After 24 h, the rats were reanesthetized, and a laparotomy was performed through the previous incision. Blood samples were collected from the heart by cannulation, immediately processed, and stored at −80°C until biochemical analyses. Animals were euthanized by cervical dislocation under anesthesia. The remnant liver was removed, and the ACL was snap frozen in liquid nitrogen and stored at −80°C until use.

### *Biochemical Analyses*

Alanine aminotransferase (ALT), alkaline phosphatase (AP), haptoglobin (HG), and bilirubin (BR) levels were measured using Modular P (Roche Diagnostics, Mannheim, Germany).

The prothrombin/proconvertin ratio (PP) was measured using the automated coagulation analyzer Sysmex CS2100i (Sysmex©, Tokyo, Japan).

### *RNA Isolation*

Each cryopreserved liver tissue was cut in a 2×2-mm piece and immediately transferred into 5-ml polypropylene tubes (BD, Franklin Lakes, NJ, USA) containing 200 µl of chilled homogenization solution and 4 µl of lysis buffer (Maxwell 16 LEV simply RNA; Promega, Fitchburg, MA, USA). While kept on ice, each tube was thoroughly mixed using a homogenizer before adding 200 µl of lysis buffer (Maxwell 16 LEV simply RNA; Promega) and vortexed at a maximum speed for 15 s before further processing according to the manufacturer's protocol. The isolated RNA was stored at 80°C.

RNA quality was evaluated using the BioAnalyzer 21000 (Agilent Technologies, Santa Clara, CA, USA). Only samples with a RIN score >7 were used.

### *Microarray Analysis*

Following the manufacturer's instructions, the Ambion® WT Expression Kit (Thermo Fischer Scientific, Waltham, MA, USA) was used for labeling 100 ng of isolated RNA. Samples were hybridized to the Affymetrix GeneChip®

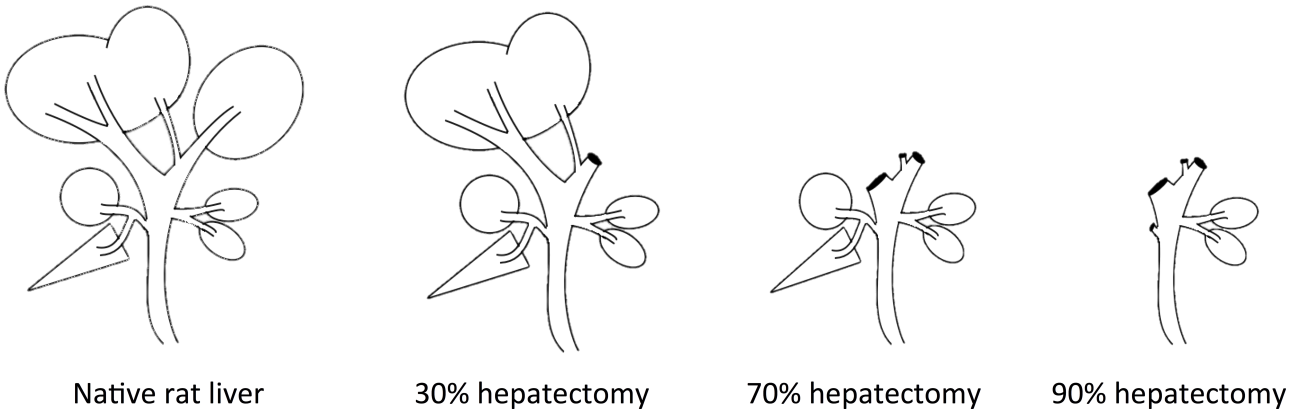


**Figure 1.** Surgical procedure.

**Table 1.** Primer Sequence Design Used for PCR Analysis

| | |
|---|---|
| Interleukin-1rn | F: TGGTGCCTATTGACTTTCGGA |
| | R: ACAGGGCTCTTTTGGTGTGT |
| Lipocalin 2 | F: AGGCTTCTGGACCGAACG |
| | R: TGTCTTTCTTTCTGGACCGCA |
| Transforming growth factor-β1 | F: CGTCAGACATTCGGGAAGCA |
| | R: CACTCAGGCGTATCAGTGGG |
| Epidermal growth factor receptor 2 | F: GGAGGCCCCTTTGATCAGAT |
| | R: GTCTGGTTTCTAGGCGCAGA |
| Nuclear receptor subfamily 1 group I | F: GGCCGATGTGTCAACCTACA |
| | R: GGTTCCTGTTTCCGTGTCGA |
| Guanylate cyclase 2C | F: GAGGTGGTGGACATGCTGAAT |
| | R: CGGTTGCCGTTTCTCATAGGC |
| Cytochrome P450 oxidoreductase | F: AGGATTTCATCACGTGGAGG |
| | R: CTCTTCAGACGGCCCATCTC |

Rat Gene 2.0 ST Array (Thermo Fischer Scientific) at 45°C and 60 rpm for 16 h and scanned with the Affymetrix GeneChip® Scanner 3000 7 G.

Quality control of the array data was performed in the Expression Console software (Affymetrix) with default RMA Gene analysis settings. Experimental data have been submitted to NBCI Gene Expression Omnibus under series record GSE97429 and will be available after March 31, 2018.

*Gene Expression Analysis*

Gene expression levels were analyzed by importing raw image files from the quantitative scanning into GeneSpring Version 13.0 (Agilent Technologies). Networks and functional analyses were generated through the use of QIAGEN's Ingenuity Pathway Analysis (IPA®; www.qiagen.com/ingenuity; QIAGEN; Redwood City, CA, USA).

*Quantitative Real-Time-PCR Expression Analysis*

Validation of microarray gene expression data was performed using quantitative real-time (qRT)-PCR expression analysis using LightCycler 480 Instrument 96-well (Roche Diagnostics, Hvidovre, Denmark). A total of 10 genes were selected for validation. Three of them were stably expressed housekeeping genes GAPDH, SDHA, and β-actin. Seven genes were selected because of different expression levels between groups (IL1rn, Lcn2, Tgfb1, Egf, Nr1I2, Gucy2c, and Por). Primer sequence details can be seen in Table 1. Analysis was performed using Multiwell Plate 96 Clear (Roche Diagnostics). All genes were analyzed in duplicates. From the isolated RNA, reverse transcription was performed in order to produce a suitable amount of cDNA. cDNA (2 µl) was mixed with 7.5 µl of SYBR Green l Master (Roche Diagnostics) and 3 µl of gene-specific primer mixture before PCR amplification according to the manufacturer's protocol. The measured expression levels were normalized to the geometric mean of the three stable expressed genes.

A comparison of expression levels of the seven selected differently expressed genes is presented in Table 2. A concurrence was found between the two methods of measuring gene expression levels, even though the PCR measurements tended to vary more for some of the genes tested. Overall, the PCR data confirmed our microarray data.

*Statistical Analyses*

Statistical analyses for gene expression data were performed using GeneSpring Version 13. By one-way ANOVA, unsupervised gene data were groupwise tested

**Table 2.** Comparison of Expression Levels of the Seven Differently Expressed Genes

| Gene Name | Baseline (PCR/MA) | PH(30%) (PCR/MA) | PH(70%) (PCR/MA) | PH(90%) (PCR/MA) |
|---|---|---|---|---|
| Interleukin-1rn | −1.69/−1.72 | 1.00/1.29 | 1.36/1.26 | 2.50/2.44 |
| Lipocalin 2 | −60.63/−13.16 | 7.09/3.48 | 19.01/4.43 | 9.19/6.65 |
| Transforming growth factor-β1 | −1.01/−1.15 | 10.02/1.48 | 16.47/1.28 | −1.74/1.14 |
| Epidermal growth factor receptor | 1.05/−1.29 | 1.48/−1.28 | 1.38/−1.67 | 1.41/−3.43 |
| Nuclear receptor subfamily 1 group i member 2 | 1.02/−1.11 | −1.87/−1.41 | −2.00/−1.34 | −1.23/1.27 |
| Guanylate cyclase 2C | −7.89/1.12 | 17.03/1.03 | 33.74/1.45 | 81.00/8.23 |
| Cytochrome P450 oxidoreductase | −2.26/−1.69 | −1.15/1.05 | −1.39/1.05 | 1.29/1.85 |

Values indicate the mean fold change in expression levels compared to the sham group. PCR, polymerase chain reaction expression analysis; MA, microarray expression analysis.

against each other and were considered significantly different with a value of $p<0.001$ (Benjamini–Hochberg corrected), and fold change (FC) of group mean expression levels was >2.0. Also, in GeneSpring, unsupervised cluster analysis was performed.

In IPA, the Benjamini–Hochberg method was used to determine significant subset from our unsupervised gene data imported from GeneSpring. Values of $p<0.05$ were considered significant. FC was set to be 1.0 as the data had already been selected with an FC>2.0 from GeneSpring. Specifically for upstream regulators, the overlap $p$ value was calculated by Fisher's exact test, measuring whether there was a statistically significant overlap between the dataset genes and the genes regulated by a transcriptional regulator. Values of $p<0.001$ were considered significant.

Statistical analyses for body weight- and liver-specific parameters were tested with regression analysis using Stata v.13.1 (StataCorp, College Station, TX, USA) and presented as mean values given with a 95% confidence interval (CI). Values of $p<0.05$ were considered significant.

## RESULTS

### *Animals and Circulating Markers*

During the time period of our study, no animals died or were euthanized prior to evaluation. No observation was made preoperatively with regard to difference in bodyweight between groups (mean: 206 g, CI: 202–210). Bodyweight decreased significantly for all intervention groups in the first 24 h after surgery (mean: 199 g, CI: 194–204). Liver regeneration was calculated as the regeneration rate (RR), which we have previously described. Twenty-four hours after the liver resection was performed, the mean fold increase in RR was 1.93 PH(90%), 0.75 PH(70%), and 0.19 PH(30%)[16].

Liver-specific parameters are presented in Figure 2. In general, the group of PH(90%) showed significantly ($p<0.001$) affected liver-specific parameters compared to all other groups with an increase in ALAT, AP, and BR and a decrease in PP. Compared with baseline, HG levels increased significantly for PH(30%) ($p<0.001$) and

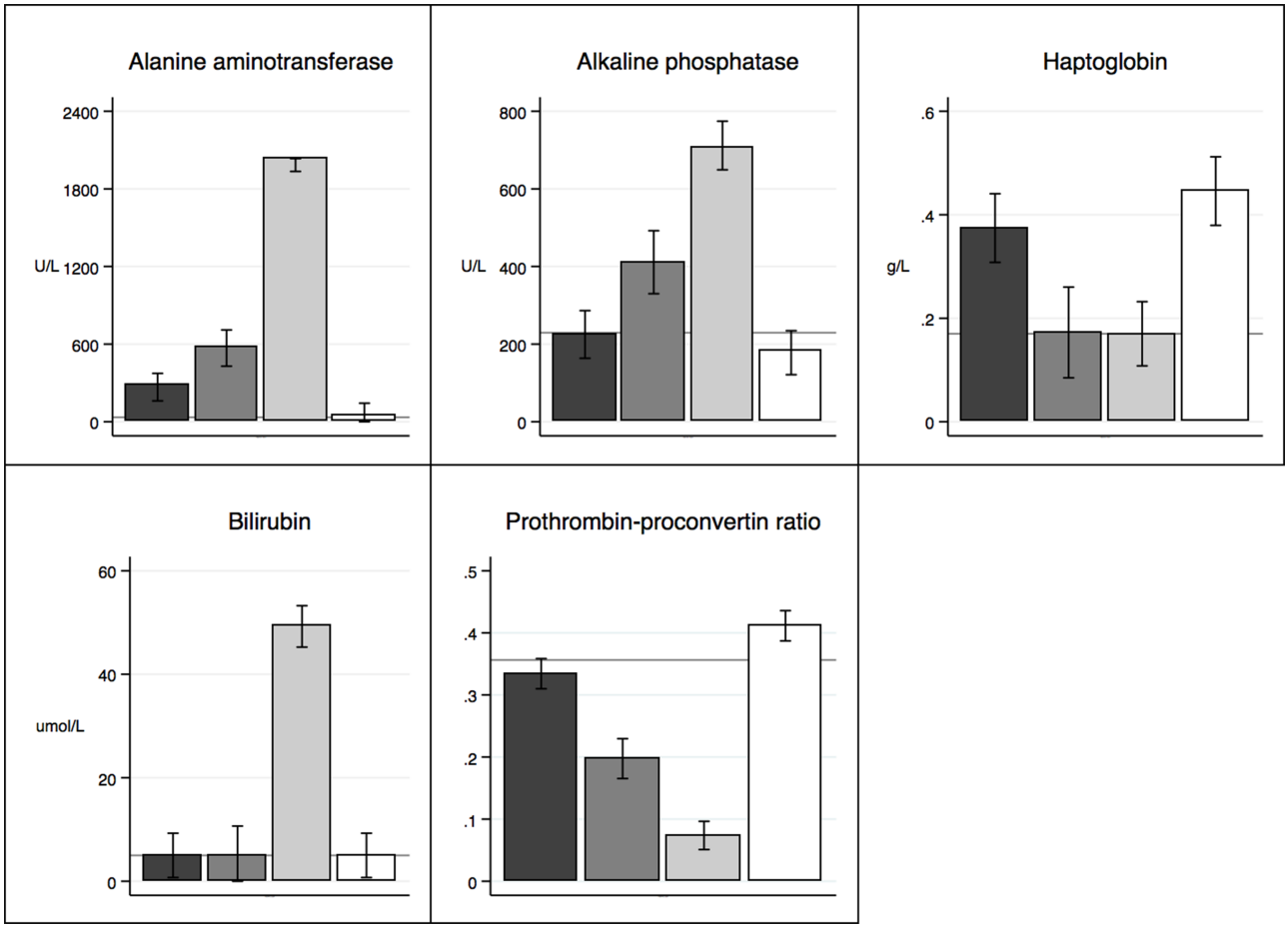


**Figure 2.** Liver-specific biochemistry: black bar, PH(30%); dark gray bar, PH(70%); light gray bar, PH(90%); white bar, sham; gray horizontal line, baseline.

sham ($p<0.001$), while it remained steady for PH(70%) ($p>0.934$) and PH(90%) ($p\geq1.0$).

*Gene Expression*

In total, microarray analysis revealed 29,489 genes. Data reduction of unsupervised gene data in GeneSpring ($p<0.001$, FC$>2$) reduced this number to 1,762 differentially expressed genes between groups, which were used for further analyses mentioned below.

Comparing each intervention group to sham revealed a total of 1,471 varying genes and an increase in the number of differentially expressed genes with the extension of PH (Fig. 3).

Figure 4 presents an unsupervised cluster analysis. From gene expression levels, groups were clustered as appearing in the study design most clearly distinguishing the PH(90%) group from the other groups.

IPA was used to identify molecular pathways and upstream regulators within the differentially expressed genes. Analyzing the affected genes and comparing the PH(90%) group to the other groups, 20 molecular pathways were found to be upregulated. Of these, five pathways were significantly upregulated throughout the groups: eukaryotic initiation factor (eIFs)2 signaling, regulation of eIF4 and p70S6K, mammalian target of rapamycin (mTOR) signaling, integrin-linked kinase (ILK) signaling, and Acyl-CoA hydrolysis. Seventy-two molecular pathways were downregulated. Each pathway was associated with its signaling pathway category(ies) shown in the IPA report (Fig. 5A–C). Regarding the downregulated pathways and the categorization of these, no remarkable differences were found between groups. A pie chart of PH(90%) versus all groups is displayed in Figure 5C and is interpretable as a generalization for the overall downregulated pathway categories. Table 3 shows all the up- and downregulated pathways. Cascades of upstream transcriptional regulators explaining the observed gene expression changes and affected molecular pathways are presented in Table 4.

## DISCUSSION

In the present study, we investigated and compared the gene expression profiles in rats subjected to increasing size of PH approaching the lower limit of the FLR. We demonstrated that the gene expression profile was significantly affected by increasing PH. Overall, we found that hepatocytes tended to express genes involved in cell proliferation and differentiation at the expense of genes involved in metabolism and thereby body homeostasis with decreasing FLR.

The bodyweight for all intervention groups was significantly lower at euthanasia compared to the bodyweight preoperatively, with a significant decrease in the

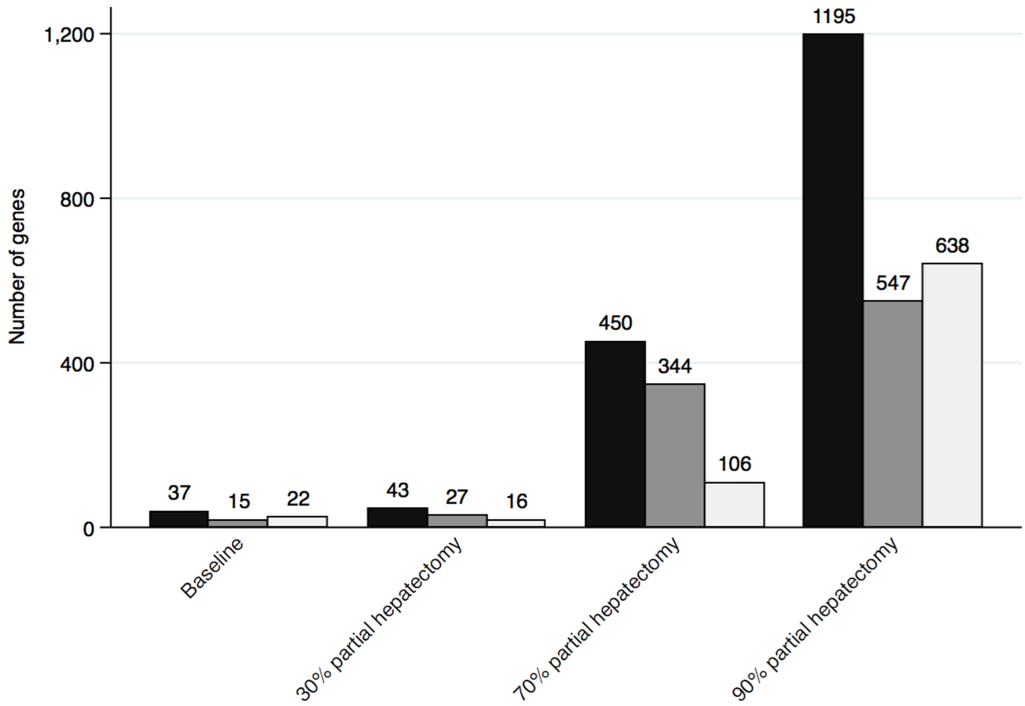


**Figure 3.** Number of genes significantly affected when comparing the sham group to all other groups. Black bar: total number of significantly affected genes; dark gray bar: number of genes significantly upregulated; light gray bar: number of genes significantly downregulated.

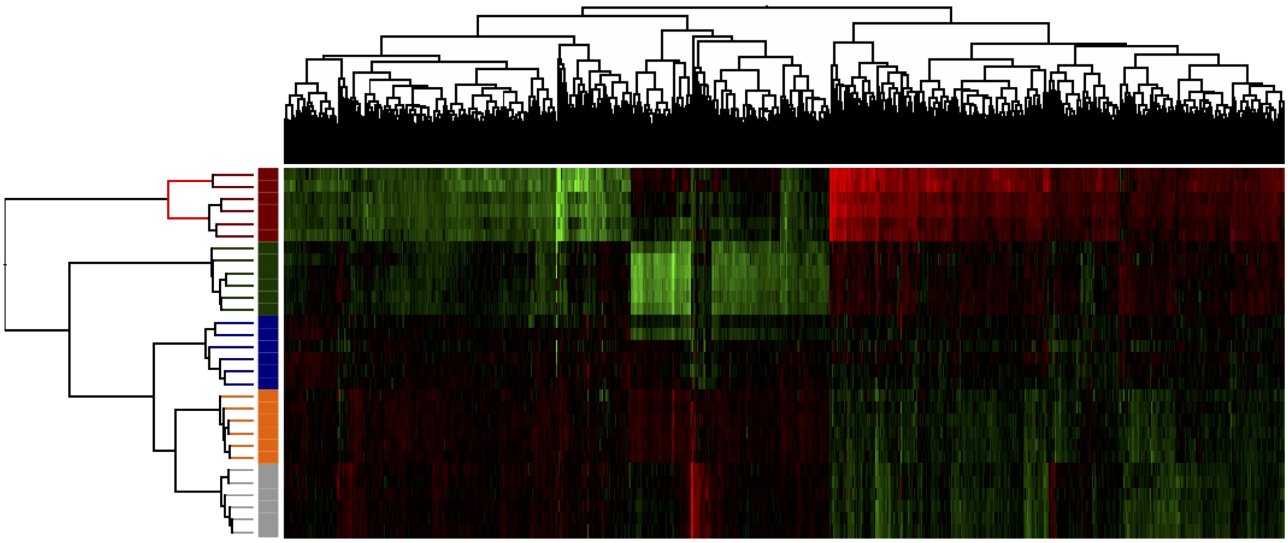

**Figure 4.** Cluster analysis (unsupervised) of the five groups: blue: PH(30%); green: PH(70%); red: PH(90%); orange: sham; gray: baseline. The range color of the analysis indicates the gene expression levels (red, decreased expression level; green, increased expression level).

resected compared to the sham group. As a change in bodyweight is a reliable marker of acute stress in rats[20], this finding indicates that PH induces a higher level of surgical stress than the sham operation.

We observed a significant increase in ALT, AP, and BR being proportional to the amount of liver parenchyma resected. As no ischemic liver parenchyma could explain this rise in liver parameters, a plausible explanation could be that the increase in portal blood flow to the FLR was proportional to the amount of liver tissue resected. This portal hyperperfusion will cause intravascular shear stress, and an associated HABR (hepatic arterial buffer response) induces constriction of the hepatic artery[21]. Enzyme leakage and PHLF seems to be linked to this phenomenon. PP was as expected, adversely affected by increasing size of the PH. We interpret this as a decrease in the FLR's synthetic capacity being inversely related to its size. The same pattern was found for HG, which was elevated in rats sustained to PH(30%) and sham operation compared to baseline, PH(70%), and PH(90%). HG is an acute phase reactant that is synthesized by hepatocytes and plays a key role in the innate immune system[22]. This finding is probably a consequence of an initially nonresponsive synthetic capacity of the FLR.

Fausto divided the gene expression after PH in rats into four phases depending on time after surgery (immediate-early genes: 0–4 h post-PH, delayed-early genes: 4–8 h post-PH, cell cycle genes: 8–20 h post-PH, and DNA replication and mitosis: 20–48 h post-PH)[23]. We analyzed the rat liver tissue at POD1 (24 h), and the gene expression profiles found are compatible with the category of cell cycle genes and genes of DNA replication and mitosis.

A comparative analysis with the sham group as a reference showed a significant increase in the number of genes significantly affected with increasing extent of PH (Fig. 3). A subsequent cluster analysis (Fig. 4) divided these genes into two main clusters: one containing the PH(90%) group and one containing all the other groups. This leads us to conclude that extended PH(90%) affects genes significantly different than minor PHs. Also, the cluster analysis revealed a subdivision of the main cluster analysis group containing all other groups than PH(90%) with a differentiation into groups based on the type of intervention. Interestingly, the influence of PH(70%) on gene expression seems to be more similar to PH(30%), sham, and baseline than the extended PH(90%). We have previously shown that the extended PH(90%) in rats leads to higher mortality than minor PHs[16], and hypothesized that the early postoperative period after PH(90%) is critical with regard to PHLF and survival. In this study, we show that there are observable and significant differences in the genetic expression between the extended PH(90%) and minor PHs in this period. The key to the sufficient regeneration of the MSFLR parallel to the continuous maintenance of liver homeostasis and survival could be a consequence in these genetic differences.

These observations prompted us to identify pathways that the liver prioritizes depending on the extent of PH. Our frame of reference was the PH(90%) group. Seventy-two pathways were downregulated in the PH(90%) group

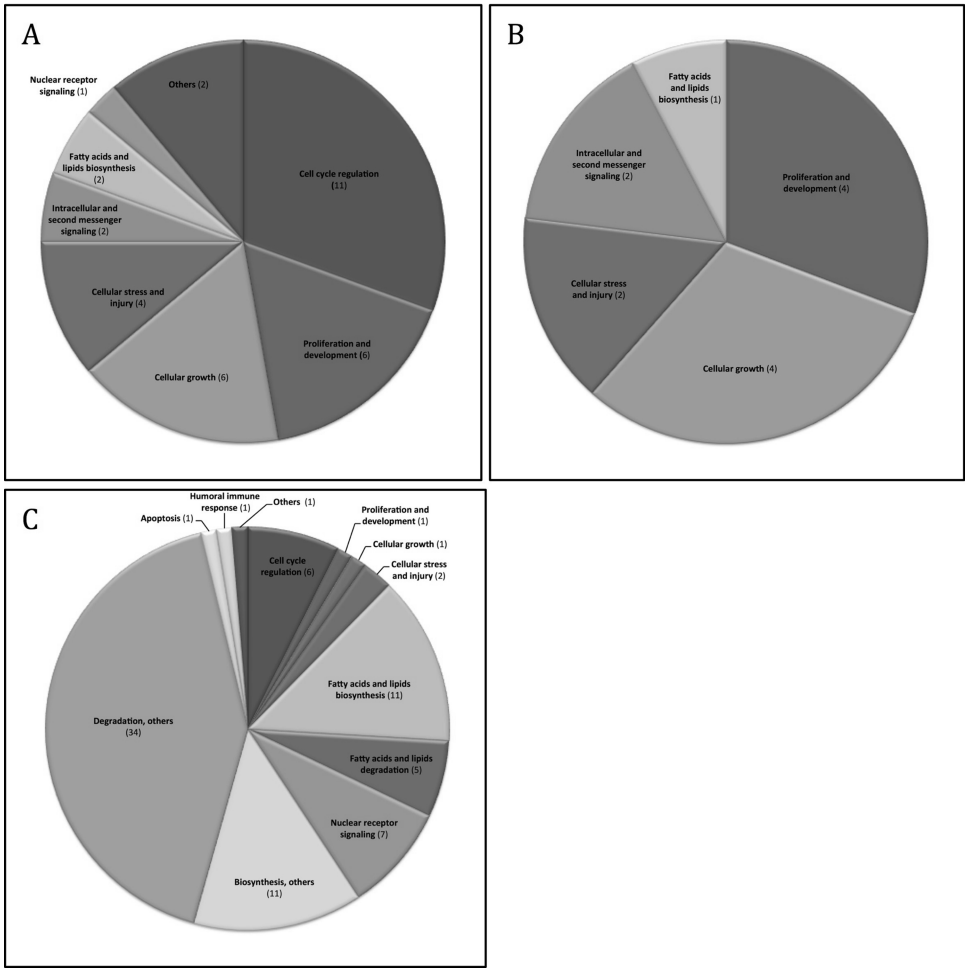


**Figure 5.** Categorized Ingenuity Pathway Analysis (IPA) molecular pathways. The categorization was performed on the 20 upregulated pathways and 72 downregulated pathways found when comparing PH(90%) to all other groups. (A) Upregulated categorized pathways: PH(90%) versus all groups ($n=20$). (B) Upregulated categorized pathways: PH(90%) versus PH(70%) ($n=5$). (C) Downregulated categorized pathways: PH(90%) versus all groups ($n=72$). The pie charts describe signaling pathway categories associated with the pathways found in IPA. The number in parentheses refers to the number of pathways clustered in the category. Several pathways are represented in more than one category. The IPA report ("Signaling Pathway Categories") for each pathway was used for categorization. Some categories were accumulated as follows: Others: cancer, disease-specific pathway; Degradation, others: hormone degradation, amino acid degradation, xenobiotic metabolism, detoxification, degradation other categories; Biosynthesis, others: hormone biosynthesis, vitamin biosynthesis, biosynthesis other categories.

compared to all the other groups. Degradation and biosynthesis were the pathway categories mainly found to be downregulated, thus indicating how the hepatostat prioritizes when the FLR approaches the lower limit.

Twenty pathways were significantly upregulated comparing PH(90%) with all other groups. The main pathway categories were found to be cell cycle regulation, proliferation and development, cellular growth, and cellular stress and injury (Fig. 5A). When comparing PH(90%) only to PH(70%), proliferation and development, cellular growth, and cellular stress and injury still appeared as significantly upregulated pathway categories, whereas cell cycle regulation is no longer significantly differently affected (Fig. 5B).

When cell cycle is active, the regulation may be independent of the size of PH. Only when PH exceeds 30% is regeneration achieved by proliferation; until then, the regeneration depends on hypertrophy only[9,16], and no cell cycle regulation is needed. On the contrary, proliferation and cellular growth seem to be alterable with the size of PH. As expected, cellular stress and injury are genetically largely expressed in the extended PH(90%) group, indicating that this extended surgical procedure is followed by a very critical phase determining survival or PHLF.

**Table 3.** Upregulated and Downregulated Pathways of Differentially Expressed Genes

**Upregulated IPA Pathways:** PH(90%) Versus All Other Groups ($n = 20$)

1. EIF2 signaling
2. Cell cycle: $G_2$/M DNA damage checkpoint regulation
3. GADD45 signaling
4. Mitotic roles of Polo-like kinase
5. Regulation of eIF4 and p70S6K
6. Cell cycle control of chromosomal replication
7. mTOR signaling
8. Estrogen-mediated S-phase entry
9. Cyclins and cell cycle regulation
10. Role of CHK proteins in cell cycle checkpoint control
11. Hereditary breast cancer signaling
12. RAN signaling
13. ILK signaling
14. ATM signaling
15. DNA damage-induced 14-3-3o signaling
16. Cell cycle: $G_1$/S checkpoint regulation
17. Stearate biosynthesis I
18. Acyl-CoA hydrolysis
19. Cell cycle regulation by BTG family proteins
20. Breast cancer regulation by stathmin 1

**Downregulated IPA Pathways:** PH(90%) Versus All Other Groups ($n = 72$)

1. Acetone degradation I (to methylglyoxal)
2. Adenosine nucleotide degradation II
3. Androgen biosynthesis
4. Aryl hydrocarbon signaling
5. Bile acid biosynthesis, neutral pathway
6. Bupropion degradation
7. Cell cycle control of chromosomal replication
8. Cell cycle: $G_2$/M DNA damage checkpoint regulation
9. Cholesterol biosynthesis I
10. Cholesterol biosynthesis II (via 24,25-dihydrolanosterol)
11. Cholesterol biosynthesis III (via desmosterol)
12. Citrulline biosynthesis
13. Complement system
14. DNA damage-induced 14-3-3o signaling
15. Dopamine degradation
16. Estrogen biosynthesis
17. Estrogen-mediated S-phase entry
18. Ethanol degradation II
19. Ethanol degradation IV
20. Fatty acid activation
21. Fatty acid α-oxidation
22. Fatty acid β-oxidation I
23. FXR/RXR activation
24. γ-Glutamyl cycle
25. γ-Linolenate biosynthesis II
26. Glucocorticoid biosynthesis
27. Glutaryl-CoA degradation
28. Glutathione-mediated detoxification
29. Glycine betaine degradation
30. Guanosine nucleotides degradation III
31. Histamine degradation
32. Leucine degradation
33. LPS/IL-1-mediated inhibition of RXR function
34. LXR/RXR activation
35. Melatonin degradation I
36. Methylglyoxal degradation III
37. Mineralocorticoid biosynthesis
38. Mitochondrial L-carnitine shuttle pathway
39. Mitotic roles of Polo-like kinase
40. Molybdenum cofactor biosynthesis
41. NAD biosynthesis II (from tryptophan)
42. Nicotine degradation II
43. Nicotine degradation III
44. Noradrenaline and adrenaline degradation
45. NRF2-mediated oxidative stress response
46. Oleate biosynthesis II
47. Oxidative ethanol degradation III
48. Proline degradation
49. Putrescine degradation III
50. PXR/RXR activation
51. Retinol biosynthesis
52. Role of BRCA1 in DNA damage response
53. Serotonin degradation
54. Stearate biosynthesis I
55. Sucrose degradation
56. Superoxide radicals degradation
57. Superpathway of cholesterol biosynthesis
58. Superpathway of melatonin degradation
59. Superpathway of methionine degradation
60. Taurine biosynthesis
61. The visual cycle
62. Thymine degradation
63. Thyroid hormone metabolism II (via conjugation and/or degradation)
64. TR/RXR activation
65. Triacylglycerol degradation
66. Tryptophan degradation III
67. Tryptophan degradation to 2-amino-3-carboxymuconate semialdehyde
68. Tryptophan degradation X (mammalian, via tryptamine)
69. Uracil degradation II (reductive)
70. Urate biosynthesis/inosine 5*-phosphate degradation
71. Xenobiotic metabolism signaling
72. Zymosterol biosynthesis

The main upregulated pathways in the PH(90%) group compared to all the other groups were eIF2 signaling, regulation of eIF4 and p70S6K, mTOR signaling, ILK signaling, and Acyl-CoA hydrolysis. The mTOR kinase is the central modulator of proliferative signal transduction. Its activation triggers a phosphorylation cascade inducing cell growth and proliferation[24]. Included in the mTOR axis are the eIFs and the p70s6K[24,25]; they are also upregulated in the PH(90%) group. Contrary to our present and previous[16] results, Zhang et al.[17] performed the extended PH(90%) and found suppressed regeneration of the FLR, mTOR downregulation, and high mortality. This could indicate that the expression of mTOR might

**Table 4.** Cascades of Upstream Transcriptional Regulators Explaining the Observed Gene Expression Changes and Affected Molecular Pathways

| IPA Top Upstream Regulators | *p* Value | | | | Name | Molecule Type (IPA) | Role in Cell (IPA Report) |
|---|---|---|---|---|---|---|---|
| | 30% PH | 70% PH | Sham | Baseline | | | |
| **Activated** | | | | | | | |
| MYC | S | S | S | S | V-Myc avian myelocytomatosis viral oncogene homolog | Transcription regulator | Cell cycle progression; apoptosis; cellular transformation |
| MYCN | S | S | S | S | V-Myc avian myelocytomatosis viral oncogene neuroblastoma-derived homolog | Transcription regulator | Tissue development |
| HGF | S | S | S | S | Hepatocyte growth factor | Growth factor | Organ regeneration; wound healing |
| FOS | S | S | S | S | FOS proto-oncogene | Transcription regulator | Cell proliferation; differentiation; apoptosis; transformation |
| TGFB1 | S | NSD | S | S | Transforming growth factor-β1 | Kinase | Cell growth; cell differentiation; apoptosis |
| F2 | S | S | S | S | Coagulation factor II; thrombin | Peptidase | Coagulation |
| POR | S | S | NSD | NSD | Cytochrome p450 oxidoreductase | Enzyme | – |
| **Inhibited** | | | | | | | |
| PPARA | S | S | S | S | Peroxisome proliferator-activated receptor α | Ligand-dependent nuclear receptor | Proliferation; abnormal morphology |
| NR1I2 | S | S | S | S | Nuclear receptor subfamily 1 group I member 1 | Ligand-dependent nuclear receptor | Cell death; morphology; phosphorylation; migration; proliferation; contact growth inhibitor; apoptosis |
| HNF1A | S | S | S | S | Hepatocyte nuclear factor 1 α | Transcription regulator | Cell growth; cell differentiation |
| RXRA | S | S | S | S | Retinoid X receptor α | Ligand-dependent nuclear receptor | – |
| SCAP | NSD | NSD | S | S | Sterol regulatory element-binding protein (SREBF) chaperone | Other | – |

The goal of the IPA Upstream Regulator analytic is to identify the cascade of upstream transcriptional regulators that can explain the observed gene expression changes in a user's dataset, which can help illuminate the biological activities occurring in the tissues or cells being studied (http://pages.ingenuity.com/rs/ingenuity/images/0812%20upstream_regulator_analysis_whitepaper.pdf).
The *p* value measures whether there is a statistically significant overlap between the dataset genes and the genes that are regulated by a transcriptional regulator. The purpose of the overlap *p* value is to identify transcriptional regulators that are able to explain observed gene expression changes. The overlap *p* value measures whether there is a statistically significant overlap between the dataset genes and the genes that are regulated by a transcriptional regulator (TR). It is calculated using Fisher's exact test, and significance is generally attributed to $p<0.01$ (http://pages.ingenuity.com/rs/ingenuity/images/0812%20upstream_regulator_analysis_whitepaper.pdf). S, significant difference, $p<0.001$; NSD, no significant difference, $p>0.001$.

be crucial to the balancing of regeneration and survival or PHLF and death.

ILK is involved in the transmission of extracellular matrix signals through integrins and is thought to have a major influence on the hepatostat[10]. If ILK is eliminated, the FLR exceeds the original liver size, indicating a defect in the termination process[26]. Findings show that ILK is a suppressor of hepatocyte growth, and the expression gradually increases toward the end of regeneration[26]. At first glance, it seems to be a paradox that ILK is significantly upregulated in the PH(90%) group compared to all the other groups. An explanation may be that the hepatostat dampens excess proliferation, which may take place at the cost of metabolism and homeostasis in an attempt to avoid PHLF. This theory is supported by a previous study we carried out on rats subjected to PH(30%), PH(70%), and PH(90%)[16]. In that study, we demonstrated an initially very low number of proliferating hepatocytes in the PH(90%) group compared to the PH(70%) group. However, this is followed by a burst in hepatocyte proliferation in the PH(90%) group, surpassing the levels seen after PH(70%) at any time point.

Based on our genetic dataset, we identified cascades of upstream transcriptional regulators (Table 4). These regulators indicate what may have been happening during the regenerative process prior to the genetic expression levels found in our dataset. Again, we chose our frame of reference to be PH(90%). Six of the seven upregulated activated genes were involved in cell proliferation, cell differentiation, and tissue development. The upregulated activation of hepatocyte growth factor (HGF) and transforming growth factor-β (TGF-β) was of special interest. The expression of immediate and delayed early genes in liver regeneration does not lead to DNA replication unless the cells can progress through the cell cycle. This progression is accomplished by growth factors such as HGF and TGF[23]. Collectively, HGF and epidermal growth factor (EGF) have a stronger proliferative effect on hepatocytes in culture than any other mitogen[10]. TGF-β was significantly activated in the PH(90%) group compared to the baseline, sham, and the PH(30%) groups, but not compared to the PH(70%) group. TGF-β has been shown to play an important role in the formation of extracellular matrix and vessels in the regenerating liver, including the sinusoids[27,28].

Of the five inhibited upstream regulators, hepatocyte nuclear factor 1-α (HFN1-α) is of special interest. HNF1-α regulates the transcription of genes into proteins that among other things are involved in glucose, cholesterol, and fatty acid transport, as well as metabolism[29,30]. Our IPA analysis further supports the theory that livers sustained to extended PH, close to the lower limit of the FLR, prioritize genes involved in cell proliferation and differentiation at the expense of genes involved in cellular homeostasis.

PH of the rat liver is a widely used model investigating liver regeneration. Despite obvious differences in rat liver size and anatomy[7] compared to the human liver, and the fact that rat liver metabolism is more rapid[31], it has been shown that the regulation of liver regeneration in rats and humans underlies the same principles[32,33]. Using the rat for experimental studies makes it possible to include a higher number of homogenous animals to the experimental groups contributing to significant data.

In previous studies, we have demonstrated that the FLR after PH(70%)[16,34] and PH(90%)[16] is almost fully regenerated (by measure of LBWR and hepatocyte proliferation) at POD5. However, with a delay in the initiation and peak of hepatocyte proliferation in the PH(90%) group at POD1, this led us to evaluate the gene expression on POD1.

Hepatocytes are the major cells in the liver constituting approximately 80% of its mass[35]. Other cells are cholangiocytes, Kupffer cells, stellate cells, blood cells, and liver sinusoidal cells. In the present study, gene analysis was performed on whole-liver biopsies, and as such, all other cells in the liver other than hepatocytes have contributed to the gene expression profiles we present. We believe that the contribution from other cells has been minor; regardless it was impossible for us to singularly isolate hepatocytes for analysis.

In conclusion, we demonstrated that the hepatostat's influence on gene expression was pronouncedly affected by the size of the PH and thereby the size of the FLR. With decreasing size of the FLR, the hepatosat tended to prioritize the expression of genes involved in cell proliferation and differentiation at the expense of genes involved in metabolism and body homeostasis. This prioritization may be a two-edged sword (i.e., cell proliferation and differentiation seem to be rational after extended PHs). However, should this take place at the expense of metabolism and body homeostasis, it may be the genetic explanation for PHLF. Future research should focus on ways to ensure that hepatocytes regenerate while at the same time preserving their metabolic and homeostatic functions to decrease the risk of PHLF.

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